Studies towards the
Sex Pheromone of the
Green Capsid Bug

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Stellingen

1. Verbindingen als (*Z*)-9-pentacoseen en (*Z*)-7-pentacoseen worden vaak ten onrechte als niet-vluchtige verbindingen geclassificeerd.
   Dit proefschrift; Schiestl et al., 2000. J. Comp. Physiol. A. 186:567-574.

2. Het nader specificeren van stoffen als 'mating stimulant pheromone', 'courtship pheromone' of 'contact pheromone' maakt hun precieze rol in een ecologisch systeem niet altijd duidelijker.

3. Veel enzymatische reacties worden onterecht als stereospecifiek geclassificeerd.
   van den Heuvel et al., 2000. Proc. Natl. Acad. Sci. USA 97: 9455-9460; Helmchen, G., 1997. Enantiomer 2:315-316.

4. Door alleen gebruik te maken van een 'library search' wordt de '*stc1*-gen specifieke verbinding' in maïs zaailingen ten onrechte door Shen et al. geïdentificeerd als β-cadineen.
   Shen et al., 2000. Proc. Natl. Acad. Sci. USA 97: 14807-14812.

5. Cortes et al. hadden (+)-cyclozonarone eenvoudiger kunnen synthetiseren door uit te gaan van epi-manool, dat gemakkelijk te isoleren is uit terpentijn van de Europese larix.
   Cortes et al., 2001. J. Nat. Prod. 64:348-349.

6. De maatregelen die door de overheid gebruikt worden in de strijd tegen BSE zijn overdreven in vergelijking met de (afwezigheid van) maatregelen die tegen de tabaksindustrie genomen worden in de strijd tegen longkanker.

7. Goede wijn behoeft geen kurk.
   Intermediair, 7 september 2000.

Stellingen behorend bij het proefschrift:

Studies towards the Sex Pheromone of the Green Capsid Bug.

Wageningen, 23 mei 2001. Falko P. Drijfhout

Introduction

1.1 Semiochemicals

For insects, chemical cues are among the most important in locating food, finding a mate and in social interactions. Such chemicals used by insects and other animals are called **semiochemicals**, which can be divided in **pheromones** and **allelochemicals**. Pheromones act between individuals of the same species. Allelochemicals act between different species and consist of two categories: **kairomones** (chemical signals that benefit the receiver of the chemical stimulus, but are deleterious to the emitter) and **allomones** (chemicals that give an advantage to the emitter e.g. defensive secretions). In Figure 1.1 the different categories of semiochemicals are shown.

Pheromones play an important role in the life of insects. The word pheromone is derived from two Greek words, i.e. *pherein,* which means "to bear, bring" and *hormôn,* which means "to excite" (Websters College Dictionary, 1997). Pheromones are defined as substances that are secreted by males and/or females to the environment (*~pherein*) and, when perceived by a second individual of the same species, trigger a specific response (*~hormôn*). Several types are known, like alarm, sex, trail and aggregation pheromones. When chemicals are produced to attract the opposite sex within a species, these chemicals are called sex pheromones. Somewhat different from sex pheromones are the aggregation pheromones, which are compounds capable of attracting both sexes. Another important class of chemicals in heteropteran species is the group of alarm pheromones; substances emitted upon approach of a predator or under stress (Blum, 1985).

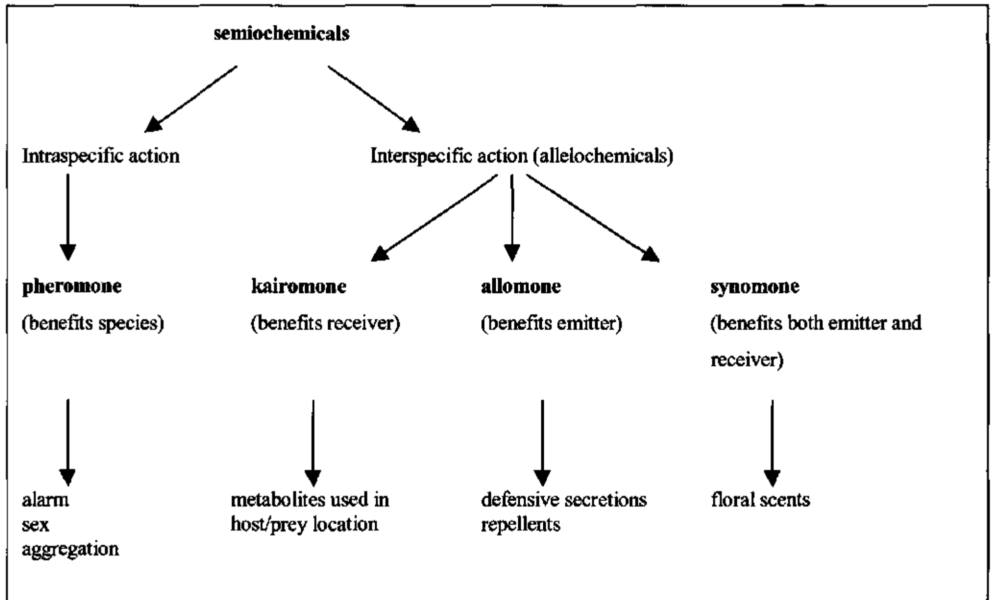

Figure 1.1 Categories of semiochemicals, with examples of their biological function (Howse, 1998). Definition of synomone according to Dicke and Sabelis (1992).

Because of the many chemicals playing a role in their orientation, the antennae of insects are highly specialised in perceiving chemicals, such as pheromones, from a far distance (Jacobson, 1972). Numerous studies have been performed on olfaction, i.e. sense of smell in relation with perceiving (sex) pheromones in Lepidoptera (e.g. Breer, 1997 and references therein; Prestwich and Du, 1997 and references therein). These studies revealed that the antenna is covered with sensory sensilla (sensory hairs), which are sensitive to certain compounds. Each sensillum has one or more olfactory receptor neurones. A neurone recognises a particular molecule through binding with a receptor protein (pheromone binding protein, PBP) on the neurone. As the molecule binds to the protein, the neurone is stimulated, which results in a flow of positive sodium ions into the cell that depolarises the cell. This depolarisation lasts for only a few milliseconds and than the cell returns to the resting membrane potential of the unstimulated neurone. These neuronal signals travel along the axons of the sensory neurone towards the olfactory lobe in the brain where this pheromonal information is processed, which can

eventually lead to a behavioural response. Although, most of the studies on understanding the molecular mechanisms of pheromone perception have been conducted in moths (Cardé and Minks, 1997 and references therein), more and more studies are conducted on perceiving pheromones in the Heteroptera order (e.g. Dickens et al., 1995; Chinta et al., 1994).

1.2 Sex pheromones

1.2.1 General

The first sex pheromone was isolated and identified by Butenandt et al. (1959). They identified the sex pheromone of the oriental silk moth, *Bombyx mori* (Lepidoptera: Bombycidae) as (*E,Z*)-10,12-hexadecadienol (bombykol). For almost 20 years it was believed that bombykol was the only component emitted by female *B. mori*, but in 1978 Kasang et al. (1978) showed that the female also produces the corresponding aldehyde, (*E,Z*)-10,12-hexadecadienal. This aldehyde was found to be part of the sex pheromone in the ratio alcohol:aldehyde of 10:1. Sex pheromones can thus be only one compound or a blend of two or more compounds. It has become clear that sex pheromones consisting of only one compound are more exception than rule and that multiple component pheromones are widely found in insect species. The ratio in which these components are present is extremely important. For example, two species of the genus *Archips* share the same four components in their pheromones, but the two species differ in the relative proportion of the components; 60:40:4:200 for *A. argyrospilus* and 90:10:1:200 for *A. mortuanus* (Evans, 1984).

Insects are extremely sensitive to sex pheromones. Male *B. mori* respond to amounts less than 10 pg (10^{-11} gram) of its sex pheromone when offered on a piece of filterpaper (Kaissling, 1979). Experiments carried out with *Adoxophyes orana* showed that males were able to locate the sex pheromone source (virgin females) over a distance of 75 meter in just one night (Noordink and Minks, 1970).

Also in non-Lepidopteran species sex pheromones exist (McBrien & Millar, 1999). The first attractant pheromone identified for a heteropteran species was in the spined soldier bug, *Posidus maculiventris* (Aldrich et al., 1978, 1984). More details on these sex pheromones are given in Section 1.4.

1.2.2 Isolation of sex pheromones

As can be seen from the flow chart in Figure 1.2, the bioassay is essential for the isolation and identification of pheromones. Only with a suitable bioassay available the isolation and analysis of sex pheromones is worthwhile. The bioassay-guided fractionation ensures that the maximum effort is concentrated on those fractions and those compounds that are involved in the behaviour of the insect.

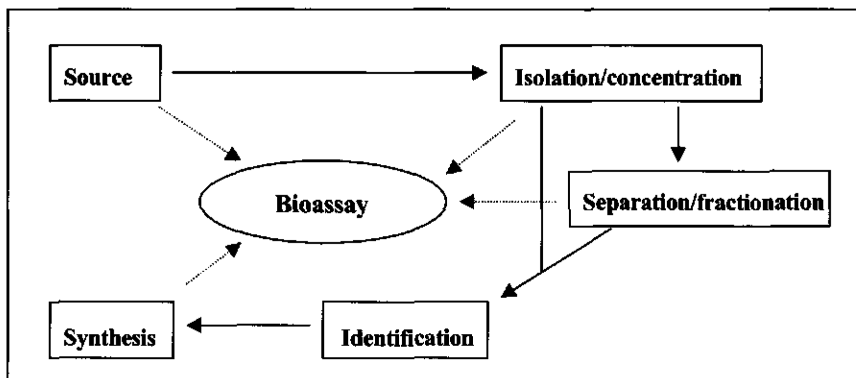

Figure 1.2 Flow chart of procedure for isolation and identification of pheromones (Stevens, 1998).

Isolation of sex pheromones is mainly achieved in two different ways. The first method is via (solvent) extraction, i.e. to extract the whole insect or body parts from the insects (e.g. pheromone glands if they are known) with an organic solvent like pentane, hexane or dichloromethane. The second method is via headspace collection, i.e. trapping of compounds emitted by living insects on a solid absorbent like Tenax, Porapak Q or activated charcoal or in a cold trap. There are many variations of both techniques. For example, Griepink et al. (2000) applied the first method not by extracting pheromone glands with a solvent, but by directly introducing the gland in the gas chromatographic

injector. The intact pheromone gland is heated in the GC, which causes the volatile compounds to evaporate, and subsequently to be trapped on the column. A different set-up of this method is described in Chapter 2. A variation on the second method was introduced in the 1990's with Solid Phase Microextraction where compounds are trapped on a 100 μm thick layer of stationary phase (Arthur and Pawliszyn, 1990).

Both isolation techniques have advantages and disadvantages. In the first method it is easy to obtain more material if necessary, by extracting more insects. When the number of insects is not limited, enough material can even be extracted to perform NMR analysis. However to obtain pure compounds, purification is necessary. This can be very time consuming, as with extracting (whole) insects, also many undesired compounds (e.g. defensive secretions or cuticular waxes) are extracted. This can be avoided by using the method of headspace collection. This method usually gives clean samples with only the volatile compounds emitted by insects (or plants if insects need to feed on plants to emit sex pheromone). However, this method is difficult to scale up and sometimes suffers from chemical background noise.

1.2.3 Identification of sex pheromones

Due to the volatility of sex pheromones gas chromatography is the ideal tool for analysis of complex mixtures obtained either via extraction or via headspace collection. Depending on the column used, compounds in the extract are separated based on their structural characteristics like carbon chain length, functional groups and/or double bonds. Information about these structural features can be obtained by comparing the retention times on two different columns e.g. a column coated with a polar polyethylene glycol phase and a column coated with an apolar dimethylpolysiloxane phase.

Instead of using the common Flame Ionisation Detector (FID), other detection techniques can be coupled to the GC (on-line). Coupled gas chromatography-mass spectrometry (GC-MS) is nowadays a standard tool in sex pheromone research. With this method compounds separated on the GC-column are transferred individually to a mass spectrometer. In the mass spectrometer these compounds are fragmented and the

different fragments can be measured, based on their mass-charge ratio (*m/z*). Based on this fragmentation pattern often a tentative structural formula of the compound can be proposed (McLafferty and Turecek, 1993).

A second detector that has been coupled to a GC is a specialised biological detector, called electroantennographic detector (EAD). Electroantennography (EAG) is a technique that relies upon the specificity and sensitivity of the olfactory system of the insect itself (Schneider, 1957), which is often located on the antennae of the insect. The simultaneous depolarisation of many receptor cells, when they are stimulated can be measured with EAG. A GC coupled to an EAG set-up (GC-EAD) (Arn et al., 1975) can be used as a screening technique (Roelofs, 1984) to determine which compounds in a complex mixture can be perceived by the insect, and which are thus likely candidates for sex pheromone constituents. Instead of testing all compounds in a bioassay only the EAD-active compounds need to be tested. One can however not always rely on EAG responses as was shown by Leal et al. (1998) where behaviourally active compounds did not elicit detectable EAG signals. It is also important to mention that EAG is restricted to whether an insect is able to recognise a certain compound or not. The behavioural response elicited by these EAD-active compounds should be tested using a separate bioassay such as windtunnel experiments, to see whether detected compounds are sex-, alarm- or aggregation pheromones or trigger no behavioural response. Thus, GC-EAD may be helpful, but results need to be thoroughly checked with additional behavioural bioassays.

When certain compounds are active in the bioassays, their identification can be pursued. Comparing the retention times of GC-MS en GC-EAD analyses can locate the position of the EAD-active compounds in the GC-EAD gas chromatogram and subsequently give information about the structure of these EAD-active compound(s). When the active compound(s) can be isolated (e.g. by thin layer –, column chromatography or preparative GC) on a microgram scale, ^{1}H-NMR-analyses can be performed to provide proof of the structure. However, as sex pheromones are normally present in very low amounts, full structure identification is mainly achieved by GC-MS

and synthesising (or buying commercially available) possible candidates and comparing retention times on GC. The final identification of a sex pheromone is to determine whether the artificial blend is able to attract the opposite sex, either in the windtunnel bioassay or in the field.

1.2.4 Use of sex pheromones in Integrated Pest Management

Since the 1940's insect pest control has been primarily achieved by the use of synthetic pesticides. Although these man-made compounds had a tremendous effect on insect populations, they also had unwanted effects on the environment. Knowing this, much research has been carried out to develop environmentally friendly alternatives; Integrated Pest Management (IPM) being one of them. The basic strategy behind IPM is to prevent insect populations from reaching their economic injury level, while avoiding ecological and sociological damage. One of the tools in IPM is the use of sex pheromones. In contrast with pesticides, sex pheromones are very specific compounds that are produced and used only by the insect species that has to be controlled. Therefore only these insects are affected, and because they use these compounds themselves for communicating with the opposite sex, there is little chance of resistance. Sex pheromones can be used in four different ways: 1) monitoring, 2) mass trapping, 3) mating disruption and 4) attract-and-kill (Jones, 1998).

Of these four different methods monitoring is most commonly used (e.g., Alford et al., 1979). With this method it is possible to obtain information about the population density of the insect species. When the population is reaching its economic threshold value, pesticides can be applied. In this case, pesticides are only used when necessary and thus a reduction of the use of these environmentally unfriendly compounds can be achieved.

Mass trapping, as the name implies, traps the pest insect and in this way prevents it from causing substantial damage to the crop. This method is only efficient when practically all the insects are trapped (Roelofs et al., 1970). As this is almost impossible,

it is not widely used, although success has been achieved in mass trapping bark beetles (Borden, 1989) and in greenhouses (Kawai and Kitamura, 1990 and references therein).

The third method, mating disruption, is successfully applied in several moth species (Cardé and Minks, 1995). With this method a high concentration of the sex pheromone is sprayed in the field thus disrupting the chemical communication and diminishing the chance that males and females can locate each other. Less copulation will thus occur, resulting in fewer offspring. As a result, the population density will be reduced till it reaches a value beneath the economic threshold.

The attract-and-kill (or lure and kill) method aims at killing the insect when it reaches its target and has been applied among others against the pink bollworm moth, *Pectinophora gossypiella* (Haynes et al., 1986). This technique consists essentially of two components: the lure, which could consist of the sex pheromone, and an affector, which could consist of a potent insecticide. The lure (sex pheromone) is formulated together with liquid that contains the affector (insecticide). This liquid is then sprayed on the plant that has to be protected. When the insect reach the plant (attracted by the sex pheromone) and eats from the plant and liquid, they will be killed.

1.3 The green capsid bug

The insect studied in this PhD thesis is the green capsid bug (*Lygocoris pabulinus* (L.), Heteroptera: Miridae). This bug (Figure 1.3a,b) is a serious pest in fruit orchards and raspberries (Hill, 1952) in North-Western Europe and is difficult to control (Blommers, 1994; Ravn and Rasmussen, 1996). *L. pabulinus* has two generations each year (Figure 1.4), of which the summer generation feeds on herbaceous plants (Petherbridge and Thorpe, 1928). In autumn, females (from the second generation) oviposit their overwintering eggs in the stem of fruit trees, after which the adults die. In spring, when trees start to bloom, nymphs emerge from the eggs (Figure 1.4). In this stage most of the economical damage occurs as these nymphs feed on shoot tips, ovaries and young fruitlets, which causes russety malformation in the fruits (Blommers, 1997).

Figure 1.3a **The green capsid bug, *L. pabulinus* (left), and appels damaged by the green cpsid bug (right). Pictures from Frankenhuyzen (1988).**

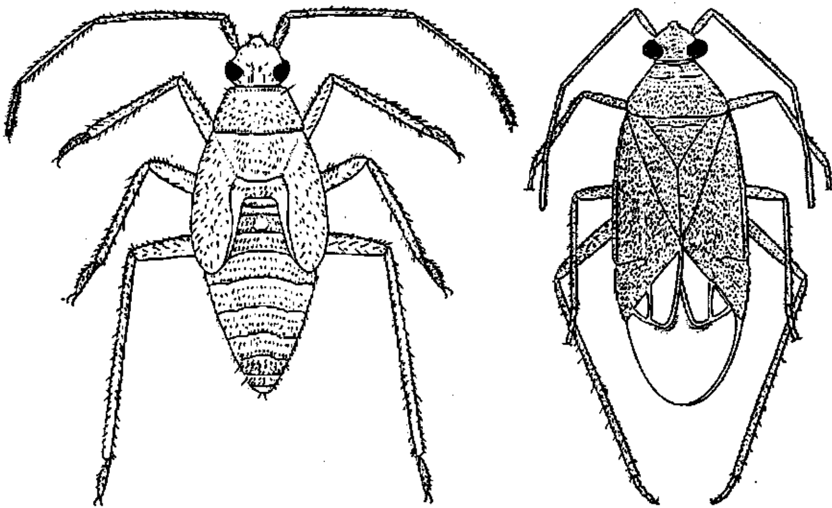

Figure 1.3b **Drawing of the green capsid bug, *L. pabulinus*. Left: fifth instar larva; Right: adult (Petherbridge and Thorpe, 1928)**

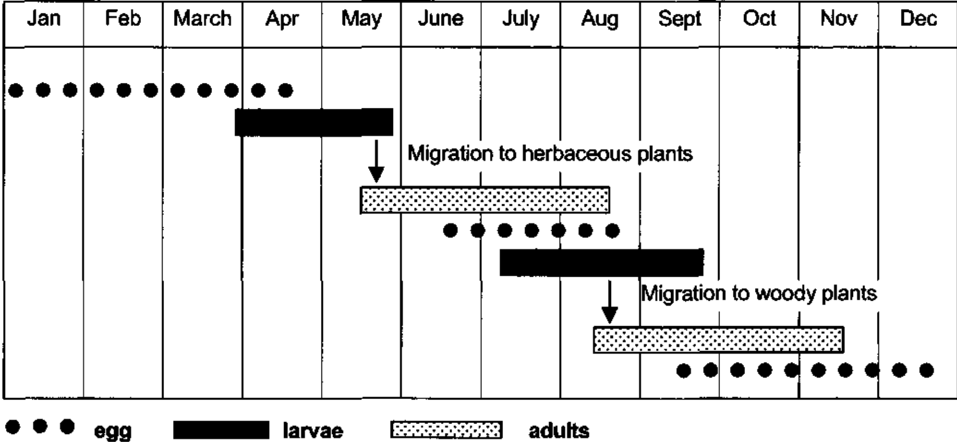

Figure 1.4 Life cycle of the green capsid bug (Frankenhuyzen, 1988). Most damage occurs during the larval stage.

Damage of the fruit will thus directly affect the yield and the quality of the crop. Consequently, economic threshold values will be exceeded soon after the nymphs have emerged. Additionally, this threshold is exceeded already at very low population densities (van den Ende et al., 1996). Fruit growers cannot predict whether or not their fruit will suffer from bug damage in the following spring.

In order to reduce the risk of damage, the growers apply insecticides against the green capsid bug before and after blooming each year, without determining the actual population density. This phenomenon is called "calendar-spraying" and requires a lot of insecticides. Additionally, there is no insecticide available that specifically kills this mirid, thus beneficial insects like natural enemies are also killed. Identification of the sex pheromone of this mirid may provide an efficient monitoring system so that the use of insecticides against this pest can be reduced.

1.4 Pheromones in true bugs (Heteroptera)

Since the identification of the first sex pheromone (Butenandt et al., 1959), there has been a continuing interest in these behaviour-modifying semiochemicals. Nevertheless, most of the attention has always been directed to lepidopterous insects. In recent years the research direction has changed and other insect orders, including Heteroptera to which *Lygocoris pabulinus* (see section 1.3) belongs, are receiving more and more attention (Hardie and Minks, 1999). Although a number of bug pheromones have been identified, pheromone identification is complex, as is shown in the review by McBrien and Millar (1999) on pheromones in phytophagous bugs. This complexity is mainly caused by a lack of knowledge about the behaviour of, and the site of production of the pheromone in these bugs.

1.4.1 Scent glands in Heteroptera; their secretions and possible role as sex pheromone glands

Chemical communication in insects requires a source of the signal molecules, such as pheromones. The source is often an exocrine gland, glands that secrete compounds through a duct to the outer part of the exoskeleton of the insect. When the compounds being released by these glands are (sex) pheromones, these glands are called (sex) pheromone glands. By far the greatest amount of work on the existence and function of these specific sex pheromone glands has been conducted with Lepidoptera. In numerous species such glands have been identified (Jacobsen, 1972). The scent glands, an important class of exocrine glands in heteropteran insects, have been studied in detail (Gupta, 1961 and references therein; Staddon, 1979, 1986). The main physiological role of these scent glands is to manufacture and store volatile substances for subsequent release (Staddon, 1979). These volatiles are usually highly odoriferous and therefore a defensive role was assigned to these compounds and the glands that produce them (Gupta, 1961). Two types of scent glands have been reported, i.e the abdominal scent gland and the metathoracic scent gland. The names given to the glands

are from the parts of the body (abdomen, metathorax) in which they are found. The abdominal glands are dorsal and they may occur up to a maximum of four. These are essentially larval glands and only occasionally continue to function in adults. The metathoracic gland exclusively occurs in adults (Staddon, 1979). Postulated functions of the compounds released by these scent glands include 1) defence against predators, 2) defence against microorganisms, and 3) induction of specific patterns of behaviour (alarm, aggregation or mating). Of all these functions, only the defence against predators has been fully proven for correid and pentatomid bugs (Aldrich, 1995), although Gupta (1961) believes that in adults these glands have primarily a sexual function.

Evidence for sex pheromone glands in Heteroptera was found with the discovery of sexually dimorphic glands. These glands were usually excessively developed in males (Aldrich, 1995), indicating that males are the producers of the sex pheromone. In some pentatomids the III-IV dorsal abdominal glands are small in females and extremely large in males (Aldrich et al., 1978). Further field studies proved that in most Heteropteran species males are attracting females. But the situation is more complicated as various species produce sex- and species-specific compounds from glands which are not sexually dimorphic (Aldrich, 1995). However, in many species no sex pheromone gland has been found yet. Isolation and identification of sex pheromones in these true bugs (Heteroptera) has thus been a challenging and difficult task. The overwhelming presence of alarm pheromones and defensive secretions makes the identification even more difficult. For an overview of the semiochemicals of true bugs, including sex, aggregation, alarm pheromones, and defensive secretions together with their production sites, several excellent reviews are available (Aldrich 1988a, 1988b, 1995).

1.4.2 Sex pheromones in plant bugs (Miridae)

In most heteropteran species in which attractant pheromones are known, males are the attractive sex. In contrast, in the Miridae family (plant bugs) the female attracts the males. Besides the evidence for a sex pheromone in *Lygocoris pabulinus* (Blommers

et al., 1988), in at least ten other mirid bugs females have been found to attract males; i.e. *Lygus hesperus* (Strong et al., 1970; Graham, 1987; Graham, 1988), *Lygus lineolaris* (Scales, 1968), *Distantiella theobroma* (King, 1973), tea mosquito bug, *Helopeltis Antonii* (Sundararaju et al., 1994), *H. clavifer* (Smith, 1977), *Lygocoris communis* (Boivin and Stewart, 1982, Graham et al., 1987); *Atractotomus mali* (Smith et al., 1994); *Campylomma verbasci* (Thistlewood et al., 1989), *Lygus rugulopennis* (Innocenzi et al., 1998), *Calocoris norvegicus* (Welter, 1991), *Neurocolpus longirostris* (Rice and Jones, 1989) and the rice leaf bug, *Trigonotylus caelestialium* (Kakizaki and Sugie, 1997). Yet of all the mirids the sex pheromone of only three species has been identified (Smith et al., 1991; Millar et al., 1997; Millar and Rice, 1998).

In spite of the extensive studies on scent glands in Heteroptera (Staddon, 1979), little is known about the production site of the sex pheromones in mirids. Moreover, most of these studies were not carried out on secretions from the abdominal or metathoracic scent glands in mirids, but in pentatomids or coreid bugs (Aldrich, 1988a, 1988b, 1995). Furthermore, studies carried out with other species than from the Miridae family cannot easily be used, as in mirids females emit the sex pheromone and not the males like in pentatomids. Authors have postulated that a site near the ovipositor may be the location of a sex pheromone producing organ (Graham, 1988) or the spermatheca (Strong et al., 1970, Aldrich, 1988b) might be the sex pheromone producing organ itself. Evidence that scent glands have a function as sex pheromone producing organs is therefore meagre, but as up to now only three (from 10,000) mirid sex pheromones have been identified, scent glands may still be the site of pheromone production.

Another difficulty in the identification of sex pheromones in Miridae is that in only two species, *D. theobroma* and *H. clafiver* a female specific calling behaviour could be observed, i.e females raise their abdomen while releasing pheromone (King, 1973; Smith, 1977). In all other mirids it is unknown when females emit their sex pheromone.

1.4.3 Chemical composition of secretions in Miridae

Typically unbranched aliphatic molecules of low molecular weight have been encountered in the secretions as major scent volatiles in Miridae. They include (*E*)-2-hexenal, (*E*)-2-octenal, and (*E*)-4-oxo-2-hexenal. The latter and its C_8 homologue are characteristic of the secretions from scent glands of Heteroptera, especially in pentatomids. In Miridae this keto-aldehyde has been encountered in only a few cases (Knight et al., 1984; Innocenzi et al., 1998). Esters form by far the majority of compounds found in volatile extracts from mirids. Males and females of the tarnished plant bug, *Lygus lineolaris*, had different amounts of two esters, hexyl butyrate and (*E*)-2-hexenyl butyrate. In males the ratio of (*E*)-2-hexenyl butyrate and hexyl butyrate is about 1:10, whereas in females equal amounts of these esters are found (Gueldner and Parrot, 1978). Esters formed also the majority of the volatile compounds present in the metathoracic gland of two capsid bugs, *Pilophorus perplexus* and *Blepharidopterus angulatus* (Knight et al., 1984). Other compounds found in the headspace from mirids are monoterpenes, like neral, neryl acetate and geranial (Hanssen and Jacob, 1982). In Figure 1.5 an overview of the compounds identified from mirid bugs is shown.

1.5 Aim and justification

The ultimate aim of this project was to develop an efficient monitoring system for *L. pabulinus* in fruit orchards, by identifying its sex pheromone. In a related species with a similar life cycle, *Campylomma verbasci* (Heteroptera: Miridae), identification of the sex pheromone and monitoring of this mirid pest has been successful (Smith et al., 1991; McBrien et al., 1994, 1996, 1997). Therefore, identification of the sex pheromone of *L. pabulinus* as well as the development of a monitoring system, based on the use of its sex pheromone, seemed feasible. Besides, *L. pabulinus* males are attracted by virgin females in the field (Blommers et al., 1988), which can be considered as additional support for this proposition.

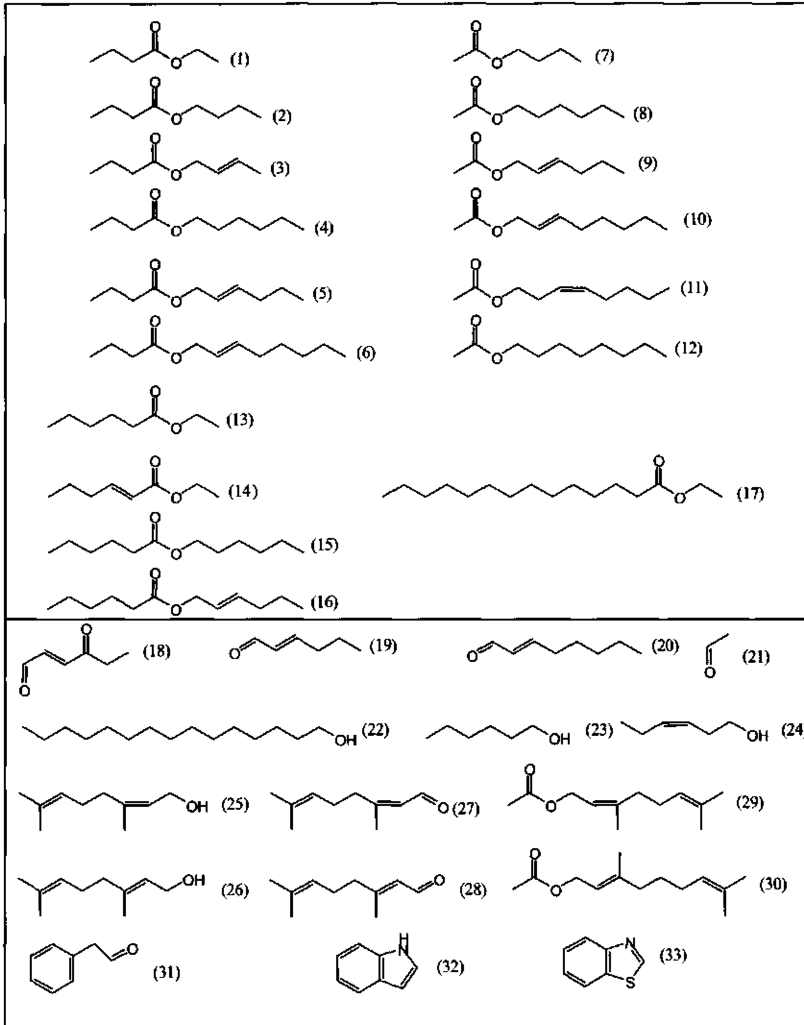

Figure 1.5 Compounds identified from headspace or scent glands in Miridae.

1: ethyl butyrate, 2: butyl butyrate, 3: (*E*)-2-butenyl butyrate, 4: hexyl butyrate, 5: (*E*)-2-hexenyl butyrate, 6: (*E*)-2-octenyl butyrate, 7: butyl acetate, 8: hexyl acetate, 9: (*E*)-2-hexenyl acetate, 10: (*E*)-2- octenyl acetate, 11: (*Z*)-3-octenyl acetate, 12: octyl acetate, 13: ethyl hexanoate, 14: ethyl 2-hexenoate, 15: hexyl hexanoate, 16: (*E*)-2-hexenyl hexanoate, 17: ethyl myristate, 18: (*E*)-4-oxo-2-hexenal, 19: (*E*)-2-hexenal, 20: (*E*)-2-octenal, 21: acetaldehyde, 22: pentadecanol, 23: hexanol, 24: (*Z*)-3-hexen-1-ol, 25: nerol, 26: geraniol, 27: neral, 28: geranial, 29: neryl acetate, 30: geranyl acetate, 31: phenyl acetaldehyde 32: indole, 33: benzothiazole. Data taken from Knight et al. (1984); Gueldner and Parrot (1978); Hanssen and Jacob (1982); Millar et al. (1997); Millar and Rice (1998) and Smith et al. (1991).

This project consisted of two components: (1) a biological study of the sexual behaviour of the green capsid bug, and (2) a chemical study to identify its sex pheromone. The biological part has been published recently in the thesis of A. T. Groot (Groot, 2000).

The first attempt to identify the sex pheromone of *L. pabulinus* using conventional techniques, dates from the 1980's, but was not successful. Therefore within the chemical part of this study, the possibility of applying some new techniques in the identification of the sex pheromone, formed an important part. Because many other semiochemicals such as alarm, aggregation pheromones and allomones are being produced by true bugs, this study also attempted to identify these and other compounds playing a role in the chemical communication of the green capsid bug. In this thesis the identification of these semiochemicals of *L. pabulinus* as well as the application of these (new) techniques is described.

1.6 Outline of thesis

Previous research in our lab on direct gland introduction into a GC-injector (Griepink et al., 2000) showed the usefulness of on-line stripping of volatiles from glands. In **Chapter 2** the application of thermal desorption of whole insects and parts of insects in pheromone research is described. This technique has been applied on two model insects, *C. verbasci* (Heteroptera: Miridae) and *Adoxophyes orana* (Lepidoptera: Tortricidae) as well as on the green capsid bug.

Chapter 3 deals with two different extraction methods, the Solid Phase Microextraction (SPME) and the Stir Bar Sorptive Extraction (SBSE). These two methods are compared and used to identify airborne volatiles emitted by female *L. pabulinus*.

One of the techniques frequently used to screen the activity of compounds produced by insects, is coupled electroantennography-gas chromatography (GC-EAD) (Arn et al., 1975, Roelofs, 1984). Studies on olfaction in male *Lygus hesperus* (Graham, 1988) showed that the antennae play an important role in locating females. Therefore,

GC-EAD was also applied in this research. In **Chapter 4** the successful application of GC-EAD in identifying compounds being perceived by male and female green capsid bugs is reported. Also the difference between male and female perception is described.

Groot et al. (1998a, 1998b) found a sex specific courtship behaviour in male *Lygocoris pabulinus*. Males start to vibrate with their abdomen when they are in the vicinity of females. This behaviour was used to identify compounds that are involved in this courtship behaviour. In **Chapter 5** the results obtained with live and dead males and females, different body parts and extracts from these body parts in this vibration bioassay, are described. Furthermore the chemical analysis of active extracts, to identify active compounds is reported.

In **Chapter 6** the testing of extracts from male and female legs, of which the latter cause vibrational behaviour in males, together with the testing of headspace extracts from male and female *L. pabulinus* in a Y-track olfactometer is described. The results obtained from GC-EAD analysis of these active extracts, on a different EAD set-up as used in Chapter 4, revealed that there is evidence for a close-range attractant in *L. pabulinus*.

In **Chapter 7** all aspects of chemical communication in green capsid bugs are discussed. Furthermore, some preliminary studies on changes in the hydrocarbon profile in females are reported. In addition, the possible effect of stress in males and females are shown. In conclusion aspects of controlling *L. pabulinus* in apple orchards with a potential sex and/or close-range pheromone are discussed.

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On-line thermal desorption-gas chromatography of intact insects*

Abstract – By using a thermodesorption system (TDS) together with a programmable temperature vaporiser (PTV) injector, the composition of the known sex pheromones of *Adoxophyes orana* (Lepidoptera: Tortricidae) and *Campylomma verbasci* (Heteroptera: Miridae) was confirmed. Only a single insect per analysis was necessary. Intact females, males or pheromone glands were placed in the oven part of the TDS, which was subsequently heated. The compounds released by this heating process were transferred to the PTV, which was cooled to −150°C. Injection took place on a dual-column GC by heating the PTV rapidly to 250°C. The major sex pheromone compounds of *A. orana* were found only in the pheromone gland of females. Male and female *C. verbasci* showed fingerprint-identical chromatograms, except for the two sex pheromone compounds, which were present only in females. No distinct differences were found in compounds released from female and male *Lygocoris pabulinus* (Heteroptera: Miridae). The advantages of this rapid method are the high sensitivity and the low degree of degradation and contamination. This technique was effective in analysing small insects by GC without prior manipulation, such as solvent extraction or distillation.

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2.1 Introduction

Insects emit sex pheromones to attract conspecific males or females for mating. Because of the volatility of these compounds, gas chromatography (GC) coupled to mass spectrometry is an ideal technique for their separation and identification. One of the difficulties in structure elucidation of sex pheromones is the small amounts in which they are produced. Collecting volatiles from insects, e.g., via headspace collection, sometimes needs to be done for several days, depending on the species (Smith et al., 1991; Millar et al., 1997; Millar and Rice, 1998), to obtain enough material for chemical analysis. An additional difficulty in the identification of sex pheromones in mirids is the presence of defensive compounds that may conceal the presence of the sex pheromone. Sampling for longer periods may increase the amounts of these defensive compounds and hamper the identification of the sex pheromones.

Many different techniques have been used to collect volatiles from insects. Bowman and Karman (1958) were the first to describe a crushing device for small samples in the injection area of the GC. A similar injection technique was described for the investigation of the constituents of single glands from insects, such as ants (Morgan and Wadhams, 1972; Morgan et al., 1979). Attygalle et al. (1987) reinvestigated the sex pheromone of *Mamestra brassicae* (Lepidoptera: Noctuidae) by using a solid sample injection technique in combination with mass spectrometry. Brill and Bertsch (1985) used a pyroprobe unit inserted into the injector of the gas chromatograph to examine the cuticular hydrocarbon profile of *Solenopsis richteri* (Hymenoptera: Myrmicinae) workers. Recently, a thermal desorption injection method was applied successfully on the pheromone glands of *Symmetrischema tangolias* (Lepidoptera: Gelechiidae) and *Scrobipalpuloides absoluta* (Lepidoptera: Gelechiidae) (Griepink et al., 2000). In this chapter, the application of a different type of thermal desorption unit is described. A Lepidopteran species, *Adoxophyes orana*, and two Miridae species, *Campylomma verbasci* and *Lygocoris pabulinus*, were used as test insects.

2.2 Materials and Methods

2.2.1 Insects *Campylomma verbasci* was reared according to Uiterdijk et al. (1997) and *Adoxophyes orana* according to de Jong (1968). *Lygocoris pabulinus* was reared under summer conditions on potted potato plants, cultivar Bintje, in wooden cages in a greenhouse at 22 ± 2°C, 65 ± 5 % R.H., L18:D6, following the procedure of Blommers et al. (1997). Every 2-3 days the newly emerged adults were collected from the rearing cages, after which the sexes were isolated in separate rearing cages. In this way, virgin males and females of known ages were continuously available for the experiments (Groot et al., 1998).

2.2.2 Chemicals Butyl butyrate, (*E*)-2-hexen-1-ol and butyryl chloride were all purchased from Acros Organics (Geel, Belgium), hexyl butyrate from Roth (Karlsruhe, Germany), and crotyl alcohol (containing ~5% of the *Z*-isomer) from Fluka (Buchs, Switzerland). (*E*)-2-butenyl butyrate and (*E*)-2-hexenyl butyrate were synthesised as described below.

2.2.3 Synthesis of (E)-2-butenyl butyrate and (E)-2-hexenyl butyrate This was done according to Vogel (1989). Esters were prepared by refluxing 1 equivalent of the acid chloride with 1 equivalent of the corresponding alcohol for 1 hour. The mixture was poured into water, washed with $NaHCO_3$ solution, then with H_2O and dried over $MgSO_4$. Esters were collected by filtering. Configuration and purity of the esters were confirmed by NMR (200 MHz; Bruker AC200) and GC-MS. (*E*)-2-butenyl butyrate contained ~5% of the *Z*-isomer, which is in accordance with the amount of *Z*-isomer in the starting material.

2.2.4 Thermodesorption System On-line thermal desorption-gas chromatography was performed by using a Thermodesorption System (TDS) (Gerstel, Mülheim am Ruhr, Germany) coupled to a GC (HP 5890) equipped with a split/splitless PTV-injector

(CIS 3, Gerstel). The TDS could also be used in split or splitless mode. A schematic drawing of the TDS equipment used is shown in Figure 2.1.

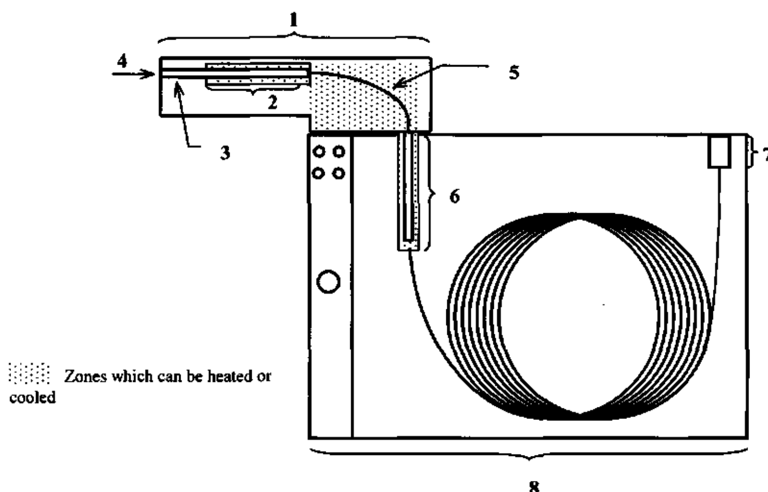

Figure 2.1 Schematic drawing of the Thermal Desorption System (TDS). 1: Thermal desorption System (TDS), 2: Oven part of TDS, 3: TDS tube, 4: gas flow (hydrogen/helium), 5: transferline (300°C), can only be heated, 6: PTV-injector, 7: FID, 8: GC

Compounds were desorbed in the ovenpart of the TDS (**2**), transferred via a transferline (**5**) (14 cm × 0.25 mm deactivated fused silica), and trapped in the liner (1.4 mm i.d.) of the PTV-injector (**6**) at −150°C by using liquid nitrogen for cooling. Injection of the compounds onto the column was done by rapid heating of the PTV-injector to 250°C (12°C/sec).

The GC was a dual column GC equipped with an apolar DB-1 column and a polar DB-WAX column (both J&W Scientific (Folsom, California), 60 m × 0.25 mm; film thickness: 0.25 μm) and two Flame Ionisation Detectors. After the PTV a Gerstel™ Graphpack-3D/2 flow splitter was installed to divide the injected sample in a ratio of 1:1 over both columns (not shown in Figure 2.1). Oven program: 40°C (2 min hold) to 238°C (10 min hold) with 4°C/min. Hydrogen was used as the carrier gas (inlet pressure 20 psi, linear velocity at 140°C: 35.5 cm/sec). Identification of the

compounds in the gas chromatograms was done by comparison of the Retention Indices (on both columns) of the unknown compounds with those of reference compounds.

2.2.5 Collecting materials from insects A living male or female mirid, freshly dissected legs from *L. pabulinus* or an excised pheromone gland from *A. orana*, (half an hour before the females started calling) were put into a glass tube (175 mm long, 6 mm O.D. and 4 mm I.D.), and two small silanised glass wool plugs were used to keep the specimen in place. The glass tube was placed into the oven part of the TDS kept at 25°C (15 min) or heated to temperatures varying from 50°C to 250°C at 1°C/sec. During this period, the carrier gas (flow rate 100 ml/min) was led over the specimen, and the volatile compounds were trapped in the PTV-injector. Compounds were then desorbed from the liner by heating the PTV to 250°C in the splitless mode.
Headspace analysis was performed by using 8 female or male mullein bugs. These were kept in a glass cage with potato leaves and pollen. Moist air was passed through the cage. Volatiles were trapped on Tenax-TA (20-35 MESH or 200-900 µm, Chrompack, Middelburg, The Netherlands; TDS-tubes were loaded with 200 mg of Tenax) for 8-24 hours at a flow rate of 60 ml/min. GC-analysis was done with the same thermodesorption system; helium was used as carrier gas.

2.3 Results

The major sex pheromone components of *Adoxophyes orana* have been identified as (*Z*)-9-tetradecenyl acetate (Z9-14:Ac) and (*Z*)-11-tetradecenyl acetate (Z11-14:Ac) (Meijer et al, 1972; Minks and Voerman, 1973). Figure 2.2 shows the difference between the contents of the female pheromone gland and the abdomen of a male *A. orana*. The female pheromone gland contained primarily these two compounds. The male abdomen was almost "empty".

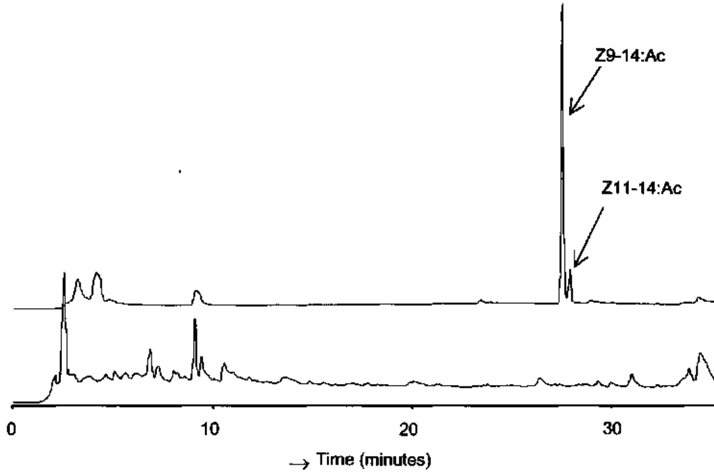

Figure 2.2 GC-profile of 2 female sex glands (upper trace) and a male abdomen (lower trace) of *Adoxophyes orana* after thermal desorption at 100°C. Column: DB1. (*Z*)-9-tetradecenyl acetate (*Z*9-14:Ac) and (*Z*)-11-tetradecenyl acetate (*Z*11-14:Ac) only present in the female.

This difference between males and females was also observed in the mullein bug, *Campylomma verbasci.* Figures 2.3 and 2.4 show similar chromatograms from a TDS-analysis (collection of the volatiles at 250°C and 25°C, respectively) of *C. verbasci* males and females. Two compounds were only present in females, and these were identified as butyl butyrate and (*E*)-2-butenyl butyrate [the sex pheromone components of *C. verbasci*, according to Smith et al. (1991)]. The 19:1 ratio, in which these two compounds were present according to the TDS analysis, is similar to that (16:1) found by Smith et al. (1991). They additionally reported the presence of hexyl butyrate in both sexes. This inactive compound was also observed in both TDS chromatograms.

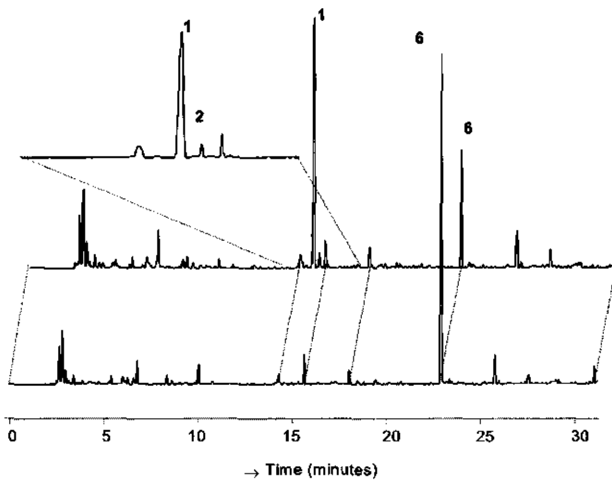

Figure 2.3 **Chromatograms of one male (lower trace) and one female (upper trace) *Campylomma verbasci* analysed with the TDS (5 min at 250°C). Column: DB1. 1: butyl butyrate, 2: (*E*)-2-butenyl butyrate, 6: hexyl butyrate.**

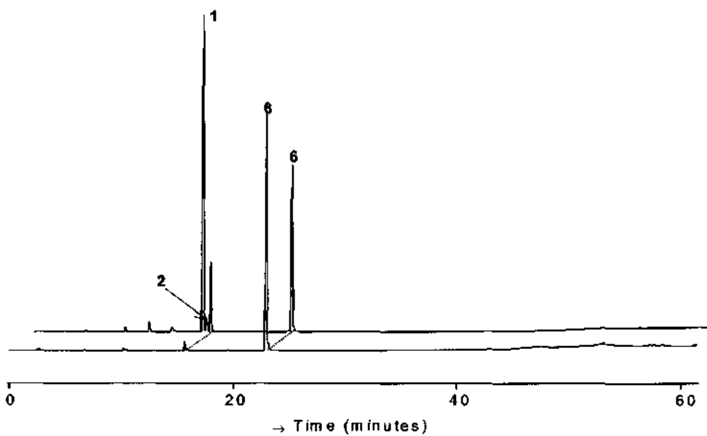

Figure 2.4 **Chromatograms of two male (lower trace) and one female (upper trace) *Campylomma verbasci* analysed with the TDS (15 min at 25°C). Column: DB1. 1: butyl butyrate, 2: (*E*)-2-butenyl butyrate, 6: hexyl butyrate.**

Figure 2.5 shows the dynamic headspace analysis of 8 female mullein bugs sampled for 8 hours. Among many other compounds, butyl butyrate and (*E*)-2-butenyl butyrate were present in the headspace extract.

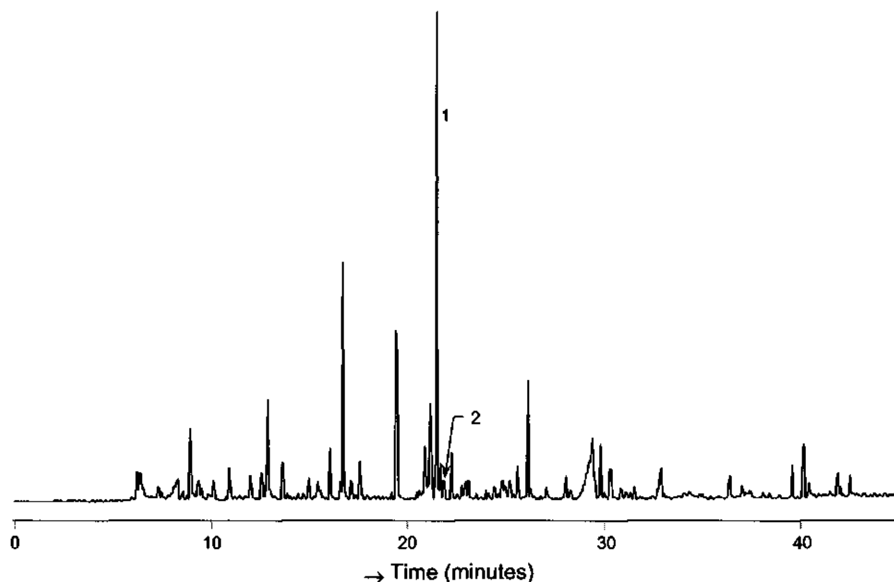

Figure 2.5 Chromatogram of dynamic headspace collection of 8 female *Campylomma verbasci*. Column:DB1. Carrier gas: helium, 1: butyl butyrate, 2: (*E*)-2-butenyl butyrate.

From the TDS-analysis of the green capsid bug, *L. pabulinus,* it is not clear which compounds may play a role in the sexual communication between females and males, because there was no distinct difference between males and females. Two chromatograms of the TDS-analysis of respectively a male and female green capsid bug are shown (Figure 2.6). Again, hexyl butyrate was present in both males and females and amounted to almost 90% of the total present. Two other compounds, (*E*)-2-hexenyl butyrate and *(E)*-4-oxo-2-hexenal, were also found in both males and females. When legs of males and females were analysed there was a distinct difference in the hydrocarbon profile (Figure 2.7). In Chapter 5 this difference will be discussed in greater detail.

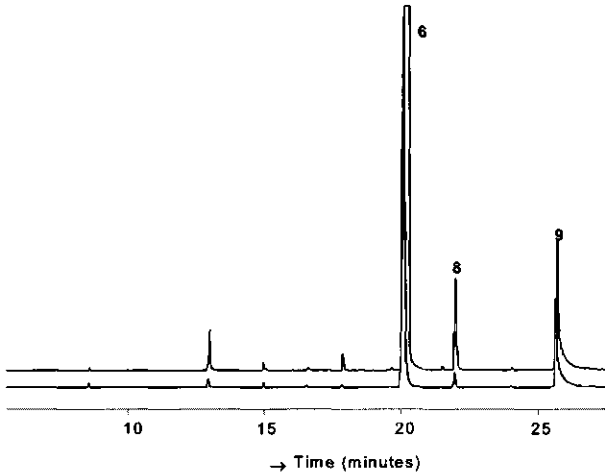

Figure 2.6 Chromatograms of a male (lower trace) and a female (upper trace) *Lygocoris pabulinus* analysed with the TDS (5 min at 250°C). Column: DB-WAX. 6: hexyl butyrate, 8: (*E*)-2-hexenyl butyrate, 9: (*E*)-4-oxo-2-hexenal.

2.4 Discussion

In the last two decades, many research groups have worked on the identification of sex pheromones in the Miridae family. However, only a few have succeeded in actual identifications (Smith et al., 1991; Millar et al., 1997; Millar and Rice, 1998). With the current method, it was possible to identify two compounds that were present only in females of *C. verbasci* using just one or two insects. These compounds were reported previously as being the sex pheromone of the mullein bug (Smith et al., 1991).

When the chromatograms are studied in detail, a clean baseline with only a few peaks is observed. The main compounds are hexyl butyrate and butyl butyrate; the latter is the main component of the sex pheromone. The minor component, (*E*)-2-butenyl butyrate, is also clearly visible. Because of the high volatility of the pheromone compounds of these mirids, sampling at room temperature is sufficient to collect material for identification. Sampling at higher temperatures, such as 250°C (Figure 2.3), resulted in the elution of many small additional peaks. However, at these high temperatures, the peaks are still sharp, and it is possible to identify individual

compounds. For the identification of cuticular hydrocarbons present in insects, sampling at these high temperatures is necessary and possible as shown in Figure 2.7.

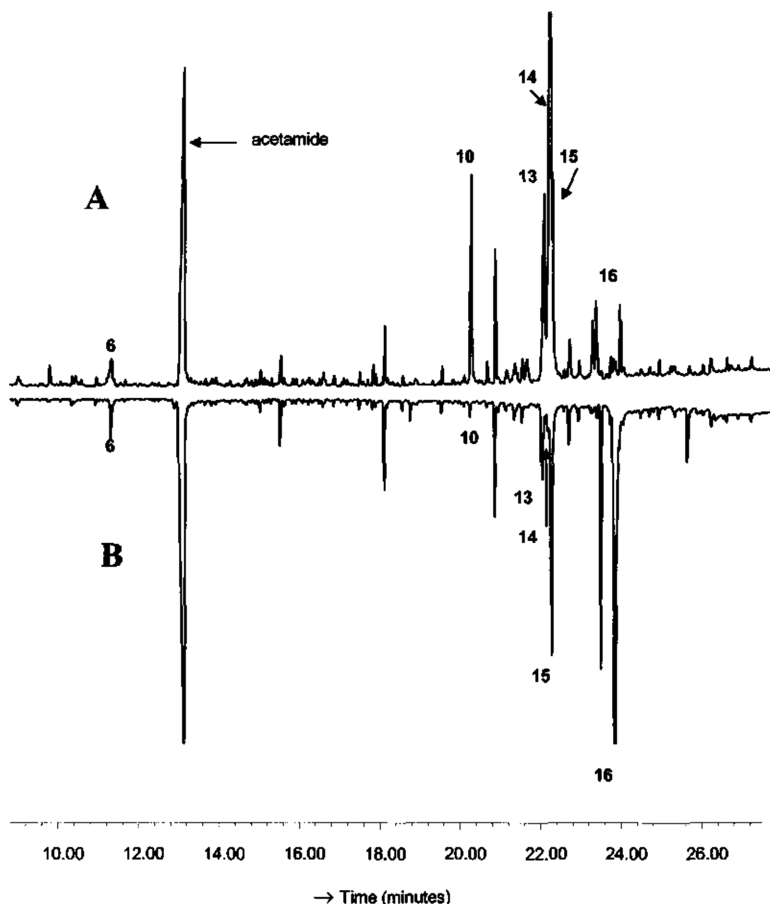

Figure 2.7 **Chromatogram of the TDS-analysis of dissected legs from a female (A) and male (B) *L. pabulinus*. Separation carried out on a DB23 column. 6: hexyl butyrate; 10: tricosane; 13: pentacosane; 14: (*Z*)-9-pentacosene; 15: (*Z*)-7-pentacosene; 16: (*Z*)-9-heptacosene. Details on the identification of the numbered compounds are given in Chapter 5.**

Another important observation is the close resemblance of the chromatograms from males and females (Figure 2.3). In fact they are virtually identical, except for the

sex pheromone components in female *C. verbasci*. In practically all TDS analyses of male and female insects, this close resemblance is apparent.

By comparing the chromatogram of the TDS analysis of only one mullein bug analysed at 250°C with a dynamic headspace analysis (Figure 2.5) one sees that there is a significant difference. Butyl butyrate and hexyl butyrate are no longer almost the only compounds present, as in the TDS-analysis of *C. verbasci*. Although butyl butyrate is still the main compound, identification of the second pheromone component, (*E*)-2-butenyl butyrate, will be more difficult. Collecting volatiles during dynamic headspace requires the use of leaves as food for the mirids to prevent desiccation. As leaves produce volatiles, even more when insects feed on them (Bolter et al., 1997), these compounds are also trapped on the Tenax. When the sex pheromone is produced in small amounts and only at certain times during the day and/or night, these plant volatiles may conceal the sex pheromone components. Another explanation for more compounds being present in the headspace could be the production of defensive compounds, emitted by the bugs themselves when they are in a stressful situation. Collecting headspace from bugs in glass cages may cause this stress. By using the TDS-method, only volatile compounds present in the insect at that particular moment are collected and injected onto the column. The bugs are kept in their rearing cages until just before analysis and the insects are, most likely, stripped of their volatiles before they can produce *de novo* stress compounds. The average time between leaving the rearing cages and death of the insect was less than 3 minutes.

In the case of the green capsid bug, it is still not clear which compounds are part of the sex pheromone. It is possible that one or more compounds from the sex pheromone are present in both sexes. This was, for instance, the case with the pheromone of two mirid bug species (*Phytocoris*) (Millar et al., 1997; Millar and Rice, 1998). On-going experiments with EAG (Chapter 4 and 6) and bio-assays (Groot et al.,

1998) should give more information about other compounds that are present in females as possible sex pheromone compounds.

With this rapid method, it is easy to determine the pheromone content from a single insect as a function of time. Normally to determine the pheromone content of insects or pheromone glands, extracts from whole insects or glands have to be made. In certain cases, many insects may be necessary. Because in the TDS-method all volatiles are directly injected onto the column, the pheromone content from a single insect can be determined. Furthermore, this technique is fast, because there is no work-up of any extracts or Tenax tubes as used during headspace analysis.

The use of a thermal desorption injection technique to determine the pheromone content of pheromone glands was demonstrated previously with the potato tuber moth, *Symmetrischema tangolias* (Griepink et al., 2000). In this case, the pheromone gland was inserted into the liner (1.4 mm i.d.) of the PTV-injector. By heating the injector to 350°C, the compounds were released and trapped on the GC-column (at 30°C). However, this approach Griepink et al., 2000) can be used only when the gland in which the sex pheromone is produced is known. In mirids no pheromonal gland has yet been found. The method described here can therefore be used to investigate the pheromonal content of these small insects, because whole insects can be used.

Coupling of this system to an EAG recording system will provide a valuable tool in the elucidation of sex pheromones. In a single GC-analysis, compounds can be "extracted" from single insects, injected into the GC, separated, and provide EAG recordings. These EAG-active compounds can then be tested in a bioassay. Replacing the FID with an MSD, creating a TDS-GC-MS-EAD set-up, will form the ultimate system. In this way compounds are not only being identified as EAD active, but structural information can be obtained as well.

This technique is, however, not suitable for measuring the emission of sex pheromones as a function of the day/night cycle. The insect dies during the collection

step. With the thermal desorption system, only the actual content of volatiles in the insect can be measured. When an insect stores its pheromone as a precursor, it will not be possible to detect the true pheromone compounds. To measure the active emission of pheromones on-line, sampling should be performed by using a different and bigger glass tube and more importantly, air has to be used. In Table 2.1, the advantages and disadvantages of the TDS are listed.

TABLE 2.1 Advantages and disadvantages of the thermal desorption system

Advantages	Disadvantages
Rapid	Only suitable for small insects (< 4 mm diameter)
Sensitive, only one insect per analysis necessary	No determination of active pheromone emission
Not only suitable for the identification of sex pheromones, but also for cuticular hydrocarbons	Not applicable for insects in which the pheromone is stored as precursors
No chemical degradation, no interference from non-volatiles, introduction of impurities is avoided	

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Solid Phase Micro Extraction versus Stir Bar Sorptive Extraction

Abstract - Two methods were used to extract compounds emitted by female *L. pabulinus*. These methods, Solid Phase MicroExtraction (SPME) and Stir Bar Sorptive Extraction (SBSE), are both based on the partition (sorption) of analytes between the gas phase (headspace) and the stationary phase. SPME has already been used in pheromone research. SBSE is a relative new technique, which has not been applied in pheromone research before. Both methods are compared with regard to their usefulness in trapping volatiles emitted by insects. SBSE was found to be much more sensitive than SPME, although SPME was easier to operate. The advantages and disadvantages of these methods are discussed.

3.1 Introduction

Solid Phase Micro Extraction (SPME) was recently developed for the extraction of organic compounds from aqueous solutions or from a gas (headspace) phase (Arthur and Pawliszyn, 1990). Since then, a number of applications have been published dealing with environmental research, volatiles in food and beverages, and water contaminants (e.g. Yang and Peppard, 1994 and references therein; Pelusio et al., 1995). SPME is by nature an equilibrium technique (Louch et al., 1992; Pwaliszyn, 1997), based on the partitioning of analytes between a stationary phase and the matrix (solvent or headspace) from which the compounds are extracted. An SPME-unit consists of a fused silica fibre coated with e.g. a polydimethylsiloxane (PDMS) phase (7-100µm) which can be drawn into a needle. Fibres can be coated with different stationary phases to enhance the sensitivity for certain compounds. SPME requires no solvent or complicated apparatus and can be used to concentrate volatile and non-volatile compounds in both liquid and gaseous phases. After the extraction of the analytes (according to their affinity to the stationary phase), they are thermally desorbed directly in the GC injector. Desorption in the GC-injector takes place by injection of the needle in the injector and pushing out the fibre in the heated zone. Because of its sensitivity, absence of solvents (that could mask compounds or introduce artefacts in GC runs) and simplicity of this method it has also been used in recent years in the identification of volatiles emitted by living plants (Vercammen et al., 2000) or in the identification of sex pheromones (Malosse, et al., 1995; Borg-Karlson and Mozuraitis, 1996; Frérot et al., 1997; Rochat et al., 2000). Using this technique, the sex pheromones of *Phyllonorcyter acerifoliella* (Lepidoptera: Gracillariidae) and *Ph. heegeralla* were identified (Mozuraitis et al., 2000). Besides its usefulness in identification of sex pheromones from insects, SPME was also found to be useful in pheromone research of animals, such as elephants (Rasmussen et al., 1997).

A comparable approach to extract compounds from liquid matrices has recently been introduced: stir bars coated with PDMS (Baltussen et al., 1999a). This technique is called Stir Bar Sorptive Extraction (SBSE). PDMS coated stir bars are now commercially available in a device called Twister™ (Gerstel, Germany). The Twister consists of a magnetic stir bar (approx. 1 cm length) enclosed in glass, coated with a polydimethylsiloxane phase (0.2 –1.5 mm thick, 10 – 350 mg PDMS). A schematic drawing of the Twister is given in Figure 3.1. The advantage of this method above SPME is that the amount of absorption material (PDMS) on the Twister is roughly 100 times more. Therefore a larger amount of material can be trapped as the sensitivity of all sorptive techniques is primarily determined by the amount of sorbent employed (Arthur and Pawliszyn, 1990; Dugay et al., 1998).

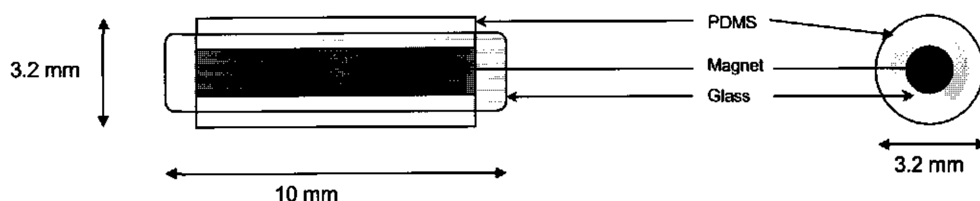

Figure 3.1 Schematic drawing of the Twister™ seen from two different sides.

As for SPME, compounds sorbed on the Twister can be thermally desorbed. After extraction of the analytes, the stir bars (Twister) can be introduced in the glass tube from the Thermal Desorption System (TDS) as described in Chapter 2 and thermally desorbed.

The SBSE-method was introduced as an extension of SPME (Baltussen et al., 1999a). In this research these two similar extraction techniques are compared in their usefulness of trapping airborne volatiles produced by female *L. pabulinus* in order to establish the pros and cons of SBSE in the identification of semiochemicals of insects relative to SPME.

3.2 Material and Methods

3.2.1 Insects *L. pabulinus* bugs were reared as described in Chapter 2.

3.2.2 Collection of emitted compounds

Twister Headspace from female *L. pabulinus* was collected on the Twister in two ways; by means of dynamic and static headspace. To extract volatiles by dynamic headspace 3-4 female *L. pabulinus* together with a potato sprout and pollen were introduced in a 250 ml flask. A Twister was placed downstream of the filtered (humidified) air that passed through this flask at 50 ml/min. The sampling was conducted in a greenhouse during 5-10 hours. In the second way (static headspace) the Twister was introduced inside a 20 ml vial with 1-3 females and pollen during 2-3 hours. In this last experiment the females could walk on the Twister.

To compare the static headspace extraction with dynamic headspace extraction the sampling time of the static headspace was prolonged to 10 hours.

SPME Before the collecting periods, the routine conditioning of the SPME fibre (100 μm PDMS phase, Supelco, Bellefonte, PA, USA) was done at 250°C for 10 min in a GC injector. Then the fibre was introduced downstream into the filtered (humidified) air that had passed through a 250 ml cage with 3-4 female *L. pabulinus* together with a potato sprout and pollen (dynamic headspace). The sampling was conducted in a greenhouse during 5-10 hours. In the static headspace situation 1-3 female *L. pabulinus* were kept in 20 ml vials with only pollen. The fibre was now inserted through a septum and sorption took place for 2-3 hours.

3.2.3 Chemical analysis

Twister Desorption of the compounds from the Twister was done by using the thermal desorption system (TDS) (Gerstel, Mülheim am Ruhr, Germany), described in the first section, mounted on a HP 6890 GC with a split/splitless PTV injector (CIS 4, Gerstel, Germany). The desorbed compounds were trapped in the PTV inlet at –100°C using liquid nitrogen. After desorption, the PTV was heated to 250°C to inject the

compounds and analyse them on a capillary column. A DB-23 column (J&W Scientific (Folsom, California), 30 m × 0.25 mm; film thickness: 0.25 μm) was used with a temperature programme from 40°C (2 min hold) to 238°C (25 min hold) at 8°C/min. At the end of the column the flow was split using an effluent splitter (Gerstel™ Graphpack-3D/2) in a 1:1 ratio to a Mass Selective Detector (HP 5973) and a Flame Ionisation Detector. The MSD system was used in full scan mode scanning from 30-500 amu. Helium was used as carrier gas (inlet pressure 20.5 psi).

SPME The compounds absorbed on the fibre were identified with GC-MS analyses using the GC-MS system as described for the analysis of the Twisters, but without the TDS. The split/splitless PTV-injector temperature was set at 250°C and the splitless period was 1 min. SPME fibres were desorbed during 1 min.

Identification of the compounds in the gas chromatograms was based on comparison of mass spectra with those present in the NIST/EPA/NIH Mass Spectral library (Version 1.6) and by comparison of the Retention Indices with those of reference compounds.

3.3 Results and Discussion

Volatiles produced by female *L. pabulinus* could be trapped on an SPME fibre as well as on a Twister. Placing more than one Twister in the glass bottles with females during the same sampling time produced an identical chromatogram for each Twister. In this study the sampling time was not varied, but similar sampling times were used for SPME and SBSE. Recently many studies have been done on the theoretical background of sorption on PDMS phases (Louch et al., 1992; Pwaliszyn, 1997; Baltussen et al., 1998, 1999b, 1999c; Dugay et al., 1998). From these studies it is evident that the number of moles of analyte absorbed by the stationary phase (n_s) depends on (1) the volume of the PDMS phase (V_s), (2) partition coefficient of the analyte (K) and (3) the sampling time (before equilibrium). The theoretical background is illustrated by equations 1 and 2 (Pawliszyn, 1997; Dugay et al., 1998):

$$n_s = \frac{KV_sV_{aq}C_{aq}}{KV_s + V_{aq}} \qquad (1)$$

$$K = \frac{n_s V_{aq}}{V_s(V_{aq}C_{aq} - n_s)} \qquad (2)$$

where C_{aq} is the initial concentration of the analyte solution (in water or air) and V_{aq} the volume of the sample. In most experiments the volume of the sample (V_{aq}) is many times more than the value of KV_s, which means that Eq. 1 can be simplified to Eq. 3.

$$n_s = KV_sC_{aq} \qquad (3)$$

From this equation it is clear that there is a linear relationship between the number of moles absorbed by the stationary phase, the concentration of the analyte and the volume of the stationary phase. When dealing with headspace analysis the volume of the sample does not consist of water but of water. Therefore the volume of the sample can better be given as V_{air} instead of V_{aq} and the initial concentration of the analyte as C_{air} instead of C_{aq}.

In this study the usefulness of Twisters in pheromone research was compared with that of SPME, and therefore the same conditions were used. To study the efficiency of SPME and the Twister it was not necessary to test different sorption times. To determine the optimal time for extracting volatiles from dynamic headspace for SBSE, further experiments are needed, but this was beyond the scope of this research.

Another remark should be that the relative concentration of compounds in the stationary phase might not correspond with the relative concentration in the headspace, as the partition coefficient (*K*-value) will not be the same for all compounds; see Eq. 1. Compounds with a higher affinity for the PDMS stationary phase will be more absorbed relative to those with a low affinity. Experiments to determine the partition coefficients for individual compounds (using Eq. 2) are therefore of much interest (Bartelt, 1997). One of the advantages of PDMS sorption (SPME and Twister) is that the stationary phase has been extensively studied in the past, as PDMS is the most common GC stationary phase. Literature data on retention of many compounds are available as Kovats' Retention Indices. Baltussen et al. (1997) presented a simple approach to calculate the partition coefficient (*K*-value) from these Retention Indices

(RI). One can now use PDMS sorption in quantitative analyses without the necessity to measure the *K*-value for each individual compound.

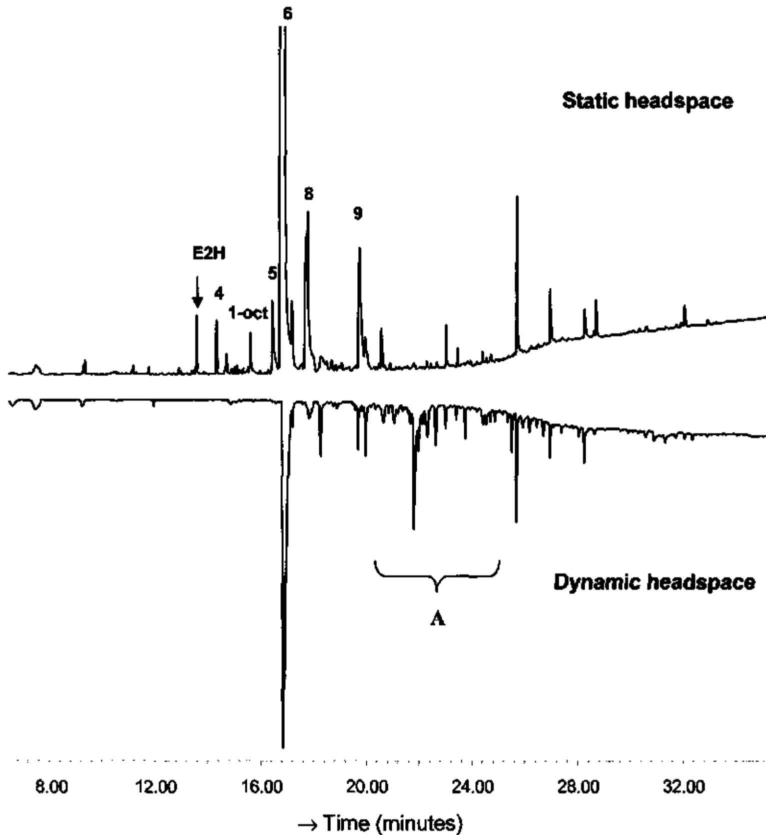

Figure 3.2 **GC-MS analysis of a Twister placed by 5 females *L. pabulinus* in resp. a static and dynamic headspace situation during 10 hours. Separation carried out on a DB23 column. A: sequiterpenoids, E2H: (*E*)-2-hexenal, 4: hexyl acetate, 1-oct: 1-octanol, 5: nonanal, 6: hexyl butyrate, 8: (*E*)-2-hexenyl butyrate, 9: (*E*)-4-oxo-2-hexenal.**

In Figure 3.2 two TDS-analyses are shown; respectively extraction of volatiles in a dynamic and a static headspace situation both with the Twister. The concentration of compounds absorbed on the Twister in the static headspace was higher, compared to the dynamic headspace situation. Due to the fact that in the static headspace situation there is more time to reach the equilibrium (compounds are not being "blown" away),

a higher concentration of compounds can probably be achieved. It might be possible that the flow in the dynamic headspace situation was too high for the highly volatile compounds to absorb on the PDMS-phase. The explanations given above are, however, very speculative. More experiments are essential to explain the differences between static and dynamic headspace analyses. Besides this quantitative difference, also some qualitative differences can be seen. Several compounds (between retention times 20 and 25 minutes in Figure 3.2) were present in the dynamic headspace analysis, but absent in the static analysis. These compounds were all sesquiterpenoids, which are typically plant-produced compounds. During the dynamic headspace analysis, a potato sprout was added to the flask. Bugs feeding on these sprouts induce the release of plant volatiles, which is observed in the dynamic headspace analysis. Therefore these compounds were absent in the static headspace analysis.

There was no consistent qualitative difference between SPME and SBSE (Figure 3.3). This was expected, as the same stationary phase (PDMS) was used in both extraction methods. In some cases, a similar feature as in Figure 3.2 was observed, where some compounds are only present in the SPME analysis, but not in the chromatogram of the Twister analysis. A straightforward explanation for this is not at hand, but one should be aware that insects do not always produce the same compounds. Due to their (social) environment, in certain cases, different compounds are emitted. Nevertheless, the amount trapped on the Twister was much higher than that on the SPME needle (Figure 3.3). Because the K-value is identical for both SPME and SBSE, almost only the volume of the stationary phase is of importance (Eq. 1), which is much higher in case of the Twister. Dugay et al. (1998) already showed that the mass of compounds extracted depends on the partition coefficient and the volume of the stationary phase. Therefore it is now possible to reduce the duration of sorption using a Twister or alternatively with the same sampling time a much higher sensitivity can be achieved.

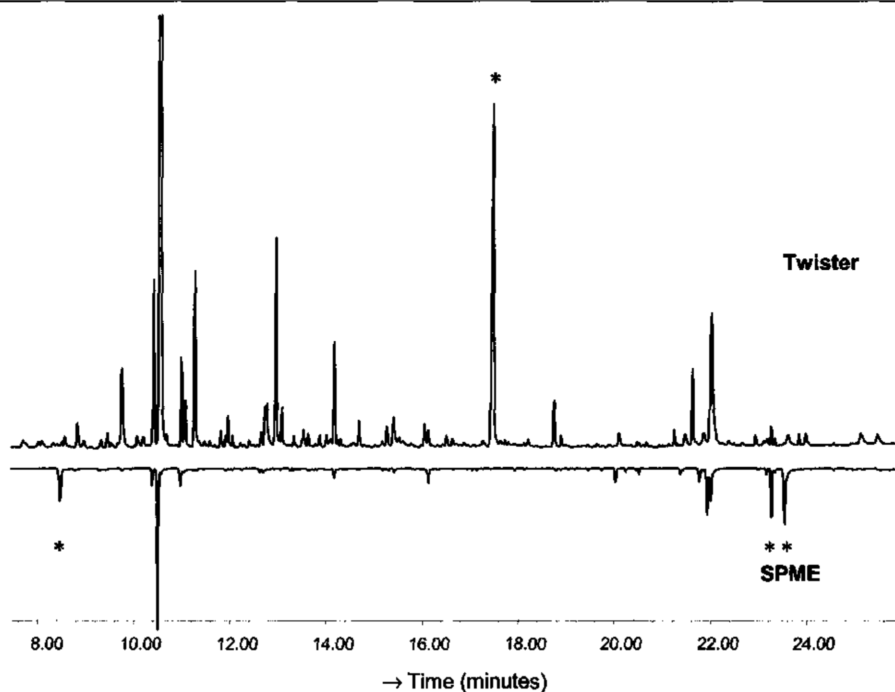

Figure 3.3 Comparison of extraction efficiency of Twister and SPME. Extraction carried out by static headspace with 3 female *L. pabulinus* in 20 ml vial with pollen. Separation was carried out on a DB23 column. *: Impurities from the column, Twister or SPME-needle.

Besides the higher sensitivity of the Twister, it has several other advantages over SPME. SPME experiments are easy to perform when insects are calling and thus emitting sex pheromone (Mozuraitis et al., 2000). The needle can then be held a few millimetres away from the tip of the abdomen or the sex pheromone producing organ, and kept there for a short time, which is enough to extract the compounds emitted by the calling insect. With insects that do not have a specific calling behaviour it is much more difficult to perform SPME, as it is not known when to place the fibre nearby the calling female. Moreover (large) living insects can easily damage the fibre. In contrast, it is easy to place a fresh Twister every 2 or 3 hours nearby a female and collect all emitted compounds, because the Twister is much more robust than the SPME-fibre. In this study, experiments were done where the SPME-fibre was placed in close vicinity of female *L. pabulinus*. This is not always successful as the insects can easily damage the fibre as they come in contact with the fibre during their flight. On the other hand,

nice results were obtained when female *L. pabulinus* were on or close by the SPME-fibre as can be seen in Figure 3.4.

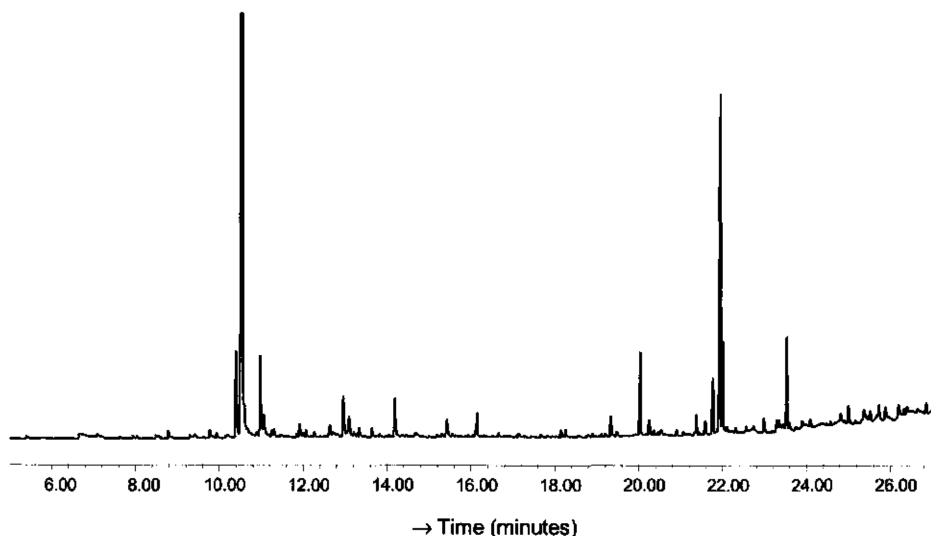

Figure 3.4 **GC-MS analysis of an SPME needle placed in a 20 ml vial with 2 female *L. pabulinus* and some pollen. Separation was carried out on a DB23 column. C_{23}-C_{27} hydrocarbons are present between 20 en 24 minutes.**

In this SPME experiment also marginally volatile compounds were absorbed on the fibre. These compounds are important in the courtship behaviour of males (Chapter 5 and 6). Because Twisters cannot be damaged, it is much easier to put one or two Twisters with female *L. pabulinus* and after 2-3 hours these can be analysed with the TDS. With the help of a magnet Twisters can be placed at almost every desirable place.

Secondly, Twisters used for the sorption of compounds can be stored in clean vials (1.8 ml). Thus there is no need for direct analysis as is necessary for SPME. This means that collection of volatiles on a Twister can be done in a different lab than the actual analysis of the Twister, since transport of Twisters from one lab to another is no problem. Although this has not been investigated, storing of the Twisters in clean vials means it might be unnecessary to clean them just before each experiment, which is

advisable for the SPME-fibre. Once they are thoroughly cleaned, they can be stored until the next sampling.

In conclusion, working with a Twister is as simple as SPME, but is much more sensitive and can be used to collect material from insects where SPME cannot be applied. SPME on the other hand can be used with any GC, while for analysing Twisters, an expensive thermal desorption unit is required.

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Coupled gas chromatographic - electroantennographic responses of *Lygocoris pabulinus* to female and male produced volatiles

Abstract – GC profiles of airborne volatiles produced by adult female and male green capsid bugs, *Lygocoris pabulinus*, showed almost no differences, although females produced a small amount of mono-alkenes. Coupled gas chromatography/electroantennogram detector (GC-EAD) recordings revealed that three compounds were consistently EAD-active. These were hexyl butyrate, (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal. Male antennae reacted strongly to these three compounds whereas female antennae gave no or little response. These compounds may be important chemical signals for male *L. pabulinus* in their long-range communication with conspecifics. This is the first report with data on successful GC-EAD recordings in Miridae.

4.1 Introduction

Since the application of the electroantennogram technique (Schneider, 1957) in coupled electroantennography-gas chromatography (GC-EAD) (Arn et al. (1975), this technique has been widely used as screening technique in Lepidoptera (Roelofs, 1984). Recently, GC-EAD has also been applied to non-lepidopteran species, such as Hymenoptera (e.g. Baehrecke et al., 1989; Thiery et al., 1990), Diptera (e.g. Guerin and Städler, 1982; Tømmerås et al., 1993), Coleoptera (e.g. White et al., 1988; Leal et al., 1992), Orthoptera (Torto et al., 1994), Trichoptera (Löfstedt et al., 1994), Thysanoptera (Pow et al., 1999), Homoptera (e.g. Dunkelblum et al., 1995) and Heteroptera (see further below).

In contrast with the knowledge about olfaction in Lepidoptera (Cardé and Minks, 1997), in heteropteran species, to which *L. pabulinus* belongs, the neural basis of reception of plant odours and pheromone compounds is poorly known (Chinta et al., 1994). Olfactory responsiveness of a single neurone of *Triatoma infestans* to human breath was the first report on bug olfaction (Mayer, 1968). Later on, electrophysiological studies on *Oncopeltus fasciatus* revealed that adult milkweed bugs have olfactory receptors on their antennae that respond to host plant odours (Pantle and Feir, 1976). Electrophysiological studies have also been performed on the pentatomid *Nezara viridula* in its relation to their olfactory perception of *cis* and *trans* isomers of bisabolene epoxides (Brézot et al., 1994). In mirids, olfactory perception of plant and insect odours has been studied in detail in *Lygus lineolaris* (Chinta et al., 1994) and *Lygocoris pabulinus* (Groot et al., 1999). However, these electroantennogram studies have been unable to shed further light on the sex pheromones in these mirids. It has been found difficult to perform GC-EAD-recordings on heteropteran species (Bjostad, 1998). EAG responses are low and together with unstable baselines result in low signal to noise ratios compared to Lepidopteran species (Brézot et al., 1994). The first report on successful GC-EAG recordings on heteropteran species was with an Alydidae spp. (Leal et al., 1995). Recently, other GC-EAG recordings were published on seed bugs (Heteroptera:

Lygidae) (Aldrich et al., 1997), and stink bugs (Heteroptera: Pentatomidae) (Leal et al., 1998; Dickens, 1999; Weissbecker et al., 2000). However, successful application of GC-EAG in mirids is scarce. Innocenzi et al. (1998) reported on GC-EAD recordings with *Lygus rugulipennis*, although no data were shown. Despite abundant evidence that males are attracted to females (see Chapter 1), the sex pheromones of many mirids remain unknown (McBrien and Millar, 1999). Application of GC-EAD in Miridae species, might enhance the change of success in the identification of their sex pheromones.

In this study coupled electroantennography-gas chromatography was successfully performed with both male and female antennae on headspace extracts from male and female *L. pabulinus*.

4.2 Materials and Methods

4.2.1 Insects *Lygocoris pabulinus* was reared as described in Chapter 2.

4.2.2 Sample collection Virgin males or females (6-9 days old) were placed in two clean 250 ml glass bottles (h. 20 cm, diam. 5 cm) (5-7 bugs per bottle) together with a potato sprout and pollen. Purified air (50 ml/min) was led through a bottle filled with water and hereafter through the two bottles. Compounds were trapped on glass tubes filled with ca. 200 mg of Tenax TA (200-900 μm, Chrompack, The Netherlands). Every 2-3 days the potato sprouts and pollen were replaced with fresh potato sprouts and pollen and every 7 days the bugs were replaced by new virgin males or females (6-9 days old). After 8-10 days the compounds on the Tenax tubes were each eluted with 4-5 ml of pentane:ether (2:1). Hereafter the two extracts were pooled and concentrated by passive evaporation to the atmosphere at room temperature and 1 atm. to ca. 1 ml.

4.2.3 Bioassays The Y-track olfactometer bioassays were performed according to Groot et al. (2001). Male and female headspace extracts were tested against the solvent used for the headspace extracts.

4.2.4 Electrophysiology (GC-EAD) 2 µl of the headspace extract was analysed by the GC-FID-EAD system described by Weissbecker et al. (2000). The GC was equipped with either a DB5 or a DB23 column, (both 30 m × 0.25 mm ID; film thickness: 0.25 µm) (J&W Scientific, Folsom, California) and a cold on-column injector. The initial oven temperature was set at 45°C. After 1 min the oven temperature rose with 8°C/min to 238°C. Helium was used as the carrier gas. Two Y-shaped GlasSeal capillary tubing connectors (Supelco, Bellefonte, Pennsylvania) were used to mix the effluent of the column with a make-up gas (helium, 30 ml/min) and to divide the resulting gas flow over two pieces of deactivated fused silica capillary leading to the FID and the EAG interface, respectively. The length and diameter of these capillaries were selected such that the flow was split in a ratio of 2 (EAG) to 1 (FID). The outflow of the column (to the EAG interface) was mixed with humidified air (600 ml/min) and was directed over a *L. pabulinus* male or female antenna *via* a 15-cm long Teflon coated stainless steel tube (5 mm ID). The antennal preparation was as follows. The head and a small part of the prothorax were cut off, and the tip of the one antenna was then clipped off. The indifferent electrode was inserted into the excised prothorax/head. The distal end of the antenna was then placed in a saline (Weissbecker et al., 1999) filled electrode connected to an Ag/AgCl-wire and hereafter via an interface box to a signal interfacing board (IDAC; Syntech, Hilversum, The Netherlands). FID and EAG signals were monitored synchronously using software and a GC/EAD interface card from Syntech.

4.2.5 Chemical identification Extracts, used for the GC-EAG recordings, were analysed on a HP 6890 GC coupled to a HP 5973 MSD. The GC was equipped with a DB-23 column (J&W Scientific, Folsom, California; 30 m × 0.25 mm; film thickness: 0.25µm). The column was split at the end using a Gerstel™ Graphpack-3D/2 flow splitter with a split ratio of ca. 1:1. One part was led to a flame ionisation detector, while the other part was led to the MSD. The initial oven temperature was set at 40°C. After 2 min hold the oven was programmed at 4°C/min to 238°C which was held for 25 min. Helium was used as the carrier gas (constant flow of 2.4 ml/min, linear velocity: 48 cm/sec). Mass

spectra (EI, 70eV) were recorded from 30 amu. to 500 amu. Identification of compounds in extracts was carried out with GC/MS using reference spectra from the NIST/EPA/NIH Mass Spectral library (Version 1.6) and by comparison of retention times with those of reference compounds. ^{1}H-NMR (200 MHz) spectra of the synthesised compounds were recorded on a Bruker AC200 spectrometer.

4.2.6 Chemicals 1-Hexanol, 2-ethylfuran and bromine were all purchased from Acros Organics (Geel, Belgium) and hexyl butyrate from Roth (Karlsruhe, Germany). (*E*)-2-hexenyl butyrate was synthesised as described in Chapter 2. (*Z*)-9-Tricosene, (*Z*)-9-pentacosene, (*Z*)-7-pentacosene and (*Z*)-9-heptacosene were all synthesised as described in Chapter 5. Both isomers of 4-oxo-2-hexenal were synthesised as described below. All the chemicals used were > 98% pure. All solvents used were distilled twice before use.

4.2.7 Synthesis

(*E*)-4-Oxo-2-hexenal was synthesised according to Pikul et al. (1987).

(*E*)-4-Oxo-2-hexenal: NMR δ ($CDCl_3$) 1.1, t, (CH_3); 2.6, q, ($COCH_2$); 6.7 (H_A), 6.8 (H_B), 9.7 (CH_XO), ABX, J_{AB} 16Hz, J_{AX} 6Hz, J_{BX} 1Hz, CH_B=CH_A-CH_XO (*E*-isomer). For mass spectrum see Figure 4.1.

(*Z*)-4-oxo-2-hexenal was synthesised in two steps. First 2-ethyl-2,5-dimethoxy-2,5-dihydrofyran was synthesised according to Hamann and Wissner (1989). Careful acid hydrolysis of this intermediate to (*Z*)-4-oxo-2-hexenal was carried out according to MacLeod et al. (1977) with the exception that the extraction was performed with CH_2Cl_2 instead of ether.

(*Z*)-4-Oxo-2-hexenal: NMR δ ($CDCl_3$) 1.1, t, (CH_3); 2.6, q, ($COCH_2$); 6.2 q, *J* 7,12 Hz; (H_A), 6.95 d, *J* 12Hz (H_B), 10.2 *J* 7 Hz (CH_XO), CH_B=CH_A-CH_XO (*Z*-isomer). For mass spectrum see Figure 4.1

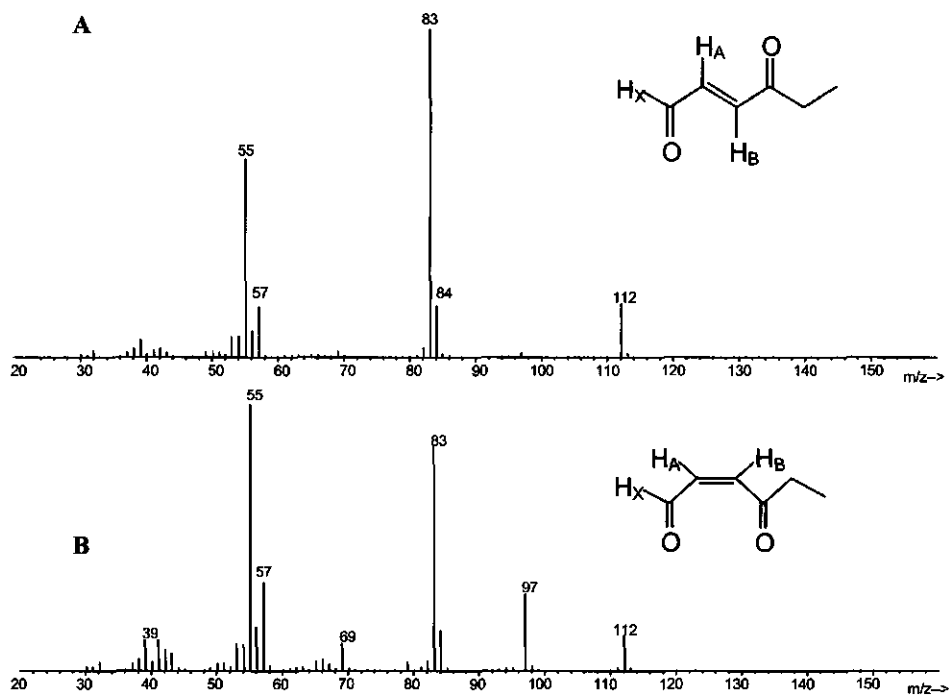

Figure 4.1 Mass spectra of (*E*)-4-oxo-2-hexenal (A) and (*Z*)-4-oxo-2-hexenal (B)

4.3 Results

Male *L. pabulinus* were only attracted to headspace extracts collected from females (Figure 4.2). When a male headspace extract was tested in the Y-track olfactometer, males did not make a choice, while when a female headspace extract was tested ca. 75% of the males walked towards the female extract.

Chemical identification of compounds present in male and female extracts revealed that, although the female extract was much more active, males and females produced a similar blend. However, only headspace from females contained a small amount of mono-alkenes. The compounds present in female extracts are listed in Table 4.1. From this Table it is clear that there was a large variation in composition of these extracts.

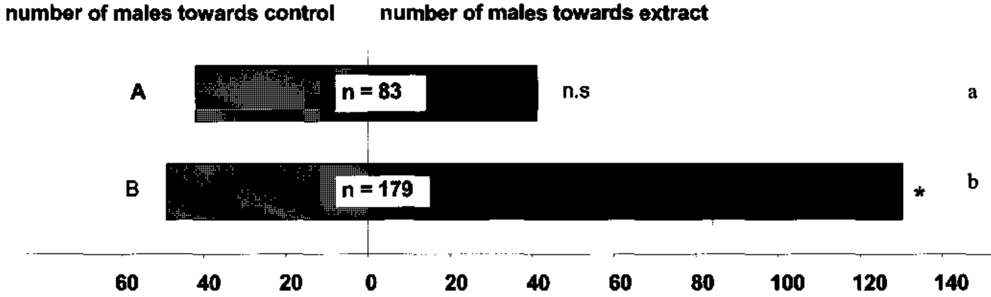

Figure 4.2 **Number of males moving towards headspace extracts from males (A) and females (B) *L. pabulinus* in the Y-track olfactometer. * P < 0.001; n.s. P > 0.3. Different letters indicate significant difference (p < 0.001).**

TABLE 4.1 ***Lygocoris pabulinus* volatile compounds identified by GC-MS in headspace extracts from females.**[a]

Compound	% ± s.e.	male EAG response[b]
butyl acetate	2.5 ± 3.9	
(*E*)-2-hexenal	0.1 ± 0.1	
1-hexanol	4.0 ± 5.0	sometimes
3-heptanone	0.6 ± 0.9	
(*E*)-4-oxo-2-hexenal	4.3 ± 3.7	strong
unknown[c]	0.5 ± 0.8	
octanal	0.1 ± 0.3	
(*Z*)-3-hexenyl acetate	0.5 ± 0.7	
hexyl acetate	1.2 ± 0.9	sometimes
limonene	0.1 ± 0.2	
pentyl butyrate	0.3 ± 0.5	
nonanal	0.9 ± 1.1	sometimes
(*Z*)-3-hexenyl butyrate	0.3 ± 0.5	sometimes
hexyl butyrate	76.0 ± 12.4	strong
(*E*)-2-hexenyl butyrate	4.5 ± 1.6	medium
decanal	0.4 ± 0.4	
α-copaene	0.3 ± 0.7	
β-caryophyllene	1.7 ± 2.6	
5,9-undecadien-2-one, 6,10-dimethyl	0.5 ± 1.0	
(*Z*)-9-pentacosene	0.5 ± 0.6	
(*Z*)-7-pentacosene	0.1 ± 0.1	

[a] The compounds are listed in order of elution on a DB5 column. The mean percentage (± s.e., n=6) of each compound is calculated according to their peak area in the gas chromatogram.

[b] EAG response from 10 GC-EAD recordings with male antennae.

[c] Mass spectrum: 55 (100), 112 (58), 83 (36), 57 (23), 97 (14)

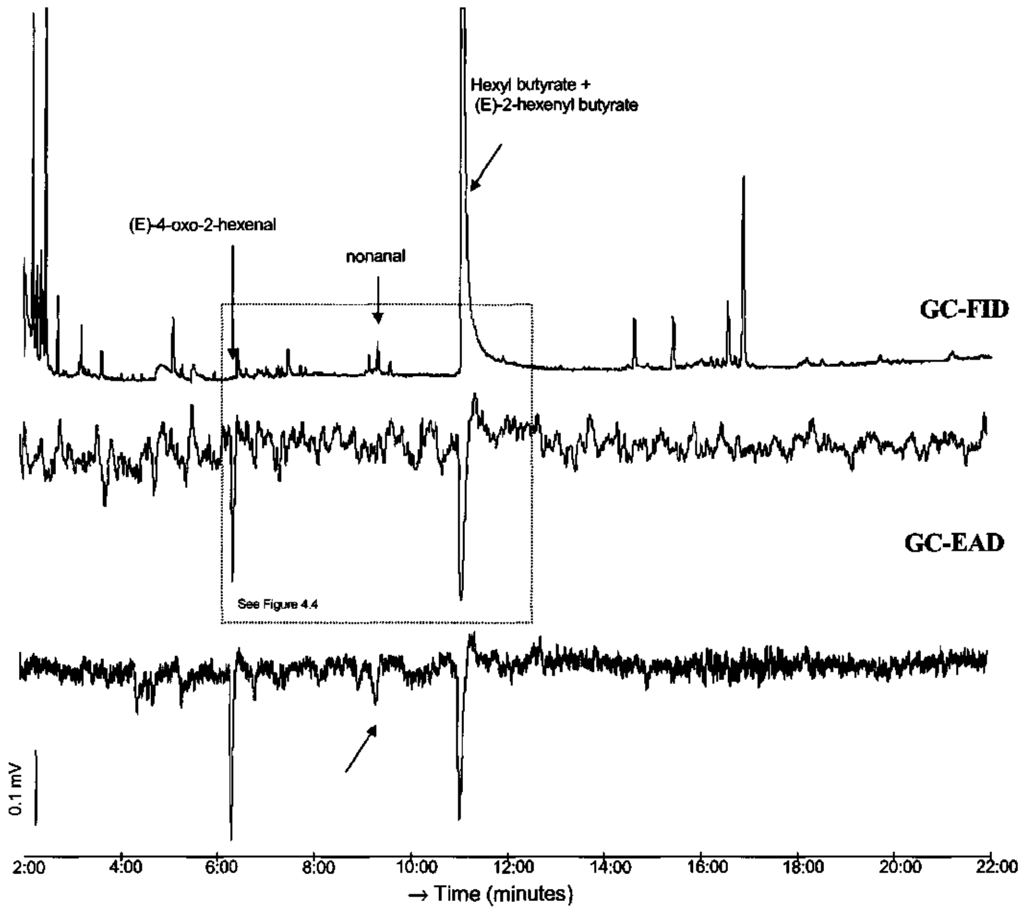

Figure 4.3 **Gas chromatogram from the separation on a DB5 column of a female headspace extract and corresponding EAG-responses from the antenna of a male *L. pabulinus*. The data represents 2 GC-injections using the same extract in the same amount.**

Coupled GC-EAG experiments with a male antenna as sensing element and separation on a DB5 column, showed two peaks that were EAG-active (Figures 4.3 and 4.4). Changing from the DB5 column to a much more polar column (DB23) showed that there were three important EAG responses (Figure 4.5).

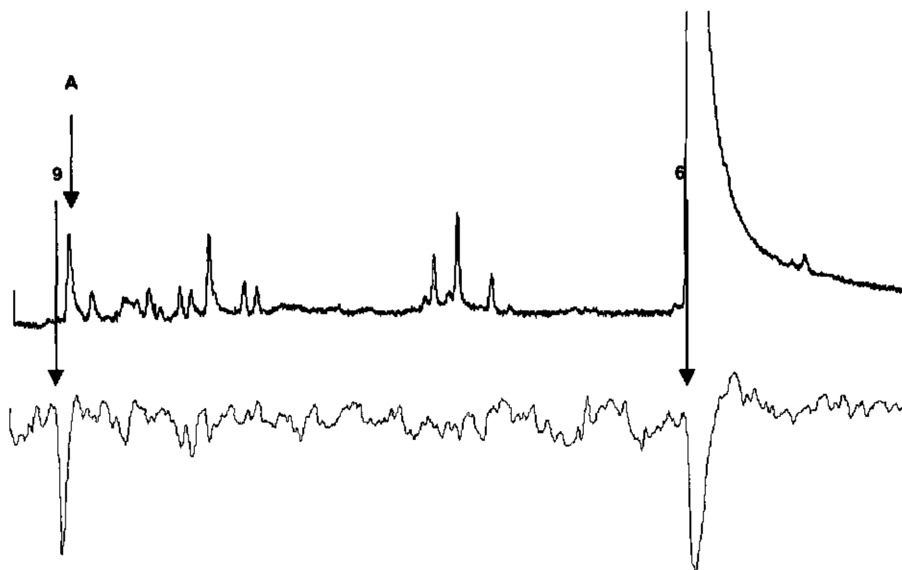

Figure 4.4 Enlargement from Figure 4.3 in which can been seen that the first EAG-response is not on peak A, but on the small peak 9 *identified as* (*E*)-4-oxo-2-hexenal.

The three compounds responsible for these responses were identified as hexyl butyrate, (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal. In a few cases nonanal, hexyl acetate, (Z)-3-hexenyl butyrate and 1-hexanol, also elicited EAG responses, as is shown in Figure 4.6. The synthesised (*Z*)-4-oxo-2-hexenal did not elicit any EAG response in both males and females.

Male and female headspace extracts showed the almost same chemical profile and also the same EAG-active compounds. Changing from an apolar to a more polar column only showed that not only hexyl butyrate, but also (*E*)-2-hexenyl butyrate was EAD-active. Female extracts contained a small amount of two alkenes, i.e. (Z)-9-pentacosene and (Z)-7-pentacosene. These alkenes were also found in the leg extracts from males and females (Chapter 5). Yet, these alkenes did not give an EAG response in the set-up used.

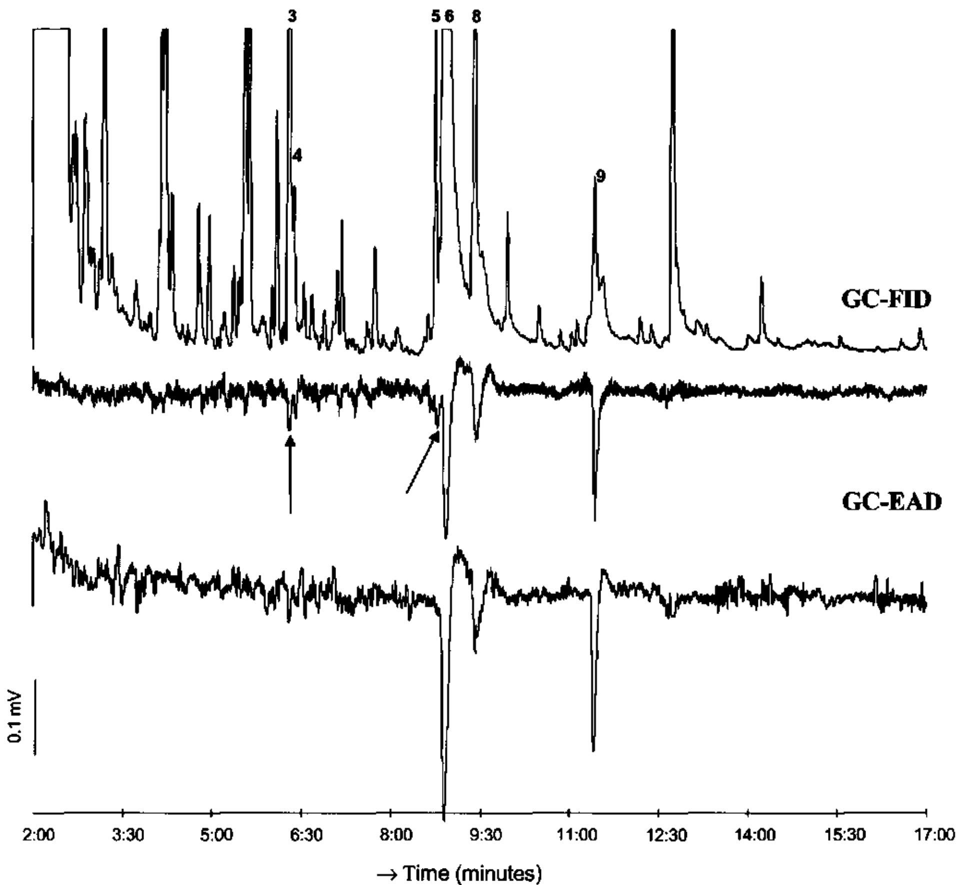

Figure 4.5 Gas chromatogram from the separation on a DB23 column of a female headspace extract and corresponding EAG-responses from the antenna of a male *L. pabulinus*. The data represents 2 GC-injections using the same extract in the same amount. 3: 1-hexanol, 4: hexyl acetate, 5: nonanal, 6: hexyl butyrate, 8: (*E*)-2-hexenyl butyrate, 9: (*E*)-4-oxo-2-hexenal. The arrows indicate that sometimes small EAG responses were obtained for 1-hexanol, hexyl acetate and nonanal.

There was a strong difference in male and female reactions to the three EAG-active compounds. From Figures 4.5 and 4.7 it is clear that the male antennae reacts much stronger to the three EAG-active compounds in the headspace extracts. (*E*)-4-oxo-2-hexenal only showed small EAG responses in a few cases with female antennae, while male antennae reacted strongly to this compound.

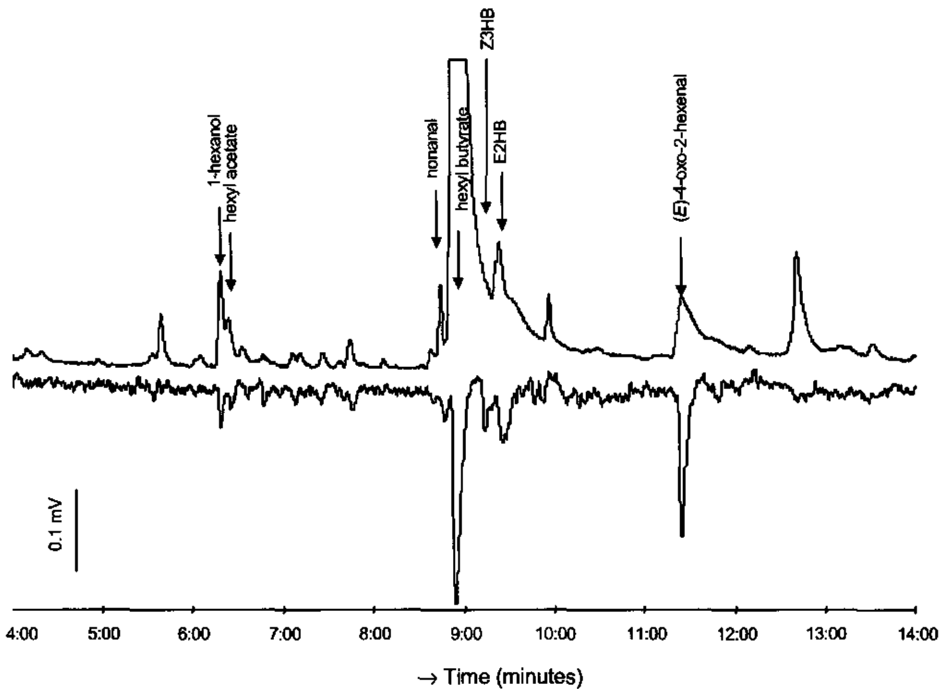

Figure 4.6 **Gas chromatogram from the separation on a DB23 column of a female headspace extract and corresponding EAG-responses from the antenna of a male L. pabulinus. Z3HB: (*Z*)-3-hexenyl butyrate, E2HB: (*E*)-2-hexenyl butyrate.**

4.4 Discussion

This study shows that it is possible to perform coupled GC-EAG recordings with the antennae of the green capsid bug, *L. pabulinus*. To my knowledge this is the first report with substantial data on GC-EAG detection in Miridae insects. It was possible to conduct GC-EAD recordings with an antennal preparation for about 30 minutes, sometimes even longer. The kind of preparation appeared to be somewhat critical as preparations with more of the thorax sometimes resulted in more unstable baselines. Preparations with the head only had somewhat lower responses and lasted shorter. However these differences were small and not consistent.

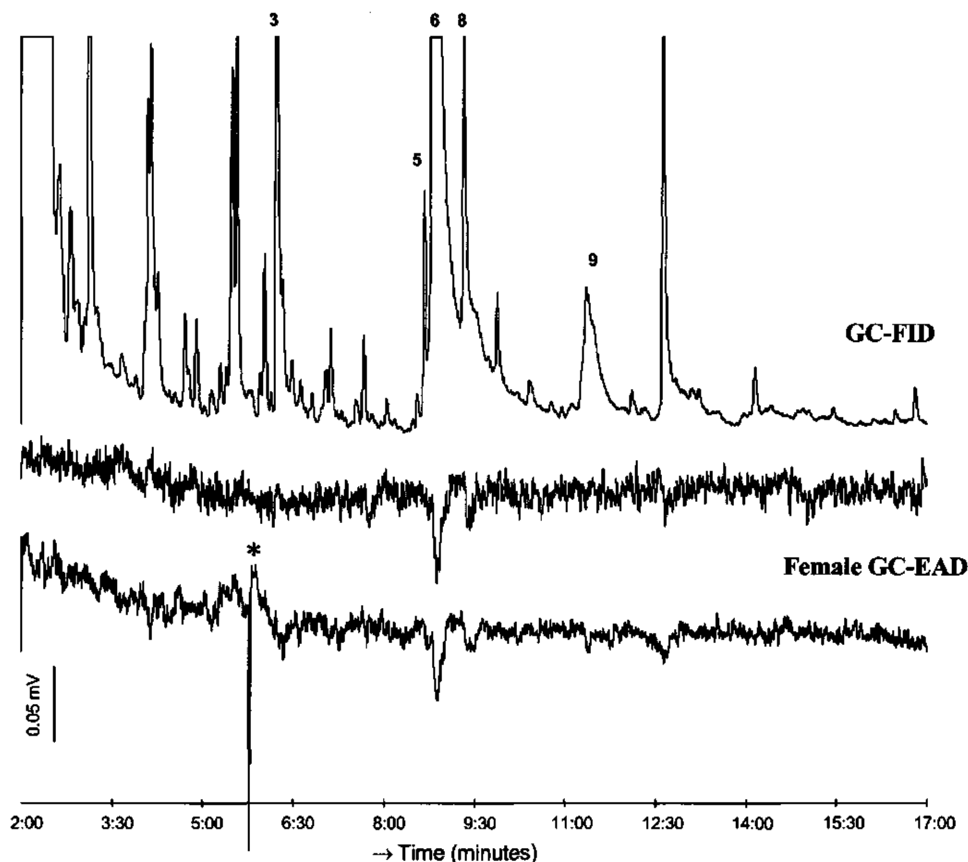

Figure 4.7 Gas chromatogram from the separation on a DB23 column of a female headspace extract and corresponding EAG-responses from the antenna of a female *L. pabulinus*. The data represents 2 GC-injections using the same extract in the same amount.
3: 1-hexanol, 5: nonanal, 6: hexyl butyrate, 8: (*E*)-2-hexenyl butyrate, 9: (*E*)-4-oxo-2-hexenal. The asterisk in the lower GC-EAD trace indicates an off-line stimulus.

Studies on male and female headspace extracts with GC showed no distinct difference between compounds being produced by the two sexes, although the headspace from females contained a small amount of alkenes (see also Chapter 6). Aldrich (1988) reported that in many *Lygus* spp. extracts do not appear to contain sex-specific compounds. However, it is still possible that sex pheromone compounds are present in very small amounts, not detectable by FID, or that they are being masked by other

compounds (McBrien and Millar, 1999). This study indicates that this is not likely in *Lygocoris pabulinus*. No EAG responses were found on "new" compounds or at times in the gas chromatogram where no or unidentified FID signals were seen. Changing from an apolar to a more polar column only showed that besides hexyl buyrate, (*E*)-2-hexenyl butyrate is also EAD-active. It was however already known that this compound is EAD-active (Groot et al., 1999) in *L. pabulinus*.

The presence (*E*)-4-oxo-2-hexenal in *L. pabulinus* was already mentioned in Chapter 2, but it did not appear to be a female specific compound. This ketoaldehyde is commonly found in secretions from the dorsal abdominal scent glands (DAG) in heteropteran species (Aldrich, 1988 and references therein; Aldrich et al., 1991; Borges and Aldrich, 1992; Pavis et al., 1994) and in *Nematinus melanaspis* and *N. pavidus* (Boeve et al., 1984) and is commonly assigned as an alarm pheromone (Aldrich and Yonke, 1975; Farine et al., 1992). Recently, this ketoaldehyde was also found in airborne volatiles from a mirid, *Lygus rugulipennis* (Innocenzi et al., 1998). Interestingly it was only found in virgin female entrainments. It was suggested that this compound is not used as an alarm pheromone, but is part of the sex pheromone of *L. rugulipennes*. Despite my research no function of this compound in *Lygocoris pabulinus* could be found yet, but as only males respond strongly to this compound and females showed little or no response, it is not likely that (*E*)-4-oxo-2-hexenal acts as a defensive compound. Furthermore, (*E*)-4-oxo-2-hexenal is being produced by the DAG's in nymphs, which are mostly absent in adults. In only a few cases this compound was found in secretions from the metathoracic gland in adult bugs (Gilby and Waterhouse, 1964; Pinder and Staddon, 1965). Due to the lack of knowledge about the secretions of the DAG's in *L. pabulinus* nymphs or in other mirid nymphs (Aldrich, 1988), the function of this compounds remains unclear.

Comparing the electrophysiological responses from male *L. pabulinus* with male *Lygus rugulipennis*, male *Lygocoris pabulinus* respond to the same three compounds, i.e (*E*)-4-oxo-2-hexenal, hexyl butyrate and (*E*)-2-hexenyl butyrate. Interestingly, all these three compounds were absent in male *Lygus rugulipennis* headspace extracts. Female *L.*

rugulipennis produce the two esters in a ratio of 39:1. In contrast, both male and female *Lygocoris pabulinus* produce these two compounds in a ratio of 20:1 in favour of hexyl butyrate. Hexyl butyrate and (*E*)-2-hexenyl butyrate are also found in *Lygus lineolaris* (Gueldner and Parrot, 1978), in which both sexes produce these esters, but in different ratios. Females produced equal amounts, whereas males produced much less of the unsaturated ester. As in *Lygocoris pabulinus*, male *Lygus lineolaris* showed stronger responses to these esters than females (Chinta et al., 1994; Groot et al., 1999). These results indicate that these esters, hexyl butyrate and (*E*)-2-hexenyl butyrate, are probably common esters in Miridae. The exact function of these compounds remains unknown as no sex pheromone of any *Lygus* or *Lygocoris* species has been identified yet. However, the fact that all the above mentioned species produce these esters sex specific, in distinct ratios or in combination with the ketoaldehyde, suggests that these compounds might play an important role in the sexual communication.

In *Lygocoris pabulinus*, these compounds may not be the only important compounds. The other compounds giving EAG responses, nonanal, hexyl acetate, (*Z*)-3-hexenyl butyrate, and 1-hexanol, should also be considered. Nonanal was found to be EAD-active in *Lygus lineolaris* when volatiles captured from females feeding on bean pods (Chinta et al., 1994) were analysed, indicating that this alcohol might be of importance in pheromonal attraction. Because of the huge amount of hexyl butyrate present in headspace extracts of *Lygocoris pabulinus*, nonanal can only be seen as a shoulder in front of hexyl butyrate. Therefore it is not always clear whether nonanal elicits an EAG response. More experiments on another column could be of help to determine the EAD activity of nonanal.

Interestingly there was a strong difference in EAG response between male and female antennae. Males reacted strongly to all three compounds especially to the ketoaldehyde, whereas females showed only a low or no response. Females also had low responses to the two esters. In other Heteropteran spp. no difference in male and female responses were observed (Leal et al., 1995, 1996, 1998; Aldrich et al., 1997) or reported (Weissbecker et al., 1999) when using male or female antennae as sensing element in

GC-EAD experiments. The fact that males reacted stronger to these compounds than females suggests that these compounds could act as a kind of aggregation pheromone in males. Groot et al. (1999) already mentioned that the sexual difference in response to a different class of compounds might be due to the fact that in mirids males are attracted to females, while females may use plant volatiles for their orientation. So male *L. pabulinus* are probably not only responding to female-produced compounds but to male and female produced compounds. Field tests with hexyl butyrate, (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal failed to catch any male *L. pabulinus*, but because of the instability of the ketoaldehyde, maybe a different dispenser should have been used (see also Chapter 7). The Y-track olfactometer bioassays revealed that males were attracted to female extracts, while male headspace extracts did not attract any males, indicating that there are still essential compounds absent in male extracts. As female extracts do contain some alkenes and these alkenes are active as a close-range attractant (Chapter 5 and 6), there is evidence that these compounds might play a role in attracting males within a certain distance. An explanation for the absence of EAG responses to these alkenes could be condensation of these compounds in the 15-cm tube leading towards the antennae. In a different GC-EAD set-up it was possible to obtain EAG-responses on the alkenes present in female headspace extracts and in female leg extracts. These results and a possible function of these alkenes, (Z)-9-pentacosene and (Z)-7-pentacosene, will be presented in Chapter 6.

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Close-range attraction in *Lygocoris pabulinus* *

Abstract – Males of the green capsid bug, *Lygocoris pabulinus*, exhibit a specific courtship behaviour, i.e. a vibration of the abdomen. When both live and dead females were offered to males, this vibration behaviour was elicited in most of the males tested. When females were dissected into separate body parts, heads, wings and legs elicited equal responses, while thorax plus abdomen elicited a much lower response. When separate body parts were extracted, the leg extracts elicited significantly stronger responses than any other extract. This suggests that female *L. pabulinus* legs are either the source of a close-range sex pheromone, or pheromone is accumulated on the legs due to grooming behaviour. The leg extracts contained several hydrocarbons such as *n*-alkenes, *n*-alkanes and some methylalkanes. Female extracts contained more (*Z*)-9-pentacosene and male extracts contained more (*Z*)-9-heptacosene. Substrates on which females had walked elicited similar responses as female legs, indicating that the pheromone is deposited on the substrate. This enlarges the functional range of low-volatility compounds, which are thought to function only when sexes are in close vicinity or in contact.

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5.1 Introduction

Sex pheromones are commonly used by insects to locate mates at long range and to stimulate mating at close range (Carlson et al., 1971; Cardé et al., 1975; Muhammed et al., 1975). Long-range sex pheromones have first been described and chemically identified in moths (Butenandt et al., 1959), and are now widely used for monitoring of lepidopterous pests (e.g. Minks and van Deventer, 1992; Cardé and Minks, 1997). In mirids (Heteroptera: Miridae), where virgin females attract males, long-range sex pheromones have been identified for three species up to now (Smith et al., 1991; Millar et al., 1997; Millar and Rice, 1998; McBrien and Millar, 1999).

Close-range sex pheromones initiate courtship behaviour. Such pheromones are usually less volatile than long-range pheromones (Blomquist et al., 1993). Despite their low volatility, close-range pheromones may play an important role in the decision of an insect to land at a certain spot (Carlson et al., 1971). Without the addition of such pheromones, arriving males may not enter a trap (Cardé et al., 1975; Kennedy, 1977). In mirids close-range sex pheromones have not been reported so far. Major focus has been on attractive and alarm compounds from the metathoracic and accessory scent glands (e.g. Carayon, 1971; Staddon, 1979; Aldrich, 1988). Compounds identified from these glands have a carbon chain length of 2 to a maximum of 15 carbon atoms, and are most commonly aliphatic acids, aldehydes, ketones, alcohols, and esters (Staddon, 1979; Aldrich, 1988). Close-range pheromones may have carbon chain lengths of 20 to 30 or even more (Blomquist et al., 1993). The source of long-range pheromones in mirids has been suggested to be the metathoracic scent gland (Aldrich, 1988), or at least the thoracic region (Millar et al., 1997), although Graham (1988) identified the ovipositor region as source of attraction. Since the chemical nature of close-range pheromones may differ completely from long-range pheromones, their sources probably differ as well.

To identify close-range sex pheromones, a specific arousal or courtship behaviour of one of the sexes should be distinguished. Males *L. pabulinus* exhibit a characteristic sex-specific courtship behaviour, namely a repeated vibration of the abdomen. Only males vibrate in the presence of females and only when they are

sexually mature (Groot et al., 1998). This vibration behaviour of male *L. pabulinus* was used to determine the source of attraction in females at close-range. Additionally, the compounds probably involved in this close-range attraction were identified.

5.2 Methods and Materials

5.2.1 Insects *Lygocoris pabulinus* were reared as described in Chapter 2.

5.2.2 Bioassays Glass Petri dishes of 5 cm diameter were cleaned with acetone, and the bottoms were covered with white filter paper discs of the same diameter. The stimuli to be tested (on filterpaper) were placed in the Petri dishes, after which one male per dish was introduced. Stimuli consisted of one bug equivalent per Petri dish, and originated from bugs that were virgin and 6-9 days old. All males were observed for 15 min. If a male in a dish started to vibrate within this period, that dish was set aside and counted as a positive response. The number of Petri dishes with positive responses was calculated as a fraction of the total number of Petri dishes in which the stimulus had been applied. One to two hours before each test, virgin males of 6-9 days old were collected from the rearing cages and isolated in small glass tubes. Different stimuli were tested at the same time, and stimuli were tested on several different days. All experiments were carried out at 19-23 °C between 10.00 and 14.00 hours.

5.2.3 Stimuli Tested First, a series of live females, dead females, live males and dead males were tested. Bugs were anaesthetised with CO_2, after which the heads were clipped off. In a following series freshly anaesthetised females were dissected into heads, wings, legs, and thorax plus abdomen. Thorax and abdomen were not subdivided, since clipping would mean cutting through several organs and glands that run from thorax to abdomen, which may then release a variety of chemicals. Thirdly, extracts were made of the different body parts of females, i.e. heads, wings, legs, and thorax plus abdomen. After anaesthetising fresh females with CO_2, the body parts were dissected and placed in 1.8 or 4 ml vials. After dissecting all available females, 15-50 μl of either

dichloromethane, pentane, pentane:ether (2:1) or water per female was added, and this amount was set as one female equivalent of the regarding extract. The extracts were stored in a freezer (–20 ± 2° C) until used in bioassays. All extracts were used 1-14 days after the initial dissections. In a fourth series one female equivalent of a synthetic mixture (2.5 µg in total) was tested. The synthetic mixture consisted of 1-hexanol (40 ng), hexyl butyrate (500 ng), (*E*)-2-hexenyl butyrate (25 ng), (*Z*)-9-tricosene (150 ng), (*Z*)-7-pentacosene (200 ng), (*Z*)-9-pentacosene (1000 ng), (*Z*)-9-heptacosene (200 ng), tricosane (80 ng) and pentacosane (80 ng) according to Table 5.1.

5.2.4 Chemical Analysis Extracts were analysed with a dual column GC (HP 6890) equipped with an apolar DB-1 column (J&W Scientific, Folsom, California; 60 m × 0.25 mm; film thickness: 0.25 µm) and a polar Stabilwax column (Restek, Bellefonte, Pennsylvania; 60 m × 0.25 mm; film thickness: 0.25 µm) and two flame ionisation detectors. Oven program: 30°C (2 min hold) to 238°C (25 min hold) with 4°C/min. Hydrogen was used as the carrier gas (constant flow of 2.4 ml/min, linear velocity: 48 cm/sec). GC/MS analysis was carried out on a Varian 3400 GC connected to a Finnigan MAT95 mass spectrometer. The BP5-column (SGE, Australia; 25 m × 0.25 mm; film thickness: 0.25 µm) was programmed from 50°C to 270°C (4 min hold) at 5°C/min. The mass spectrometer was operated in the 70 eV EI mode and scanning was done from mass 24 to 500 at 0.7 sec/dec. ^{1}H NMR (200 MHz) spectra were recorded on a Bruker AC200 spectrometer. FT-IR spectra were recorded on a Perkin-Elmer 1725X spectrometer. Identification of compounds in extracts was carried out by GC/MS using reference spectra from the NIST/EPA/NIH Mass Spectral library (Version 1.6) and by comparison of retention times of reference compounds on the dual column GC. The position of the double bond in the alkenes was determined by derivatisation with DMDS according to Carlson et al. (1989).

5.2.5 Chemicals Hexanol, tricosane, pentacosane, 1-bromotetradecane, 1-bromohexadecane, 1-bromooctadecane, triphenylphosphine, nonanal, heptanal, n-butyllithium in hexane, DMDS and urea were all purchased from Acros Organics

(Geel, Belgium), hexyl butyrate was purchased from Roth (Karlsruhe, Germany). (*E*)-2-hexenyl butyrate was synthesised as described in Chapter 2. (*Z*)-9-Tricosene, (*Z*)-9-pentacosene, (*Z*)-7-pentacosene and (*Z*)-9-heptacosene were all synthesised as described below. All the chemicals used were more than 98% pure. All solvents used were distilled twice before use.

5.2.6 Synthesis

Alkyltriphenylphosphonium bromide: A mixture of 0.3 mmol of the alkylbromide (1-bromotetradecane, 1-bromohexadecane or 1-bromooctadecane) and 0.3 mmol of triphenylphosphine was heated to 140°C under a nitrogen atmosphere for 5 hours. The reaction mixture formed a solid when cooled down and 5 ml of dry acetone and 12 ml of dry diethyl ether was added to the solid and cooled to –20°C (overnight). After filtration the alkyltriphenylphosphonium bromides were obtained as white crystals.

Alkenes: A slurry of 5 mmol of powdered alkyltriphenylphosphonium bromide in 10 ml of THF was prepared under nitrogen. The mixture was cooled in an ice bath and 5 ml of DMSO was added after which 5 mmol of *n*-butyllithium in hexane was injected. The butyllithium was added at such a rate that the temperature of the mixture remained at 10-15 °C. After 5 min, 5 mmol of the aldehyde (nonanal or heptanal) was injected, and the resulting mixture was stirred for 30 minutes at ambient temperature. The mixture was diluted with water and extracted with petroleum ether 40/60. The extract was dried ($MgSO_4$), filtered and concentrated to give the alkene in 90% yield. The alkene was further purified with column chromatography on silica gel and eluted with hexane to give a mixture of *Z*- and *E*-isomers in a ratio of 85:15.

5.2.7 Separation of Z- and E-isomers The two isomers of the alkenes obtained from the synthesis were separated making use of their different complexation with urea (Leadbetter and Plimmer, 1979). One part of alkene and 5 parts of urea were dissolved in 20 parts of methanol and the solution was left to crystallise at room temperature. The white crystals were separated by filtration. The methanol from the filtrate was

evaporated to obtain the (*Z*)-isomer in 97% purity. To obtain the (*Z*)-isomer in even higher purity the procedure was repeated. If the alkene could not be dissolved in methanol, isopropanol was added.

(*Z*)-9-Tricosene:

^{1}H NMR δ ($CDCl_3$) 0.83-0.89, t, (6 H, $-CH_3$); 1.2-1.6, m, (34 H, $-CH_2CH_2-$); 2.0, m, (4 H, $=CH-CH_2$), 5.3-5.35, m (2 H, CH=CH); MS: *m/z* = 322 (M^+); Kovats' Indices: 2320 on Stabilwax and 2271 on DB-1. IR (film): $\nu_{HC=CH\,(cis)}$ 722 (m) cm^{-1}

(*Z*)-7-Pentacosene:

^{1}H NMR δ ($CDCl_3$) 0.83-0.89, t, (6 H, $-CH_3$); 1.2-1.4, m, (38 H, $-CH_2CH_2-$); 1.9-2.0, m, (4 H, $=CH-CH_2$), 5.3, m (2 H, CH=CH); MS: *m/z* = 350 (M^+); Kovats' Indices: 2526 on Stabilwax and 2477 on DB-1; IR (film): $\nu_{HC=CH\,(cis)}$ 722 cm^{-1}

(*Z*)-9-Pentacosene:

^{1}H NMR δ ($CDCl_3$) 0.83-0.89, t, (6 H, $-CH_3$); 1.2, m, (38 H, $-CH_2CH_2-$); 1.9-2.2, m, (4 H, $=CH-CH_2$), 5.3-5.4, m (2 H, CH=CH); MS: *m/z* = 350 (M^+); Kovats' Indices: 2519 on Stabilwax and 2470 on DB-1; IR (film): $\nu_{HC=CH\,(cis)}$ 722 cm^{-1}

(*Z*)-9-Heptacosene:

^{1}H NMR δ ($CDCl_3$): 0.9, t, (6 H, $-CH_3$); 1.1-1.5, m, (40 H, $-CH_2CH_2-$); 2.2, m, (4 H, $=CH-CH_2$), 5.3, m (2 H, CH=CH); MS: *m/z* = 378 (M^+); Kovats' Indices: 2721 on Stabilwax and 2671 on DB-1; IR (film): $\nu_{HC=CH\,(cis)}$ 722 cm^{-1}

5.2.8 Statistical Analysis If males responded to a source, differences in responses towards the different sources were statistically analysed by fitting a logit regression model with overdispersion to the daily observed counts of responses of a test (McCullagh and Nelder, 1989), using the computer programme Genstat 5 (release 4.1, PC/Windows NT, 1997). In the model, source was taken as explanatory variable and the variance was assumed to be proportional to the binomial variance. First a chi-square test

for the residual deviance was conducted to determine overdispersion. Overall effect of treatments was determined by performing an F-test for the ratio of the mean deviance for treatment and the mean deviance of the rest. If the overall test was significant ($p < 0.05$), pairwise comparisons between treatment means on the logit scale were conducted, using the t-test.

5.3 Results

Live and dead females elicited similar responses, the fractions of vibrating males being 0.88 ± 0.08 and 0.74 ± 0.09 (mean ± s.e.), respectively (Figure 5.1A). Live and dead males elicited vibration responses in few males. When the bodies of females were dissected, the head, wings and legs were equally attractive and as attractive as dead females, while the thorax plus abdomen of females were significantly less attractive (Figure 5.1B). Responses to freshly dissected wings and heads may be due to grooming, which spreads attractive compounds over the body surface. For confirmation of the presence of attractive compounds on the whole body surface, small pieces of filter paper were rubbed over female bodies (after anaesthesia and clipping off heads). When these pieces of paper were offered in clean Petri dishes, almost half of the tested males (0.41 ± 0.07, mean ± s.e.) started vibrating ($n=46$).

After extraction of the separate body parts of females with an organic solvent, leg extracts elicited significantly more vibrational response than all other extracts (Figure 5.1C). Extracts from thorax plus abdomen did not elicit a response from males, which may be due to defensive compounds in the metathoracic gland. Therefore, extracts were also made of females' thorax plus abdomen, from which the metathoracic gland was removed by gently cutting the cuticle with two sharp tweezers, trying to destroy as little tissue as possible. Few males did respond to this extract (Figure 5.1C). When differences in responses between freshly dissected body parts and their corresponding extracts were statistically compared, male responses to the leg extracts were not significantly different from responses to freshly dissected legs, while wing and head extracts elicited significantly lower responses ($P < 0.05$) than freshly dissected wings and heads.

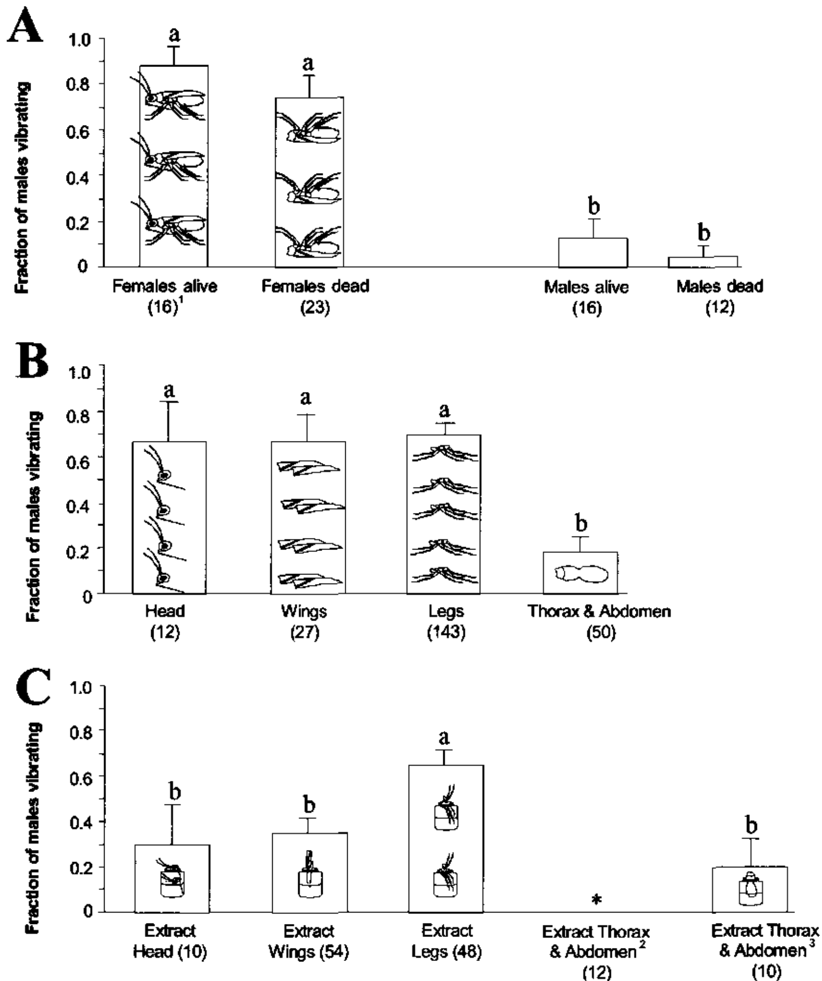

Figure 5.1 **Male *L. pabulinus* responses (mean ± s.e.) to different stimuli. A: whole insects, B: body parts of females, C: extracts of female body parts in dichloromethane.**

[1] Total number of males tested,

[2] Metathoracic gland left in thorax,

[3] Metathoracic gland removed from thorax.

Significant differences were determined between sources within one group (A, B, C). Different letters above the bars indicate significant differences in each group at the 5 % level. See text for statistical methods used. *Not included in the statistical analyses, as no males responded. Figures in the bars kindly provided by A.T. Groot.

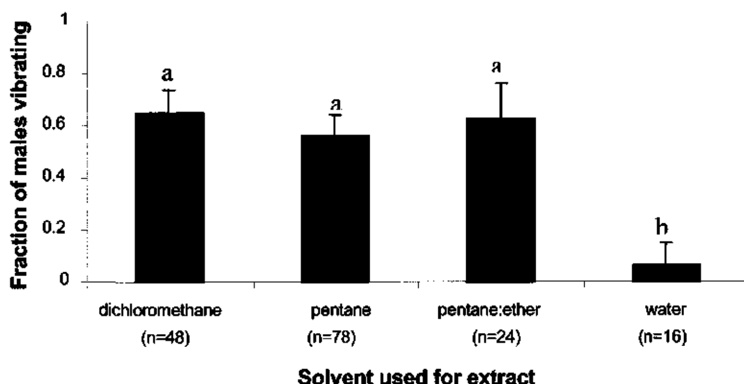

Figure 5.2 Response of male *L. pabulinus* to extracts of female legs prepared in different solvents. Mean ± S.E. (n= number of males tested). Different letters above bars indicate significant differences at $p \leq 0.05$ (2-tailed).

Extracts prepared with organic solvents stimulated significantly more males to vibrate than aqueous extracts (Figure 5.2). The graph indicates that aqueous extracts caused only minimal vibration in males. Legs from female bugs, used for these aqueous extracts, still elicited vibratory behaviour in males. No significant differences in male vibration occurred using dichloromethane, pentane or a mixture of pentane and ether (Figure 5.2).

In both male and female leg extracts the major part of the compounds consisted of hydrocarbons (Figure 5.3A). These hydrocarbons consisted of alkenes (75%), alkanes (20%) and some methylalkanes (5%). (*Z*)-9-Pentacosene was the most abundant alkene in females, while (*Z*)-9-heptacosene was the most abundant in males. The ratio of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene was opposite in males and females.

Furthermore, sometimes the female extracts contained more (*Z*)-9-tricosene than male extracts (Figure 5.3B, C). Both male and female extracts contained three oxygen containing compounds: hexyl butyrate, (*E*)-2-hexenyl butyrate and sometimes 1-

hexanol. Other minor compounds identified in the leg extracts of male and female *L. pabulinus* are listed in Table 5.1. Table 5.2 lists some characteristics used to identify the most abundant alkenes present in the extracts.

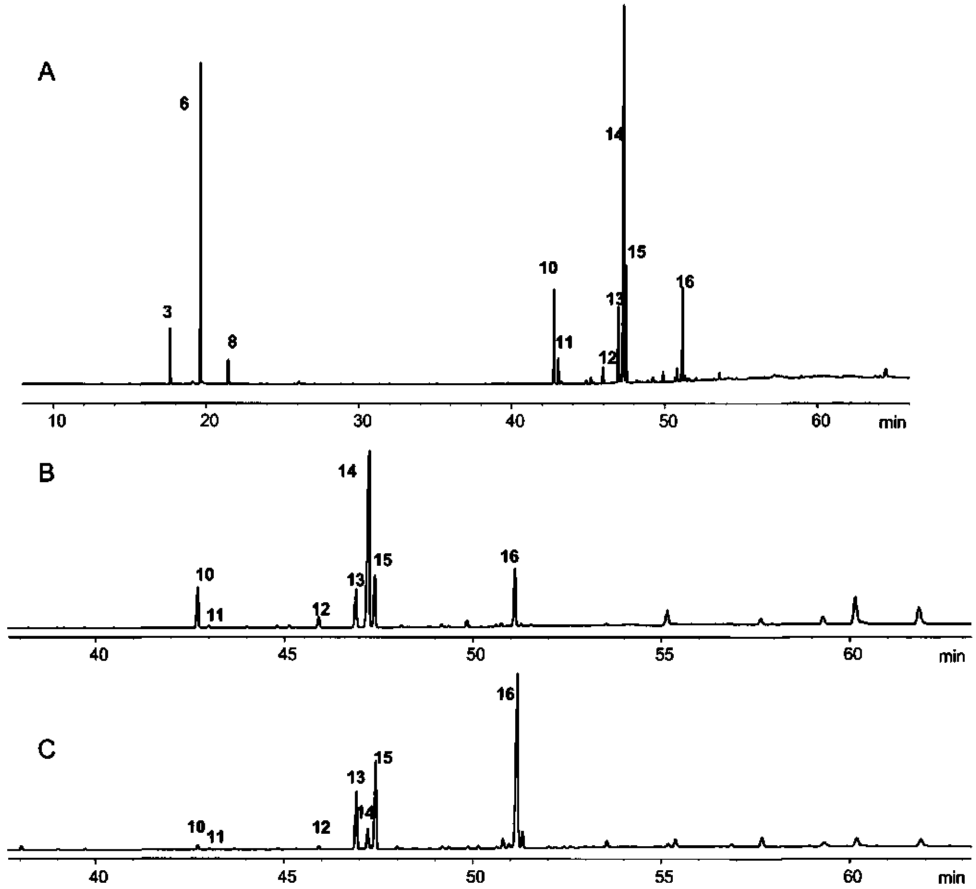

Figure 5.3 Gas chromatograms of *L. pabulinus* leg extracts. A: female leg extract. Enlargement of the cuticular hydrocarbon part of female (B) and male (C) extracts. Separation carried out on a DB-Wax column, Detector: FID. For explanation of the numbers see Table 5.1.

TABLE 5.1 Average composition (%) of extracts of legs from female and male *L. pabulinus*.

No[a]	Compound	Females	Males
3	Hexan-1-ol[b]	1.7[c] ± 1.8	0.6 ± 0.4
6	Hexyl butyrate[b]	19.2 ± 13.7	22.3 ± 23.4
8	(*E*)-2-Hexenyl butyrate[b]	0.8 ± 0.5	0.8 ± 0.9
10	Tricosane[b]	6.3 ± 1.9	0.4 ± 0.7
11	(*Z*)-9-Tricosene[b]	3.1 ± 1.7	0.1 ± 0.1
	(*Z*)-9-Tetracosene	≤ 0.2	≤ 0.2
	Tetracosane	≤ 0.2	≤ 0.2
12	2-Methyltetracosane	3.3 ± 1.5	1.8 ± 0.2
13	Pentacosane[b]	8.6 ± 2.8	10.6 ± 4.9
14	(*Z*)-9-Pentacosene[b]	40.8 ± 9.2	3.4 ± 2.3
15	(*Z*)-7-Pentacosene[b]	8.7 ± 1.9	20.5 ± 4.8
	9-Hexacosene	≤ 0.2	≤ 0.2
	2-Methylhexacosane	≤ 0.2	≤ 0.2
16	(*Z*)-9-Heptacosene[b]	7.6 ± 4.6	39.6 ± 13.2
	(*Z*)-7-Heptacosene	≤ 0.2	≤ 0.2
	Heptacosane	≤ 0.2	≤ 0.2
	(*Z*)-9-Nonacosene	≤ 0.2	≤ 0.2

[a] Numbers according to Figure 5.3 and Figure 5.4

[b] Compounds used in the synthetic mixture

c All values reported are mean % ± SD.

TABLE 5.2 Characteristics used in identification of the four major alkenes present in leg extracts of *L. pabulinus*.

Compound		Kovats' Indices		MS Characteristics	
Name	Number in Table 1	DB1	Stabilwax	M^+	*m/z* of parent peak and major fragments after DMDS derivatisation
(*Z*)-9-tricosene	11	2271	2319	322	173 (C_1-C_9), 243 (C_{10}-C_{23}), 416 (M^+)
(*Z*)-9-pentacosene	14	2470	2518	350	173 (C_1-C_9), 271 (C_{10}-C_{25}), 444 (M^+)
(*Z*)-7-pentacosene	15	2477	2526	350	145 (C_1-C_7), 299 (C_8-C_{25}), 444 (M^+)
(*Z*)-9-heptacosene	16	2670	2719	378	173 (C_1-C_9), 299 (C_{10}-C_{27}), 472 (M^+)

TABLE 5.3 Male vibration response to different parts of female legs

Source	fraction of males responding ± s.e.	n		
female legs	0.70 ± 0.05	143		c[2]
forelegs	0.76 ± 0.14	17	a[1]	
middle legs	0.71 ± 0.15	17	a	c
hindlegs	0.88 ± 0.10	17	a	
coxae + femorae	0.45 ± 0.14	22	b	d
tibiae + tarsi	0.41 ± 0.14	22	b	

[1] different letters indicate significant differences between pairs ($p < 0.05$),

[2] different letters indicate significant differences between groups ($p < 0.05$).

See text for statistical methods used.

The vibrational bioassays suggest that female legs are the source of a close-range sex pheromone. To determine whether the source of attraction could be defined more precisely, female legs were subdivided into (A) forelegs, middle legs and hindlegs, or into (B) coxae plus femorae, and tibiae plus tarsi. In series A one pair of forelegs, middle legs or hindlegs of three females was placed in one Petri dish, so that 6 legs per dish were offered. In series B the 6 coxae plus femorae of one female were placed in one Petri dish, and the 6 tibiae plus tarsi in another. Table 5.3 shows that all parts of the legs were equally attractive, no significant differences were found between any pair. However, when the overall response to fore, middle and hind legs was compared to the overall response to coxae plus femorae and tibiae plus tarsi, responses to entire legs were significantly stronger than to those of parts.

When legs contain attractive compounds, these compounds may be deposited on the substrate on which female *L. pabulinus* walk. To determine possible deposition of attractive compounds, three different substrates were tested: a piece of potato leaf (cultivar Bintje), a piece of green bean leaf (*Phaseolus vulgaris*, cultivar Miracle) and the glass of an empty Petri dish. One *L. pabulinus* female was allowed to walk in each dish for 75-140 min. As a control pieces of potato leaf or empty dishes on which males had walked for 60-120 min were tested, as well as pieces of potato leaves on which no bug had walked. In Table 5.4 is shown that males did respond to substrates on which

females had walked, while no males showed vibration behaviour in any of the control dishes.

TABLE 5.4 Male vibration response to substrate on which females had walked

Substrate	**Fraction of males responding ± s.e.**	**n**	
potato leaf on which **female** had walked for 75-140 min	0.68 ± 0.10	39	a[1]
bean leaf on which **female** had walked for 75-140 min	0.64 ± 0.13	22	a
empty Petri dish in which **female** had walked for 75-140 min	0.37 ± 0.09	46	a
potato leaf on which **male** had walked for 60-120 min	0	15	*
empty Petri dish in which **male** had walked for 60-120 min	0	10	*
potato leaf	0	10	*

[1] different letters indicate significant differences ($p < 0.05$). See text for statistical methods used. *Not statistically analysed, as no male responded.

During the 75-140 min that females walked around in the dishes, a characteristic pheromone-laying behaviour was not observed. In a second experiment a solid-phase microextraction (SPME)-needle [100 μm polydimethylsiloxane coating from Supelco (Bellefonte, USA)] was positioned on the bottom of the Petri dish through a hole in the side. A metal strip was placed in a V-shape around the needle to reduce the amount of space the bug had, thus increasing the chance of bug-needle contact. One or two bugs were then placed in the Petri dish for 2-3 hours. Desorption of the SPME-needle (250°C) revealed the same hydrocarbon profile as found in the leg extracts of the corresponding sex. From Figure 5.4 it is clear that when females walked on or near the needle, a high concentration of (*Z*)-9-pentacosene was deposited on the needle. The volatile compounds 1-hexanol, hexyl butyrate and (*E*)-2-hexenyl butyrate were not always present.

When the synthetic mixture, containing compounds derived from female leg extracts, was tested in the vibration bioassay, no males started to vibrate. A few males showed vibratory behaviour when male legs, loaded with (*Z*)-9-pentacosene, were offered (Table 5.5). Adding (*Z*)-9-tricosene to these legs did not result in more males

vibrating. When female legs were loaded with a high dose of (*Z*)-9-heptacosene, a similar amount of males showed vibration behaviour as when untreated female legs were offered (Table 5.5).

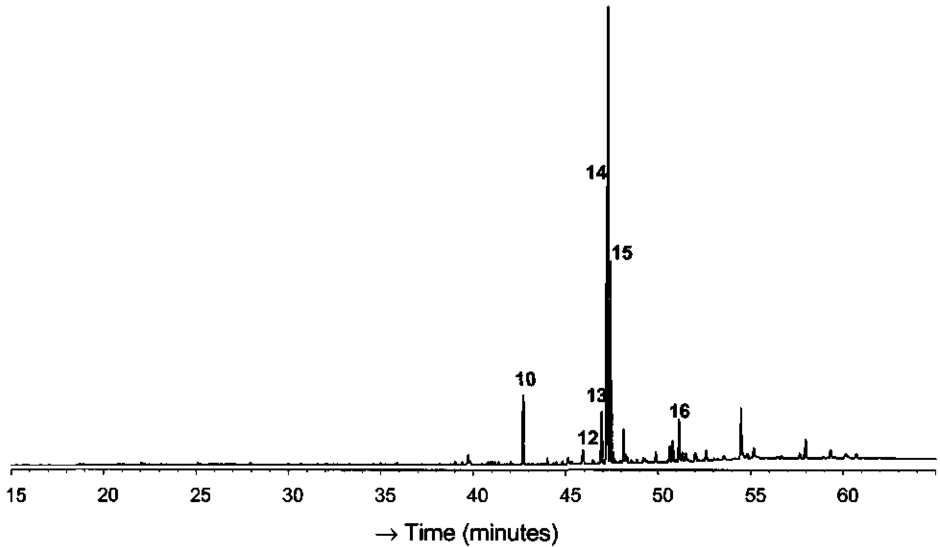

Figure 5.4 Gas chromatographic analysis of an SPME-needle on which a female bug had walked. Separation carried out on a DB-Wax column, Detector: FID. For explanation of the numbers see Table 5.1.

TABLE 5.5 Male vibratory response to freshly dissected legs, with or without alkene added (n = number of males).

Source	Fractions of males responding ± s.e.	n	
synthetic mixture	0	32	a*
male legs	0	5	a
male legs + 5 µg (*Z*)-9-pentacosene	0.19 ± 0.11	29	a
male legs + 5 µg (*Z*)-9-pentacosene + 1 µg (*Z*)-9-tricosene	0.05 ± 0.06	10	a
female legs + 5-8 µg (*Z*)-9-heptacosene	0.76 ± 0.13	21	b
female legs	0.70 ± 0.06	143	b

*** different letters indicate significant difference at $p \leq 0.05$ (2-tailed).**

5.4 Discussion

The legs of *L. pabulinus* females consistently elicited vibration behaviour in males, suggesting that legs are the source of compounds involved in this close-range attraction in this species. Legs have been recognised as the site of sex pheromone release in the aphid *Megoura viciae* (Marsh, 1972), the mosquito *Culiseta inornata* (Lang, 1977), the tsetse fly *Glossina morsitans morsitans* (Carlson et al., 1978), the housefly *Musca domestica* (Schlein et al., 1980), and the parasitoid braconid *Ascogaster reticulatus* (Kainoh and Oishi, 1993). In some species, specific glands in the legs have been identified as the site of sex pheromone excretion (Marsh, 1972; Schlein et al., 1980). In *L. pabulinus*, responses to fore, middle and hind legs were equally strong. The lower response to parts of the legs compared to entire legs may be due to the lower amount of leg biomass per Petri dish in the latter group. From these experiments no specific site of possible glands in legs became apparent.

Perhaps contact sex pheromones are not synthesised in specific glands in the legs. Cuticular hydrocarbons are probably synthesised by oenocytes, large cells in which smooth endoplasmatic reticulum and mitochondria are abundantly present, which appear to be restricted to epidermal tissue in thorax and abdomen (Gu et al., 1995; Schal et al., 1998). After synthesis, attractive hydrocarbons may be deposited at specific target sites, as in the German cockroach *Blattella germanica*, where the wings accumulate large amounts of pheromone (Gu et al., 1995). Hence, the cuticle of legs may be the specific target deposition site of attractive compounds.

The presence of close-range sex pheromone on the legs may also be due to grooming. Grooming may either accumulate pheromone from other body parts on the legs (Howard and Blomquist, 1982), or it may spread the pheromone from leg glands over the whole body surface, as in polistine wasps (Beani and Calloni, 1991), whose territorial marking pheromones from leg glands function as sex attractants as well. *L. pabulinus* males and females groom frequently (Groot et al., 1998) and the attractive compounds are not only present on female legs, but also on other body parts, as is

shown by the male response to female wings and heads, and to pieces of filter paper rubbed over female bodies. In short, the site of sex pheromone production does not have to be the site of pheromone release, specific glands are not necessarily involved, and grooming may enhance chemical dispersion or accumulation at specific sites.

In mirids, close-range or contact pheromones have not been studied so far, and to my knowledge this is the first study on cuticular hydrocarbons of a mirid species. In *L. pabulinus* the major part of the hydrocarbons consisted of mono-alkenes with double bonds at Δ^9 ($C_{23:1}$-$C_{29:1}$), followed by *n*-alkanes (C_{23}-C_{27}) and some 2-methyl alkanes (2-MeC_{24} and 2-MeC_{26}). All compounds were C_{23}-C_{29} hydrocarbons. This is also the first time that in *L. pabulinus* a clear-cut difference was found between male and female derived compounds, i.e. females produce a high amount of (*Z*)-9-pentacosene, whereas males produce high amounts of (*Z*)-9-heptacosene. Because of the low amount of (*Z*)-9-pentacosene present in males, the ratio of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene is opposite in males and females. To my knowledge, there are only two other studies on cuticular hydrocarbons in heteropteran species, the milkweed bug, *Oncopeltus fasciatus* (Lygaeidae) (Jackson, 1983), *Triatoma infestans* and *T. mazzotti* (Reduvidae) (Juárez and Blomquist, 1993). In all these species mostly *n*-alkanes, branched methylalkanes and dimethylalkanes were found. Furthermore hydrocarbons up to 41-43 carbon atoms were found. More importantly, male and female *O. fasciatus, T. infestans* and *T. mazzotti* had similar profiles, whereas *L. pabulinus* males and females produce a different blend of hydrocarbons.

The hydrocarbons of *L. pabulinus* females appear to elicit vibratory behaviour in males, because the aqueous extracts from legs (in which no hydrocarbons are present) were not active. Furthermore, female legs used for water extraction remained active, indicating that the compounds were still present on the legs. Fractionation of the extracts on silica gel, eluting with subsequently pentane and a mixture of pentane:ether (2:1), lead to two fractions; Fraction 1 containing the apolar hydrocarbons and Fraction 2 containing more polar oxygenated compounds. Vibration

bioassays with these two fractions suggested that both fractions were needed to elicit vibration behaviour in males, although only a slight increase was observed (Figure 5.5).

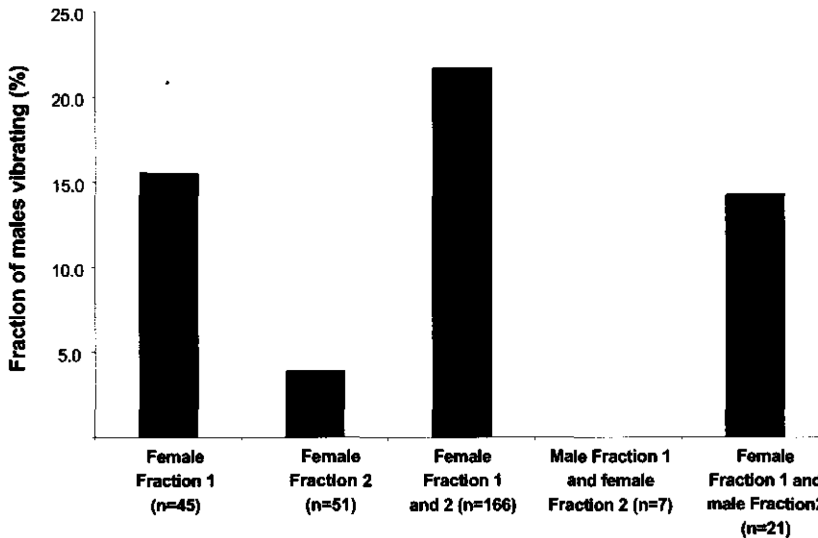

Figure 5.5 Response of male *L. pabulinus* to different fractions from male and female leg extracts after fractionation on a silica gel column. Fraction 1: eluted with pentane; containing hydrocarbons. Fraction 2: eluted with pentane:ether; containing oxygenated compounds

The composition of the fraction with oxygenated compounds of males and females did not show any difference. The similarity of this fraction between males and females was supported by the finding that this fraction from males combined with the hydrocarbon fraction of females did cause some vibratory behaviour in males. These results are however very preliminary as only a few males vibrated. Further research on the identification of active compounds is described in Chapter 6.

In various species cuticular hydrocarbons have been identified as contact pheromones (e.g. Muhammed et al., 1975; Carlson et al., 1978; Bolton et al., 1980; Dillwith et al., 1981; Blomquist et al., 1993; Gu et al., 1995; Fukaya et al., 1996; Doi et al., 1997). Especially alkenes seem to be involved in the sexual communication (Howard and Blomquist, 1982). More precisely, (*Z*)-9-pentacosene is often the main

hydrocarbon present in beetles (Baker et al., 1979), ants (Morgan et al., 1992) and bees (Paulmier et al., 1999). In *L. pabulinus,* (*Z*)-9-pentacosene, in spite of being the only compound obviously present in lower amounts in males than in females, on its own was not active in bioassays. As the synthetic mixture did not elicit vibration behaviour in males either, probably more compounds are part of the contact pheromone. The most abundant male alkene, (*Z*)-9-heptacosene, did not act as a repellent, because female legs sprayed with a high dose of this alkene still elicited vibratory behaviour in males (Table 5.5). More experiments (which are described in Chapter 6) are needed to determine if there are other compounds present in the extracts that act as a vibration elicitor.

The attractive compounds on female legs may be deposited on a substrate. Male *L. pabulinus* showed strong responses to substrates on which females had walked. The fraction of males responding to potato leaves, on which females had walked, was even similar to responses to female legs. When females were allowed to walk on an SPME-needle, a similar chemical profile was found as in attractive female leg extracts. These results support the hypothesis that these hydrocarbons are deposited on the substrate. As a characteristic pheromone-laying behaviour was not observed, I suspect that deposition on the substrate occurs passively, or that pheromone is adsorbed to the substrate. Adsorption or deposition of attractive compounds on the substrate increases the probability of sex encounters, as it elicits intensive searching by males in these areas (Colwell et al., 1978; Fauvergue et al., 1995). Depending on their volatility, these pheromones are active at some distance, as in *Megoura viciae* (Pickett et al., 1992), or they elicit response at close range or upon contact, as in the other species mentioned. Adsorption of pheromone to a substrate also increases the surface area from which pheromone evaporates, thereby increasing both the degree of volatilisation and the possible communication distance (Colwell et al, 1978). Males may follow a gradient of intensity, created by the release of the compounds over time, to orient their movements towards females (Fauvergue et al., 1995). In this way, the functional range of low-volatile cuticular hydrocarbons would be greatly enlarged.

Identification of the sex pheromones of mirid species is a challenging task. This study indicates that the sex specific cuticular hydrocarbons, probably in combination with esters, may play a role in the sexual communication of these bugs. Evidence is accumulating that such hydrocarbons not only function as contact pheromones, but are also involved in attraction at a (short) distance. Uebel et al. (1978) demonstrated that field catches of male *Fannia canicularis* and *F. pusio* increased slightly when alkenes were loaded on the lures. Connor et al. (1980) reported that the pheromone of the arctiid moth *Utetheisa ornatrix* (*Z,Z,Z*)-3,6,9-heneicosatriene plus a small quantity of an unidentified C_{21} tetraene), perhaps serves as a close-range orientation cue for locating the female. Recently, Schiestl et al. (1999, 2000) reported that flowers of *Ophrys* orchids also mimic the odour profile of bees by using this class of alkenes in order to attract these bees. Successful pheromone trapping of mirids might also have to take these close-range cues into account.

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Mate location in the green capsid bug, *Lygocoris pabulinus*

Abstract – Headspace extracts from female *L. pabulinus*, as well as female leg extracts attracted males in Y-track olfactometer bioassays. In contrast, only female leg extracts were active in the vibration bioassay. Male extracts had no activity at all in either bioassay. When the leg extract was analysed by coupled gas chromatography-electroantennography (*Z*)-9-pentacosene and (*Z*)-7-pentacosene were EAD-active. (*Z*)-9-pentacosene and sometimes (*Z*)-7-pentacosene were also observed in female headspace extracts. EAG responses could be obtained for (*Z*)-9-pentacosene. A mixture of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene in the ratio 5:1 elicited vibrational behaviour in males. These results indicate that these alkenes are important cues for male *L. pabulinus* in mate location behaviour.

6.1 Introduction

Although some sex pheromones of the Miridae-family have been identified (Smith et al., 1991; Millar et al., 1997; Millar and Rice, 1998), little is known about the biology of these insects. It is still unknown where the pheromones are produced and the actual communication between sexes is also a black box. The courtship behaviour of some bugs has been studied (e.g. Dunbar, 1972; Borges et al., 1987; Rodriguez and Eberhard, 1994; Wang and Millar, 1997), but not in relation to the production site or identification of their sex pheromone. Recently, the sexual behaviour of *Lygocoris pabulinus* has been studied in detail in order to identify its sex pheromone (Groot, 2000). However, the sex pheromone and its production site remained unclear. Field and laboratory tests have shown that males are attracted to females (Blommers et al., 1988; Groot et al., 1996). Nevertheless, no specific calling behaviour of the females was observed (Chapter 5). Recently a sex specific behaviour of males was discovered, i.e. a vibration of the abdomen. Hence, a vibration bioassay was developed to test the activity of different extracts from males and females (Groot et al., 1998a, 1998b). In Chapter 5 the chemical composition of these active extracts has been described, however, full identification of the active compounds eliciting the vibration behaviour was not accomplished.

Apart from this vibration bioassay, Groot et al. (2001) described a different bioassay: the Y-track olfactometer. In this bioassay, females were found to be attractive to males, whereas males did not attract other males. In the present research the leg extracts used in the vibration bioassay, as well as headspace extracts from males and females were tested using this Y-track olfactometer. The results from the Y-track olfactometer bioassay and the vibration bioassay are compared. The activity of the two important alkenes, (*Z*)-9-pentacosene and (*Z*)-7-pentacosene (reported in Chapter 5) were also tested in the vibration bioassay. A possible system for mate location in male *L. pabulinus* is postulated.

6.2 Methods and materials

6.2.1 Insects *Lygocoris pabulinus* was reared under summer conditions as described in Chapter 2.

6.2.2 Bioassays

Olfactometer tests The set-up used for conducting these experiments was as described by Visser and Piron (1998) with specific adjustments according to Groot et al. (2001) (see Figure 6.1).

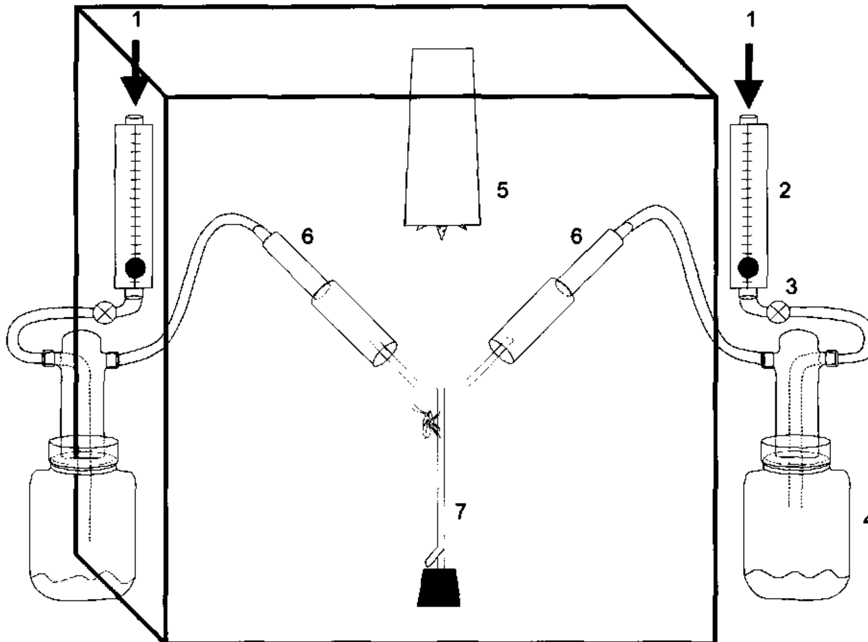

Figure 6.1 Schematic drawing of open Y-track olfactometer. The open front was covered by a black cloth during experiments. 1) incoming clean air, 2) air flow control, 3) open/close valve, 4) glass bottle under a tungsten lamp (25 W), used to test volatiles produced by living insects, 5) lamp in black cylinder with red filter, 6) glass tube with gauze, place where the filterpaper with extract or control (solvent) was placed, 7) brass rod (diameter: 4 mm, length to junction 13 cm) with extension at base for placing a male bug (reproduced with permission from Groot et al., 2001)

The Y-track was placed in a dark box under a halogen lamp (4-12V DC, 10 VA). To suppress flight intention of males, this lamp was placed in a black socket sealed with a red filter, so that the light intensity at the base of the Y-track was 6.3-6.5 lux only. 30-40 μl of the extract to be tested was pipetted on a piece of filter paper (5 × 0.5 cm), which was placed in the outlet of the glass tube, approximately 10 cm from the Y-branch (of fork). One to two hours before each test, virgin males of 6-9 days old were collected from the rearing cages and isolated in small glass tubes. The extracts tested were (I) female headspace extract, (II) male headspace extract, (III) extract from female legs and (IV) extract from male legs. These were all tested against a control that consisted of filter paper loaded with 40 μl of the solvent used in preparing the extracts, i.e. pentane or a mixture of pentane:ether (2:1).

Vibration Bioassays This bioassay was carried out as described in Chapter 5. The filterpapers (5 cm diameter) to be tested were loaded with 1 FE of female leg extract or ca. 70 μg of a mixture of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene in a ratio of 5:1. In a second series hexyl butyrate and (*E*)-2-hexenyl butyrate (25 μg in a ratio of 20:1) were added to the alkene-mixture.

6.2.3 Sample collection

Headspace extracts Volatiles from males and females *L. pabulinus* were collected as described in Chapter 4.

Leg extracts After anaesthetising fresh *L. pabulinus* with CO_2, the legs were dissected and placed in 1.8 ml vials. After dissecting all available insects, 15-25 μl of pentane per bug was added, the amount of which was set as one male or female equivalent of the regarding extract. The extracts were stored in a freezer (–20 ± 2° C) until used in bioassays. All extracts were used within 1-14 days after the initial dissections.

6.2.4 Coupled gas chromatography-electroantennography (GC-EAD) Extracts were analysed by a GC-FID-EAD system. A Varian 6000 GC, with two injector-ports and a Flame ionisation detector (FID), was equipped with a DB23 column, (40 m x 0.20 mm

ID x 0.25 μm film thickness) (J&W Scientific, Folsom, California). 2 μl of the extract was injected splitless (during 0.8 min) at an injector temperature of 250°C. The initial oven temperature was set at 50°C. After 1 min the oven was programmed at 8°C/min to 238°C. Hydrogen was used as the carrier gas. The column effluent was split 1:1 (using a Y-connector) into two 35 cm long deactivated fused silica columns (0.25 mm ID), one connected to the FID and the other to the EAD. Make-up gas (nitrogen, 15 ml/sec) was added just before the split point (also using a Y-connector) to accelerate the effluent through the deactivated columns. The end of the column to the EAD passed through the second injector-port on the GC (T=250°C) and hereafter through a heating device (T=250°C) and extruded 1-2 mm out of the heating device into a Liebig condenser (45 cm long). Humidified air (ca. 900 ml/min), which was cooled by leading the tubing for the air, upstream of the Liebig condenser, through a bucket of ice-water, was directed over the *L. pabulinus* antennal preparation. Additionally, cold water (3-7°C) was led through the Liebig condenser to further cool the humidified air so that the temperature at the antennal preparation was approximately 15°C (see also Figure 6.2).

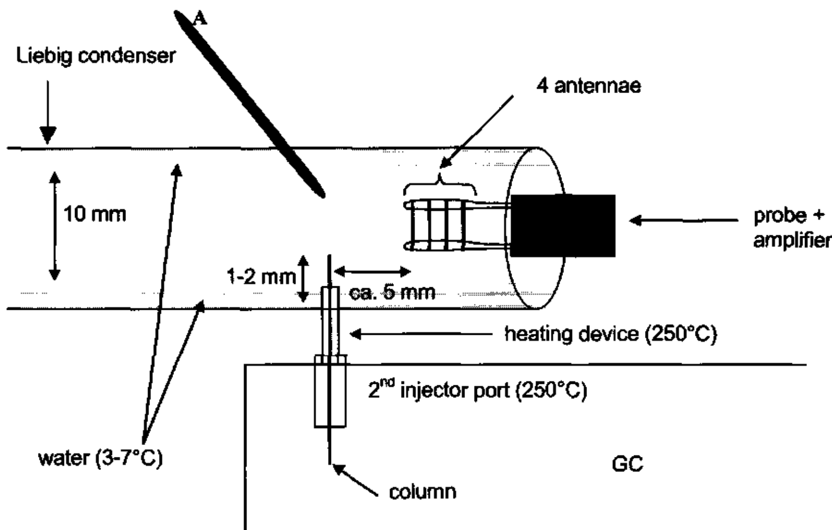

Figure 6.2 GC-EAD set-up. A: Pipet for off-line stimuli

The antennal preparation was prepared as follows: four excised male antennae were placed between two gold wires (probe) (gold kindly provided by Dr. M.A.

Posthumus). This probe was subsequently attached to the input of an amplifier (Syntech, Hilversum, The Netherlands). The end of each gold wire was spoon-shaped and a drop of electrode gel (Spectra 360, Parker Laboratories Inc., USA) was placed on the spoon part. The tips of the four antennae were then inserted into the gel thus establishing electrical contact and holding them in place. Hereafter the probe was connected to the amplifier and inserted into the Liebig condenser so that the antennae were within 5 mm of the end of the column. FID and EAG signals were monitored synchronously using software and a GC/EAD interface card from Syntech.

6.2.5 Chemicals Hexyl butyrate was purchased from Roth (Karlsruhe, Germany). (*E*)-2-hexenyl butyrate was synthesised as described in Chapter 2. (*Z*)-9-pentacosene and (*Z*)-7-pentacosene were synthesised as described in Chapter 5.

6.2.6 Chemical analysis The analysis of extracts from male and female legs has been described in Chapter 5. The headspace extracts were analysed by gas chromatography and coupled GC/MS as described in Chapter 4.

6.2.7 Statistical analysis Results from the Y-track olfactometer and the vibration bioassay were analysed by the software programme SAS (version 8), procedure GENMOD for logistic regression (1997). The following comparisons were made:

A1: response towards the control versus response towards each of the tested extracts in the Y-track olfactomter.

A2: response towards the female leg extract versus response towards the female headspace extract.

B1: response towards each of the tested extracts or chemicals in the vibration bioassay.

6.3 Results

In Figure 6.3 the response of males in the Y-track olfactometer towards the different sources is shown. Males walked significantly more towards either the female headspace

extracts or towards the female leg extracts. In total 130 (73%) males walked towards the female headspace extract versus only 49 males who walked towards the control. In case of the female leg extract 93 (72%) of the males walked towards the extract and 37 towards the control. In case of the male headspace extract or the male leg extract, equal amounts of males walked towards the extract and the control. There was no significant difference between the female headspace extract and the female leg extract.

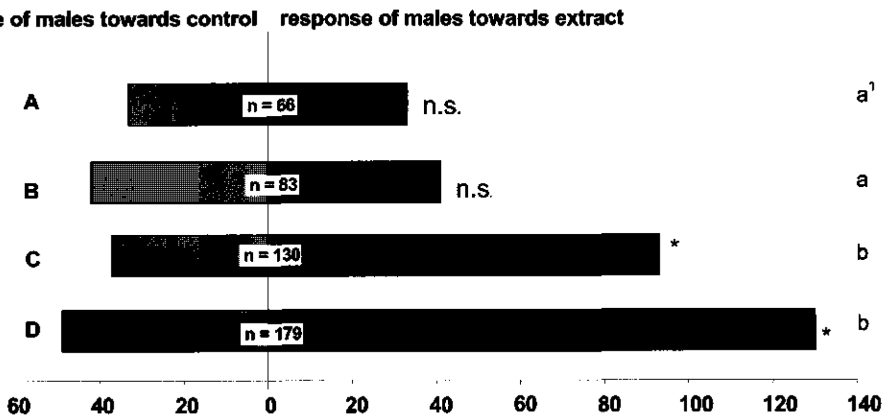

Figure 6.3 **Responses of male L. pabulinus towards different sources in the Y-track olfactometer. A: male leg extract, B: male headspace extract, C: female leg extract, D: female headspace extract. * $P < 0.001$; n.s. $P > 0.3$.**

[1] Different letters indicate significant differences ($P < 0.001$).

Males did elicit the sex specific vibration behaviour when a mixture of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene was introduced in the bioassay. About 50% of the males vibrated when (*Z*)-9-pentacosene and (*Z*)-7-pentacosene were used in the ratio 5:1 (Table 6.1). Adding hexyl butyrate and (*E*)-2-hexenyl butyrate to the stimuli did not effect the percentage of males vibrating. There was no significant difference between the female leg extract and the mixture of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene. Extracts made from male legs were inactive.

TABLE 6.1 Male vibration response to male and female *L. pabulinus* leg extracts and synthetic compounds

Source		Fraction of males Responding ± s.e.	n	
1)	Female leg extract	0.53 ± 0.28	201	a[1]
2)	(*Z*)-9-pentacosene + (*Z*)-7-pentacosene (5:1)	0.52 ± 0.17	83	a
3)	(*Z*)-9-pentacosene + (*Z*)-7-pentacosene (5:1) + hexyl butyrate and (*E*)-2-hexenyl butyrate (20:1)	0.43 ± 0.17	30	a
4)	male leg extract	0.03 ± 0.05	33	b

[1]Different letters indicate significant differences ($p < 0.001$).

The chemical composition of the leg extracts from males and females has been described in Chapter 5. In summary, females contained a high concentration of (*Z*)-9-pentacosene and males had a high concentration of (*Z*)-9-heptacosene present. Furthermore the ratio of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene was 5:1 in females and ca. 1:5 in males. In Figure 6.4 the gas chromatogram of a female leg extract is shown. The female leg extract contained very few highly volatile compounds, but a high amount of hydrocarbons. GC-EAD recordings shown in Figure 6.5 revealed that of these hydrocarbons two alkenes were EAD-active.

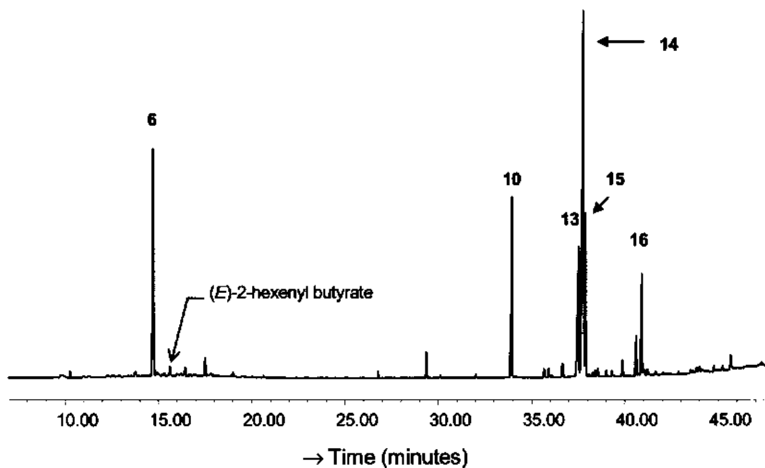

Figure 6.4 Chromatogram of GC-MS analysis of a female *L. pabulinus* leg extract. Column: DB23. 6: hexyl butyrate, 10: tricosane, 13: pentacosane, 14: (*Z*)-9-pentacosene, 15: (*Z*)-7-pentacosene, 16: (*Z*)-9-heptacosene.

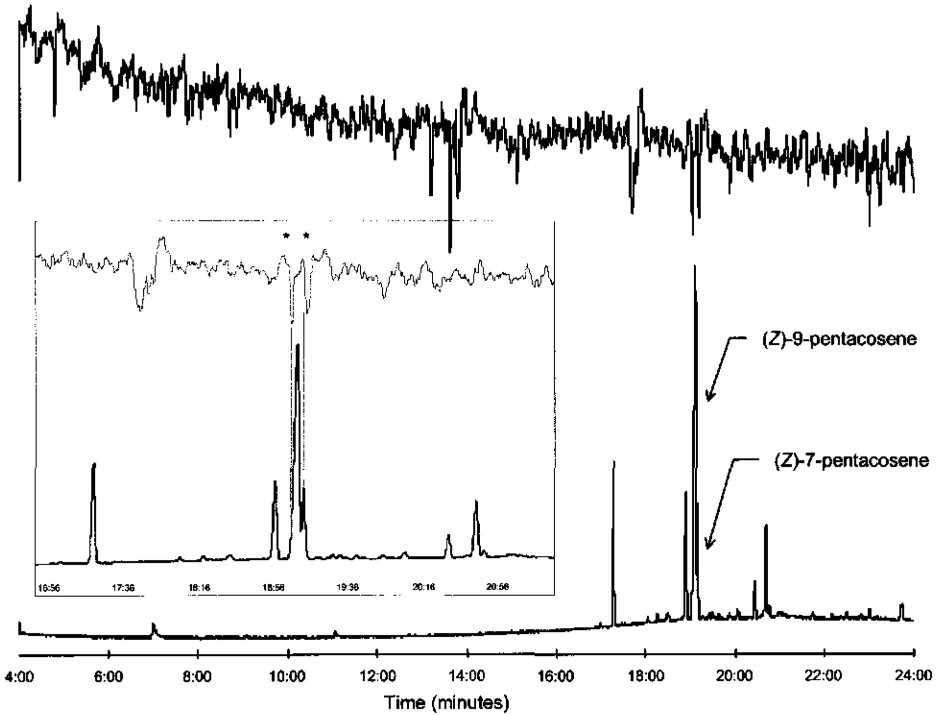

Figure 6.5 **Gas-chromatogram from the separation (on a DB23 column) of a female leg extract and corresponding EAG-responses from 4 male *L. pabulinus* antennae. * indicates consistent EAG responses. Other spikes/"signals" were not consistently showing up.**

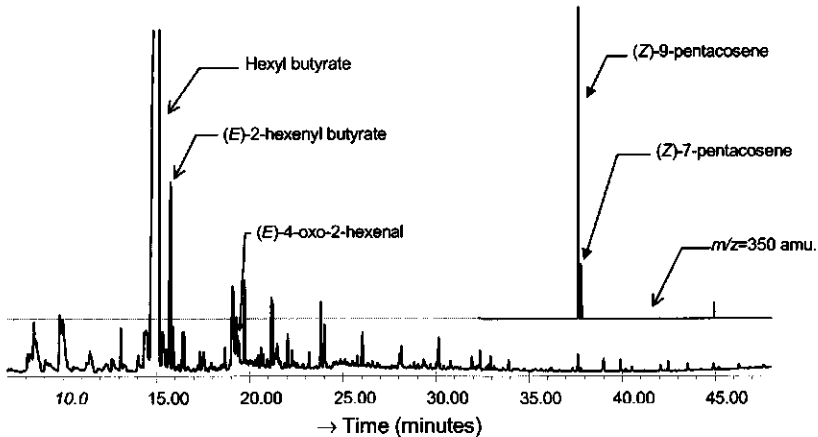

Figure 6.6 **Chromatogram of GC-MS analysis of headspace extract from female *L. pabulinus*. Insert: Reconstructed Ion Chromatogram with *m/z* value of 350 amu (M^+ of both (*Z*)-9-pentacosene and (*Z*)-7-pentacosene).**

From Figure 6.6 it is clear that female headspace extracts contain a small amount of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene.

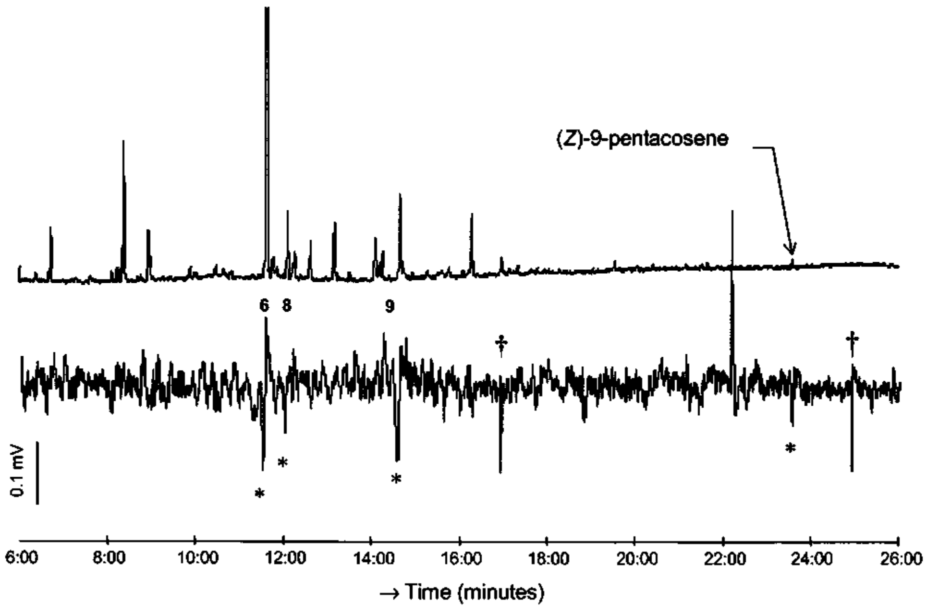

Figure 6.7 **Gas-chromatogram from the separation (on a DB23 column) of a female headspace extract and corresponding EAG-responses from 4 male *L. pabulinus* antennae. 6: hexyl butyrate, 8: (*E*)-2-hexenyl buyrate, 9: (*E*)-4-oxo-2-hexenal. * indicates EAD-active compounds. † indicates spikes.**

GC-EAD recordings of these female headspace extracts in this set-up also revealed that (*Z*)-9-pentacosene is EAD-active (Figure 6.7). Although the signals are low and some false positive EAG-responses are seen, the EAG response on (*Z*)-9-pentacosene was consistent in most of the recordings and can therefore not be characterised as a false positive EAG-response.

6.4 Discussion

All the male extracts tested in the two bioassays were neither attractive to males nor did any vibration occur in males. This is in accordance with results of Blommers et al. (1988), i.e. males are only attracted to females, and hence there is evidence for a female produced sex pheromone. Interestingly, both female headspace and female leg extract attracted males in the Y-track olfactometer. When these two different extracts were chemically compared, a distinct difference can be seen. The headspace extracts contain primarily volatile compounds, while the leg extracts contain mostly hydrocarbons (C_{23}-C_{27}), which are only marginally volatile. In contrast, female and male headspace extracts were almost identical. Variations of compounds present (Table 4.1 in Chapter 4) and different ratios between these compounds were not sex specific. Variation between males and females was similar to that between females themselves and males themselves. In addition, both male and female headspace extracts contained the three most EAD-active compounds, i.e. hexyl butyrate, (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal (Chapter 4) of which only the latter was absent in all leg extracts. However, hexyl butyrate and (*E*)-2-hexenyl butyrate failed to attract any males in the Y-track olfactometer or in the field. Furthermore, these two compounds were present in similar concentrations and ratios in the headspace and leg extracts from males, which were not attractive to males.

The only distinct difference between male and female headspace extracts was the presence of a small amount of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene in female headspace extracts (Figure 6.6). These two compounds comprised also the main difference in male and female leg extracts (Chapter 5). Interestingly (*Z*)-9-pentacosene and (*Z*)-7-pentacosene were also EAD-active in female leg extracts (Figure 6.5). Only (*Z*)-9-pentacosene elicited EAG responses in GC-EAD recordings of female headspace extracts (Figure 6.7), but this is probably due to the low concentration of (*Z*)-7-pentacosene in these extracts. Earlier GC-EAD recordings (Chapter 4) did not show any EAG responses on these alkenes, probably due to the long distance (15 cm) between the end of the column and the antennal preparation used. In the current set-up this distance is

very small (maximum of 5 mm), and therefore there is no chance of condensation of these alkenes. The signal-to-noise ratios of the responses obtained were however small compared to those with the set-up used earlier because of the unstable baseline in the current set-up. A different antennal preparation, like the one used in Chapter 4, could give a more stable baseline resulting in a higher S/N value. In order to accomplish this some technical adjustments regarding the antennal preparation have to be carried out. Therefore, a hole should be made in the Liebig condenser, near the outlet of the column, where the glass electrodes can be inserted. Although these alkenes are marginally volatile compounds, this is not the first time that EAG responses of such compounds have been recorded (Shiestl et al., 2000; Connor et al., 1980).

In *L. pabulinus*, (*Z*)-9-pentacosene and (*Z*)-7-pentacosene elicit vibration behaviour in males. Earlier (Chapter 5) no activity was found using these alkenes, although some activity was found when male legs were loaded with a 5 times higher amount of (*Z*)-9-pentacosene than present in female legs. The amounts used in the current bioassays were 10-15 times higher than in the natural samples. Why males do vibrate when a much higher dose is offered is not clear. It might be that the actual concentration on the legs is higher if the amounts are plotted per area, because the piece of filterpaper has a larger surface area than legs. In addition, compounds may be absorbed by the filterpaper, hampering their release. Little is known about the actual release rate of these rather involatile compounds, and therefore in bioassays higher amounts than present in natural samples are used (Schiestl et al., 2000).

There is still no evidence that the ratio of 5:1 is important for eliciting this vibration behaviour in males. Due to the lack of insects no experiments could be carried out with different ratios of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene. It is even not certain whether (*Z*)-7-pentacosene is really necessary to elicit vibrational behaviour. More important is that a mixture of these two alkenes induced vibration behaviour in males.

Alkenes or alkadienes are commonly used as recognition cues in (social) insects. Many reviews have been published on the function of alkenes and alkadienes in insects

(Howard and Blomquist, 1982; Blomquist et al., 1987; Lockey, 1988; Howard, 1993; Singer, 1998; Paulmier et al., 1999). It is thus clear that alkenes have different functions in insect communication. They can function as a mating stimulant pheromone, e.g. (*Z*)-9-pentacosene in the little house fly (Uebel et al., 1977), contact pheromone or even an attractant at a short distance (Carlson and Beroza, 1973; Uebel et al., 1978; Schietsl et al., 1999, 2000).

In *L. pabulinus* these two alkenes probably do not only elicit vibration behaviour, but might also act as an attractant, as the female leg extract attracted as many males as the female headspace extract in the Y-track olfactometer. As (*Z*)-9-pentacosene and (*Z*)-7-pentacosene are present in both female headspace and female leg extracts, it is likely that (*Z*)-9-pentacosene and probably (*Z*)-7-pentacosene play a role in attracting males in the Y-track olfactometer bioassay. This Y-track olfactometer is probably not suitable to distinguish between long-range and close range communication, due to the small distance (ca. 10 cm) between source (filterpaper) and insect. Because of this small distance, the Y-track olfactometer is probably a close range bioassay rather than a long-range bioassay. Attraction of males in this bioassay is thus probably due to the presence of the alkenes in female extracts. This can explain why extracts from female legs and headspace extracts are equally attractive to males, although they contained compounds of different volatility. The absence of the alkenes, or their incorrect ratio, explains why male extracts are not attractive to males. Olfactometer tests with a mixture of the two active alkenes should provide proof whether they attract males either alone or in combination with volatile compounds present in headspace extracts.

Nevertheless, it is unlikely that they serve as long-range cues in mate location. Still males could be trapped in the field (Blommers et al., 1988) indicating a long-range mate location behaviour in *L. pabulinus*. An explanation for this discrepancy could be that communication in *L. pabulinus* is indeed divided in two distinct steps: a long-range (Blommers et al., 1988; Groot et al, 1998a, 1999), and a close range step (Groot, 2000; Chapter 5). Long-range and close range communication has been found in the green

stink bug, *Nezara viridula* (Hemiptera: Pentatomidae) (Borges et al., 1987), i.e. long-range mate location and a close range courtship behaviour. When extracts from males were used instead of males, only long-range female behaviour was observed. Borges et al. (1987) concluded that this close range behaviour involved sound signals, which has also been found by other authors (Ota and Cokl, 1991; Ryan and Walter, 1992; Jeraj and Walter, 1998). Yatsynin and Rubanova (1997) reported that various extracts from the female sunn pest (*Eurygaster integriceps*) did not cause long-distance attraction in males, but grooming and copulation were provoked in the immediate vicinity. Doi et al. (1997) proposed a similar long-range communication and a close range courtship behaviour in *Drosophila ananassae*. The sex pheromone was identified as (*Z,Z*)-5,25-hentriacontadiene. It was supposed that after aggregation of males and females on the plant, involving (*Z*)-11-octadecenyl acetate and (*Z*)-11-eicosenyl acetate as aggregation pheromone (Schaner et al., 1989), male flies recognise conspecific females by this involatile hydrocarbon. Likewise, Syvertsen et al. (1995) found that once a male parasitoid *Cardiochiles nigriceps* (Hymenoptera: Braconidae) has located a female, antennation and mounting of the female are in part mediated by the alkadiene (*Z,Z*)-7,13-heptacosadiene. Thus, compounds being produced by both male and female *L. pabulinus* may act as semiochemicals in the males to locate other green capsid bugs. Once they are in the vicinity of other *L. pabulinus* they might use (*Z*)-9-pentacosene and (*Z*)-7-pentacosene to locate the females. These alkenes may trigger the decision of males to make a certain choice, as was found in the olfactometer test with the headspace extracts from females (Figure 6.3, entry C and D compared to A and B), or males will probably not enter the trap (Cardé, 1975). Furthermore field studies are necessary to investigate if males need both volatile compounds (present in headspace extracts) and alkenes (present in leg extracts) to actually enter the trap.

Alternatively, as females deposit these alkenes on the substrate (Chapter 5) males may perceive these compounds and follow the trail towards the female or start to vibrate. It is not likely that the vibration behaviour in male *L. pabulinus* is elicited by sound signals from females, as males also vibrate when dead females or only legs from fe are

offered (Chapter 5). Whether females react on this vibration by males in a way as was found in *N. viridula* (Ota and Cokl, 1991), remains unclear.

6.5 References

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Final results and discussion

7.1 Techniques

In this thesis the isolation and the identification of semiochemicals, which are being produced by *Lygocoris pabulinus* are described. Different methods (Chapter 2, 3 and 4) were used to accomplish this, and to determine whether males and females produce the same or different compounds. The sex specific compounds could be involved in the sex pheromone of *L. pabulinus.* Very promising results were obtained with the Thermal Desorption System (TDS) regarding the sex pheromone of *Campylomma verbasci.* The two sex pheromone compounds, butyl butyrate and (*E*)-2-butenyl butyrate (Smith et al., 1991), were only produced by female *C. verbasci.* Volatiles stripped from male and female *L. pabulinus* however did not show any differences, but when legs from males and females were analysed a different hydrocarbon profile was found in the two sexes. A similar difference was found when a solvent extraction method was used to make the leg extracts (Chapter 5). Trapping airborne volatiles from males and females on Tenax (Chapter 4) did not reveal sex specific compounds, which was in accordance with the TDS method. TDS analysis revealed three major peaks in male and female *L. pabulinus*: hexyl butyrate, (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal, which were also found in the headspace extract together with other (plant) products. These results are illustrative of the potential of the TDS-method. The (apparent) absence of sex specific compounds is thus not the result of the method used, but caused by the fact that there are probably no sex specific compounds in the volatile secretions of the green capsid bug, this in contrast with *C. verbasci.*

Stir Bar Sorptive Extraction (SBSE) is a useful way to extract volatiles from insects. Although the optimal conditions for using the Twister have not been investigated, similar compounds were present in analyses of Twisters compared with the

classical headspace collection via Tenax. In my opinion there are several ways (see also below) in which the Twister can be applied in pheromone research, of which only a few have been studied in Chapter 3. Additional studies to determine the optimal conditions are essential. On the other hand, a Thermal Desorption System is expensive and for this reason, SPME is likely to remain the most widely used method.

7.2 Female *Lygocoris pabulinus*

Female *L. pabulinus* did not show any calling behaviour, in contrast with two other mirids, *Distantiella theobroma* (King, 1973) and *Helopeltis clafiver* (Smith et al., 1977). Yet, this calling behaviour is more exception than rule, as in no other mirids, where sex pheromones are involved, calling behaviour could be observed or has been studied (McBrien and Millar, 1999). Because of the absence of this calling behaviour, it is still unknown when females are emitting pheromone. L.H.M. Blommers (pers. communication) found that females are active in the afternoon leading to the conclusion that they probably emit their sex pheromone at that time. In my experiments, volatiles were trapped during several days, disregarding the emission periods of females. As these extracts were as active as live females in the Y-track olfactometer (Chapter 6), the compounds involved in the attraction must also have been trapped. If females only emit sex pheromone during a short period, these compounds will be present only in small amounts, and may be overwhelmed by other compounds. Thus by comparing headspace from males and females these compounds might be overlooked. Therefore GC-EAD recordings with these extracts were carried out, but these experiments did not reveal additional active compounds, other than those already identified in the analyses of all the extracts. These results may be explained in two ways; either one or more of the EAD-active compounds (Chapters 4 en 6) attract males towards females, or attractive compounds do not give detectable EAG responses (Leal et al., 1998).

Aldrich (1988) suggested that the metathoracic scent gland (MTG) might be the site of pheromone production in mirids. Therefore the content of the metathoracic scent glands in females was studied using the TDS-method. Hexyl butyrate and (*E*)-2-hexenyl

butyrate (20:1) comprised almost 95% of the compounds present in this gland, but (*E*)-4-oxo-2-hexenal was not found. The two esters, alone or in combination, failed to attract any males; hence the metathoracic scent gland is not likely to be the sex pheromone-producing gland in *L. pabulinus*. Furthermore, hexyl butyrate and (*E*)-2-hexenyl butyrate are found in many other mirids (Gueldner and Parrot, 1978), indicating that at least more compounds must be involved in the sex pheromone. Little droplets excreted by female (and male) *L. pabulinus* at the legs were found to consist of almost pure (*E*)-4-oxo-2-hexenal, but nothing is known about the origin. Hypothetically (*E*)-4-oxo-2-hexenal can be biosynthesised from (*E*)-2-hexenyl butyrate via hydrolysis a reduction and an oxidation step and in this way it may be derived from the metathoracic scent gland. These droplets were formed when handling the insects, suggesting that they may have a defensive role (Aldrich, 1988). On the other hand, the sex specific olfactory response caused by (*E*)-4-oxo-2-hexenal, described in Chapter 3, is not typical for defensive compounds.

As little is known about when female *L. pabulinus* produce sex pheromone, it is also unknown if and when females stop producing pheromone. Groot et al. (2001) suggested that female *L. pabulinus* probably stop emitting pheromone when they are exposed to high concentrations of hexyl butyrate. Traps baited with live females and 2 dispensers (each loaded with 20 mg hexyl butyrate) failed to catch any males. In order to study the behaviour of females in the vicinity of high amounts of hexyl butyrate, headspace was taken from females in the presence of 1 dispenser with 20 mg hexyl butyrate. Because of the large amount of hexyl butyrate it was difficult to compare the results with headspace taken from undisturbed females. It was also not possible to expose the females for longer periods to hexyl butyrate, as within 15 hours all the females died. According to A.T. Groot (pers. comm.) females stayed alive during the field experiments, indicating that the concentration of hexyl butyrate in the field was probably lower. Apparently when females are exposed to high concentrations of hexyl butyrate this leads to drastic changes in their physiological state and ultimately even to death. Probably long before this time this leads to a stop in pheromone production. It

may therefore be premature to conclude (Groot, 2000) that in the presence of high concentrations of hexyl butyrate females have a choice in starting or halting the active release of sex pheromone. In addition, one should be careful in stating that the unattractiveness of females is specifically caused by hexyl butyrate. Other esters of similar volatility, such as pentyl pentanoate or octyl acetate should be tested in a similar way to determine whether the observed effect can be specifically contributed to hexyl butyrate.

This is not to say that hexyl butyrate has no effect at all on the sexual behaviour in females. Therefore more studies were carried out on females under stress conditions, maybe induced by hexyl butyrate produced by conspecifics. Some preliminary TDS-analysis of female legs showed that disturbed females (5 females in a 20 ml vial), sometimes showed a different hydrocarbon profile. Most females have a pattern as described in Chapter 5 with (*Z*)-9-pentacosene and (*Z*)-7-pentacosene present in a ratio of 5:1 and low amounts of (*Z*)-9-heptacosene. However in a few cases it was observed that females had similar amounts of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene present, or that even more (*Z*)-9-heptacosene was present than (*Z*)-9-pentacosene resembling the situation found in males.

Figures 7.1 and 7.2 show the difference in hydrocarbon pattern in an undisturbed female and a disturbed female. Unfortunately there was no possibility to test individual females (disturbed or undisturbed) in bioassays and afterwards to analyse their legs to determine the hydrocarbon pattern. One thing that became clear from these preliminary experiments is that females can have different hydrocarbon patterns, which may affect on their attractiveness to males. The hydrocarbons are thus not present in fixed amounts, but their ratio can change. This provides evidence that these hydrocarbons in females do have more functions than only to constitute a waxy layer to prevent water loss. If prevention of desiccation should be the only function, one would not expect a differentiation between males and females.

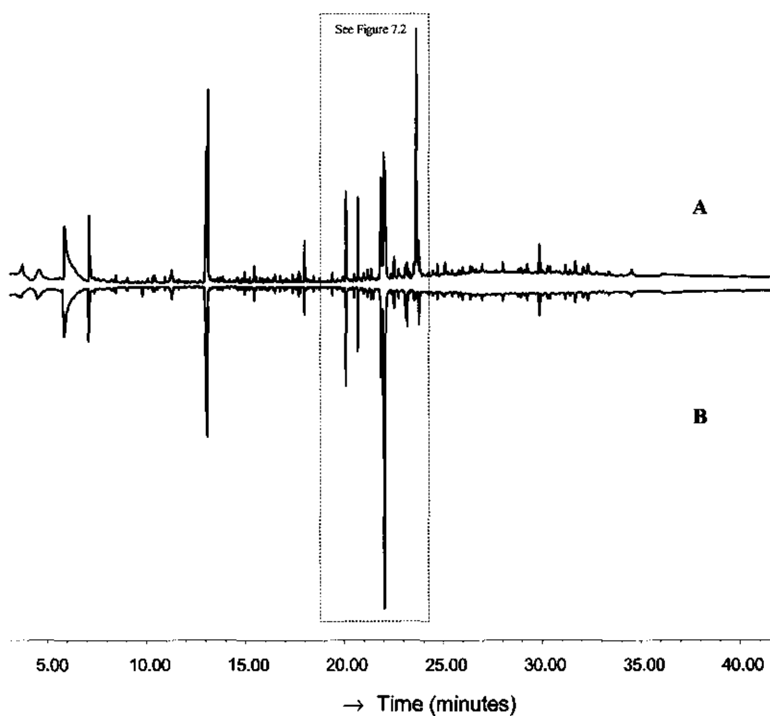

Figure 7.1 TDS analyses from legs from A: disturbed females and B: undisturbed females. Separation was carried out on a DB23 column.

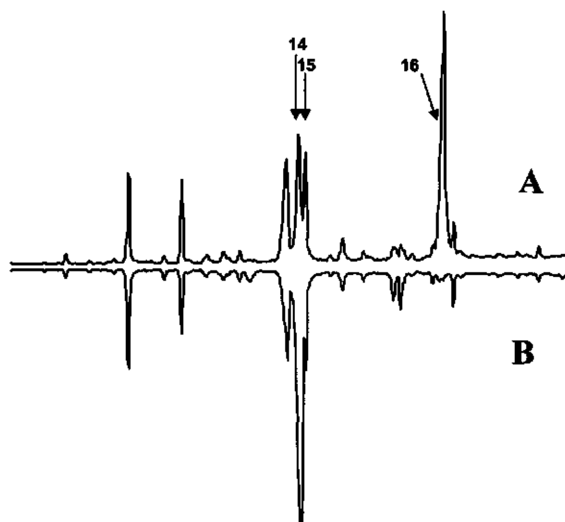

Figure 7.2 Enlargement of the dashed blocked area in Figure 1. A: Disturbed females; B: undisturbed females. Note the difference in amounts of (*Z*)-9-pentacosene (14) and (*Z*)-9-heptacosene (16). 15: (*Z*)-7-pentacosene

For numerous insects, analyses have shown that cuticular hydrocarbons are modified during the course of development (Brown et al., 1992; Provost et al., 1993 and references therein; Jurenka et al., 1998; Desena et al., 1999) or influenced by the social environment (Trabalon et al., 1988; Benziane and Campan, 1993; Howard, 1998). Although diapausing face flies (*Musca autumnalis*) had a hydrocarbon profile different from reproductive insects, males and females had similar profiles (Jurenka et al. 1998). Nevertheless, Jurenka et al. (1998) mentioned that it would be interesting to determine the hydrocarbon profiles before and just after mating, as Uebel et al. (1975) reported that female face flies use several alkenes as sex pheromones. Literature about changing hydrocarbon profiles influenced by their social environment is sparse. Trabalon et al. (1988) showed that the total hydrocarbon level of unreceptive female *Calliphora vomitoria* dropped more than 70% compared to receptive females. Additional research (Benziane and Campan, 1992) showed that there was also a slight increase in both the relative proportions of the cuticular hydrocarbons and the concentrations of all hydrocarbons when female *C. vomitoria* were reared under isolated conditions. A reduced mating success might thus be mediated by changes in cuticular hydrocarbons.

Before concluding too much from these results, it should be tested if females have the normal pattern before disturbance and if the profile changes during disturbance. Changes in the female hydrocarbon profile do not necessarily have to occur during disturbance; it can be the result of the rearing conditions (Benziane and Campan, 1992). Irrespective of when the hydrocarbon profile changes, it should be determined whether female *L. pabulinus* are receptive or unreceptive in relation to their hydrocarbon profile. If females with a different hydrocarbon profile are indeed unreceptive this will provide more evidence that the alkenes play an important role in the communication between male and female *L. pabulinus*.

Further research to determine when these changes occur is necessary. To accomplish this the following experiment might be carried out: undisturbed females should walk on or nearby a Twister, which can then be analysed. Hereafter the same female should be disturbed and again allowed to walk on or nearby a Twister. After

analysing the second Twister, and comparing it with the previous one, the hydrocarbon pattern of the same individual female can be compared in a disturbed and undisturbed situation.

7.3 Male *Lygocoris pabulinus*

Like females, male *L. pabulinus* are also subjected to stress and may therefore not always be responsive to pheromones. Extracts tested twice in bioassays did not always give the same results, indicating that males (and females) must be in an appropriate physiological state to respond to an offered stimulus (or to produce pheromone). It is however unknown which conditions make *L. pabulinus* males become unresponsive to pheromones. Once during EAG experiments males did not respond electrophysiologically to (*E*)-4-oxo-2-hexenal (Figure 7.3).

In Chapter 4 (*E*)-4-oxo-2-hexenal was found to be an EAD-active compound giving high EAG responses. Because the peak consisting of hexyl butyrate and (*E*)-2-hexenyl butyrate (6+8 in Figure 7.3) did give clear EAG-responses, as normal (see Chapter 4), one cannot conclude that something was wrong with the EAG set-up. Besides, a mixture of (*E*)-4-oxo-2-hexenal, (*E*)-2-hexenyl butyrate and (*Z*)-3-hexenyl butyrate showed only EAG responses for the two esters.

It is very peculiar that only one compound is not EAD-active at all, while normally this compound gives the best EAG response, even at low concentrations (see Figure 4.3 and Figure 4.4). The males used for this experiment all came from one rearing cage. A possible explanation for males not responding is a blockage of the receptor on the male antenna. This intriguing result can explain why in some cases males do not react positively in the Y-track olfactometer or in the vibration bioassay.

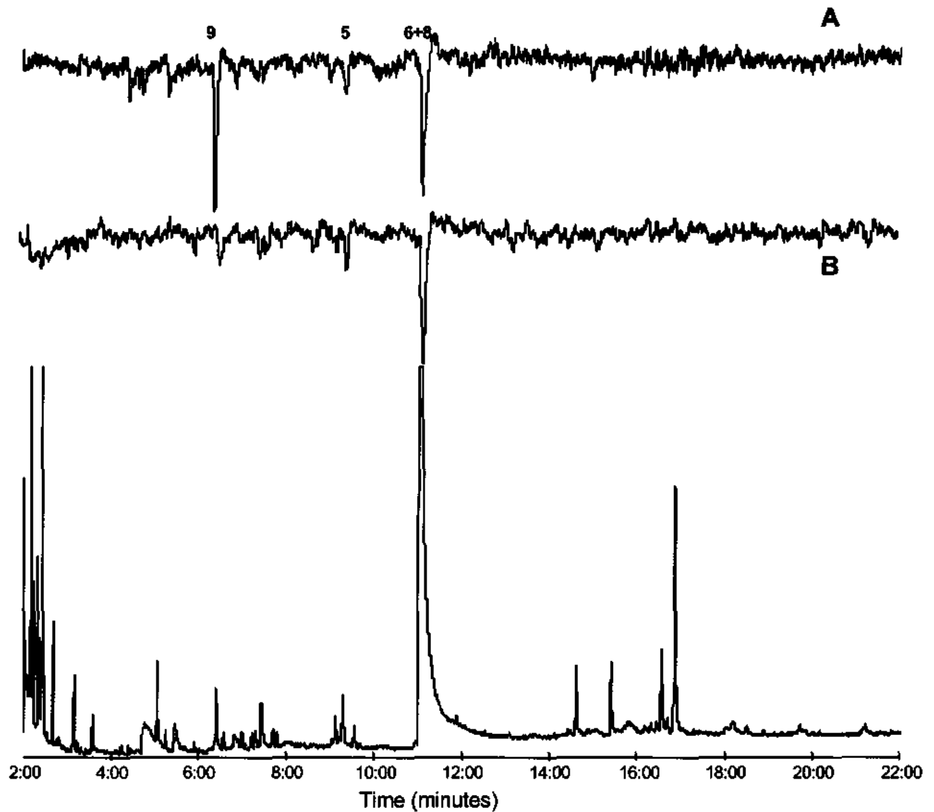

Figure 7.3 Gas chromatogram from the separation on a DB5 column of a female headspace extract and corresponding EAG-recordings from the antenna of a male *L. pabulinus* as described in Chapter 4. The data represents 2 GC-injections using the same extracts in the same amount. A: antenna responding to hexyl butyrate and (*E*)-2-hexenyl butyrate (6+8), (*E*)-4-oxo-2-hexenal (9) and small response on nonanal (5). B: antenna responding to hexyl butyrate and (*E*)-2-hexenyl butyrate (6+8) and small response on nonanal (5) but NOT responding to (*E*)-4-oxo-2-hexenal (9).

Blocking of receptors has been noticed in other insects as well. Roelofs and Comeau (1971) did extensive field studies on compounds having synergistic or inhibitory effects in traps baited together with the sex pheromone of the red-banded leaf roller, *Argyrotaenia velutinana*. Other studies on pheromone perception in *Trichoplusia ni* and *Mamestra brassicae* moths showed that N-alkylmaleimides are able to block the receptor irreversibly (Berger and Estes, 1987; Renou and Brousset, 1994). No response

recovery was found in treated insects after 24 h, although no lethality was observed. Although high concentrations of the sex pheromone also caused inhibition of the EAG response, this was reversible within 15 minutes (Renou and Brousset, 1994). Studies done on pheromone detection with EAG in the processionary moth, *Thaumetopoea pityocampa*, showed an inhibition of the EAG-response up to 95 % when insects were exposed to pheromone inhibitors just before EAG-recordings (Parrilla and Guerrero, 1994). Tests in the field with traps baited with the sex pheromone formulated together with pheromone analogues, showed a significant reduction of trap catches in several species (Riba et al., 1994; Parrilla and Guerrero, 1994) suggesting that males were unable to sense the sex pheromone with their antennae. It is however premature to explain this discovery solely by a blocking effect. To better understand the behaviour of *L. pabulinus*, with respect to stress, additional research is essential.

From the results described above it can be concluded that both male and female *L. pabulinus* are sensitive to stress. This stress may affect the behaviour in females and stop the production of sex pheromone or change the hydrocarbon pattern. Stress may also affect the behaviour of males, by not responding to offered stimuli or by not responding positively in bioassays. Furthermore, it became clear that identification of the sex pheromone of the green capsid bug was not straightforward and that chemical communication between males and females in *L. pabulinus* is more complex than originally assumed.

7.4 Applications in the field

In Chapter 6 the role of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene as attractants was already discussed. The question arises whether males can perceive these alkenes from a certain distance or only by making contact. Experiments done with SPME showed that a relatively high amount of these alkenes were found on the fibre. As the fibre was always in the vicinity of the insects, female *L. pabulinus* could easily walk on the needle, and deposit low-volatile compounds on the fibre. Walking on the fibre was

also observed with a video camera. Even when the fibre was held nearby dead females without touching them, these hydrocarbons were found on the needle (Figure 7.4).

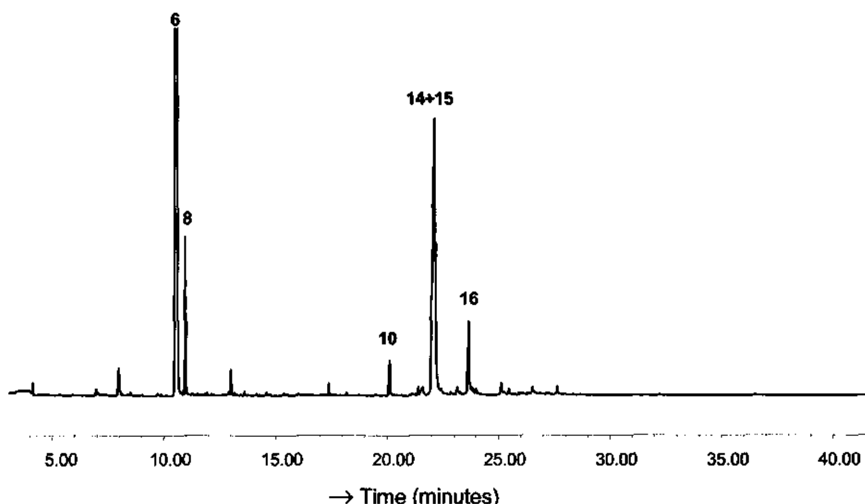

Figure 7.4 Volatiles from 6 dead female *L. pabulinus* analysed with SPME on a DB23 column. The SPME fibre was placed with females in 4 ml vial during 18 hours and did not contact the dead bodies. 6: hexyl butyrate, 8: (*E*)-2-hexenyl butyrate, 10: tricosane, 14: (*Z*)-9-pentacosene, 15: (*Z*)-7-pentacosene, 16: (*Z*)-9-heptacosene.

This gives evidence that (Z)-9-pentacosene and (Z)-7-pentacosene are present in the headspace of dead females, which leads to the conclusion that males also should be able to perceive these marginally volatile compounds, without touching females or leaves on which females had walked. Various studies have shown that cuticular hydrocarbons are involved in sexual attractiveness (for a review see Blomquist et al., 1987). Some of these compounds have been described as pheromones involved in the sexual communication e.g. (Z)-9-tricosene in *Musca domestica* (Carlson et al., 1971), (Z)-7-tricosene in *Drosophila stimulans* (Howard and Bomquist, 1982) or (Z)-11-hentriacontene in *Fannia pusio* (Uebel et al., 1978).

To establish the real function of the alkenes together with the EAD-active compounds windtunnel and field studies are essential. A mixture of all possibly EAD-active compounds, i.e. hexyl butyrate, (*E*)-2-hexenyl butyrate, (*E*)-4-oxo-2-hexenal and

probably nonanal, 1-hexanol, hexyl acetate and (*Z*)-3-hexenyl butyrate, should be tested both in the windtunnel as in the field to determine whether males are attracted to these compounds. Addition of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene should reveal if the number of males respond or trapped increases. Apart from these EAD-active compounds it remains unclear if tricosane plays a role in the (sexual) communication in *L. pabulinus*. From the Figures 1.7 and 5.3 it is clear that this alkane is more present in females than in males. Tricosane may function as an aggregation (or alarm) pheromone, as was proposed for tridecane in nymphs from *Nezara viridula* (Lockwood and Story, 1985).

Earlier experiments with dispensers in the field had no success in trapping males. Compounds used for these dispensers are listed in Table 7.1. Yet, several aspects have to be taken into account, before drawing any conclusions. First of all, the control traps (baited with three live females) caught an average of only 4 males per trap per week in 1998 or even worse, 1-2 males per trap per week in 1999 (Groot, 2000). Secondly, the instability of (*E*)-4-oxo-2-hexenal should be considered. Although some antioxidants were added to the dispensers it is not certain whether this unstable keto-aldehyde will not decompose too soon. Thirdly, almost no variation in the total amount of active ingredients in the dispenser was tested, although this can be of importance (Millar and Rice, 1997). Finally, only the delta traps used by Blommers et al. (1988) were used. When the alkenes are essential, it may be important to investigate the trap-design. Carlson and Beroza (1973) used seven different traps baited with 0.5 – 100 mg of (*Z*)-9-tricosene (muscalure). The electric grid traps were most effective, probably not because of a better release rate, but because flies caught in the other traps could escape from these traps. In addition Schiestl et al. (2000) were able to attract bees from a distance of about 50 cm with dummy-bees which were scented with C_{20}-C_{29} hydrocarbons. Because little is known about the exact release rate of these alkenes, a different trap might be necessary to catch the green capsid bug. Further research is essential to define the release rate of the alkenes in relation to the trap.

TABLE 7.1 Cmpounds used for lures in the field [a]

(*E*)-2-hexenyl butyrate	pentyl butyrate
(*E*)-2-octenyl butyrate	(*E*)-2-hexenal
(*E*)-4-oxo-2-hexenal	nonanal
(*Z*)-3-hexenyl butyrate	1-hexanol
hexyl butyrate	(*Z*)-9-tricosene
butyl acetate	(*Z*)-7-pentacosene
hexyl acetate	(*Z*)-9-pentacosene

[a] Compounds were used in different ratios

Although the ultimate aim formulated in Chapter 1 has not been reached, substantial data on the semiochemicals of both male and female *L. pabulinus* have been gathered. In addition, important knowledge about the function of the hydrocarbons in *L. pabulinus* was obtained. This research also showed that GC-EAD is a valuable tool in order to determine electrophysiological compounds present in secretions from bugs. Besides GC-EAD, several new techniques, i.e TDS, SBSE and SPME could be applied in the identification of both volatile and marginally volatile compounds from mirids. Especially the TDS has been found useful to identify marginally volatile compounds, such as cuticular hydrocarbons present in males and females (Chapter 2 and Figure 7.1). Several studies have shown that these hydrocarbons are useful compounds in chemotaxonomy (Lockey, 1991). Application of TDS in chemotaxonomy has therefore a wide perspective.

Nevertheless, the sex pheromone has not been identified yet, but several compounds have been found in male and female *L. pabulinus* eliciting either an EAG response or a behavioural response in males (Chapter 4 - 6). Unfortunately, only a few experiments in the field were conducted with these compounds. There is still a lot of work to be done before final conclusions can be drawn on the application of the sex pheromone of *L. pabulinus* in IPM. In principle, monitoring the pest with its sex pheromone is possible, but Groot (2000) feels that in practice sex pheromone traps will

probably not be efficient or reliable tools. To support this statement, Groot (2000) refers to the low trap catches with caged females. Monitoring the pest with its sex pheromone is therefore probably difficult and may not be feasible as only one male per trap already indicates that damage thresholds will be exceeded (van den Ende, 1996). This does not mean that *L. pabulinus* cannot be controlled by means of its sex pheromone. Mating disruption might still be a promising possibility. The sex pheromone of *Campylomma verbasci* could be identified, yet catches in the field were not high. Caged females caught between 1-2 males per day (Smith et al., 1991). Blommers et al. (1988) caught an average of about 5 male *L. pabulinus* per week, which is more or less similar to the trap catches for *C. verbasci*. In spite of the low number of male *C. verbasci* trapped, mating disruption with the sex pheromone of *C. verbasci* has been successful (McBrien et al., 1996, 1997). According to these results mating disruption of *L. pabulinus* might be feasible, although there is still a long way to go.

In conclusion monitoring the green capsid bug by means of its sex pheromone needs to be further evaluated once the right blend of essential compounds is known. In addition, controlling this mirid pest by mating disruption might also be feasible. Several candidate compounds are now available to be tested in the field, to determine which of these compounds are necessary for controlling *L. pabulinus*.

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Summary

The green capsid bug, *Lygocoris pabulinus* (L.) (Heteroptera: Miridae) is a serious pest in fruit orchards, which is difficult to control. Because it is difficult to determine the actual population density, fruit growers apply insecticides against the green capsid bug on regular times to reduce the risk of crop damage (calendar spraying). Previous studies have shown that males are attracted to females and this might open the possibility to monitor this mirid pest with its sex pheromone. This ultimately would lead to a reduction in the usage of insecticides. Therefore this study to unravel the chemical structure and composition of the sex pheromone of this mirid was undertaken.

Several methods were used to collect and identify volatiles emitted by male and female *L. pabulinus*. First of all, a thermodesorption system (TDS) was used. Intact females, males or pheromone glands were placed in the oven part of the TDS, which was subsequently heated, thereby stripping volatile compounds from the insect. With this method the composition of the known sex pheromones of *Adoxophyes orana* (Lepidoptera: Tortricidae) and *Campylomma verbasci* (Heteroptera: Miridae) were confirmed, using only a single insect per analysis. The advantages of this rapid method are its high sensitivity and the low degree of degradation and contamination of the stripped off compounds. This technique was effective in analysing volatiles from small insects by gas chromatography without prior manipulation, such as solvent extraction or distillation.

Secondly, two similar methods were used to extract compounds from the headspace of female *L. pabulinus*. These methods, Solid Phase Microextraction (SPME) and Stir Bar Sorptive Extraction (SBSE) were both successful in trapping volatiles from *L. pabulinus*. SBSE was found to be much more sensitive than SPME, but SPME was easier to operate.

Bioassays play an important role to determine the biological activity of extracts or compounds. A disadvantage of the methods described above is that no material can be gathered to use in any bioassay. Therefore samples from both sexes of *L. pabulinus* were collected in two other ways. Firstly, headspace extracts were obtained by trapping volatiles from males and females on Tenax and subsequently eluting the Teanx with an organic solvent. Secondly, extracts were made from different body parts of the green capsid bug.

In order to determine the biological activity of these extracts and of the pure compounds, two different bioassays were used, 1) a vibration bioassay and 2) a Y-track olfactometer.

In the so-called vibration bioassay the specific courtship behaviour, i.e. a vibration of the abdomen, of male *L. pabulinus* was used. When both live and dead females were offered to males in this bioassay, vibration behaviour was elicited. When females were dissected into separate body parts, heads, wings and legs elicited equal responses, while thorax plus abdomen gave a much lower response. When these separate body parts were extracted with an organic solvent, the leg extracts elicited significantly stronger responses than any other extract. This suggests that female *L. pabulinus* legs are either the source of a (close-range) sex pheromone, or that female *L. pabulinus* accumulates the pheromone on the legs by grooming behaviour. Live and dead males or male legs did not elicit any vibration behaviour in males. Substrates on which females had walked elicited similar responses in males as female legs, indicating that the female deposits the pheromone on the substrate. This occurs passively as no depositing behaviour was observed in the females.

In the Y-track olfactometer, males had to choose between two different sources of volatiles: the male or female extract to be tested and the solvent used to make these extracts as a control. Headspace extracts from male and female *L. pabulinus*, as well as male and female leg extracts were tested in the Y-track olfactometer. Both the female leg and headspace extracts attracted males. Male extracts had no activity at all.

All the extracts were analysed by Gas Chromatography/Mass Spectrometry (GC/MS) and coupled Gas Chromatography-Electroantennography (GC-EAD). Male and female headspace had an almost similar profile, except for a small amount of mono-alkenes, sometimes present in female headspace extracts. Chemical analysis of the leg extracts showed that these contained several hydrocarbons such as *n*-alkenes, *n*-alkanes and some methylalkanes. Female leg extracts contained more (*Z*)-9-pentacosene while male leg extracts contained more (*Z*)-9-heptacosene. Furthermore, two alkenes were present in different ratios in males and females. (*Z*)-9-pentacosene and (*Z*)-7-pentacosene were present in the ratio of 5:1 in females, but in about 1:5 in males. (*Z*)-9-pentacosene and (*Z*)-7-pentacosene were also the two alkenes observed in female headspace extracts.

GC-EAD recordings with the headspace extracts from both males and females revealed that three compounds were consistently EAD-active. These were hexyl butyrate, (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal. These compounds were also found with the thermal desorption system (TDS) in males and females. Besides these compounds, sometimes EAG responses were obtained for 1-hexanol, hexyl acetate, nonanal and (*Z*)-3-hexenyl butyrate. In a different GC-EAD set-up the female leg extracts as well as the female headspace extracts were analysed. These recordings showed that also (*Z*)-9-pentacosene and (*Z*)-7-pentacosene were EAD-active.

Although female headspace and female leg extracts differ much in composition, both attracted males in the Y-track olfactometer. On the contrary, male leg extracts did not attract males in this bioassay. The only difference between male and female leg extracts was the ratio of various alkenes. A mixture of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene in the ratio 5:1 elicited vibrational behaviour in males. The results indicate that these alkenes are important cues for male *L. pabulinus* and are probably also responsible for the attraction of males in the Y-track olfactometer by female headspace extracts.

Male antennae reacted strongly to hexyl butyrate, (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal whereas female antennae gave little or no response, suggesting that these compounds may be important chemical signals as well for male *L. pabulinus* in

their communication with conspecifics. These three compounds, with or without any of the other compounds giving irregular EAG-responses, together with the alkenes are probably used by male green capsid bugs to locate females. Further research is needed to determine exactly which compounds are needed in a particular ratio to attract male *L. pabulinus*.

Samenvatting

De groene appelwants, *Lygocoris pabulinus* (L.) (Heteroptera: Miridae) is een lastig te bestrijden plaag insect in de fruittteelt. Een betrouwbare methode om de populatiedichtheid te bepalen, ontbreekt. Daarom spuiten fruittelers elk jaar op geregelde tijden tegen dit insect. Eerder onderzoek heeft aangetoond dat de mannetjes van de groene appelwants gelokt worden door vrouwtjes. Daarom is een studie verricht naar het seksferomoon van deze wants met het doel synthetisch seksferomoon bij de bestrijding van deze plaag in te zetten.

Diverse methoden werden in dit onderzoek gebruikt om de stoffen die door mannetjes en vrouwtjes van de groene appelwants worden afgescheiden te identificeren. Als eerste werd een zogenaamd Thermisch Desorptie Systeem (TDS) gebruikt. Met deze methode is het mogelijk om stoffen van het insect op de kolom van een gaschromatograaf (GC) te brengen door middel van verhitting van het monster. Intacte mannetjes en vrouwtjes, dan wel delen van het insect werden in een buis in de oven van de TDS gebracht. Na verhitting van de oven werden de stoffen die vrijkwamen geconcentreerd door middel van een zgn. koude val (−150°C) in de injector van de GC. Door snelle verhitting van de koude val (injector) werden de stoffen op de kolom gebracht, waarna deze stoffen gescheiden kunnen worden. Met deze methode werd de samenstelling van het seksferomoon van zowel de toortswants, *Campylomma verbasci* (Heteroptera: Miridae) als de vruchtbladroller, *Adoxophyes orana* (Lepidoptera: Tortricidae) bevestigd. Slechts een enkel insect was hiervoor nodig. Het voordeel van deze snelle methode is de hoge gevoeligheid en kleine kans op ontleding van de vrijgekomen stoffen en verontreinigingen. De methode is vooral geschikt voor de analyse van stoffen die door kleine insecten worden geproduceerd.

Als tweede werden twee verschillende methoden getest om stoffen uit de headspace van de groene appelwants te extraheren. Deze methoden, Solid Phase MicroExtraction (SPME) en Stir Bar Sorptive Extraction (SBSE), zijn met elkaar vergeleken om te bepalen 1) of ze geschikt zijn als concentratie stap en 2) of er een voorkeur voor één van de twee methoden is. Beide methoden waren geschikt als

concentratie stap. SBSE is veel gevoeliger dan SPME, maar SPME kan veel gemakkelijker toegepast worden.

Biotoetsen zijn van groot belang om de biologische activiteit van extracten en zuivere stoffen aan te tonen. Een nadeel van de hierboven genoemde methoden is dat er niet voldoende materiaal verzameld kan worden om biotoetsen mee uit te voeren. Daarom werden op twee andere manieren stoffen verzameld voor de biotoetsen. Dit werd in de eerste plaats gedaan door de vluchtige stoffen die door de wantsen werden afgescheiden op een absorbens (Tenax) te verzamelen en vervolgens met een organisch oplosmiddel van de Tenax af te spoelen. Bij de tweede methode werden bepaalde delen van het insect, waaronder de poten geëxtraheerd met een organisch oplosmiddel.

Twee biotoetsen werden toegepast om de activiteit van deze extracten te testen. De eerste biotoets berustte op het sekse specifieke vibratiegedrag van de mannetjes. Mannetjes gaan namelijk trillen met het achterlijf als ze in de buurt van vrouwtjes komen. Mannetjes bleken dit trillingsgedrag te vertonen als levende of dode vrouwtjes werden aangeboden. Verschillende delen van het insekt, zoals poten, vleugels en kop gaven een zelfde respons te zien. Het borststuk met het achterlijf gaf een veel lagere respons. Wanneer deze verschillende delen met een organisch oplosmiddel werden geëxtraheerd, induceerde de extracten van de poten duidelijk veel meer trillingsgedrag dan de andere extracten. Dit zou erop kunnen wijzen dat de poten een bron van een zogenaamd korte afstand feromoon zijn. Een andere mogelijkheid is dat deze stoffen ergens anders geproduceerd worden, maar dat ze door het poetsgedrag van de vrouwtjes op de poten terechtkomen. Mannetjes van de groene appelwants, levend of dood, veroorzaakten geen enkel trillingsgedrag als ze aan andere mannetjes werden aangeboden. Als een bepaald substraat, bijvoorbeeld blad, waarop een vrouwtje van de groene appelwants een tijd lang had gelopen, aan mannetjes werd aangeboden, werd dit trillingsgedrag ook waargenomen. Dit geeft aan dat deze stoffen ook door de vrouwtjes aan het substraat worden afgegeven. Dit gebeurt waarschijnlijk passief, aangezien geen enkel actief depositie gedrag in vrouwtjes werd waargenomen.

In de tweede biotoets, een verticale Y-staaf olfactometer, konden de mannetjes kiezen uit twee aangeboden geurbronnen; het te testen extract en als controle het oplosmiddel gebruikt voor het maken van het extract. De headspace van vrouwtjes en mannetjes, evenals de extracten van de poten van vrouwtjes en mannetjes werden in de Y-staaf olfactometer getest. Zowel de headspace als de extracten van de poten van vrouwtjes waren aantrekkelijk voor de mannetjes. Geen van de mannetjesextracten was aantrekkelijk, dat wil zeggen de mannetjes maakten geen keuze tussen de extracten en de controle.

Alle extracten werden geanalyseerd met gekoppelde gaschromatografie en massa spectrometrie (GC/MS) en gekoppelde gaschromatografie en electroantennografie (GC-EAD). Uit de GC/MS analyses bleek dat er bijna geen verschil was tussen de headspace van mannetjes en vrouwtjes; een aantal alkenen werden echter alleen in de headspace van de vrouwtjes gevonden. Chemische analyse van de potenextracten van mannetjes en vrouwtjes wees uit dat deze diverse koolwaterstoffen bevatten, zoals *n*-alkenen, *n*-alkanen en methylalkanen. Er was een duidelijk verschil in verhouding van deze koolwaterstoffen in mannetjes en vrouwtjes. De extracten van de poten van de vrouwtjes bevatten meer (*Z*)-9-pentacoseen dan de extracten van de pootjes van de mannetjes, terwijl de extracten van de pootjes van de mannetjes juist meer (*Z*)-9-heptacoseen bevatten. Verder was de verhouding waarin (*Z*)-9-pentacoseen en (*Z*)-7-pentacoseen in de extracten van de poten van mannetjes en vrouwtjes voorkwam niet hetzelfde. In vrouwtjes was deze verhouding 5:1 ten gunste van (*Z*)-9-pentacoseen terwijl dat in mannetjes ongeveer 1:5 was. Deze twee alkenen werden soms ook in de headspace van vrouwtjes gevonden.

Uit de GC-EAD afleidingen, van zowel mannetjes als vrouwtjes headspace extracten, kan worden afgeleid dat drie verbindingen constant goede EAG responsen geven. Deze verbindingen werden geïdentificeerd als hexyl butyraat, (*E*)-2-hexenyl butyraat en (*E*)-4-oxo-2-hexenal. Deze verbindingen werden al in een eerder stadium met de TDS-analyses gedetecteerd. Naast deze drie verbindingen werd een aantal andere verbindingen gevonden die alleen af en toe een EAG respons gaven. Deze

verbindingen zijn 1-hexanol, hexyl acetaat, nonanal en (*Z*)-3-hexenyl butyraat. Tijdens GC-EAD metingen op een andere opstelling werden ook EAG responsen gevonden voor (*Z*)-9-pentacoseen en (*Z*)-7-pentacoseen.

Alhoewel de potenextracten van vrouwtjes en de headspace van vrouwtjes erg van elkaar verschilden in samenstelling, waren beide aantrekkelijk voor mannetjes in de Y-staaf olfactometer. Het extract van de pootjes van mannetjes was dat echter niet. Vergelijking van de extracten van de poten van mannetjes en vrouwtjes leverde een verschil in de alkenen samenstelling. Verder werd gevonden dat een synthetisch mengsel van (*Z*)-9-pentacoseen en (*Z*)-7-pentacoseen in de verhouding 5:1 eveneens trillingsgedrag in mannetjes opwekte. Deze resultaten geven aan dat deze alkenen een belangrijke rol spelen bij het gedrag van de mannetjes van de groene appelwants. Het is heel goed mogelijk dat deze alkenen ook verantwoordelijk zijn voor de aantrekking van de mannetjes in de Y-staaf olfactometer proeven aangezien deze alkenen ook in de headspace van vrouwtjes gevonden werden.

De antennes van de mannetjes waren beduidend gevoeliger voor de EAG actieve stoffen dan de antennes van vrouwtjes. Hexyl butyraat, (*E*)-2-hexenyl butyraat en (*E*)-4-oxo-2-hexenal gaven sterke EAG signalen met de antennes van mannetjes, terwijl de antennes van de vrouwtjes weinig tot geen EAG signalen gaven. Dit geeft aan dat deze drie stoffen juist voor de mannetjes erg belangrijk zijn in de communicatie met andere soortgenoten. Deze drie stoffen, met of zonder de stoffen die alleen af en toe een EAG respons gaven, in combinatie met de alkenen worden waarschijnlijk door de mannetjes gebruikt bij het zoeken naar vrouwelijke soortgenoten. Verder onderzoek zal moeten uitwijzen welke combinatie van deze verbindingen in welke verhouding in staat is mannetjes van de groene appelwants te lokken.

List of abbreviations used in this thesis and numbering of compounds

Abbreviations

DMDS	dimethyl disulphide
EAG	electroantennogram
FE	female equivalent
FID	flame ionisation detector
FT-IR	Fourier transform infra red spectroscopy
GC	gas chromatograph
GC/MS	coupled gas chromatography-mass spectrometry
GC-EAD	coupled electroantennography-gas chromatography
MTG	metathoracic gland
NMR	nuclear magnetic resonance
PTV	programmable temperature vaporiser
SBSE	stir bar sorptive extraction
SPME	solid phase microextraction
TDS	thermal desorption system

Numbering of compounds

1	butyl butyrate
2	(*E*)-2-butenyl butyrate
3	1-hexanol
4	hexyl acetate
5	nonanal
6	hexyl butyrate
7	(*Z*)-3-hexenyl butyrate
8	(*E*)-2-hexenyl butyrate
9	(*E*)-4-oxo-2-hexenal
10	tricosane
11	(*Z*)-9-tricosene
12	2-methyltetracosane
13	pentacosane
14	(*Z*)-9-pentacosene
15	(*Z*)-7-pentacosene
16	(*Z*)-9-heptacosene

Curriculum Vitae

Falko Pieter Drijfhout werd op 18 augustus 1971 geboren in Pretoria, Zuid-Afrika. Zijn middelbare schoolloopbaan werd gestart op Hoërskool "De Kuilen" in Kuilsrivier (Zuid-Afrika). Na verhuizing naar Nederland in 1987 werd het schoolleven voortgezet aan het "Gomarus College" in Groningen en in 1991 afgerond aan de Gereformeerde Scholengemeenschap "Guido de Brès" in Amersfoort met een VWO-diploma.

Academische vorming werd genoten aan de Landbouwuniversiteit in Wageningen, thans Wageningen Universiteit geheten. Binnen de studie moleculaire wetenschappen werden bij de volgende vakgroepen afstudeervakken gedaan: Bio-Organische Chemie (prof. Ae. de Groot, dr. T.A. van Beek en dr. M.A. Franssen), Biochemie (prof. C. Veeger en dr. W.H. van Berkel), Entomologie (prof. J.C. van Lenteren en dr. J.J.A. van Loon). Het diploma werd behaald in augustus 1996.

Van september 1996 tot oktober 2000 werd als onderzoeker in opleiding in dienst van NWO een promotieonderzoek verricht aan de vakgroep Organische Chemie van Wageningen Universiteit, onder leiding van prof. Ae. de Groot en dr. T.A. van Beek, hetgeen beschreven is in dit proefschrift.

List of Publications:

Fraaije, M.W., **Drijfhout, F.P.**, Meulenbelt, G., and van Berkel, W.J.H., 1997. Vanillyl-alcohol oxidase from *Penicilium simplicissimum*: Reactivity with *p*-cresol and preliminary structural analysis, pp. 261-264. In: K. Stevenson, V. Massey and Ch. Williams, Jr (eds), Flavins and Flavoproteins XII. University Press, Calgary, Canada.

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Drijfhout, F.P., Groot, A.T., van Beek, T.A., and Visser, J.H. Coupled Gas Chromatographic - Electroantennographic responses of *Lygocoris pabulinus* (L.) to female and male produced volatiles. In preparation.

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