THE CHEMICAL DIVERSITY OF MIDGE PHEROMONES

LAKMALI AMARAWARDANA

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ABSTRACT

Midges (Diptera: Cecidomyiidae) are pests of many economically valuable agricultural crops. The female sex pheromones of several midge species have been identified and the hypothesis was that midge sex pheromones could be chemically more diverse in structure than previously thought. This hypothesis was tested in the context of four midge species of importance to UK horticulture: pear leaf midge, *Dasineura pyri,* pear midge, *Contarinia pyrivora,* blackcurrant midge, *D. tetensi* and blackberry midge, *D. plicatrix*. The major component of the pheromone of *D. pyri* was identified as (2*R*,13*R*, 8*Z*)-2,13-diacetoxy-8-heptadecene. Four isomers were separated by high performance liquid chromatography (HPLC) and in field tests the first eluting isomer only was attractive to male midges. Addition of the second eluting isomer in 1:1 ratio greatly reduced the catches. The minor component is yet to be identified. Analysis of volatile collections from female *C. pyrivora* by gas chromatography (GC) coupled to electroantennographic recording (EAG) showed two consistent responses from male midges and they were identified as 2,7-diacetoxyundecane and 7-acetoxyundecane-2-one respectively. The field testing with isomers of 2,7-diacetoxyundecane separated by HPLC revealed that the first and the third eluting isomers were attractive. The racemic 7-acetoxyundecane-2-one was active as well as the first eluting isomer from HPLC. Combination of the active isomer of 7-acetoxyundecane-2-one with that of 2,7-diacetoxyundecane in 1:10 ratio did not show a significant increase in the trap catch. Two EAG active components were detected in *D. tetensi* female volatile collections. The major component was identified as (*Z*)-2,12-diacetoxy-8-heptadecene and after separation of stereoisomers by HPLC the third eluting isomer has shown to be attractive to male *D. tetensi* in the field. The structure for the minor component was proposed as a keto-acetate homologue of the corresponding major component. Preliminary work carried out on identification of the female sex pheromone of *D. plicatrix* indicated two responses from conspecific males. These were shown to be 15-carbon acetates with the acetate function at C-2, probably with two and one double bonds respectively. New or improved methods for rearing midges, collecting midge pheromones, setting up EAG preparation and separating stereoisomers by HPLC are reported. The pheromones identified are all novel structures; but related to those previously identified as midge sex pheromones with only one representation of the new class of components, keto acetates pheromones will provide tools for growers to monitor the pests as part of integrated pest management programmes.

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INTRODUCTION

1.1. CHEMICAL COMMUNICATION

1.1.1. Communication in insects

Exchange of information among individuals is an essential aspect of social interactions. Like other members of the animal kingdom, insects communicate with each other. They may convey messages by making a noise, releasing odours, beating wings or by changing surface chemistry. The sensory organs are specialised to acquire signals from the vicinity, as movements, vibrations, incidence of light and smell. There are varying types of sensory organs located on different body parts. Mechanoreceptors (specialised hair) detect movements, vibrations (chordothonal organ), chemoreceptors (sensilla) detect the presence of chemical substances in the air by antennae, taste buds on palps are for tasting, and the presence and quality of incident light is detected by photoreceptors (compound eyes) located on the head (Chapman, 1982).

In insects, conveying information through chemical signals is of profound importance in all aspects of their life cycle and ecological interactions. The ability to detect chemical cues from the environment is involved in mediating a wide range of behaviours such as locating members of opposite sex for mating, distribution of individuals and maintaining territory, setting off an alarm in order to warn of possible dangers, recognising feeding sites and to give directions to food sources. These chemical compounds are called semiochemicals (Wyatt, 2003).

1.1.2. Semiochemicals

Chemical compounds which mediate interaction between organisms, within species or between different species, are called semiochemicals, from the Greek word *semeion* meaning sign or signal (Regnier, 1971). These chemical compounds help to understand the behaviour, development, population dynamics and the evolution of organisms. Semiochemicals are classified into two major classes on the basis of species interaction and further divided into several subclasses, taking into consideration their function. Pheromones govern intra-specific interactions and allelochemicals mediate inter-specific communication (Regnier, 1971; Nordlund and Lewis, 1976).

1.1.3. Pheromones

Pheromones are chemical compounds that enable members of the same species to communicate with each other (in order to establish an intra-social relationship). The term pheromone is derived from Greek words, "*pherein*" for carrying and "*horman*" for stimulation (Regnier and Law, 1968).

Volatile pheromones are biosynthesised in exocrine glands and the site of pheromone production varies among insects. In a large number of moths, pheromones are produced in epidermal cells located in the abdomen (Jurenka, 2004) and in coleopterans (bark beetles) pheromone is biosynthesised in the midgut (Hall et al., 2002 and Nardi et al., 2002) and some other insects it is the tip of the abdomen (Leal, 1997). The amount of pheromone released by an individual differs from species to species and can be in picogram-nanogram range (Regnier, 1971; Regnier and Law, 1968).

In any chemical communication there are three important factors involved in the process: the emitter who emits the chemical signal, a medium which transmits the signal and a receiver who receives the chemical signal and responds accordingly. Furthermore, the chemical messages which alter the behavioural pattern of the receiver may induce a quick or a delayed reaction after

perception. Pheromones that cause comparatively immediate behavioural response/s in the receiver are known as releaser pheromones and pheromones that cause enduring responses are known as primer pheromones. Alarm, sex, aggregation, oviposition, trail and nest building pheromones are categorised as releaser pheromones and the states of sexual maturation, development and physiology are determined by primer pheromones (Regnier and Law, 1968; Shorey, 1973).

Alarm pheromones

Defensive secretions that cause dispersion, agitated walking and dropping from the host are categorised as alarm pheromones. For example, when attacked by predators, aphids secrete an alarm pheromone, (*E*)-*β*-farnesene, which causes dispersion of other aphids in the vicinity (Nault et al, 1976). Also, several defensive chemicals are released when male and female tarnished bugs (*Lygus lineolaris*) are disturbed, mainly, (*E*)-2-hexenal, 1-hexanol, (*E*)-2-hexenol, hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal (Wardle, et al, 2003). Similar components have been identified from other plant bugs such as soya bean stink bug, *Piezodorus guildinii* (Zarbina, 2000) and western conifer-seed bug, *Leptoglossus occidentalis* (Blatt et al, 1998).

Sex pheromones

Sex pheromones are chemical cues produced by males or females that attract a conspecific partner for mating (Section 1.2).

Aggregation pheromones

Aggregation pheromones cause conspecific males and females to assemble around the pheromone source either by attraction, arrestment or both ways. Wertheim et al. (2005) has summarised the ways which insects get benefit from aggregation pheromones, such as mate finding, protection from natural enemies, efficient use of resources and overcoming host resistance. A blend of ipsenol, ipsdienol and *cis*-verbenol identified as the aggregation pheromone of *Ips acuminatus* attracted conspecific males and females (Bakke, 1978). Also (+)-exo-brevicomin from female frass and myrcene from the host plant attracted both males and females to the host tree (Byers and Wood 1980).

Oviposition pheromones

The chemical markers used by females after laying eggs are called oviposition or host marking pheromones. This would prevent other conspecific females laying eggs on the same site. Such a marker was isolated from adult body extracts of cigarette beetle, *Lasioderma serricorne,* and identified as α-serricorone (Imai et al, 1990).

Trail pheromones

Chemical markers that lead insects to food sources are known as trail pheromones. A foraging insect returns to the nest and on the return journey it lays a chemical trail. It is a complex process which induces insects to leave the nest, follow the trail and return to the nest (Camazine et al, 2003). This type of behaviour is common in ants (Blatrix et al, 2002).

1.1.4. Allelochemicals

Whittaker and Feeny (1971) described allelochemicals as chemicals produced by one species that affect the growth, behaviour or population biology of another species. Allelochemicals are further divided into kairomones and allomones according to whether the receiver or the emitter of the chemical signal benefits from the interaction respectively (Regnier, 1971; Nordlund and Lewis, 1976).

These compounds could be either volatile substance, exudates from a root system or percolated substances from plant litter as a result of precipitation (Field et al., 2006).

Allomones

Allomones are defined as compounds which benefit the producer but which modify the behaviour of the receiver, for instance, defensive secretions and repellent compounds. Wasps, *Polistes fuscatus,* produce a defensive secretion,

methyl palmitate, to protect the nest from foraging ants which were repelled (Posy et al, 1984).

Kairomones

Those compounds benefitting the receiver but detrimental to the producer are kairomones. The pear-derived volatile, ethyl (2*E*,4*Z*)-2,4-decadienoate, was identified as an attractant for codling moth males and females, both virgin and mated. It is highly effective against codling moths in walnut and apple orchards. (Light et al., 2001)

Synomones

Synomones benefit both the producer and the receiver. The volatiles produced by herbivore-damaged plants attract predators or parasitoids that are natural enemies of those herbivores. Larvae of European apple sawfly, *Hoplocampa testudinea* feed on fruitlets. Infested fruitlets emit large quantities of (*E,E*)-α-farnesene, (*E*)-β-ocimene and another terpenoid which attract a specialised egg parasitoid, *Lathrolestes ensator* (Boevé, 1999).

1.1.5. Insect chemical perception

Chemically-induced behaviour plays a critical role in survival and reproduction of insects. Insect chemical odorant messages are translated into neuronal electrical impulses through specialised organs, generally the antennae, and processed in the brain to elicit behavioural and physiological responses. Detection of these chemical substances is mediated by specialized sensory neurons or olfactory receptor neurons in sensilla which are located along the antennae. These sensilla contain dendrites of olfactory receptor neurons, sensilum lymph which protects from dehydration, odorant-binding proteins and odour-degrading enzymes (Leal, 2005)

Transduction

The cascade of events from capturing odorant molecules to the activation of neurons and generation of electrical signal is known as transduction. Chemicals are detected at the dendritic end and the axonal end of the olfactory neuron

receives the signal. Organic volatile molecules enter sensilla through pore tubules in the sensillum wall. Then these hydrophobic odour molecules are bound by odorant-binding proteins and the complex crosses the sensillum lymph and reaches the olfactory receptor neurons. At the receptor cell membrane, the odorant-odorant binding protein complex interacts with the molecules in the receptor proteins (G-protein coupled receptors) which couples to transmembrane proteins. This process activates the secondary messenger components (cyclic adenosine monophosphate or inositol 1,4,5, triphosphate) involved in the opening up of the ion channels (Schoonhoven et al., 2005; Vogt, 2005; Kaissling, 1996; Krieger et al., 1997). This depolarises the dendritic membrane resulting in generation of an action potential that travels over the axonal membrane to glomeruli in the antennal lobe where they are integrated and behavioural responses are elicited.

1.2. SEX PHEROMONES OF INSECTS

1.2.1. Sex pheromones

Sex pheromones are odours produced by either males or females that stimulate one or more behavioural reactions in the opposite sex, bringing the sexes together for the purpose of mating. Sex pheromones may attract mates over long distances. Insects fly upwind towards the source and aggregate near the pheromone-emitting individual. After the two sexes come together, compounds acting at a close range stimulate the courtship behaviour (Shorey, 1973).

Sex pheromones are species-specific and present in single (Lacey et al., 2004) or multi-component blends (Sasaerila et al., 2000; Picimbon et al., 1997; Witzgall et al., 2001). The most abundant component in the pheromone blend is regarded as the major pheromone component and the component/s which exist in smaller quantities are regarded as minor pheromone components.

In closely related species the pheromone blends are often composed of the same or similar components. The specificity of these is achieved by mixing individual components in different ratios or addition of trace amounts of different components. *Heliothis zea* (Klun et al., 1980) and *Heliothis virescens* (Tomlinson et al., 1975; Klun et al., 1980) are two related species. Female gland extracts of both species contain large amounts of (*Z*)-11-hexadecenal and traces of (*Z*)-9-hexadecenal, (*Z*)-7-hexadecenal, and hexadecanal. In addition to the above pheromone components *H. virescens* blend consists of tetradecanal, (*Z*)-9-tetradecenal, and (*Z*)-11-hexadecen-1-ol. This would prevent inter-breeding of *H. zea* and *H.virescens*.

Further female gland extracts of two subspecies of *Hemileuca electra* contain (10*E*,12*Z*)-hexadeca-10,12-dienal, hexadecyl acetate and (10*E*,12*Z*)-hexadeca-10,12-dien-1-ol. The presence of (10*E*,12*Z*)-hexadeca-10,12-dienal in *H. electra mojavensis* was eight time more than in *H. electra*. Hexadecyl acetate was half as much that found in female gland of *H. electra* (McElfresh and Millar, 1999). The specificity of blends in different species containing similar components is achieved by changing functionalities or by altering the chain length (Roelofs 1995) or the geometry or the position of the double bonds (Meijer et al., 1972). Sex pheromone components of two species of nettle caterpillars, *Setothosea asigna* (Sasaerila et al., 1997) and *Setora nitens* (Sasaerila et al., 2000) mainly consist of 9-dodecenal and 9,11-dodecadienal. In the former and the latter species, the active components appeared to be *E* and *Z* isomers of above components respectively. The relative ratio of the components in the blend is also important. (*E*)- and (*Z*)-11-tetradecenyl acetate are the two components in the pheromone blend of asiatic leafroller, *Archippus breviplicanus* (Sugie et al., 1977). Only certain mixtures of (*E*)- and (*Z*)-11-tetradecenyl acetate are attractive to males. For example, 70:30 or 65:35 blends of (*E*): (*Z*)-isomers are strongly attractive to conspecific males of the asiatic leafroller.

1.2.2. Chemistry of sex pheromones

Since the identification of very first pheromone, "bombykol" from *Bombyx mori* in the 1950s (Butenandt et al., 1961) numerous other pheromones belonging to several different classes of insects have been identified. The basic skeleton consists of carbon and hydrogen. About 10-23 or more carbons atoms are arranged in linear or cyclic pattern with or without branches attached (Howse et al., 1998; Bayer, 2006; Ando et al., 2004). Insects perceive odour components through the air and therefore volatility is an important aspect of pheromone compounds, especially substances involved in long range communication. As a result molecular weight is a critical factor and therefore, pheromones have molecular weights in the region of 300 or less (Regnier, 1971).

Ando et al. (2004) categorised Lepidopteran sex pheromones as Type I and Type II which are derived by fatty acid synthetic pathways. Type I are straight chain compounds with an even number of carbons, 0-3 double bonds and containing a functional group which could be an alcohol, acetate or aldehyde located at the terminal end. Type II are long chain polyunsaturated hydrocarbons and epoxy derivatives with an odd number of carbon atoms. Other than that Howse et al. (1998) summarised pheromone components into several classes, terpenes, amides and heterocyclic compounds (ethers, lactones and ketals).

Chirality has long been discussed relating to insect sex pheromone chemoreception (Mori, 1989). Stereoisomers occur due to the presence of asymmetric or chiral centre/s in the compound. Insect behaviour upon exposure to stereoisomers has been reported in the literature. Amongst chiral compounds there are many cases in which only one enantiomer is bioactive. Campbell et al. (2007) reported that sex pheromone of Current Pug moth, *Eupithecia assimilata* is 3*Z*,6*Z*-*cis*-9,10-epoxyheneicosadiene and the males were attracted to the (9*S*,10*R*)-enantiomer but not to the opposite enantiomer or the racemic 3*Z*,6*Z*-*cis*-9,10-epoxyheneicosadiene. The activity of the naturally occurring isomer is inhibited by those isomers which are not present in nature. The female sex

pheromone of *Popillia japonica* was identified by Tumlinson et al. (1977) as (*Z*)-5-(1-decenyl) oxacyclopentane-2-one. The *R*-enantiomer attracted conspecific males while the presence of the opposite enantiomer in the lure at as little as 1 % significantly reduced the attraction.

Mori (1989) has grouped chiral pheromone components into different categories based on the bioactivity of the stereoisomers: (a) a large number of pheromones identified have a one active enantiomer and the antipode is not inhibitory; (b) only one enantiomer is active and the opposite enantiomer is inhibitory; (c) all enantiomers are active and the insect does not discriminate among the stereoisomers; (d) species of the same genus use different enantiomers; (e) both enantiomers are needed for biological activity; (f) only one enantiomer is active and its activity can be enhanced by addition of less active stereoisomer; (g) one enantiomer is the pheromone of the male while the antipode is the pheromone of the females and (h) only the meso-isomer is active.

1.3. ISOLATION AND IDENTIFICATION OF PHEROMONES

1.3.1. Pheromone isolation

Isolation of the pheromone is needed for structure elucidation and bioassays. A number of isolation techniques are described in the literature, solvent extraction, (dissection and gland extraction, ovipositor or whole body wash) (Rivault et al., 1998; Eiras, 2000), entrainment, solid phase micro extraction (SPME) (Mozuraitis, 1999) and direct injection of biological material using a specially-built GC injector port (Attygalle and Morgan, 1988). The method of extraction varies from insect to insect.

Solvent extraction

Solvent extraction is by far the most commonly used method. Normally organic solvents like pentane, hexane and dichloromethane are used in extraction. The downside of solvent extraction is the incorporation of contaminants (cuticular

materials, and insect body fluid) into the extracts (Attygalle and Morgan, 1988; Jones and Oldham, 1999; Leal et al., 2001; Rivault et al., 1998).

Volatile collection

Entrainment is employed for the collection of air-borne pheromone compounds from live insects. Air is filtered through activated charcoal, drawn over the insects held in a glass chamber and volatiles are collected into filters containing porous adsorbent material such as Porapak-Q (Byrne et al., 1975; Chang et al., 1988; Hall et al., 2006), Super-Q (Dickens et al., 2002) or activated charcoal (Ho and Millar, 2002). Volatiles can be collected for extended periods from living insects and even food can be supplied during the process. The trapped volatiles then can be extracted by eluting solvent through the filter containing adsorbent material or by thermal desorption (Howse et al., 1998).

The composition in the gland extracts of pheromones can be slightly different from exact composition due to addition of impurities, loss of material during extraction and ineffective trapping of volatiles during aeration by the adsorbent. As an alternative to conventional pheromone isolation techniques, solid-phase microextraction (SPME) has been introduced in analysis of insect volatiles. It extracts volatiles from the gland surface during pheromone release (Frérot et al., 1997; Malosse et al., 1995) into a thin film of immobilised matrix (liquid polymeric or solid porous phase) coated over the surface of a fused-silica fibre (Augusto and Valente, 2002). The volatiles are then thermally desorbed in the injector of a gas chromatograph or GC-MS (Theodoridis et al., 2000).

1.3.2. Column chromatography

Liquid column chromatography is widely used in separation and purification of compounds (Colegate and Molyneux, 1993). In column chromatography, a glass column is packed with stationary phase or adsorbent (silica gel or alumina) and the liquid phase is allowed to flow through under gravity or low pressure. The sample is loaded onto the top of the silica bed and gradient or isocratic solvents are passed through. The eluent is collected fraction wise and

analysed by gas chromatography or thin layer chromatogarphy. The size of the column is determined by the amount of the sample to be chromatographed (Skoog and Leary, 1992; Millar and Haynes, 1998). The purification and fractionation of crude insect pheromone extracts by column chromatography has been adopted in many cases of pheromone related studies (Subchev and Jurenka, 2001; Tumlinson et al, 1969).

1.4. PHEROMONE ANALYSIS

1.4.1. Gas Chromatography (GC)

In insect pheromone analysis gas chromatography (GC) is a widely used tool due to the high resolution and the ability to couple it to a variety of detection techniques such as electroantennography and mass spectrometry. In GC the mobile phase is gaseous and the stationary phase is a high boiling point liquid coated onto a porous support or the inner wall of a glass, metal or fused-silica column. Analytes are partitioned between gaseous mobile and liquid immobilised phases during separation. Carrier gas is normally chemically inert and includes helium, nitrogen or hydrogen. The choice of carrier gas is associated with the type of detector used. Fused silica capillary columns are preferred over packed columns as capillary columns give a higher degree of resolution although only low volumes can be analysed at a time. The column is housed in a thermostatically controlled oven and temperature can be programmed taking optimum temperature of the liquid stationary phase into consideration (Skoog and Leary, 1992).

The sample is vaporised in the inlet and carried into the column by the carrier gas. Separation of components is brought about depending on the boiling point, molecular weight and relative solubility in stationary phase. The GC can be fitted with different types of detectors. The flame ionisation detector (FID) is the most widely used detector.

1.4.2. Gas chromatography linked to electroantennography (GC-EAG)

GC-EAG is an instrument set-up used in electrophysiological studies of insects. The insect's antennal responses are measured upon exposure to volatile chemicals in a form of an electrical signal which enables detection and identification of chemical compounds used by insects in communication. Gas chromatography (GC) separates the components in a volatile extract and EAG determines the activity of each component using an insect antenna as a detector (Moorhouse et al., 1969).

The effluent from the GC column is split between flame ionisation detector (FID) and the other half is collected, mixed with nitrogen and passed over an antennal preparation as a form of a puff. In that way, the recording signal in the electoantennogram (EAG) can be correlated with peaks on the gas chromatogram (Cork et al., 1990). Arn et al. (1975) introduced a method in which humidified air was used for delivering GC effluent over the antennal preparation. This is the most widely practised method in pheromone detection.

The type of antennal preparation depends on the insect and an excised antenna (Subchev et al., 2003), head with antennae intact (Gries et al., 2002) or the whole insect can be used. In the case of small soft bodied insects like midges, the whole insect is used in the preparation as they are difficult to handle.

The antennae are mounted on glass capillaries filled with electrolyte solution placed between micromanipulators of the EAG instrument. The insect's antennal response when exposed to volatiles is recorded as a change in the potential which arises due to depolarisation of individual receptors along the antennae (Howse et al., 1998).

1.4.3. Gas chromatography coupled to mass spectrometry (GC-MS)

Detection and analysis of pheromone compounds can be achieved by gas chromatography linked to a mass spectrometer. A mass spectrometer is an analytical instrument which converts molecules into ionic fragments and brings about separation according to the mass-to-charge ratio (*m/z*) of the fragments. It generates a mass spectrum for a given compound showing the relative abundance of ion species vs. *m/z*. It is widely used for determination and confirmation of structure based on fragmentation, occurrence of isotopes, and quantification. It is also used in monitoring reactions in synthesis and micro-scale reactions which can't be detected by other means (Jones and Oldham, 1999).

Basically, the instrument consists of several components: ion source, the mass analyser, the detector and the recorder. The ion source converts neutral molecules to charged species and this can be brought about either by electron impact (EI) or chemical ionisation (CI) modes (Skoog and Leary, 1992). The vaporised sample is introduced from the outlet of the GC and in EI mode the vaporised sample is bombarded with a beam of electrons (70 eV). The interaction of fields of both electrons and sample molecules causes knocking off an electron from the sample or the parent molecule. The resulting ion is called the radical-cation ($M^{\cdot+}$) as a result of loss of an electron. Larger ions release excess energy by fragmenting into smaller but stable units and forming charged fragments and radical species (Lambert et al., 1998).

Chemical ionisation is a less vigorous technique. It imparts a small amount of energy therefore fragmentation is reduced to a certain extent. CI is a method used for confirming the molecular mass. Different types of CI reagents are available, e.g. liquid (methanol and acetonitrile) or gaseous (methane, isobutane and ammonia) (Bouchonnet et al., 2004). Molecules interact with the CI reagent and undergo a series of reactions. Fragment formation can vary according to the CI reagent. Nevertheless the most useful piece of information is the molecular ion $[M+H]^+$ (Johnstone and Malcolm, 1996). Low pressure CI

with liquid reagent has an advantage over the gaseous reagents as it eliminates the contamination from reagent gas and simply gives the [M+1] fragment (Bouchonnet et al, 2004). After ionisation the stream of positive ions is accelerated into the mass analyser where ions are separated according to their mass-charge ratio.

There are several types of analysers and the most commonly used ones are quadrupole analysers and ion trap detectors. The ion-trap detector is considered to be more sensitive than a quadrupole analyser and can detect nanogram-picogram level of compounds (Skoog and Leary, 1992). The inlet system, ion source, mass analyser and the detector are located in a low pressure (10^{-5} torr) chamber. At atmospheric pressure the filament can get oxidised and charged ions lose their charge as they collide with gas molecules in the air and lose the ability to accelerate towards the detector (Barker, 2000).

The mass spectrum gives reliable information about the chemical structure, for example, molecular mass (only in some cases) and functionalities. Particularly, characteristic fragments such as retro Diels–Alder and McLafferty rearrangement show the connectivity of the fragments in the parent molecule (Mohan, 2004). Further, computer based library search and comparison of retention index with that of known compounds have made identification feasible.

1.5. PHEROMONE IDENTIFICATION

1.5.1. Micro-scale reactions

Microchemical reactions are employed as a part of the structural confirmation and elucidation of nanogram quantities of natural products. The products are analysed by coupled GC-MS. Valuable information can be obtained about the structure: presence and the location of the functionalities, assigning absolute configuration to chiral centres, functional group modification in structure determination and for enhancing the chromatographic properties (Jones and

Oldham, 1999).

Hydrogenation, preparation of dimethyl disulphide derivative (DMDS), ozonolysis and epoxidation (Tumlinson and Heath, 1976) are some micro analytical reaction used in structure elucidation. Catalytic hydrogenation determines the degree of unsaturation of the compounds. The GC-MS analysis of hydrogenated compounds shows increase of *m/z* of ions by 2 units per double bond (Zhang et al., 2004). The DMDS derivative, ozonolysis and epoxidation are employed to determine the position of the carbon-carbon double bond of a compound.

However, some reactions are best suited for certain types of compounds only (Jones and Oldham, 1999; Attygalle and Morgan, 1988). For example, the addition of two methyl-thio groups across the double bond of long chain monounsaturated alcohols, acetates and aldehydes increases the molecular weight by 94 (Leonhardt and Devilbiss, 1985; Buser et al., 1983). In that case those compounds with high molecular weight need a longer retention time to be eluted from the GC. Micro-ozonolysis brings about the oxidation of the carbon-carbon double bond/s of linear compounds in the presence of ozone and oxygen. The cleavage at the double bond position results in the formation of smaller fragments of aldehydes and ketones. However, the GC chromatographic identification of product may be difficult as low molecular weigh products can overlap with the solvent peak of the GC (Beroza and Bierl, 1967). Epoxidation is a useful reaction for the conformation of multi-unsaturation in compounds. The drawback of this reaction is that the reaction by-products can interfere during chromatographic analysis (Jones and Oldham, 1999).

1.5.2. Retention Index (RI)

Retention index is the retention time data from gas chromatography expressed in relation to a set of standard compounds. This technique was first proposed by E. Kovats and he mentioned that the retention behaviour of a substance can be expressed with respect to retention properties of a series of closely related

compounds (Kovats, 1958). In, Kovats index calculation, linear alkanes are employed as standards. The standard substances, the *n*-alkanes were assigned an index of 100 times the carbon number (for example 100 for methane, 200 for ethane etc) at any temperature on a given GC phase (Poole, 2003). Other than a series of *n*-alkanes, saturated fatty acid methyl esters or acetates can be used as reference compounds (Cork et al., 1991).

The retention index for a compound is obtained by co-analysis of series of *n*-alkanes and the compound of interest (or unknown) by GC. Typically RI is calculated relative to *n*-alkanes. The RI value is reproducible in any other GC system equipped with the same type of column and this allows comparison of data from different chromatographic systems (Millar and Haynes, 1998). This would allow use of published data for the identification of unknowns. Retention index provides information on polarity, size of the molecule and functional groups (Kovats, 1961).

1.5.3. High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a multipurpose instrument used both in qualitative and quantitative analysis in pharmaceutical, agrochemical and biochemical industries. HPLC is extensively used in both analytical and preparative scale separation.

The instrument comprises an injector, column, pump (which delivers the solvent), detector (that monitors the eluting compounds) and software for handling data. As its name implies, compounds are separated by passing through the stainless steel column tightly packed with tiny porous particles at a high pressure. Either silica or silica based material, particle size of 3-5 μm and pore size of 100-300 Å is used as the column packing/ stationary phase. The columns are categorised on the basis of the chemistry of the packing material, normal and reverse phase, ion exchange and gel filtration. The choice of the eluting solvent/s depends on the properties of the stationary phase. The selection of the detector is based on the properties of compounds to be

analysed, for instance ultraviolet (UV) detector can be used for monitoring UV-active compounds only (Adamovices, 1990). Refractive Index Detector is considered as a universal detector and fluorescence detectors, conductivity detectors and mass spectrometers are also used depending on the requirement.

Enantioseparation is becoming increasingly important as optical isomers possess different biological activities (Leffingwell, 2003). HPLC separation seems to be a convenient and reproducible method for resolution of the racemates in semi-preparative scale. A number of chiral stationary phases are available, some of which are cyclodextrin, polysaccharides (amylase and cellulose), monocyclic antibiotic, synthetic chiral macrocycles (crown ether), chiral synthetic polymers, chiral imprinted polymers and protein based phases (Gübitz and Schmid, 2001).

HPLC has been used in isolation of insect pheromone compounds from crude pheromone extracts (Gries et al., 2006; Giblin-Davis et al., 1997; Pomonis et al., 1993) and the separation of diasteromeric derivatives of the racemic mixture (Gries et al., 2002). However, there are only a few known cases of HPLC chiral separation of enantiomers of insect pheromones. The polysaccharide based chiral stationary phase combined with normal phase solvents is used for separation of enantiomers of insect pheromones (Pu et al., 1999; Wakamura et al., 2005; Yamamoto et al., 2000; Campbell et al., 2007). The two most commonly used polysaccharide base stationary phases are Chiralpak-AD that is an amylose backbone with phenylcarbamate moiety and Chiralpak-OD (cellulose instead of amylose backbone). These phases are prepared by coating macroporous silica gel with synthesised derivatives of triesters and tricarbamates of cellulose and amylase (Okamoto and Kaida, 1994). It has been shown that the enantioselectivity on polysaccharide stationary phases involves intermolecular interactions (hydrogen bonding, dipole-dipole and π-π interactions) between analytes and stationary phase (Yashima and Okamoto, 1995). These interactions are relatively weak and more effectively bring about the separation of enantiomers under low polarity organic solvent with an alcoholic modifier (Gübitz and Schmid 2001; Chankvetadze et al., 2001;

Chankvetadze et al., 2002; Tachiban and Ohnishi 2001; Wen-zhi et al., 2004).

1.6. APPLICATION OF INSECT PHEROMONES IN PEST MANAGEMENT

1.6.1. Monitoring

Pests can be monitored in an environmentally friendly way using pheromone compounds or other semiochemicals. The knowledge of presence or absence of a pest in the field is vital in pest management, so that timely application of insecticides or any other controlling methods can be implemented (Wall, 1990).

Monitoring requires a sensitive trapping device for detection as well as estimating the threshold level and pest population density. There are many types of traps used in monitoring, e.g. sticky traps, delta traps and funnel traps are ones that used commonly. In some cases the shape and the colour of the trap can have an influence the trap catch (Strom and Goyer, 2001). Other than that, factors as such as trap layout in the field, plot size, direction of the wind, and aging of release devices contribute to the performance of traps (Cork, 2004).

The pheromone must be loaded in suitable a dispenser to control the release rate and protect it from degradation (Weatherston, 1990; Brown and McDonough, 1986; Vrkoč et al., 1988). Various types of devices are used for releasing synthetic pheromones for monitoring and controlling. Cotton dental wicks, glass reservoirs, sachets made from polymer films, polyethylene caps and tubes, rubber septa, hollow fibre, puffers, sprayable microcapsules, paraffin wax formulation are some of the releasing devisers used (Cork, 2004; Welter et al., 2005). For any system it is important to determine the release rate of the pheromone to maximise the attractiveness and detect how long it will last in the field.

The information from the trap catches can be used for early warning, surveying and quarantine purposes. Treatment programmes can be implemented based on the population density of the pest. For example application of insecticides on cabbage and cauliflower against diamond back moth *Plutella xylostella* carried out based on the sex pheromone trapping of males was found to be significantly more effective than calendar date base application of insecticides (Reddy and Guerrero, 2001). In addition to trap catches, the biology of the pest and climatic condition which predict the occurrence of the susceptible stage of the life cycle are also required for timing a treatment strategy (Wall, 1990).

Pheromone monitoring traps are species specific, need little maintenance, are not labour intensive and little expertise is required for operation. However, successful monitoring program requires the interpretation of trap catches which seems to be the limiting factor and trap saturation (Howse et al., 1998).

1.6.2. Control

Mating disruption

Mating disruption is a technique that is applied in pest management. During the process synthetic sex pheromones interfere in the mechanisms of male insects locating calling females for mating. If the technique is effective, a decrease in successive generations can be observed due to reduced incidence of mating. Essentially a high concentration of sex pheromone of the target species is dispersed throughout the selected area that disrupts the upwind flight of males responding to calling females. There are several mechanisms of mating disruption: sensory fatigue, competition between natural and the synthetic sources, camouflage of the natural plume, imbalance of sensory input, antagonists to attraction and pheromone mimics (Sanders, 1997; Cardé, 1990).

Mating disruption is an application which is more suitable at low population densities and the other important factor is that the population should be relatively isolated, especially where mated females can disperse into the treated area. Further, species with flightless females such as gypsy moth are potential candidates for control by mating disruption (Leonhardt et al, 1996). The

efficiency of the technique depends upon the reproductive characteristics, species dispersal, population density, the concentration of the pheromone (dose per unit area) and pheromone release rate (Cardé, 1990). Codling moth, *Cydia pomonella* L. was one of the serious pests in pome fruit orchards in Western North America as reported by Thomson et al. (2001) and the major tactic for controlling codling moth was mating disruption. Witzgall et al. (2008) mentioned in his review that over the years application of mating disruption of codling moth has expanded and indicated that in orchards treated with 100 g of synthetic codlemone per hectare effective control of the codling moth population could be acheived over the entire growing season. Several products are registered in US such as Isomate C+, CheckMate CM, Nomate CM and disrupt CM for commercial purposes (Thomson et al., 2001).

Mass trapping

Another technique in semiochemical based pest management is mass trapping. A large numbers of pest insects are removed and a massive reduction in the population ultimately protects resources.

The success of mass trapping depends on the odour source which determines the efficiency of mass trapping. The odour source can be an attractant such as aggregation, sex pheromone or host plant volatile in combination with pheromone or alone (Ladd and Klein, 1986; Yang et al., 2004). Aggregation pheromones as the attractant reduce both males and females in the population whereas sex pheromone attracts only one sex (either males or females), therefore this technique is sometimes less effective.

Mass trapping depends on several important factors such as number of traps needed for a given area, efficiency of lures, ability of traps to catch and retain insects, trap saturation and cost effectiveness and labour requirement (El-Sayed et al., 2006).

Attract and kill

The concept behind this technique is the same as in mass trapping. However it is slightly modified to overcome the problem of trap saturation and labour

intensity. A combination of volatile attractant/s draws insects towards the source and insects die by coming into contact with the insecticide-treated surface. Sticky formulations of many contact insecticides such as deltamethrin and cyfluthrin are used in trapping (Ebbinghaus et al., 2001). This strategy has shown much promise in tackling codling moth (Lösel et al., 2000), olive fruit fly (Mazomenos et al., 2002) and grape root borer (Weihman and Liburd, 2006).

1.7. GALL MIDGES (Diptera: Cecidomyiidae)

1.7.1. General biology

Gall Midges are plant feeders belonging to the order Diptera and family Cecidomyiidae. There are three important traits in the life cycle of these plant feeding midges; (1) the formation of galls due to larval feeding, (2) the prolonged diapause shown by the third instar that occurs due to climatic factors (Barnes, 1956) and (3) production of unisexual progenies (individual females produce either female or male insects). Harris et al. (2006) showed cellular changes occurred due to larval feeding on epidermal and mesophyll cells of wheat infested by Hessian fly, *Mayetiola destructor* larvae. Epidermal and mesophyll cells located at the base of the leaf showed signs of becoming nutritive, that is increased in numbers of cellular organelles such as mitochondria, proplastids, golgi and rough endoplasmic reticulum.

Early in their evolution, Cecidomyiidae larvae fed on fungi. However, several lines evolved as vascular plant feeders and the other lines developed into predators of mites, aphids and various other arthropods. There are about 5,000 described species of Cecidomyiidae world-wide (Gagné, 1994).

The size of the adult may not be more than a few millimetres (1-4 mm) and larvae are spatula bodied of 1-5 mm in length. The colouration of adults, eggs and larvae may differ according to species (Barnes, 1948; Alford, 1984). The adults are small and fragile bodied with hairy wings with distinctive venation,

hairy segmented antennae (flagella) and long thin legs. Adults have a very short life span (1-2 days) and are considered as the reproductive phase (Barnes, 1948). Newly-emerged adults mate following pheromone secretion by calling female midges. During calling females settle on a surface and extend the ovipositor bearing the pheromone gland to full length. Calling may last for several hours or in spells of a few minutes (Van Lenteren et al., 2002). Male midges detect the pheromone and fly upwind towards the source.

Normally eggs are elongated and hardly visible to the naked eye. Larvae are legless and cylindrical and typically have three instars. The larval stage is the feeding phase. Larvae directly feed on plant tissue and the feeding niche can vary from species to species, for instance, flower buds, leaves, stems and fruits.

Many economically important pests are included in this family with a diverse host range (cereals, fruits, vegetables, ornamental and herbal crops). They are predominantly monophagous and some are oligophagous (Gagnē, 1994).

1.7.2. Chemical Ecology of Gall midges

Response to host plant volatiles

Phytophagous insects use volatile cues for locating sites for feeding and oviposition (Foster and Harris, 1997). Volatiles from the host plant were tested for activity against females of several Cecidomyiidae species. Galanihe and Harris (1997) demonstrated in a wind tunnel that females of *Dasineura mali* can distinguish apple from pear foliage, fly upwind and land on apple foliage. Anfora et al. (2005) observed EAG responses from *D. mali* female antennal preparation for olfactory stimulants identified from immature apple leaves, hexanol and (*Z*)-3-hexen-1-ol. Similarly females of *Sitodiplosis mosellana* were attracted to volatiles from panicles of intact wheat and several components responsible for attraction were identified as acetophenone, (*Z*)-3-hexenyl acetate, 3-carene, 2-tridecanone, 2-ethyl-1-hexanol and 1-octen-3-ol. The mixture of the six-component blend and three-component mixture consisting of acetophenone, (*Z*)-3-hexenyl acetate and 3-carene was found attractive to females in the field

in the same ratio and concentration as in nature (Birkett et al., 2004). Volatiles from wounded raspberry canes were found to be attractive to female *Resseliella theobaldii*. Mated females strongly preferred volatiles emanating from freshly split canes than those from old ones (Gordon and Williamson, 1991). Headspace SPME extractions were analysed by GC and ten components were identified to be produced only from newly made splits. Three components, 6-methyl-5-hepten-2-ol, myrtenal and myrtenol elicited EAG responses from mated female antennae. On the contrary Crook et al. (2001) reported that females of *Dasineura tetensi* did not show olfactory discrimination to resistant and susceptible black currant varieties and suggested that *D. tetensi* may have a different mechanism for identifying the resistant/susceptible varieties.

Sex pheromones of gall midges

Until recently, little was known about sex pheromones of Cecidomyiidae in comparison to those of other insect families (Hardie and Minks, 2000) even though the existence of long-range sex pheromones in several midge species had been reported. The presence of a female-produced sex pheromone was demonstrated in Hessian fly, *M. destructor* (McKay and Hatchett, 1984), pea midge, *Contarinia pisi* (Wall et al., 1985), blackcurrant leaf midge, *D. tetensi* (Garthwaite et al., 1986), sorghum midge, *Stenodiplosis sorghicola* (Sharma and Vidyasagar, 1992), orange blossom wheat midge, *S. mosellana* (Pivnick, 1993) and apple leaf midge, *D. mali* (Harris et al., 1996; Heath et al., 2005) in laboratory bioassays as well as field experiments with live virgin female or female extracts as baits.

Foster et al. (1991) identified the first Cecidomyiidae sex pheromone from *M. destructor* as (2*S*,10*E*)-2-acetoxy-10-tridecene. Since then 10 more midge sex pheromones have been identified (Table 1.1). Although, the sex pheromone of sorghum midge has not been fully identified, the structure has been suggested as a combination of acetoxy and butyroxy functionalities (Riolo et al., 2006).

Table 1.1. Composition of female sex pheromones of midges (categorised according to major component of pheromone)

Name	**Pheromone components**	**Reference**
Mono-functional unsaturated compounds		
Hessian fly, *Mayetiola destructor*	(2*S*,10*E*)-2-acetoxy-10-tridecene (2S)-2-acetoxytridecane (2*S*,10*Z*)-2-acetoxy-10-tridecene (2*S*,10*E*)-10-tridecen-2-ol (2*S*,8Z,10*E*)-2-acetoxy-8,10-tridecadiene (2*S*,8*E*,10*E*)-2-acetoxy-8,10-tridecadiene	Foster et al., 1991 Andersson et al., (2009)
Chrysanthemum midge, *Rhopalomyia longicauda*	(2*S*,8*Z*)-2-butyroxy-8-heptadecene (2*S*,8*Z*)-8-heptadecen-2-ol	Liu et al., 2009
Douglas fir cone Gall midge, *Contarinia oregonensis*	(2*S*,4*Z*,7*Z*)-2-acetoxy-4,7-tridecadiene	Gries et al., 2002
Locust bean midge, *Dasineura gleditchiae*	(2*R*,8*Z*)-2-acetoxy-8-heptadecene	Molnár et al., 2009
Di-functional saturated compounds		
Aphidophagous gall midge, *Aphidoletes aphidimyza*	(2*R*,7*S*)-2,7- diacetoxytridecane	Choi et al., 2004
Orange blossom wheat midge, *Sitodiplosis mosellana*	(2*S*,7*S*)-2,7- dibutyroxynonane	Gries et al., 2000

Table 1.1. Composition of female sex pheromones of midges (cont.)

Pea midge, *Contarinia pisi*	(2*S*,11*S*)-2,11-diacetoxytridecane (2*S*,12*S*)-2,12-diacetoxytridecane (2*S*)-2-acetoxytridecane	Hillbur et al., 2001
Raspberry cane midge, *Resseliella theobaldi*	(2*S*)-2-acetoxyundecan-5-one Undecan-2-one (2*S*)-2-acetoxyundecane (2*S*)-undecan-2-ol	Hall et al., 2009
Swede midge, *Contarinia nasturtii*	(2*S*,9*S*)-2,9-diacetoxyundecane (2*S*,10*S*)-2,10-diacetoxyundecane	Hillbur et al., 2005
Red cedar cone midge, *Mayetiola thujae*	(2*S*,12*S*)-2,12-diacetoxyheptadecane (2*S*,13*S*)-2,13-diacetoxyheptadecane (2*S*,14*S*)-2,14-diacetoxyheptadecane	Gries et al., 2005
Di-functional unsaturated compounds		
Apple leaf midge, *Dasineura mali*	(13S,8Z)-13-acetoxy-8-heptadecen-2-one	Cross and Hall, 2009

Cecidomyiidae pheromones are comprised of a straight carbon chain with odd number of carbon atoms (9, 11, 13 and 17) unlike the majority of Lepidopteran sex pheromones. Another prominent feature in Cecidomyiidae pheromones is the occurrence of acetoxy, butyroxy or keto functionality on C2, not the terminal carbon (C1) as in Lepidopteran type I pheromones (Ando et al., 2004; Millar, 2000).

The midge pheromones can be categorised on the basis of number of functional groups in the major component/s and the presence or absence of unsaturation (Table 1.1). Unsaturation seems to occur in compounds having 13 or 17 but not 9 or 11 carbon atoms in the chain. Mostly, the unsaturation is due to the presence of a single double bond. However, *C. oregonensis* sex pheromone is considered to be an exceptional case as it has two double bonds.

The existence of multi-component blends as well as single component pheromones can be found amongst Cecidomyiidae species. The components in the pheromone blends are closely related as observed in Lepidopteran sex pheromones (Ando et al., 2004; Millar, 2000).

1.7.3. Pest management of midges

Species of the family Cecidomyiidae have been identified as pests affecting the economical viability of their hosts. A range of control methods to curb population development has been practised. Chemical treatment of soil and plant debris (Guo Yuan and Zhong-Qi, 1997; Qin-Fuu et al., 1997), crop sanitation, fumigation (Barnes, 1948) and planting resistant varieties are some ways of controlling midges (Smith et al., 2004).

The application of insecticide at the time of the flying period and larval stage protects the crop from midge attack. Bunting and Hudson (1991) reported that ovipositing females and neonate larvae of Hessian fly on winter wheat were effectively controlled using foliar application of insecticides. However, concerns are mounting over the application of insecticide due to the adverse effects caused to human health and other beneficial organisms, persistence of residues

and development of resistant strains (Thacker,2002). In contrast chemical control is more efficient if the susceptible life stages of midges are targeted. Therefore a proper monitoring system is required for detecting midges in the field (Harris and Foster, 1999).

Presently midges are monitored by several different ways such as sampling soil, detection of eggs by dissection of buds (Finn and Liburd, 2002; Sarzynski and Liburd, 2003) counting eggs on leaves (Harris et al., 1996) or conelets (Gries et al., 2002). In *D. tetensi,* the time of oviposition and emergence are predicted on a model based on variation of air temperature and developmental rate (Cross and Crook, 1999). However, soil sampling and counting eggs on leaves are tedious, time consuming and not always reliable in predicting outbreaks.

Pheromone based monitoring tools could be used in managing midges in the integrated pest management (IPM) approach. Pheromone monitoring systems are species specific, less labour intensive and do not require any special skills for operation. Female produced sex pheromones from a number of midge species have been identified and synthesised. The wind tunnel tests (Hillbur, et al., 1999; Foster et al., 1991; Harris and Foster, 1991) indicated the stimulatory behaviour by male midges for synthetic pheromone components and field tests were also promising (Hillbur et al., 2000). The efficacy of midge pheromones was demonstrated with different types of lures, traps and blend compositions (Cross and Hall, 2009; Hillbur et al., 2005).

The Natural Resources Institute in collaboration with East Malling Research has been developing pheromone traps for monitoring apple leaf midge, *D. mali* (Cross and Hall., 2009; Cross et al., 2009), and raspberry cane midge, *R. theobaldi* (Cross et al., 2008) in UK. Recently, a pheromone monitoring system for orange wheat blossom midge, *S. mosellana*, has also been introduced for UK wheat growers (Bruce et al. 2007).

1.8. AIMS AND OBJECTIVES

The overall aim of this work was to explore further the chemical diversity of midge pheromones by identifying the female sex pheromones of midge species of importance to UK horticulture.

The majority of Cecidomyiidae sex pheromones identified so far are mono- or di-acetates. Recently acetoxy-ketone structures have been found in the pheromones of two midge species – the apple leaf midge, *D. mali*, and the raspberry cane midge, *R. theobaldii* (Table 1.1). Thus the hypothesis was that a much wider chemical diversity of sex pheromones can be found within the family Cecidomyiidae, and, in particular, that the acetoxy-ketones might be widespread. This hypothesis was tested by investigating the sex pheromones of four midge species: pear leaf midge, *Dasineura pyri;* pear midge, *Contarinia pyrivora;* blackcurrant midge, *Dasineura tetensi* and blackberry midge, *Dasineura plicatrix.*

The identification of the female sex pheromones of these pest species will also make a basis for development of methods for monitoring and possibly control of these species.

CHAPTER 2
GENERAL METHODS AND MATERIALS

2.1 INSECT COLLECTION

Late larvae of pear leaf midge, *Dasineura pyri,* pear midge, *Contarinia pyrivora,* blackcurrant leaf midge, *Dasineura tetensi,* and blackberry midge, *Dasineura plicatrix* were collected from heavily infested pear shoots, pear fruitlets, blackcurrant shoots and blackberry shoots respectively with assistance from staff at East Malling Research.

Plant materials were stored in clear plastic boxes (19 cm x 10.6 cm x 7.5 cm; Sarstedt, Aktlengesellachaft & Co., Germany) with the base covered with a wet kitchen towel in order to keep the plant material in a fresh condition. Mature larvae crawled out from the leaves for pupation, and these were collected and reared individually in clear plastic tubes (1.5 cm i.d. x 2.3 cm: Sarstedt, AG, Germany) containing a piece of wet filter paper which acted as the substratum for pupation and also for retaining moisture. Tubes were closed with plastic caps and stored under controlled conditions at 23°:18°C and 16L:8D light cycle.

Adults emerged from cocoons after 7-12 days in those species reared except for *C. pyrivora* which is described in Chapter 3. Although a large number of midges were reared, the number of adults emerging was variable. Males and females were separated on the basis of antennal morphology. The male antenna consists of segments, each separated by a small tubular stalk and the segments are densely covered by hair-like sensilla. The segments in the female antennae have a thin cover of sensilla and the segments are directly fused together (Figure 2.1). These features can easily be observed under a hand lens.

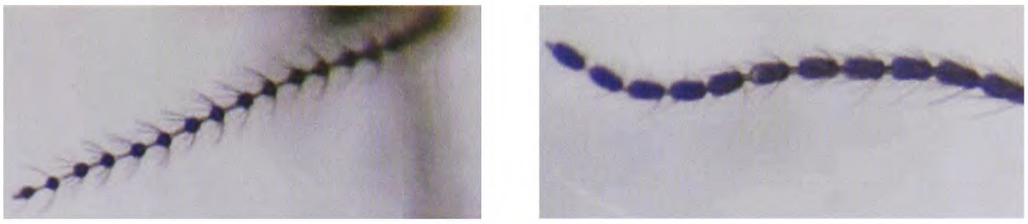


Figure 2.1 Antennal morphology of male (left) and female (right) of *C. pyrivora*.

2.2 PHEROMONE COLLECTION

Volatiles from male and female midges were collected separately by air entrainment under the same controlled conditions as used for insect rearing (Figure 2.2). Midges were placed in a specially made glass vessel (5.3 cm i.d. x 13 cm; Hamilton Laboratory Glass Ltd, Margate, UK) with a glass frit at the upwind end. Air was drawn into the vessel (0.5 L/min) with a vacuum pump (M 361C, Charles Austen Pump Ltd, UK) through an activated charcoal filter (20 cm x 2 cm; 10-18 mesh, Fisher Chemicals, UK) and out through a collection filter consisting of a Pasteur pipette (4 mm i.d.) containing Porapak Q (200 mg; 80-100 μm, Waters Associates Inc., USA) held between two glass wool (Field Instruments Co. Ltd, Twicekham, UK) plugs. The Porapak was purified by Soxhlet extraction with chloroform (Fisher Scientific) and prior to use filters were cleaned with dichloromethane (2 ml, pesticide grade, Fisher Scientific) and dried by a stream of nitrogen. Midges were introduced at 24 hr intervals for 3-7 days. Collection filters were extracted with dichloromethane (1.5 ml), concentrated under a stream of nitrogen to approximately 0.2 ml and refrigerated prior to analysis.

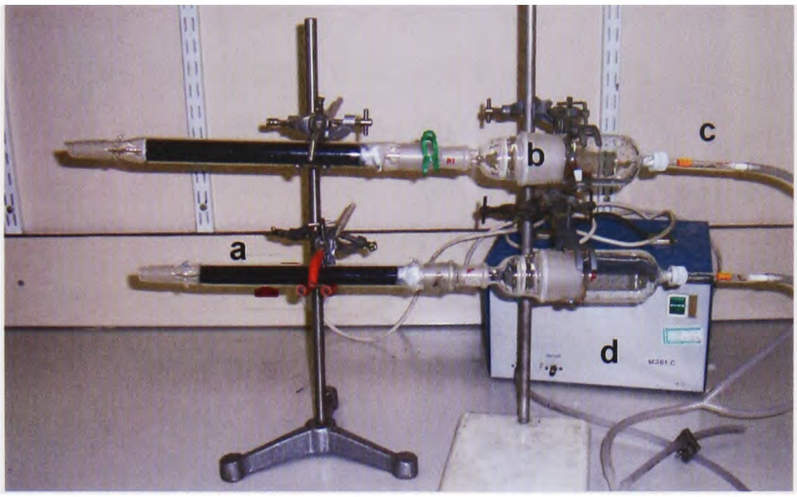


Figure 2.2 Entrainment apparatus for collecting volatiles from midges in the laboratory (a: charcoal filter, b: entrainment chamber, c: Porapak Q filters and d: pump)

2.3 GAS CHROMATOGRAPHY WITH FLAME IONISATION DETECTION

Quantification, comparisons and analysis of synthetic pheromone components were done with a Agilent HP6850 instrument fitted with a fused silica capillary column (30 m x 0.32 mm i.d. x 0.25 µm film thickness) coated with polar phase (Supelcowax-10, Supelco, USA) used in the analysis. The splitless injector was set at 220°C. The temperature of the oven was held at 50°C for 2 min then programmed at 10°C/min to 250°C and held for 5 min. Helium was used as the carrier gas (1.0 ml/min). Compounds were detected by FID and data was processed with EZChrom software (Elite v3.0).

2.4 GAS CHROMATOGRAPHY LINKED TO ELECTROANTENNOGRAPHIC RECORDING (GC-EAG)

Male antennal responses to female volatiles were analysed by gas chromatography (GC) linked to an electroantennograph (EAG) (Cork *et al.*, 1990). The GC used was a HP6890 instrument (Agilent Technologies) with a flame ionisation detector and fused silica capillary columns (30 m x 0.32 mm x 0.25 µm film thickness) coated with polar (Supelcowax-10, Supelco, USA) and non polar (SPB-1, Supelco, USA) phases. The column ends were connected to

a push-fit Y-connector, the outlet of which was connected to a second Y-connector. This was connected with identical pieces of deactivated silica capillary column, one going to the flame ionisation detector (FID) and the other to a glass T piece. A stream of nitrogen (200 ml/min) blew the contents of the T piece directly over the antennal preparation for 3 sec every 17 sec. The oven temperature was maintained at 50°C for 2 min, then programmed at 10°C/min to 250°C and held for 5 min. Injection was splitless at 220°C and helium was used as carrier gas (2.4 ml/min).

EAG responses were recorded using a portable recording unit (INR-2, Syntech, The Netherlands) comprising integrated electrode holders and amplifier. The insects were anaesthetised with carbon dioxide and the legs and wings were removed using a scalpel blade in order to keep the preparation stable during the recording. Movements from the legs and wings interfered with the recording, giving very poor signal-to-noise ratio making it difficult to identify the actual EAG response for active pheromone components. Freshly pulled glass capillary electrodes filled with electrolyte solution of 0.1 M KCl (Hopkin & Williams Ltd, Essex England) and 1% polyvinylpyrrolidine (BDH Chemicals Ltd, England) were used for mounting the insects. Both antennae were inserted into the recording electrode and the abdomen into the reference electrode (Figure 2.3). Recording from the male antenna and the flame ionisation detector was obtained simultaneously and components responsible for antennal responses further examined. Signals were amplified and analysed with EZChrom software (Elite v3.0).

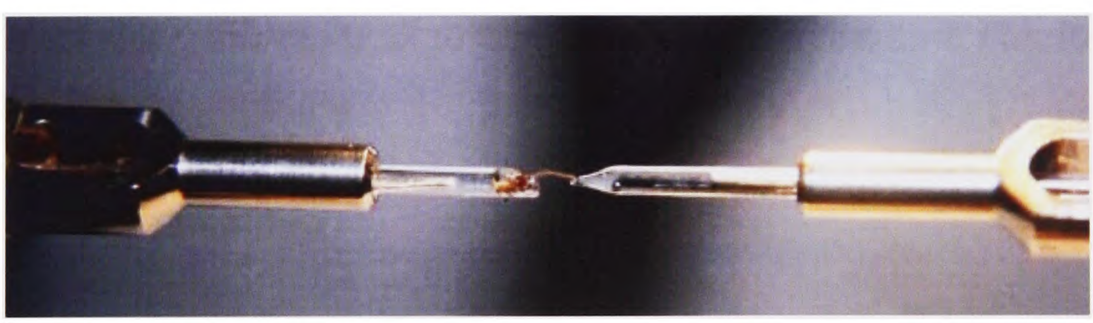


Figure 2.3 Male antennal preparation used in the electroantennographic analysis of *C. pyrivora*

The original volatile samples were reduced to approximately 200 µl under a gentle stream of nitrogen and 3 µl samples were used in routine analysis. The retention index was calculated relative to series of linear acetate esters with even numbers of carbon atoms (6-20) for each EAG active component.

2.5 GAS CHROMATOGRAPHY LINKED TO MASS SPECTROMETRY (GC-MS)

The identification of compounds giving EAG responses from male midges was carried out using three different GC-MS systems and the following conditions were used in the analysis.

2.5.1 HP 5973 Quadropole mass spectrometer

The GC was a HP6890 (Agilent Technologies) coupled to a HP 5973 quadropole mass spectrometer (Agilent Technologies) operated in electron impact (EI) mode. The source temperature was at 230°C and the quadropole was set at 150°C. The temperature of the transfer line was maintained at 250°C.

Injection was splitless (220°C) with fused silica capillary columns (30 m x 0.25 mm i.d.) coated with polar (Supelcowax-10, Supelco, USA) or non-polar (SPB-1, Supelco, USA) phases as above. The temperature of the oven was held at 60°C for 2 min then programmed at 10°C/min to 250°C and held for 5 min. Helium was used as the carrier gas (1.0 ml/min).

2.5.2 ITD 700 Ion Trap

Volatiles were also analysed in a GC-MS ion trap instrument. The GC (Carlo Erba 5300) was fitted with a polar capillary column (25 m x 0.32 mm i.d.; Supelcowax-10, Supelco, USA) linked to a Finnigan ITD 700 Ion Trap Detector and the oven temperature was programmed initially at 60°C for 2 min then at 6°C/min to 250°C. Injection was splitless at 200°C and helium was used as

carrier gas (0.8 kg/cm^2). Normally, for a single analysis 2-3 µl sample volumes were injected. However, in some cases there were no traces of potential pheromone components seen on GC-MS traces and an aliquot (20µl) of volatile collection was concentrated under a stream of nitrogen to 2µl and analysed.

2.5.3 Saturn 2200 Ion trap

GC-MS analysis was carried out with Varian CP 3800 gas chromatograph linked to Varian Saturn 2200 ion trap mass spectrometer. Samples were analysed on fused capillary columns (30 m x 0.25 mm i.d.) coated with polar (Supelcowax-10, Supelco, USA) and non-polar (30 m x 0.25 mm i.d.; VF5, Varian) phases. Oven temperature was programmed at 50°C for 2 min, then 6°C/min to 250°C and held for 5 min. Helium was used as the carrier gas (1.0 ml/min) and the injection was splitless. The chemical ionisation (CI) spectrum was obtained on the same apparatus equipped with the polar column under same conditions with liquid reagent, methanol.

GC retention times of potential pheromone components were converted to Retention Indices (RI) relative to those of acetate esters with a linear carbon chain (6-20 carbons).

2.6 MICRO-HYDROGENATION OF NATURAL PHEROMONE COMPONENTS

An aliquot (20-40 µl) of the collection of volatiles from female midges in dichloromethane was reduced just to dryness in a gentle stream of nitrogen. The residue was taken up in petroleum spirit (bp 40-60°C; 10µl). To this were added a few granules of 10% palladium on carbon (Aldrich Chemicals) catalyst suspended in petroleum sprit that had previously been washed in petroleum spirit. Hydrogen gas was bubbled through the suspension via a piece of fused silica capillary tubing (20cm) for 1 min. The resultant reaction mixture was analyzed by GC-MS.

2.7 SYNTHESIS

All midge pheromone components used in field testes were synthesised by Prof. Hall (See Appendix 1).

2.8 SEPARATION OF ISOMERS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The HPLC system used was a pump (Jasco PU-2080 *plus*) and a manual injector with a loop capacity of 20 µl. A volume of 10µl of a racemic mixture (1 mg/ml in hexane) was analysed at a time on Chiralpak AD-H column (150 mm x 4.6 mm i.d.; Daicel Chemical Industries Ltd.). Elution of compounds was monitored by Jasco UV-2075 *plus* UV detector at 200 nm or 210 nm and the data was processed by EZChrom software (Elite v3.0). Separation of peaks was achieved by isocratic elution with normal phase solvent systems containing propan-2-ol and hexane (HPLC grade Fisher Scientific). The flow rate and the solvent composition were adjusted according to analytes to give spectrum separation. The isomers were collected by hand separately into sample vials. The quantification of HPLC separated isomers was carried out by gas chromatography. Each sample was reduced to a volume of 1 ml, an aliquot of 1 µl was injected to the GC and the amount in each sample was worked out by comparison of peak area with that of 10 ng of racemic material injected.

2.9 FIELD TESTS

HPLC separated individual isomers, racemates and binary mixtures of synthetic pheromone components of (*D. pyri, C. pyrivora and D. tetensi*) were tested in the field. Mainly the field tests were focussed on (1) identifying the active stereoisomer, which was assumed to be the one produced by the insect, (2) the activities (inhibitory, inactive or partially active) of the stereoisomers which are probably not produced by the insect and (3) the extent of the activity of the minor component (active or inactive) and how it effects the activity of the major component (enhance the attractiveness or becomes inhibitory).

All treatments were replicated three or more times and a randomised complete block design was used in all field tests. The exact methodology of each field test is discussed in detail under separate chapters.

2.9.1 Dispenser

A hexane solution of the synthetic pheromone of known concentration was prepared and added in aliquots of 100 µl per dispenser. Impregnated rubber septa (Z10,072-2;Sigma Aldrich, Gillingham, UK) were allowed to dry under a fume hood.

2.9.2 Traps

White delta traps (28 cm long × 20 cm sides; Agrisense, Treforest, UK) with sticky bases baited with dispensers containing pheromones were used in the all field tests. Control traps were baited with rubber septa without the pheromone components.

2.9.3 Data analysis

Statistical analyses were done by Genstat (Genstat release 10.1, 2008, Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Data were transformed to log (x+1) to fit the assumptions of homogeneity of analysis of variance. The data field tests were subjected to two way analysis of variance (ANOVA). If significant differences ($p < 0.05$) among treatments were revealed by ANOVA, means were differentiated with the least significant different (LSD) test. See Appendix for details of the data analyses.

CHAPTER 3

IDENTIFICATION OF THE FEMALE SEX PHEROMONE OF THE PEAR LEAF MIDGE, *Dasineura pyri*

3.1. INTRODUCTION

Pear leaf midge, *Dasineura pyri* is a pest of pear, *Pyrus communis* (Rosaceae), and causes severe damage to nursery stocks and developing pear trees. It is an important pest in UK pear orchards as well as many other European countries. Male and female midges emerge from the soil in early spring and after mating the females lay eggs in the folds of young pear leaves. The eggs hatch and the larvae feed on leaves which prevent them from unfolding. As the larva matures the leaves turn brownish and later become black and brittle causing stunted growth in young plants. *D. pyri* has 3-4 generations per year. However, the number of generations greatly depends on the length of the season and availability of new growth on pear trees (Alford, 1984; Barnes, 1948).

Although the existence of a female produced sex pheromone has not been demonstrated in this species, by comparison with other *Dasineura* species (Harris et al., 1996) it was assumed that one exists and work was carried out to identify female specific components that caused an electroantennographic response from the male midge.

3.2. MATERIALS AND METHODS

3.2.1. Insect Collection

Late larvae of *D. pyri* were collected from heavily infested pear shoots in the pear orchards of Broadwater Farm, West Malling, UK in May 2006 and July

2007 with assistance from EMR staff. For further analysis more larvae were obtained from pear shoots received from New Zealand (Peter Shaw, Hortresearch) in November 2006 and January 2007 (Table 3.1). Over 20,000 larvae were collected in total and details were given in the Results Section 3.3.1. These were maintained in individual tubes until their emergence as adults, as described in General Materials and Methods, Section 2.1

3.2.2. Observation of calling behaviour of female pear leaf midge

Virgin females (40), i.e. those that emerged at the beginning of the photophase, were used for the experiment. Female midges remained inside the individual plastic tubes under controlled conditions in an insectary at 23°C:18°C and 16L:8D light cycle during the experiment. The observations were made at one hour intervals and the number of females calling was counted for the 8 hour period of the photophase.

3.2.3. Pheromone collection

Volatiles were collected from males and females separately by air entrainment as explained under General Materials and Methods in Chapter 2 Section 2.2.

3.2.4. Gas chromatography linked to electroantennographic recording (GC-EAG)

Male antennal responses to female volatiles were analysed by gas chromatography (GC) linked to electroantennography (EAG) as described in General Materials and Methods Section 2.4. Insect preparations were made with the whole insect suspended between two newly pulled glass electrodes. The male preparation was exposed to column effluent from the GC which carried the components of female *D. pyri* volatiles and signals from the antennae were recorded and processed by Ezchrom software.

3.2.5. Gas chromatography linked to mass spectrometry (GC-MS)

GC-MS analyses were carried out as described in section 2.5.2 and 2.5.1 of General Methods and Materials. The Finnigan ITD 700 Ion Trap Detector with polar GC column and HP 5973 quadropole mass selective detector with nonpolar GC column were used.

3.2.6. Micro-hydrogenation of natural pheromone component

As described in General Methods and Materials 2.6, a few granules of 10% palladium on carbon catalyst were added in to an aliquot (20 µl) of volatiles from female midges and hydrogen gas was bubbled through the reaction mixture using a piece of fused silica capillary tubing for 1 min. The reaction mixture was analyzed by GC-MS.

3.2.7. Liquid chromatography of pheromone collections

The volatile collections from female midges were fractionated on silica gel (200 mg) contained in a Pasteur pipette. The silica gel was dry packed and petroleum spirit (bp 40-60°C) passed through while tapping the column with a piece of rubber tube in order to remove any gas bubbles trapped inside. Two volatile collections (F1 and F3) were combined and evaporated to dryness under a stream of nitrogen gas. The residue was taken up in petroleum spirit (bp 40-60°C) and the solvent was evaporated twice before dissolving the residue in petroleum spirit (100 µl). The extract was loaded onto the silica gel and another 2 ml of petroleum sprit was passed through. Compounds were eluted using a gradient solvent system of diethyl ether and petroleum spirit in increasing order of polarity (1 ml each of 5, 10, 20, 50, 100% diethyl ether: petroleum spirit). Fractions (1 ml) were collected and analysed by coupled GC-EAG and GC-MS.

3.2.8. Synthetic pheromone

Racemic (*Z*)-2,13-diacetoxy-8-heptadecene was prepared by Prof. Hall from racemic (*Z*)-13-acetoxy-8-heptadecene-2-one by reduction with lithium aluminium hydride in ether and acetylation with acetic anhydride in pyridine (Appendix 1).

3.2.9. Separation of stereoisomers by high performance liquid chromatography (HPLC)

The two pairs of enantiomers of (*Z*)-2,13-diacetoxy-8-heptadecene were separated by HPLC on a Chiralpak AD-H column as described in Chapter 2 Section 2.7. Elution of compounds was monitored by UV detector at 200 nm and the data was processed by Jasco Borwin software. The racemate was separated into four peaks by isocratic elution with 0.4% propan-2-ol in hexane and flow rate of 0.4 ml/min.

3.2.10. Absolute configuration of stereoisomers

Three of the four saturated analogues of stereoisomers of (*Z*)-2,13-diacetoxy-8-heptadecene were synthesised by Prof. Hall. The three stereosiomers were (*R,R*)-2,13-diacetoxyheptadecane, (*S,S*)-2,13-diacetoxyheptadecane and (*R,S*)-2,13- diacetoxyheptadecane.

The racemic (*Z*)-2,13-diacetoxy-8-heptadecene was hydrogenated. Approximately 200 µl of 1 mg/ml of the racemate was mixed with 10 drops of the catalyst (10% palladium on carbon in hexane) and hydrogen was bubbled through the mixture for 10-12 min. The experimental procedure and apparatus used were as described in Chapter 2 Section 2.6. The reaction was monitored by GC-MS. The hydrogenated reaction mixture was filtered through a filter (Acrodisc ® CR 13 mm Syringe Filter with 0.45 µm PTFE Membrane; Gelman Laboratory) and the filtrate was analysed.

The hydrogenated racemic mixture was co-analysed with each of the synthetic (*R,R*)-2,13-diacetoxyheptadecane, (*S,S*)-2,13-diacetoxyheptadecane and (*R,S*)-2,13-diacetoxyheptadecane by HPLC on the chiral phase which was used in separating the isomers of (*Z*)-2,13-diacetoxy-8-heptadecene. Equal volumes (7.5 µl; 1 mg/ml) of hydrogenated racemic mixture and each of the saturated analogues of known stereochemistry were co-injected. The method described under 3.2.9 was slightly modified in the analysis. Four peaks of the racemic (*Z*)-2,13-diacetoxyheptadecane were separated by isocratic elution with 0.4% propan-2-ol in hexane at 0.3 ml/min monitored at 220 nm in the UV detector.

The order of elution of the isomers in the hydrogenated racemic mixture was confirmed as follows. Collections of HPLC separated four stereoisomers of (*Z*)-2,13-diacetoxy-8-heptadecene (A, B, C, and D) were hydrogenated individually as described in Chapter 2 Section 2.6. The reaction was monitored by GC-MS. Co-analyses of racemic (*Z*)-2,13-diacetoxyheptadecane with each of the hydrogenated stereoisomers by HPLC on a chiral phase were carried out. The same conditions used in the analysis of stereoisomer with known configuration were used.

3.2.11. Field tests

First field test

Preliminary field tests were carried out at East Malling Research Station and Broadwater Farm, West Malling, UK from 25th July - 22nd September 2006. Rubber septa were impregnated with synthetic racemic (*Z*)-2,13-diacetoxy-8-heptadecene (10 µg) by adding a hexane solution (0.1 mg/ml; 100 µl) and allowing to dry under a fume hood. White delta traps with sticky bases baited with lures were deployed in four separate plots of pears. In each plot an unbaited control and a pheromone-baited trap were hung on the 10th and the 20th pear trees on the centre row. The sizes of the plots varied from 7 ha to 0.25 ha. Insect counts were taken once a week.

Second field test

The four stereoisomers of the synthetic pheromone were tested in St Leonard pear orchard (7 ha) West Malling, UK in September, 2007. Approximately 12 µg of each of four stereoisomers was collected from the HPLC chiral separation (Section 3.2.9). Three rubber septa lures of each individual isomer (A, B, C and D) containing 3 µg were made. An aliquot of 100 µl was used for septa from a stock solution of 0.03 µg/µl. White delta traps were used as in the first field experiment; and traps were placed in a single row (ninth from the border) on every seventh tree in a randomised complete block design. Three replicates of each treatment were made.

Third field test

Stereoisomers, B, C and D were combined with A in the ratio of 1:1 in order to investigate the inhibitory effects of individual components. The rubber septa lures were impregnated with binary mixtures (A+B, A+C and A+D) containing approximately 4 µg of each component and tested against the active stereoisomer A alone (Table 3.2). Four lures from each type were made and the test was carried out in early October, 2007.

Fourth field test

The activity of HPLC separated individual stereoisomers was tested in pear orchards in Nelson, New Zealand, from November, 2007 to January, 2008. Stereoisomers A, B, C and D were tested against racemic mixture of (*Z*)-2,13-diacetoxy-8-heptadecene. The first eluting isomer from HPLC, A was tested twice due to erroneous labelling of dispensers. Dispensers were loaded with 5 µg of individual stereoisomers (A, B, C or D) and 10 µg of the racemate of (*Z*)-2,13-diacetoxy-8-heptadecene. All treatments were replicated five times and trap counts were taken once every week.

Fifth field test

Binary mixtures of stereoisomers of A with B, C and D were tested in pear orchards in Nelson, New Zealand, from November, 2007 to January, 2008. Rubber septa dispensers were impregnated with 5 µg of each component in 1:1 ratio and tested alongside A with loading of 5 µg per dispenser.

Data analysis

Data were transformed to log(x+1) in order to achieve the normality of the data set. If analysis of variance (ANOVA) showed significant differences among treatments ($p<0.05$), then the means were differentiated with the LSD test.

3.3. RESULTS

3.3.1. Insect Collection

Approximately 20,000 larvae were collected and reared. Although a large number of insects was reared, only 30.5% females emerged. The highest percentage of adults emerged from the May 2006 collection with males and females at 32.3% and 41.3% respectively (Table 3.1).

Table 3.1. Numbers of larvae of *D.pyri* reared and females and males emerged from each collection.

Collection	Origin	Number of larvae reared	Number of males emerged	Number of females emerged
May, 2006	UK	6299	2037	2603
November, 2006	NZ	1356	210	348
January, 2007	NZ	3636	349	539
July, 2007	UK	~9000	-	2964

3.3.2. Calling behaviour of female *D. pyri*

The calling behaviour of newly emerged females was observed for eight hours from the beginning of the photophase in the insectary. Newly emerged female midges initiated calling in the early hours of the photophase. This continued throughout the observation period and declined at the end of the day after 8 hr (Figure 3.1). Few midges (15%) were found to be calling in the early hours, after commencing the light regime, and a maximum number of callings (45%) occurred after 4 hr into the photophase.

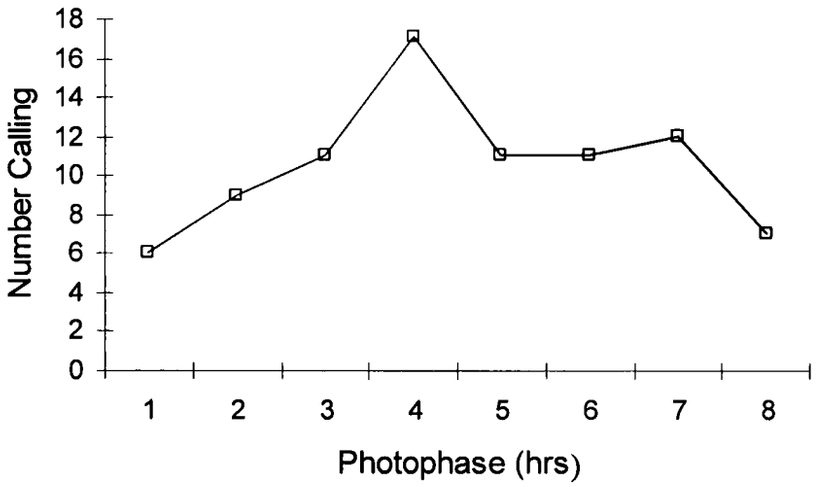


Figure 3.1. Calling behaviour of *D. pyri* females (n = 40)

During the observation period 67.5% of the midges called at least once. Calling behaviour was not continuous, and about 25% out of the total number called uninterruptedly for 3 hours or more during the time observations were taken. These results indicated that pheromone collection should be started early in the photophase to have the time of maximum calling frequency.

3.3.3. Pheromone collection

Eighteen sets of female (F1-F3, NZF1- NZF6, EMRF1- EMRF8 and NRIF1) and nine sets of male (M1-M3 and NZM1-NZM6) volatile samples were collected with batches ranging from 30 to 1160 midges (Table 3.2).

Table 3.2. Number of *D. pyri* males and females entrained in 2006 and 2007

Filter ID	Number of insects emerged	Filter ID	Number of insects emerged
May, 2006 (UK)			
F1	830	M1	401
F2	1159	M2	474
F3	614	M3	332
November, 2006 and January, 2007			
NZF1	107	NZM1	105
NZF2	180	NZM2	105
NZF3	61	NZM3	26
NZF4	354	NZM4	220
NZF5	113	NZM5	93
NZF6	72	NZM6	36
July, 2007 (UK)			
EMRF1	210	EMRF6	470
EMRF2	228	EMRF7	470
EMRF3	233	EMRF8	472
EMRF4	256	NRIF1	209
EMRF5	252		

3.3.4. GC-EAG analysis

GC-EAG analyses of volatiles from female *D. pyri* midges collected during May 2006 indicated the presence of two compounds eliciting consistent EAG responses from male antennae (Figure 3.2). The minor component (EAG response with lower intensity) appeared at 18.42 min and 19.26 min on polar and non polar columns respectively and the major component (EAG response with higher intensity) appeared at 21.90 min and 20.37 min respectively (Table 3.3). These EAG active compounds were assumed to be components of the female sex pheromone.

Midges were reared in 2006 and 2007 and several female volatiles collections (F1-F3, NZF1- NZF6, EMRF1-EMRF8 and NRIF1) were made. All these female volatile collections were analysed by GC-EAG and GC-MS. Male response to the major component was seen when all these volatile collections were tested by GC-EAG. Also, the presence of the minor component was observed in May 2006 (F1-F3) and November and January 2007 (NZF1-NZF6) collections when these were tested by GC-EAG. However, the minor response was not noticed from male midge EAG preparations for volatile collections, EMRF1- EMRF8 and NRIF1 (Table 3.2).

Although the retention time of the response from male midges varies slightly, the response seems to be within a spread of a puff (20 sec). This is because the nitrogen puff containing volatiles blows over the EAG preparation after every 17 sec by the puff delivery system.

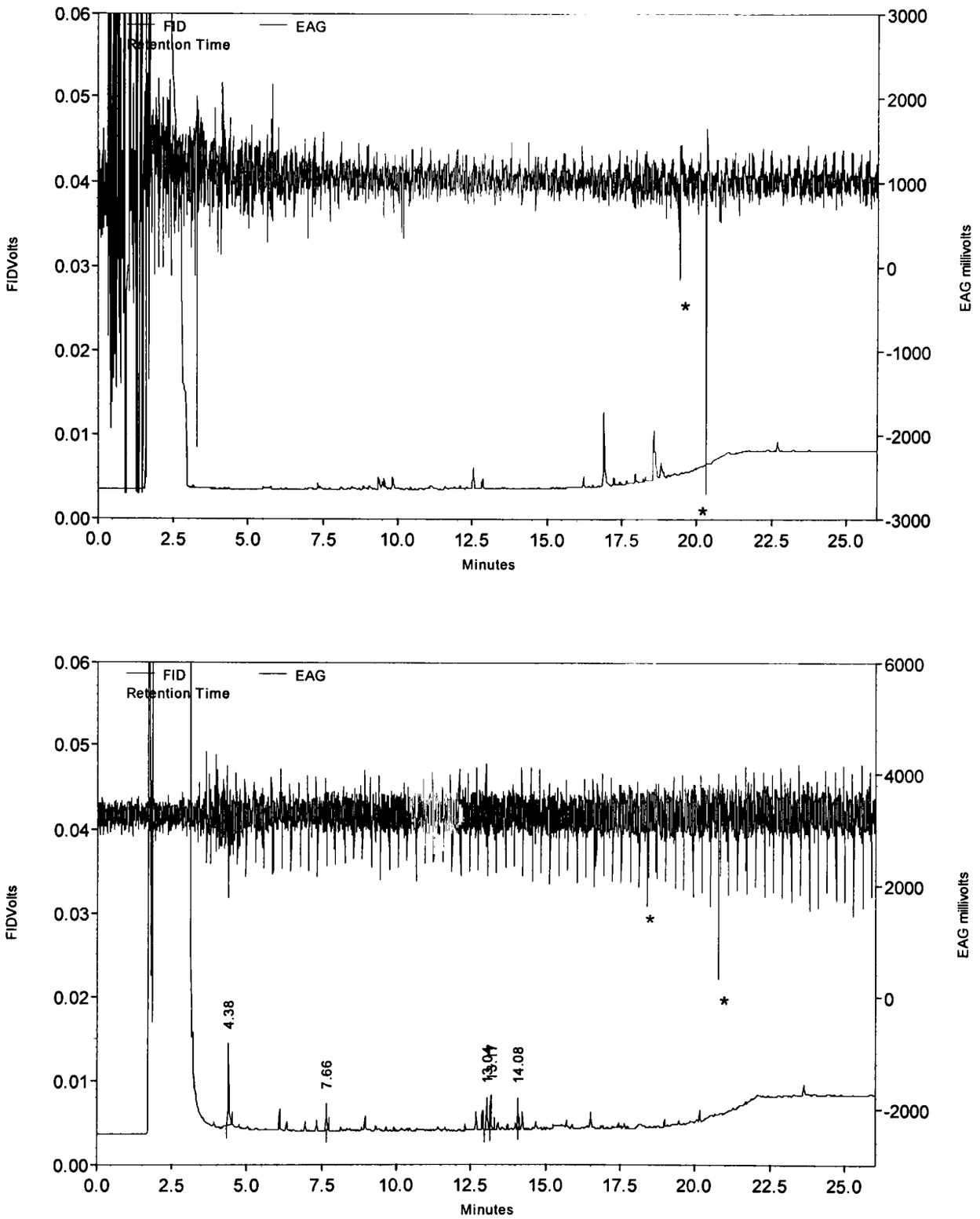


Figure 3.2. Male response (*) to volatile collections from females on non-polar GC column (upper) and on polar column (lower)

Table 3.3. Retention times (RT) and retention indices (RI) of the natural pheromone components of *D. pyri* and synthetic standards analysed on non-polar and polar GC columns in GC-EAG and GC-MS

Compound	Non-polar GC column		Polar GC column		Δ^1
GC-EAG	**RT (min)**	**RI**	**RT (min)**	**RI**	
Response to major component	20.37	1854	21.90	2087	233
Response to minor component	19.26	1726	18.42	1770	44
Heptadecan-2-ol		1512		1726	114
Heptadecan-2-one		1799		1537	38
2-acetoxyheptadecane		1621		1576	-45
NZF1- NZF6	19.40	1746	18.33	1753	7
GC-MS					
Major component	33.86	1854	24.33	2082	228
Hydrogenated major component	34.24	1881	21.80	2051	170
(*Z*)-2,13-diacetoxy-8-heptadecene	33.88	1857	24.34	2083	226
Z)-2,13-diacetoxyheptadecane (Hydrogenated)	34.23	1889	21.85	2056	167
(*Z,E*)-2-acetoxy-10,12-heptadecadiene	18.85	1688	18.13	1736	48

[1] Difference between RI on polar column and RI on non polar column.

3.3.5. GC-MS analysis

In GC-MS analysis of volatiles from female *D. pyri*, a peak was observed having the same RI as the major pheromone component detected in the GC-EAG analysis on both polar and non polar GC columns. This peak was not observed in GC-MS analysis of volatiles from male *D. pyri* (Fig.3.3).

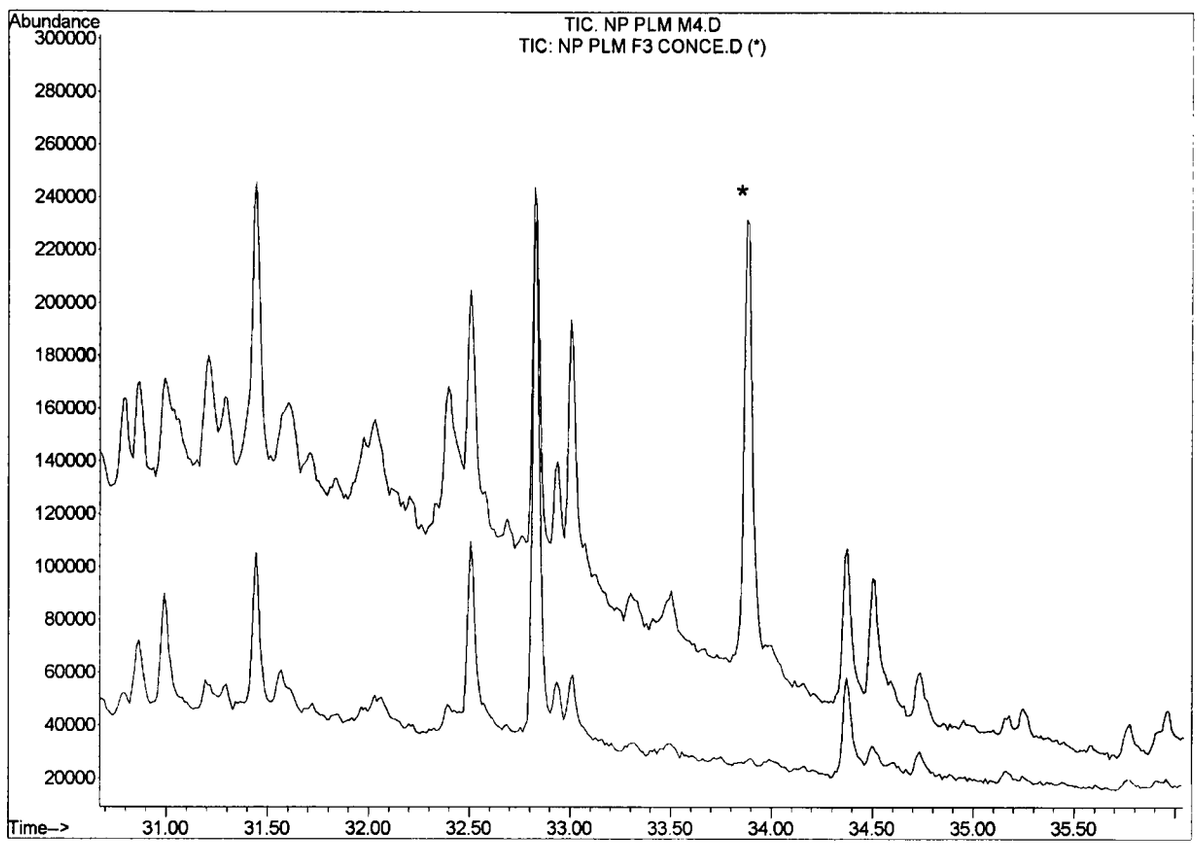


Figure 3.3. Comparison of volatile collections from female (upper) and male *D. pyri* (lower) in GC-MS analysis on non polar column.

No consistent female-specific component was detected in GC-MS analyses corresponding to the minor component observed in GC-EAG analysis on polar and non polar GC columns.

Retention data of the major component and the synthetic standards on GC-EAG polar and non polar column were used to make an approximation of the elution of the major component on GC-MS relative to synthetic standards on the relevant GC column. Also, scanning the mass spectrum for selected ions was carried out in discovering the pheromone components on mass

spectrum. Further, the major component and the MS fragmentation pattern and GC retention indices were compared with those of known compounds.

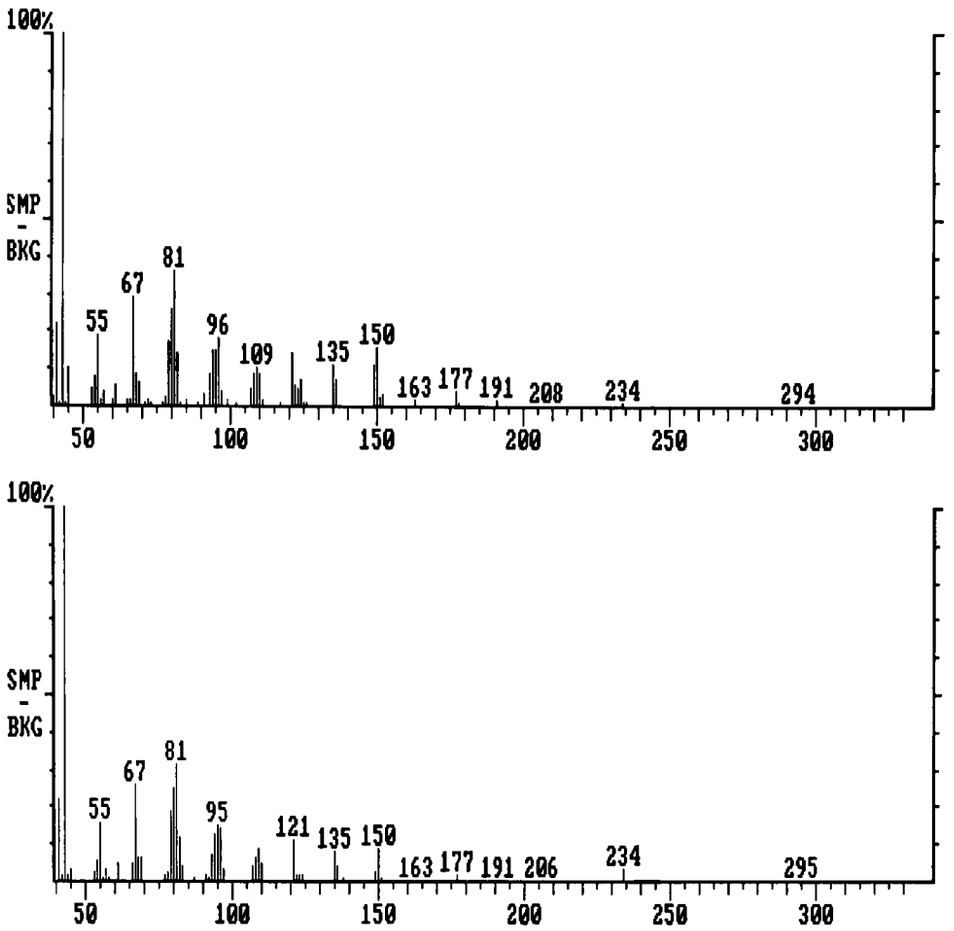


Figure 3.4. Mass spectrum of major pheromone component (upper) and synthetic (*Z*)-2,13-diacetoxy-8-heptadecene (lower).

The mass spectrum (Figure 3.4) showed ions at *m/z* 43 and *m/z* 61 which are diagnostic peaks for acetate esters. Mass spectral data of the pheromone components of *Mayetiola thujae,* (2*S*,12*S*)-2,12-diacetoxyheptadecane, (2*S*,13*S*)-2,13-diacetoxyheptadecane and (2*S*,14*S*)-2,14-diacetoxy-heptadecane (Gries et al., 2005) were compared with those of *D. pyri.* Amongst three pheromone components of *Mayetiola thujae*, (2*S*,13*S*)-2,13-diacetoxyheptadecane shows similar fragments which are of *m/z* 2 units higher than *D. pyri* pheromone component, for instance, *m/z* 236, 179, 152, and 123.

Mass spectral data of the above components are very similar but each

component has one or two unique ions. Thus *m/z* 179 only appeared in the mass spectrum of 2,13-diacetoxyheptadecane, resulting from α-cleavage of 13-14 bond followed by removal of two acetate groups from the parent molecule of 356.

This indicated that the *D. pyri* pheromone component was a 17-carbon diacetate with one double bond. The fragment of low intensity at *m/z* 234 was consistent with loss of two acetic acid units from 354. The ion at *m/z* 177 (2 %) was formed due to similar bond cleavage at C13 indicating that one acetoxy group was located on C13. The similarity of the mass spectrum suggested that the other acetoxy group was located on the second carbon atom as in (2*S*,13*S*)-2,13-diacetoxyheptadecane.

Ions from the possible allylic cleavage were not observed on the mass spectrum. However, ions resulting from the subsequent fragmentation of the above were seen. This was a strong indication of the position of the double bond on the eighth carbon. The ion at *m/z* 150 was formed due to removal of acetoxy group on 13th carbon. The ion *m/z* 211 due to allylic cleavage of 6-7 carbon–carbon bond. Also, the elimination of acetic acid unit from resulting ion m/z 157 of allylic cleavage produced m/z 96 (Figure 3.5).

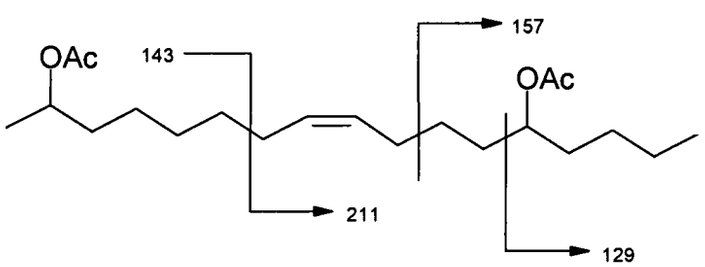


Figure 3.5. Possible mass fragmentations of candidate pheromone component, (Z)-2,13-diacetoxy-8-heptadecene

3.3.6. Micro-hydrogenation of natural pheromone component of pear leaf midge

The mass spectrum after the hydrogenation of volatiles collected from female *D. pyri* (F3) showed that several ions increased by two mass units of the major component, indicating the presence of one double bond (Figure 3.6). The retention index difference of the hydrogenated major component and the mono unsaturated component indicated a difference of -27 and +31 units on non-polar and polar columns respectively. In the identification of the sex pheromone from Douglas fir cone gall midge, *Contarinia oregonensis*, (2*S*,4*Z*,7*Z*)-2-acetoxy-4,7-tridecadiene (Gries et al., 2002), the retention indices of all possible isomers of the monounsaturated analogues were tabulated on different GC phases. Gries et al. (2002) compared those retention indices with that of saturated analogues of *C. oregonensis* pheromone component on a similar non-polar GC phase that we used in this work showed that for the pheromone component of *D. pyri* the double bond should be in the middle not at the end of the chain. The retention index differential of *D. pyri* on polar column matched those values of Gries et al. (2002), suggesting that the double bond may be on the *Z*8 position.

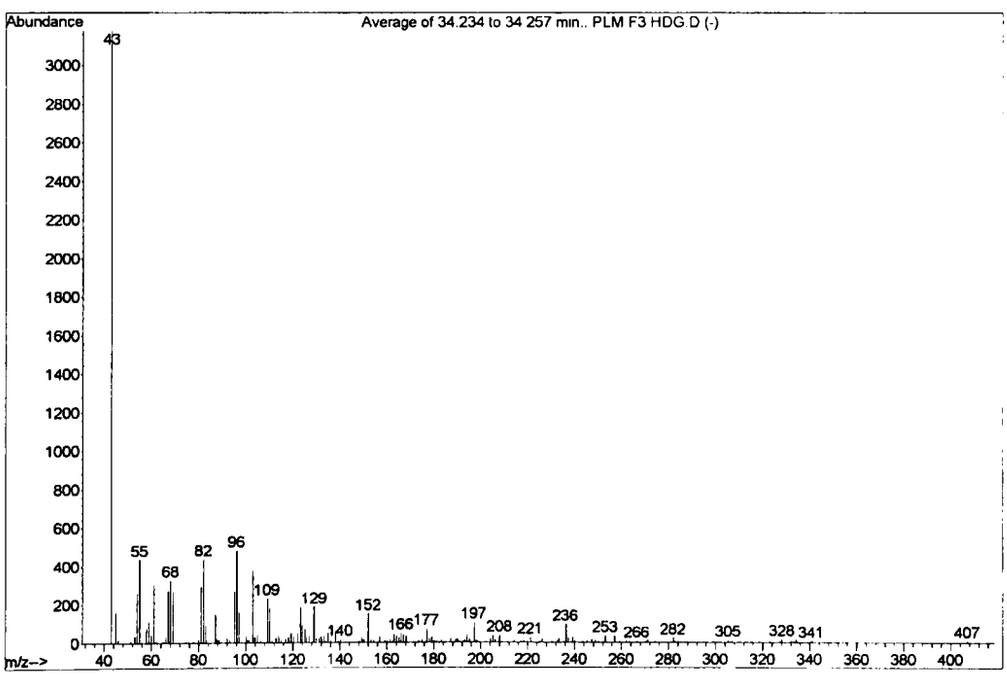


Figure 3.6. Mass spectrum of the hydrogenation product of the major natural pheromone component of *D. pyri.*

(*Z*)-2,13-diacetoxy-8-heptadecene was synthesised as a model 17-carbon, monosaturated diacetate, from (*Z*)-13-acetoxy-8-heptadecene-2-one, the pheromone of the apple leaf curling midge, *D. mali* (Appendix 1). The retention indices and the GC mass spectrum of both on polar and non-polar columns were found to be identical with those of the major EAG active component in volatiles collected from female midges of *D. pyri* (Table 3.4).

3.3.7. Attempt to identify the minor EAG-active component

The volatile collections F1 and F3 were combined as both extracts appeared to contain the minor and the major components according to GC-EAG analysis and in comparison of GC traces of F1 and F3 they seemed to be similar.

Three known compounds, heptadecan-2-ol, 2-acetoxyheptadecane and heptadecan-2-one which were closely related to the major component, (*Z*)-2,13-diacetoxy-8-heptadecene were tested on GC polar and non-polar columns. Retention data indicated that none of the three compounds were the minor component (Table 3.3).

Liquid chromatography (LC) was carried out on volatiles from female *D. pyri* in an attempt to purify the minor EAG active component and to compare its behaviour in LC with that of known synthetic compounds. A mixture of synthetic compounds, heptadecan-2-ol, heptadecan-2-one and the synthetic major component (*Z*)-2,13-diacetoxy-8-heptadecene was separated by LC. In the gradient elution of diethyl ether/petroleum spirit, the order of elution of components was heptadecane-2-one, heptadecane-2-ol and (*Z*)-2,13-diacetoxy-8-heptadecene (Table 3.4). The gradient elution of diethyl ether/petroleum spirit separated the diacetoxy component from other synthetic mono functional constituents.

Based on the above observation, volatile collections from females were fractionated. Six fractions were obtained from liquid column chromatography and analysed by GC-EAG and GC-MS. GC-EAG analysis revealed male

midges responded to major and the minor components eluting in the later fractions (Table 3.4).

Table 3.4. Fractions obtained by LC separation of a mixture of synthetic compounds, heptadecan-2-ol, heptadecan-2-one and the synthetic major component (*Z*)-2,13-diacetoxy-8-heptadecene and female volatile collection (+ EAG response or presence of the component; - no EAG response from *D. pyri* males or absence of the component)

Fraction diethyether: hexane %	17:2OH[1]	17:2keto[2]	Z8-17:2,13 OAc[3]	EAG response	
				Minor component	Major component
0:100	-	-	-	-	-
5:95	-	-	-	-	-
10:90	-	+	-	-	-
20:80	+	+	+	-	+
50:50	+	-	+	+	+
100:0	-	-	+	+	+

[1] heptadecan-2-ol
[2] heptadecan-2-one
[3] synthetic (*Z*)-2,13-diacetoxy-8-heptadecene

If the minor component was related to any of above synthetic components (heptadecan-2-ol and heptadecan-2-one) it would have been present in the earlier fractions, most likely in the third fraction (20% diethyl ether/petroleum spirit).

Although two collections, F1 and F3 were combined and impurities were removed to a certain extent by LC, it was not possible to acquire a mass spectrum for the minor component.

A model component was synthesised based on the retention indices of the minor component. GC-EAG analysis of (*Z,E*)-2-acetoxyheptadeca-10,12-diene was carried out. A male *D. pyri* EAG antennal preparation did not respond to the synthetic compound. When retention times and retention indices of (*Z,E*)-2-acetoxyheptadeca-10,12-diene on polar and non polar GC columns were compared with those of the male EAG response for the minor

pheromone component, the inter-column differential of the synthetic (*Z,E*)-2-acetoxyheptadeca-10,12-diene was fairly close to that of the natural minor component, but not identical (Table 3.3).

3.3.8. HPLC separation of isomers of synthetic (*Z*)-2,13-diacetoxy-8-heptadecene

(*Z*)-2,13-Diacetoxy-8-heptadecene has two chiral centres and therefore possess four diastereomers. All four isomers were separated by HPLC on a chiral column (A, B, C and D) (Figure 3.7).

In unreplicated GC-EAG analysis of the separated isomers, male midges showed electrophysiological activity only to the first three stereoisomers (A, B and C) but not to the last peak, D, suggesting the naturally occurring pheromone component was amongst the first three. The last eluting stereioisomer from HPLC did not give an electrophysiological response.

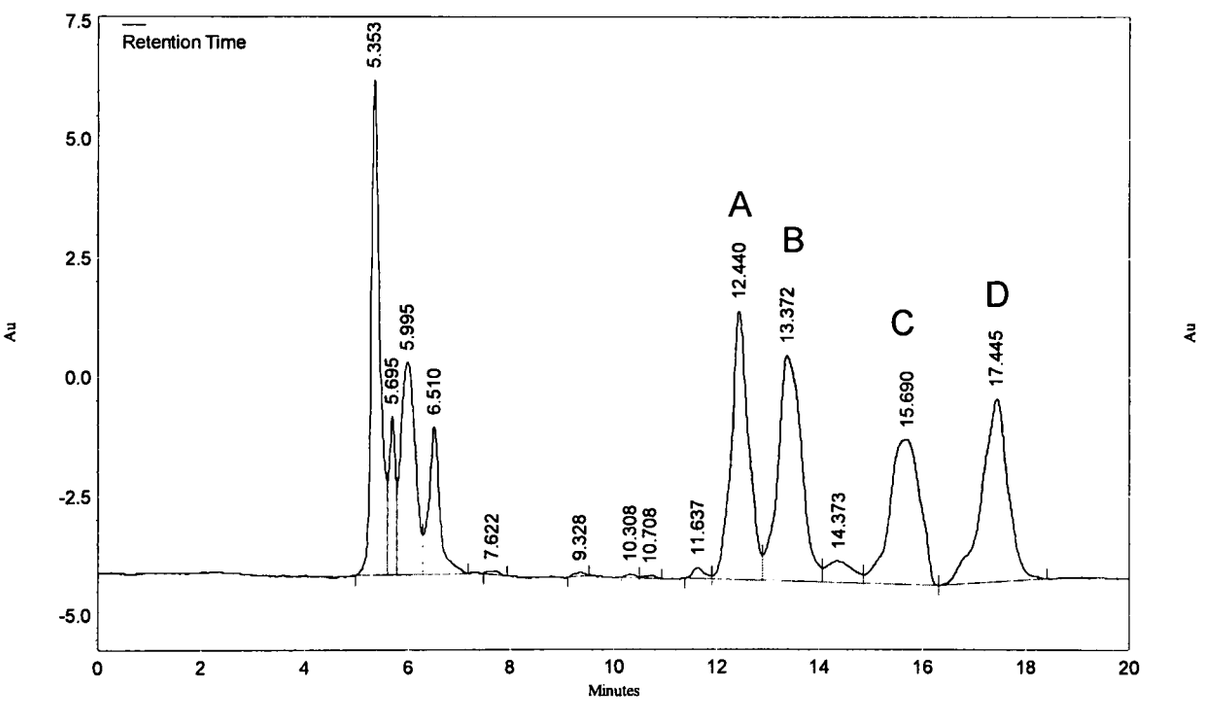


Figure 3.7. HPLC separation of isomers of (*Z*)-2,13-diacetoxy-8-heptadecene

3.3.9. Absolute configuration of stereoisomers

Racemic 2,13-diacetoxyheptadecane was prepared by catalytic hydrogenation of (Z)-2,13-diacetoxy-8-heptadecene and was separated into four peaks, HA, HB, HC and HD by HPLC on the chiral column (Figure 3.8). Peaks HA, HB, HC and HD were collected and GC analysis confirmed that all stereoisomers had the same GC retention time as racemic 2,13-diacetoxyheptadecane. The third eluting peak (HC) split into two peaks, only one of which was the stereoisomer of 2,13-diacetoxyheptadecane and the other was an impurity (Figure 3.8).

Co-analysis of racemic 2,13-diacetoxyheptadecane with three synthetic stereosisomers of known stereochemistry suggested that the first, second and the fourth eluting isomers had (*R,R*), (*R,S*) and (*S,S*) configuration at the chiral centres respectively (Figure 3.9 a, b and c). Therefore, the configuration of the third eluting stereoisomer from the HPLC on the chiral column should be (*S,R*).

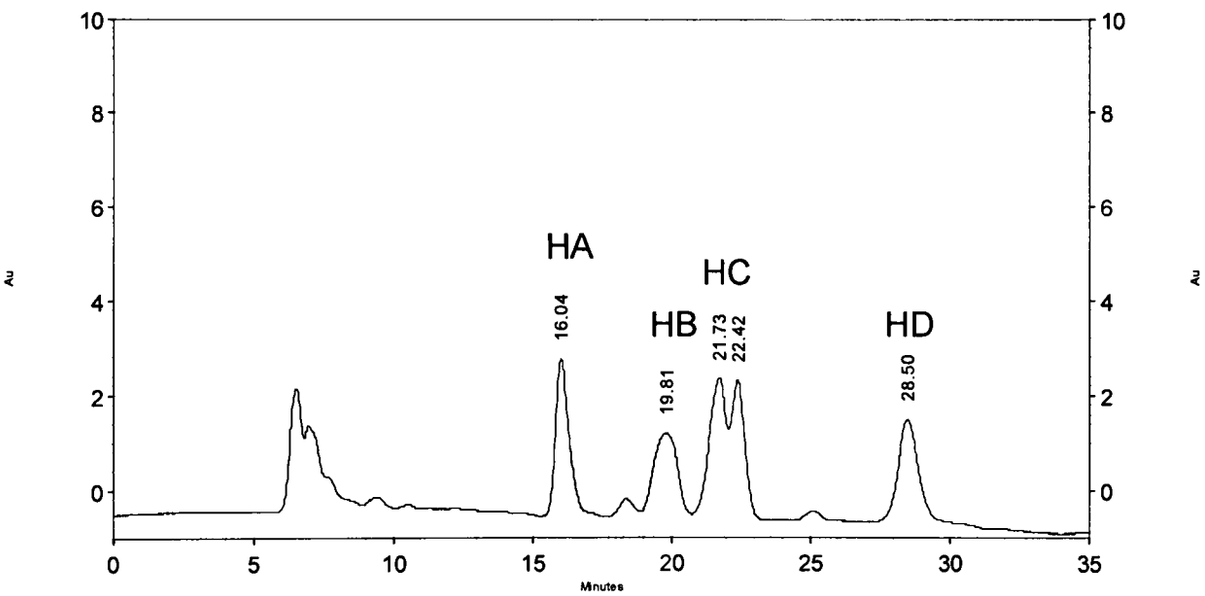


Figure 3.8. HPLC separation of racemic 2,13-diacetoxyheptadecane

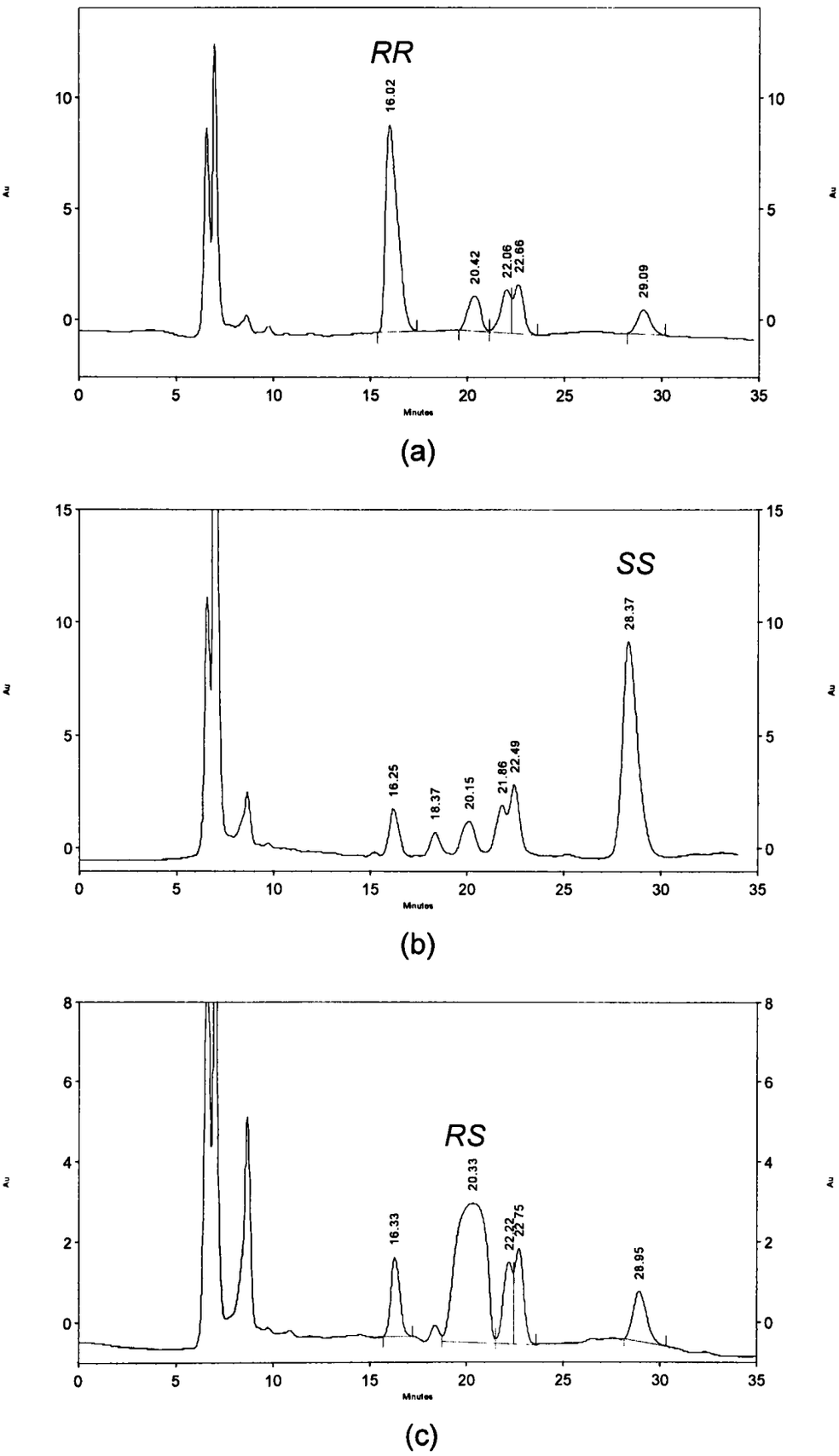


Figure 3.9. Co-analysis of the racemic (*Z*)-2,13-diacetoxyheptadecane with that of (a): (*R,R*)-2,13-diacetoxyheptadecane,(b): (*S,S*)-2,13-diacetoxy-heptadecane and (c): (*R,S*)-2,13-diacetoxyheptadecane.

The four stereoisomers of (*Z*)-2,13-diacetoxy-8-heptadecene were separated by HPLC and hydrogenated individually. Analysis by HPLC on the chiral column demonstrated that the elution order of the stereosiomers of 2,13-diacetoxyheptadecane (HA, HB, HC, and HD) was the same as that of stereoisomers of (*Z*)-2,13-diacetoxy-8-heptadecene (A, B, C and D; Figure. 3.8 and Figure 3.9 a, b, and c). In co-chromatography, increase of the area of one peak of the racemate indicates co-elution of the isomer with known stereochemistry (Figure 3.10 a, b, c and d).

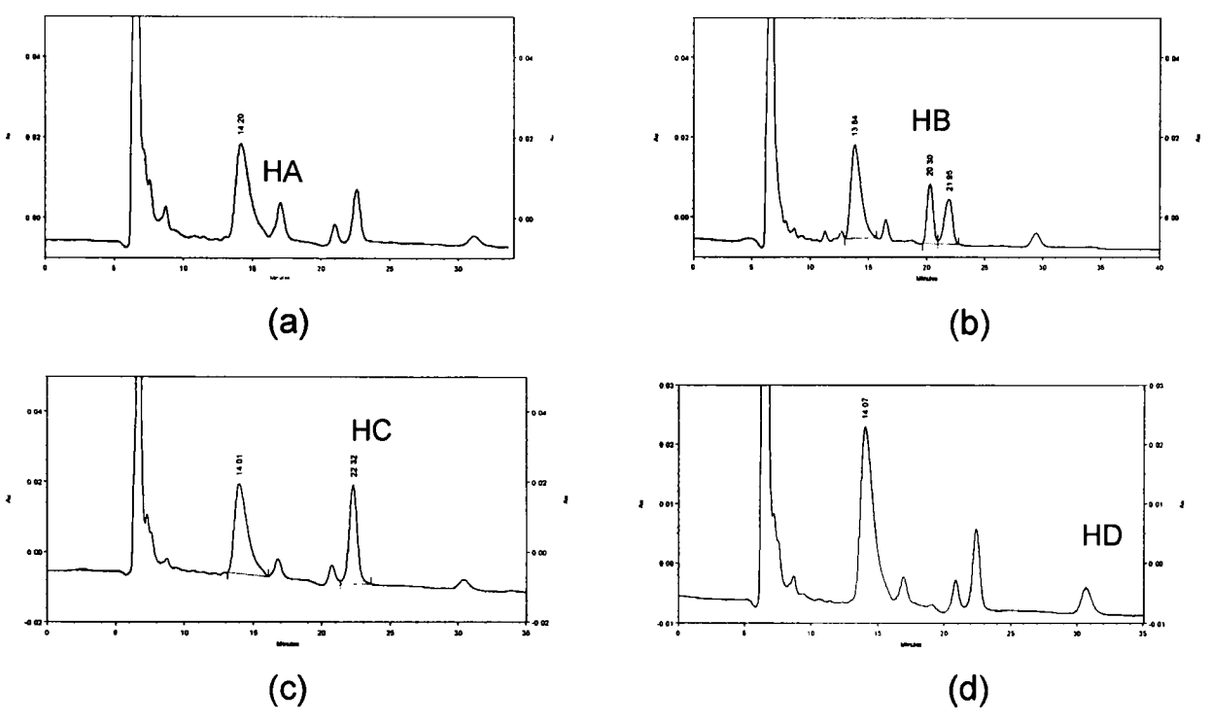


Figure 3.10. Coanalysis of the racemate (*Z*)-2,13-diacetoxyheptadecane with each of hydrogenated individual stereoisomers of (*Z*)-2,13-diacetoxy-8-heptadecene (a) hydrogenated A+hydrogenated racemate; b) hydrogenated B+ hydrogenated racemate; c: hydrogenated C+hydrogenated racemate and d: hydrogenated D+hydrogenated racemate)

3.3.10. Field tests

First field test

The racemic (*Z*)-2,13-diacetoxy-8-heptadecene was tested in August-September, 2006. Lures containing 10 µg were used in baited traps and tested with unbaited traps. The presence of the midge was detected in the orchard, as upon examining the damaged shoots larvae were found feeding inside the galls. However, traps baited with the racemic mixture did not catch

any males of *D. pyri*.

Second field test

The four isomers of synthetic *(Z)*-2,13-diacetoxy-8-heptadecene were separated by HPLC and individual components (A, B, C and D) were tested in the field. Trap catches for two consecutive weeks revealed that A is the attractive isomer for male *D. pyri*. More males per trap were caught in traps baited with isomer A (F=81.58, df= 4, 8, P<0.001) than traps baited with B, C, D or unbaited. B and D did not catch any male midges (Figure 3.11) and there were no significant differences between catches with isomers B, C and D compared with catches in the unbaited traps.

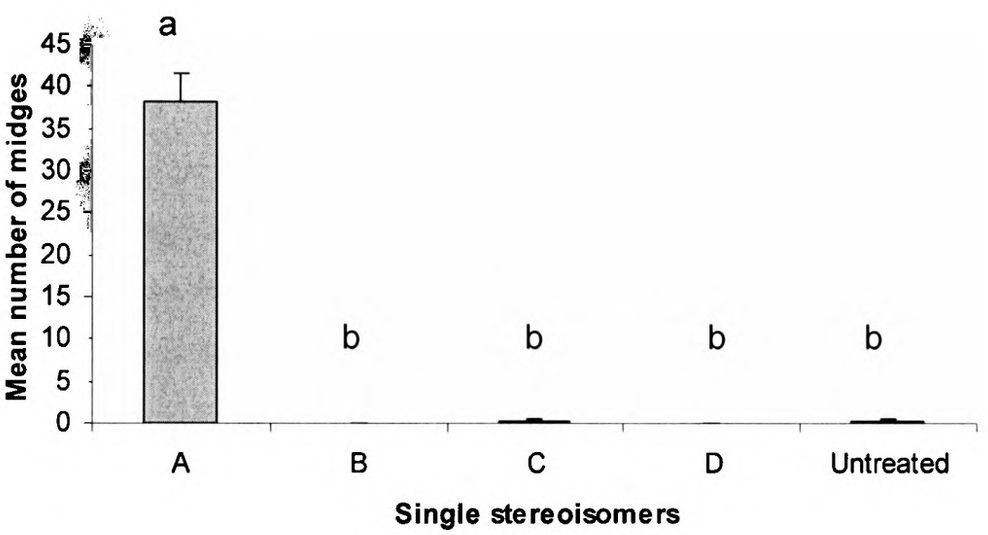


Figure 3.11. Mean trap catch (± SE) of *D. pyri* males during two weeks (12-24/09/2007; 3reps) with single stereoisomers (A, B, C, D) of (*Z*)-2,13-diacetoxy-8-heptadecene. (Data from trap catches were transformed to log(x+1) and statistically analysed by ANOVA followed by LSD test at (α<0.05)).Means followed by the same letter were not significantly different (p>0.05).

Third field test

This field test was carried out at the end of the season and catches were too low for statistical analysis. However male *D. pyri* midges were caught in traps baited with A alone and mixtures of A+C and A+D. No midges were caught in the unbaited traps and those baited with mixtures A+B suggesting that isomer B may be the inhibitory isomer (Figure 3.12).

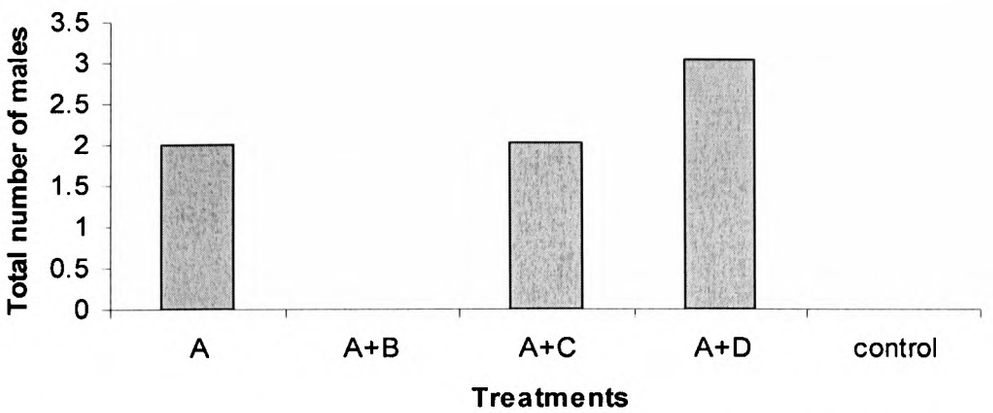


Figure 3.12. Total catch of *D.pyri* males over two weeks (2-8/10/2007; 3 reps) in traps baited with binary mixtures of stereoisomers (A+B, A+C and A+D)) and stereoisomer A of (*Z*)-2,13-diacetoxy-8-heptadecene.

Fourth field test

The first field test carried out in New Zealand confirmed the results from the UK that indicated traps baited with A attracted significantly more male midges than traps baited with the other isomers, B, C, D, racemate or control (*F*=46.56, *df*=6,24 *P*<0.001) (Figure 3.13). The numbers of males caught in traps baited with B, C, D or racemate were not significantly different from the numbers caught in unbaited traps. A few males were attracted to traps baited with the racemate. However, statistical analysis showed the numbers caught by the racemate were not significantly different from the catches in B, C, D or the control.

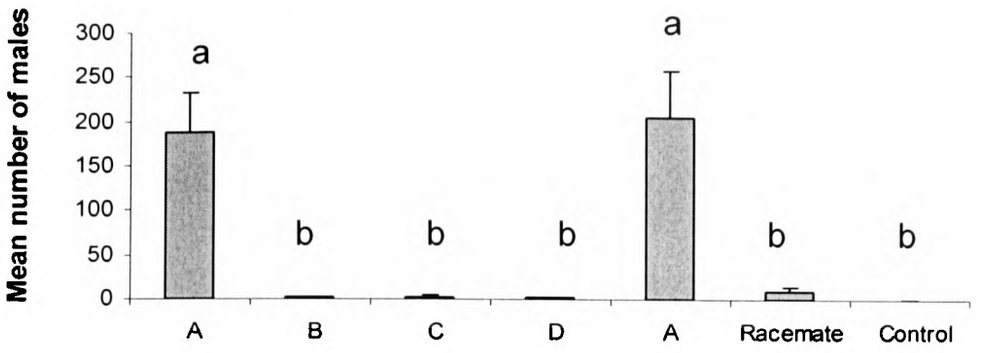


Figure 3.13. Mean trap catch (± SE) of *D. pyri* males during three weeks (14/11-12/12/2007; 5 reps) with HPLC separated single stereoisomers (A, B, C, D) and racemate of (*Z*)-2,13-diacetoxy-8-heptadecene carried out in Nelson, New Zealand (Data from trap catches were transformed to log(x+1) and statistically evaluated by ANOVA followed by LSD test at (α<0.05)). Means followed by the same letter were not significantly different (p>0.05). Stereoisomer A was tested twice due to an error in labelling dispensers.

Fifth Field test

When traps baited with lures containing binary mixtures of A and B, C or D alongside A and unbaited control were tested, higher numbers of males (F=66.85, df=4, 16, P<0.001) were caught in A, A+C and (A+D) than A+B or control (Figure 3.14). Combining C or D with A did not affect the attractiveness of A to male midges and all three treatments attracted males to the same extent. However, attractiveness of isomer A was strongly inhibited by B which was not attractive to males when tested singly in the fourth field test.

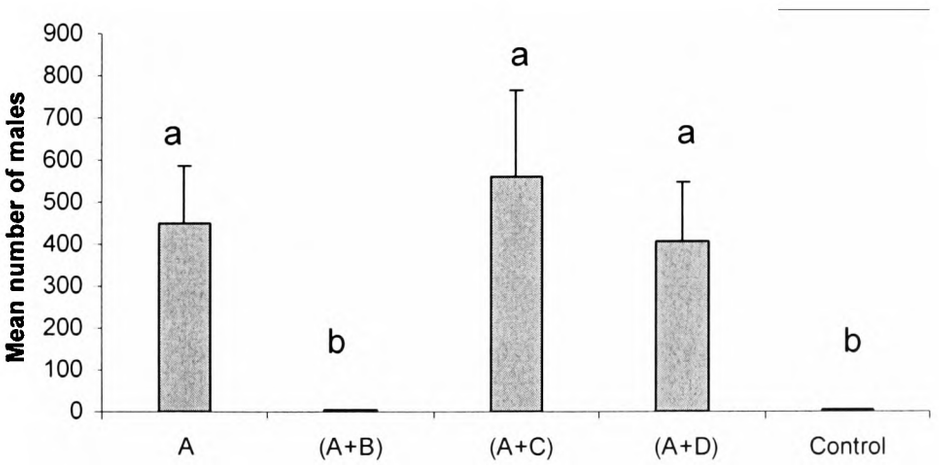


Figure 3.14. Mean trap catch (± SE) of *D. pyri* males during three weeks (14/11-12/12/2007; 5 reps) with A alone and binary mixtures of A+B, A+C and A+D of (*Z*)-2,13-diacetoxy-8-heptadecene made from HPLC separated stereoisomers carried out in Nelson, NZ (data from trap catches were transformed to log(x+1) and statistically evaluated by ANOVA followed by

LSD test at (α<0.05)). Means noted by the same letter are not significantly different (p>0.05).

3.4. DISCUSSION

Calling behaviour and pheromone emission of females are synchronised in midges. Foster et al (1991b) indicated that virgin female Hessian flies produced significantly large amounts of pheromone soon after emergence and pheromone production declined in aged females after eight hours of the photophase. Similarly a peak calling period was noted in newly emerged virgin females of *D. pyri* three hours after emerging and calling was less in aged females. Therefore, pheromone collection from virgin females was started as early as possible in the photophase.

GC-EAG analysis of volatiles collected from female *D. pyri* showed two EAG responses from males and they were assumed to be components of the female produced sex pheromone. The mass spectrum and the retention indices of the compound causing the large EAG response suggested that it was a 17-carbon diacetate with one double bond. Possibilities for the positions of the acetoxy substituents and the double bond were deduced from the GC retention times and mass spectra of both the pheromone component and the analogue obtained by catalytic hydrogenation. Synthetic (*Z*)-2,13-diacetoxy-8-heptadecene was shown to have identical GC retention times on polar and non-polar columns and mass spectrum to those of the natural pheromone component.

When preliminary tests were conducted in UK, males of *D. pyri* showed no preference for the racemic (*Z*)-2,13-diacetoxy-8-heptadecene at 10 µg lure loadings when it was first tested. The same reaction was observed when the racemate was tested in New Zealand. Fewer numbers of males found in traps in the UK may have been due to the higher level of population in NZ where the test was carried out.

The first stereoisomer eluted from the HPLC column (A) attracted significant number of male *D. pyri* midges while the other three remained unattractive. Also, results suggested that the second eluting isomer (B) was strongly inhibitory and responsible for the unattractiveness of the racemic mixture. Attraction of A was completely inhibited by B stereoisomer. The absolute configuration of the attractive isomer was found to be (2*R*,13*R*)-2,13-diacetoxy-8-heptadecene and the inhibitory isomer was having *RS* configuration at the chiral centres. Thus the *R*-configuration at C-2 is essential for biological activity with the 13*R*-isomer being attractive and the 13*S*-isomer inhibitory. On the other hand, any route for synthesis of an attractant must fix the configuration at C-13 as *R* but the configuration at C-2 is not critical as a mixture of (2*R*,13*R*)- and (2*S*,13*R*)-isomers will be attractive.

(2*R*,13*R*)-diacetoxy-8-heptadecene is the first unsaturated diacetate to be found as a component of a midge sex pheromone, although it is closely related to (*Z*)-13-acetoxy-8-heptadecan-2-one, pheromone of the apple leaf midge *D. mali* (Cross and Hall, 2009). The saturated analogue of the *D. pyri* pheromone component, 2,13-diacetoxyheptadecane, was found to be one of the three components of the female sex pheromone of the red cedar cone midge, *Mayetiola thujae* (Gries et al., 2005).

It was not possible to obtain a mass spectrum for the compound causing the smaller EAG response because of the small amount present, even after attempted purification by liquid chromatography. The retention data on polar and non polar GC columns indicated it was much less polar than the major component and unsaturated monoacetate was considered to be a possible structure. However, in liquid silica gel chromatography the minor component eluted after the major component indicating that it is more polar.

CHAPTER 4

IDENTIFICATION OF THE FEMALE SEX PHEROMONE OF THE PEAR MIDGE, *Contarinia pyrivora*

4.1. INTRODUCTION

Pear midge, *Contarinia pyrivora* (Riley), is a pest of pear fruitlets. Damaged fruitlets swell abnormally and then blacken and die. The growth of unattacked fruitlets in the same cluster is also slowed. *C. pyrivora* causes crop loss in many countries including Europe, USA, Canada and China. In the UK it is reported from every county except those in Scotland (Barnes, 1948).

The overwintering pupae end their pupal stage in mid-March. The adults emerge from soil when flower buds are in the pre-blooming stage. After mating, the females lay eggs (10-30) on buds and a few days later the eggs hatch and the larvae emerge and start feeding on the fruit pulp and eventually form a cavity. Attacked fruitlets grow rapidly and get noticeably rounder than normal ones. The growth is arrested after two weeks and the blackened fruitlets crack and fall. Healthier fruitlets in the same cluster get affected owing to the immense competition for assimilation of nutrients (Barnes, 1948; Alford, 1984).

Emergence and oviposition of pear midge coincide with the blooming of pear buds. Females oviposit on buds at the pre-blooming stage or when buds are white. However, these two events, the oviposition and the blooming of pear buds, are not synchronised. There are late flowering varieties which can be planted as an alternative. In general, emergence of overwintering larvae is triggered by two critical factors - the moisture content in the soil and the temperature (Franzmann et al., 2006).

C. pyrivora is normally controlled by applications of insecticides either at the

beginning of the pupal hatching stage or at the green bud stage of pear blossoms which last for a few days. Application of an insecticide is ineffective in later stages during the white bud stage and thus the timing of the insecticide application is crucial. If the pest is carefully monitored, the unnecessary spraying of insecticide can be avoided (QinFuu, 1997).

This study was carried out to identify the female sex pheromone of C. *pyrivora* to provide a basis for development of improved monitoring and control strategies.

4.2. MATERIALS AND METHODS

4.2.1. Collecting and rearing midges

Fruitlets infested with *C. pyrivora* were collected from a pear orchard at Elmston Farm, Preston, in Kent during early June 2006. Mature larvae emerging from damaged fruitlets were collected and potted individually in tubes (approximately 3000) containing moistened bulb fibre, cotton wool, wood fibre or paper towel in mid June 2006. Half of the tubes were stored in a box in an outdoor insectary under natural conditions and the other half in an incubator at 18°-23°C and 16L:8D hr.

Tubes were inspected once a month and those that turned mouldy were removed. Some larvae in the outdoor insectary pupated by mid February 2007. Pupae were removed from the outdoor insectary and incubated at 20°C under 10L:14D hr in the laboratory in order to accelerate post-diapause development. At higher temperatures pupae terminate diapause and emerge as adults.

4.2.2. Pheromone collection

Males and females of *C. pyrivora* were separated based on morphological features of the antennae. Volatiles from males and females were collected

separately using the experimental set up described in Section 2.2., i.e. collecting volatiles from males and females on Porapak Q, followed by extracting with dichloromethane.

4.2.3. Gas chromatography linked to electroantennography (GC-EAG)

GC-EAG analyses were carried out on both polar and non polar GC columns using male *C. pyrivora* EAG preparations as described in Section 2.4.

4.2.4. Mass spectrometry linked to gas chromatography (GC-MS)

Volatile collections from male and female *C. pyrivora* were analysed on a non polar GC column by GC-MS using a HP 5973 quadrupole mass spectrometer and polar GC column with an ITD 700 Ion Trap instrument as described in Section 2.5.

Synthetic samples were also analysed by GC-MS on the Varian Saturn 2200 ion trap mass spectrometer using the non-polar VF5 GC column as described in Section 2.5. For the purpose of retention index calculations, an aliquot of 1 µl from acetate standards (10 ng/µl) and synthetic pheromone components (10 ng/µl) were co-injected. Details of the procedure were given in 2.5.1.

4.2.5. HPLC separation of stereoisomers of 2,7-diacetoxyundecane and 7-acetoxyundecane-2-one

HPLC separation of the stereoisomers of 2,7-diacetoxyundecane on a chiral column was carried out as described in Section 2.7 using 0.5% propan-2-ol in hexane at 0.3 ml/min and eluting compounds were detected at 210 nm on the UV detector.

Despite the above conditions bringing about the separation of four isomers of 2,7-diacetoxyundecane, it was observed that continuous application of these conditions reduced the resolution of B and C. In order to minimise the problem, the column was subjected to regular conditioning with 10% propan-2-ol in hexane at 0.6 ml/min.

The two stereoisomers of 7-acetoxyundecane-2-one were separated on the chiral column with 1% propan-2-ol in hexane at a rate of 0.5 ml/min and the peaks were detected at 210 nm. Collections of the four individual isomers of 2,7-diacetoxyundecane and the two isomers of 7-acetoxyundecane-2-one were quantitatively analysed by GC as described in Section 2.7 and used in the field tests.

4.2.6. Release rate experiment

The release rates of 2,7-diacetoxyundecane and 7-acetoxyundecane-2-one from rubber septa dispensers (Z10,072-2; Sigma Aldrich, Gillingham, UK) containing 100 µg were measured in the wind tunnel at 27°C and a wind speed of 8 km/hr. At intervals these were then entrained for 24 hrs at 27°C using the same apparatus employed for entraining midges (Section 2.2). Purified air was drawn through a charcoal filter at 1.5 L/min and over the rubber septa lure in a glass chamber and volatile components were trapped on a Porapak-Q filter (200 mg). After 24 hr, filters were eluted with dichloromethane (1 ml; Pesticide Grade, Fisher Scientific) and eluent was collected into a glass vial to which was added a known amount of 1-acetoxyhexadecane as an internal standard. To eluents of 2,7-diacetoxyundecane and 7-acetoxyundecane-2-one, 2 µg and 5 µg of 1-acetoxyhexadecane were added, respectively. Aliquots of 2 µl were analysed by GC-FID (Section 2.3). Pheromone containing dispensers were entrained on the 2nd and 5th day and thereafter weekly from November 2008 to December 2008.

4.2.7. Field tests

First field test

The two stereoisomers of 7-acetoxyundecane-2-one (minor 1 and minor 2), two individual stereoisomers (major A and D) and a mixture of two isomers (major B+C) of 2,7-diacetoxyundecane at 4 µg loadings were tested in the field alongside racemates of the minor and the major components (16 µg). The tests were carried out at Elmstone Court, Preston and Mole End Farms, Maidstone, Kent in early March 2008. Traps were put in the field at the time when pear buds were green. At each site, three replicates of each treatment were tested. Traps were hung at 10 m intervals (three pear trees) approximately 1 m above the ground in the tree. Traps were monitored from 4-25 March 2008.

Second field test

The second field test compared catches of *C. pyrivora* in traps baited with four stereoisomers of 2,7-diacetoxyundecane (A, B, C and D; 4 µg), the active isomer of 7-acetoxyundecane-2-one (minor 1; 0.4 µg/dispenser) and the binary mixtures of minor 1 (0.4 µg) with isomers of major A and D (4 µg/dispenser) in 1:10 ratio. At the end of March 2008, traps of the *first field test* at Elmstone Court were replaced with treatments of second field test in new delta traps. Each treatment was replicated four times. Traps were monitored from 26 March - 1 April 2008.

Third field test

Loadings of 3 µg/dispenser of the individual stereoisomers of 2,7-diacetoxyundecane (A, B, C and D) and mixtures of A in combination with B, C and D in 1:1 ratio were prepared and tested against the control. Traps were deployed at Court Lodge Farm, East Farleigh, Kent. Four replicates of each were tested and the experimental layout was as described in the first field test. Traps were monitored from 8-24 April 2008.

Fourth Field test

The attractiveness of the racemate and four individual stereoisomers, A, B, C, and D of 2,7-diacetoxyundecane was re-tested in the field in March 2009 in pear orchards at Mole End Farm, Maidstone, Kent. Rubber septa lures with loadings of 4 µg in green delta traps with white sticky bases were hung on pear trees 1 m above the ground 10 m apart in a row. The test was a randomised complete block designed with 5 replicates of each treatment and trap counts were taken for two weeks.

4.3. RESULTS

4.3.1. Collecting and rearing midges

C. Pyrivora larvae survived on all four types of material both in the out-door insectary and in the incubator. Approximately 200 larvae in tubes containing paper towels and a few in tubes containing cotton wool and wood fibre (~30) pupated. None of the larvae in the incubator pupated whereas larvae kept in the outside insectary pupated, suggesting the best substratum for larval rearing is wet paper towels and the chances of obtaining adults are much higher if they are reared under natural outdoor conditions. Although a large number of larvae was reared (~3000), only 135 reached adulthood.

4.3.2. Pheromone collection

Volatiles were collected from both male and female *C. pyrivora* separately. Four collections from female midges (PMF1-PMF4) and two collections from male midges (PMM1 and PMM2) were made (Table 4.1).

Table 4.1. Numbers of female and male *C. pyrivora* used in volatile collections.

Filter ID	No. females	Filter ID	No. males
PMF1	20	PMM1	27
PMF2	7	PMM2	7
PMF3	32		
PMF4	7		

4.3.3. Gas chromatography linked to electroantennography (GC-EAG)

Analysis of female volatile collections by GC-EAG showed two responses from male midges on both polar and non-polar GC columns (Figure 4.1). Results were consistent when the experiment was repeated using different EAG preparations of *C. pyrivora* males.

On the polar column the responses appeared at 14.73 and 14.34 min and on the non-polar column at 15.22 and 13.56 min (Table 4.2). Overlayed GC-MS traces of male and female volatiles were compared and the active components were present only in female volatile collections which confirmed the GC-EAG results (Figure 4.2). The peaks on the GC trace of male volatile collections appearing just before where the minor and the major components ought to be were impurities as these appeared on female traces as a shoulder on the pheromone peak. The male stimulatory activity was observed when male midges were exposed to female volatile extracts only. The two components causing the EAG responses were presumed to be components of the female sex pheromone. The amounts of the major and the minor components emitted by a female of *C. pyrivora* were approximately 0.4 and 0.07 ng respectively.

Table 4.2. Retention times (RT), retention indices (RI) and intercolumn differentials (Δ) of the major and the minor natural and synthetic pheromone component of *C. pyrivora* on non-polar and polar GC columns in GC-EAG and GC-MS analyses.

	Non-polar GC column		Polar GC column		
Compound	**RT (min)**	**RI**	**RT (min)**	**RI**	Δ
GC-EAG					
Major response	15.22	1311	14.73	1477	166
Minor response	13.56	1161	14.34	1435	274
GC-MS					
Major pheromone component	22.92[1]	1296	15.95[4]	1462	166
Minor pheromone component	20.54[1]	1169	15.62[4]	1431	262
2, 7-diacetoxyundecane	24.41[2]	1287	26.21[3]	1461	174
2-acetoxyundecan-7-one	22.73[2]	1195	25.12[3]	1423	228
7-acetoxyundecan-2-one	22.15[2]	1168	25.44[3]	1431	263
(*Z*)-13-acetoxy-8-heptadecen-2-one		1730		2054	324
(*Z*)-2,13-diacetoxy-8-heptadecene	33.86	1854	24.33	2082	228

[1] SPB-1(HP 5973 Quadropole mass spectrometer); [2] VF5 and [3] Supelcowax-10 (Varian GC-MS); [4] Supelcowax-10 (ITD700 Ion Trap)

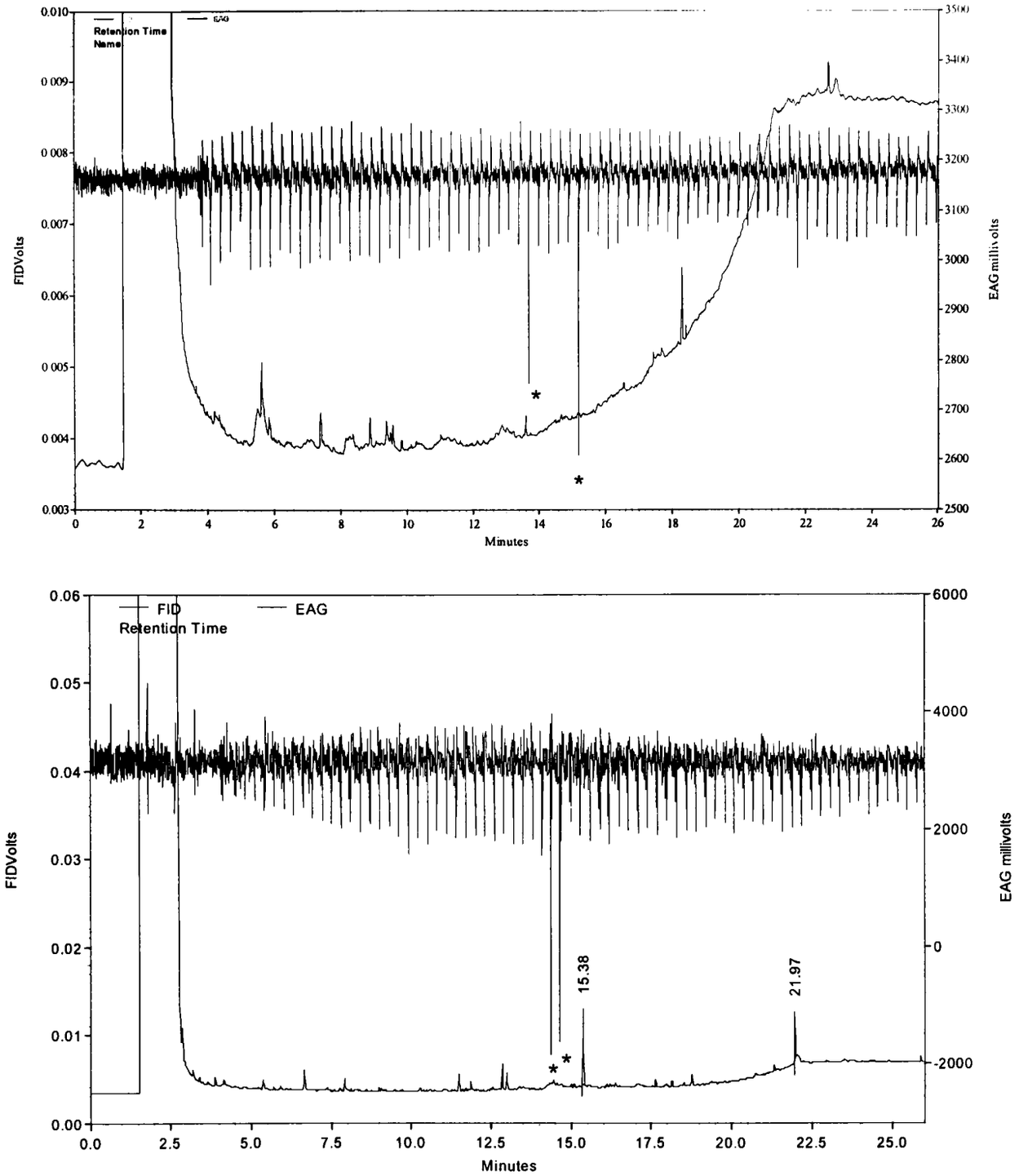


Figure 4.1. GC-EAG analysis of volatile collections from female *C. pyrivora* with male EAG preparations on non polar (upper) and polar (lower) GC column. EAG responses are marked with *

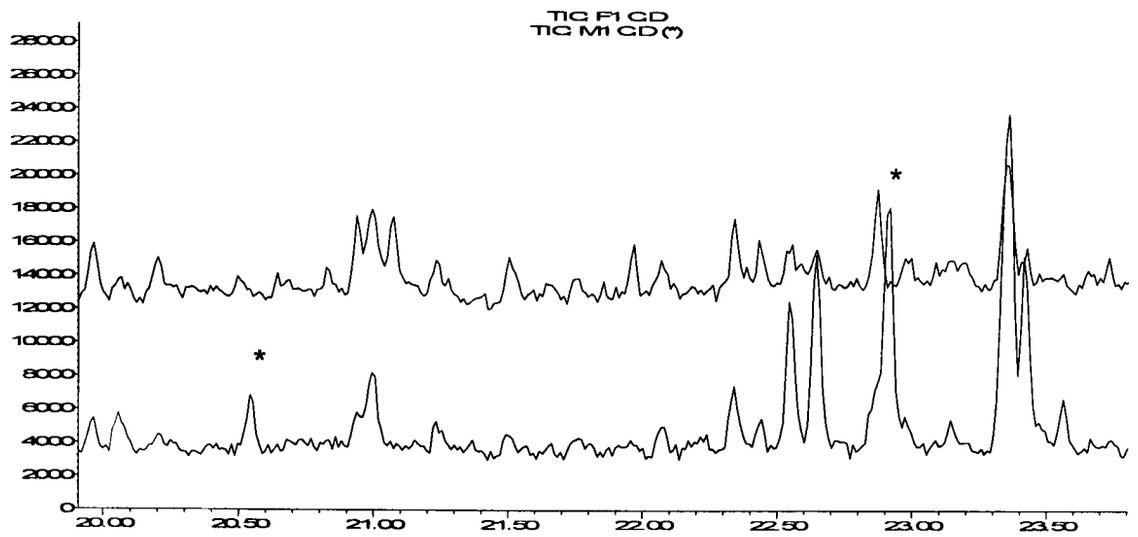


Figure 4.2. Comparison of GC-MS traces of volatiles from male (upper) and female (lower) *C. pyrivora* midges on non-polar column in Agilent GC-MS; * peaks for minor and the major components

4.3.4. Gas chromatography linked to mass spectrometry (GC-MS)

The peak responsible for the major EAG response was explored using mass fragmentation pattern and retention indices. The mass spectrum suggested it was an acetate which was evident from the presence of diagnostic peaks for acetate at *m/z* 61 ($CH_3COOH_2^+$), due to protonated acetic acid as well as m/z 43 (CH_3CO^+). The peak at m/z 152 is possibly formed after elimination of two acetic acid units from the parent molecule with the molecular weight of 272 (Figure 4.3).

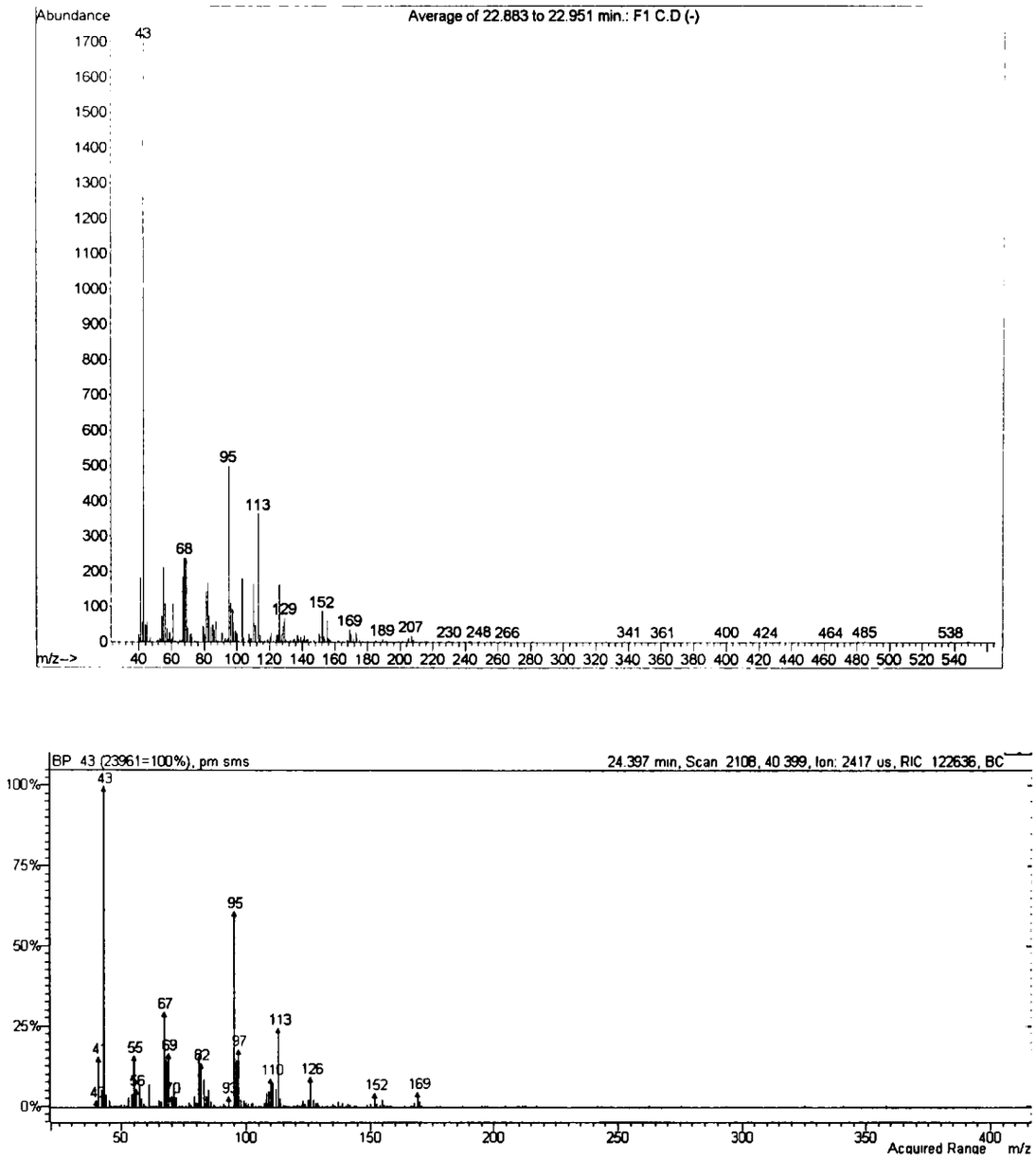


Figure 4.3. Mass spectra of natural major pheromone component of *C. pyrivora* (upper) and synthetic 2,7-diacetoxyundecane (lower) on HP and Varian mass spectrometers respectively.

In the absence of the molecular ion, the chain length and the presence of other functionalities in the molecule were determined by comparing retention indices of the major component with those of known pheromone compounds.

The female sex pheromone component of pear leaf midge, *Dasineura pyri*, has been identified as of 2,13-diacetoxy-8-heptadecene with RI's of 1854 and 2082 on non polar and polar columns respectively (Chapter 3; Table 3.3) giving a RI differential of 228. The corresponding figures for the major pheromone component of *C. pyrivora* were 1311 and 1477 and 166 respectively (Table 4.2) suggesting a diacetate structure with six fewer carbon atoms i.e. 11 carbon

atoms.

The assumption that the major component was a diacetoxyundecane was strongly supported by the GC-MS fragmentations. Thus the ion at *m/z* 152 is consistent with loss of the acetic acid units from $C_{15}H_{28}O_4$ with molecular weight 272.The position of the acetate groups was established on the basis of mass spectrometry. The removal of $CH_3CH_2CH_2CH_2$- (*m/z* 57) from the C11 carbon chain produced a quite intense fragment at m/z 95 (60%) a strong indication that one of the acetate groups was located on seventh carbon (Figure 4.4).

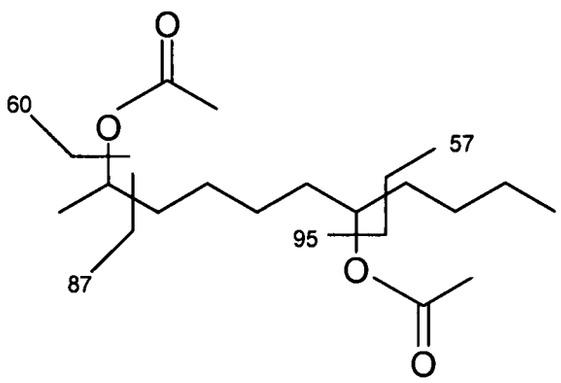


Figure 4.4. Mass fragmentation of the major component 2,7-diacetoxyundecane of *C. pyrivora*

The location of the second acetoxy group was established as C2, based on the fact that an oxygenated functionality can be found on the second carbon atom in all known midge pheromones. This was confirmed by of the presence of *m/z* 126 in the mass spectrum. The elimination of *m/z* 87 and the acetoxy group on C7 leads to the formation of *m/z* 126 (Figure 4.4).

The structure for the major component of *C. pyrivora* was therefore suggested to be 2,7-diacetoxyundecane. This was synthesised by Prof. Hall (Appendix 1) and the mass spectra and the retention times of the synthetic 2,7-diacetoxyundecane and natural major component were found to be identical (Figure 4.3).

4.3.5. Identification of minor component

The comparison of GC profiles of male and female extracts indicated a peak for the potential minor pheromone component (Figure 4.2). The inter-column differential of *C. pyrivora* minor component was compared with those of known pheromone components and it was found that of the *C. pyrivora* minor component was quite close to pheromone component (*Z*)-13-acetoxy-8-heptadecen-2-one of *D. mali* (Table 4.2). Therefore, the minor component of *C. pyrivora* is likely to be a keto-acetate.

The retention index differentials of the minor pheromone components of *C. pyrivora* vs. *D. mali* were 569 and 619, respectively (Table 4.2). The retention indices on both non polar and polar columns indicated that the minor pheromone component of *C. pyrivora* had six carbons atoms less than (*Z*)-13-acetoxy-8-heptadecen-2-one, i.e., 11 carbon atoms.

According to the retention index data, the minor component was more likely a keto-acetate with 11 carbons and it was assumed that the functionalities are located on the second and the seventh carbons on the chain as in the major component of *C. pyrivora*, 2,7-diacetoxyundecane. The mass spectrum of the natural component indicated the presence of *m/z* 43, 61, 168 which is consistent with the 11 carbon keto-acetate. The fragment *m/z* 168 was formed due to the loss of acetate group from the parent molecular with a molecular weight of 228 (Figure 4.6 upper). Ion *m/z* 111(25%) was formed due to the removal of *m/z* 57 ($CH_2CH_2CH_2CH_3$) from *m/z* 168 suggesting that the acetate group is located on C7 as in the major component of *C. pyrivora* (Figure 4.5) and the existence of the keto group. However, a similar type of bond cleavage (α bond cleavage) can occur in the presence of a keto functionality on C7 forming *m/z* 111 (168-57). Nevertheless *m/z* 58 is very characteristic of keto on C2 and the substitution pattern of 7-acetoxyundecan-2-one is consistent with the major component.

The two possible analogues of the minor component 7-acetoxyundecan-2-one,

and 2-acetoxyundecan-7-one were synthesised by Prof. Hall (Appendix 1). 7-Acetoxyundecan-2-one has a retention index (Table 4.2) and mass spectrum (Figure 4.6 lower) identical to that of the natural occurring component (Figure 4.6 upper).

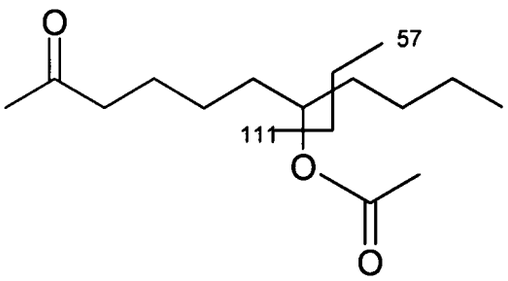


Figure 4.5. Suggested mass fragmentation of the minor component, 7-acetoxyundecane-2-one of *C. pyrivora*,

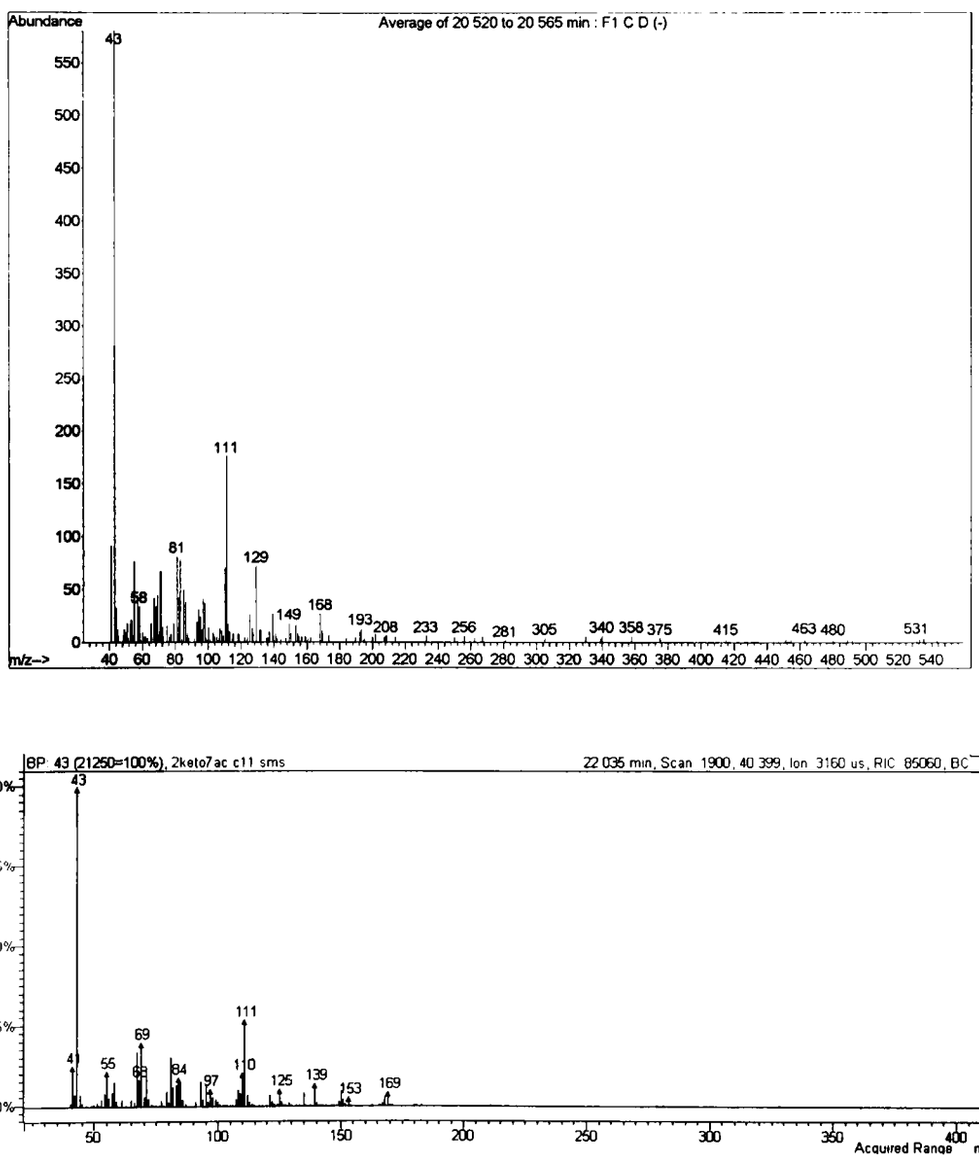


Figure 4.6. Mass spectra of natural minor pheromone component 7-acetoxyundecane-2-one (upper) and synthetic of *C. pyrivora* (lower) . Upper: on HP 5973 Quadropole (SPB-1) and lower: on Varian (Supelcowax-10) mass spectrometers.

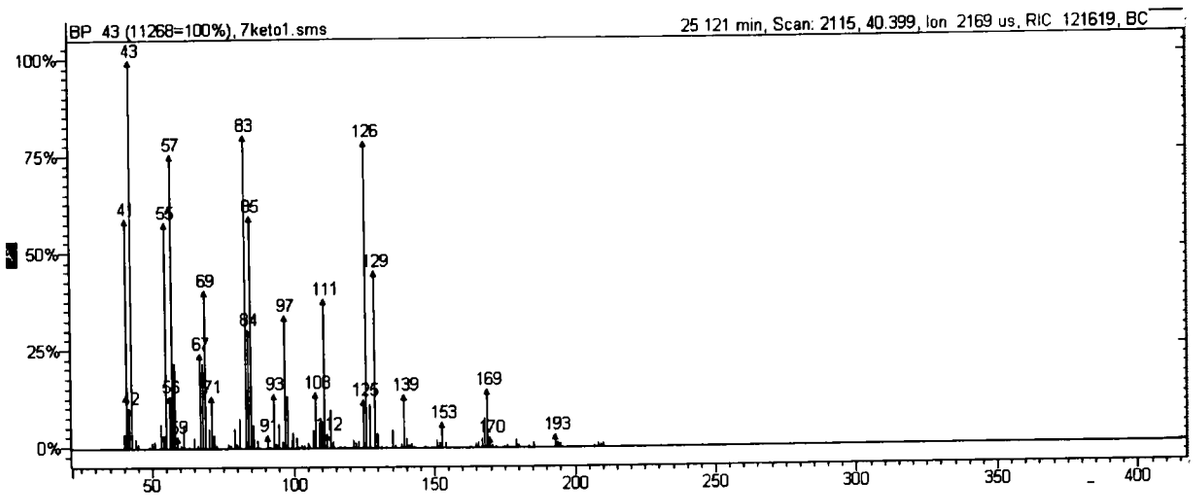


Figure 4.7. Mass spectrum of synthetic 2-acetoxy undecane-7-one

The other isomer, 2-acetoxyundecan-7-one is clearly distinguished by retention index and mass spectra (Figure 4.7). Ion *m/z* 126 was formed due to McLafferty rearrangement of 2-acetoxy undecane-7-one at the keto group followed by removal of the acetic acid unit. Cleavage of C6-C7 of 2-acetoxy undecane-7-one resulted *m/z* 143 and elimination of acetate group yield *m/z* 83 (77%). As the retention indices and the mass spectrum of the synthetic and natural component were found to be similar, 7-acetoxyundecane-2-one was suggested as the possible structure for the minor component.

4.3.6. HPLC separation of stereoisomers of 2,7-diacetoxyundecane and 7-acetoxyundecane-2-one

Resolution of the racemic 2,7-diacetoxyundecane into its four stereoisomers was not fully satisfactory as two of the four stereoisomers eluted together (Figure 4.8 upper). Although the exact reason for the poor resolution of B and C peaks was not known, HPLC separation continued as field testing of individual isomers was a necessity during the flight of *C. pyrivora*.

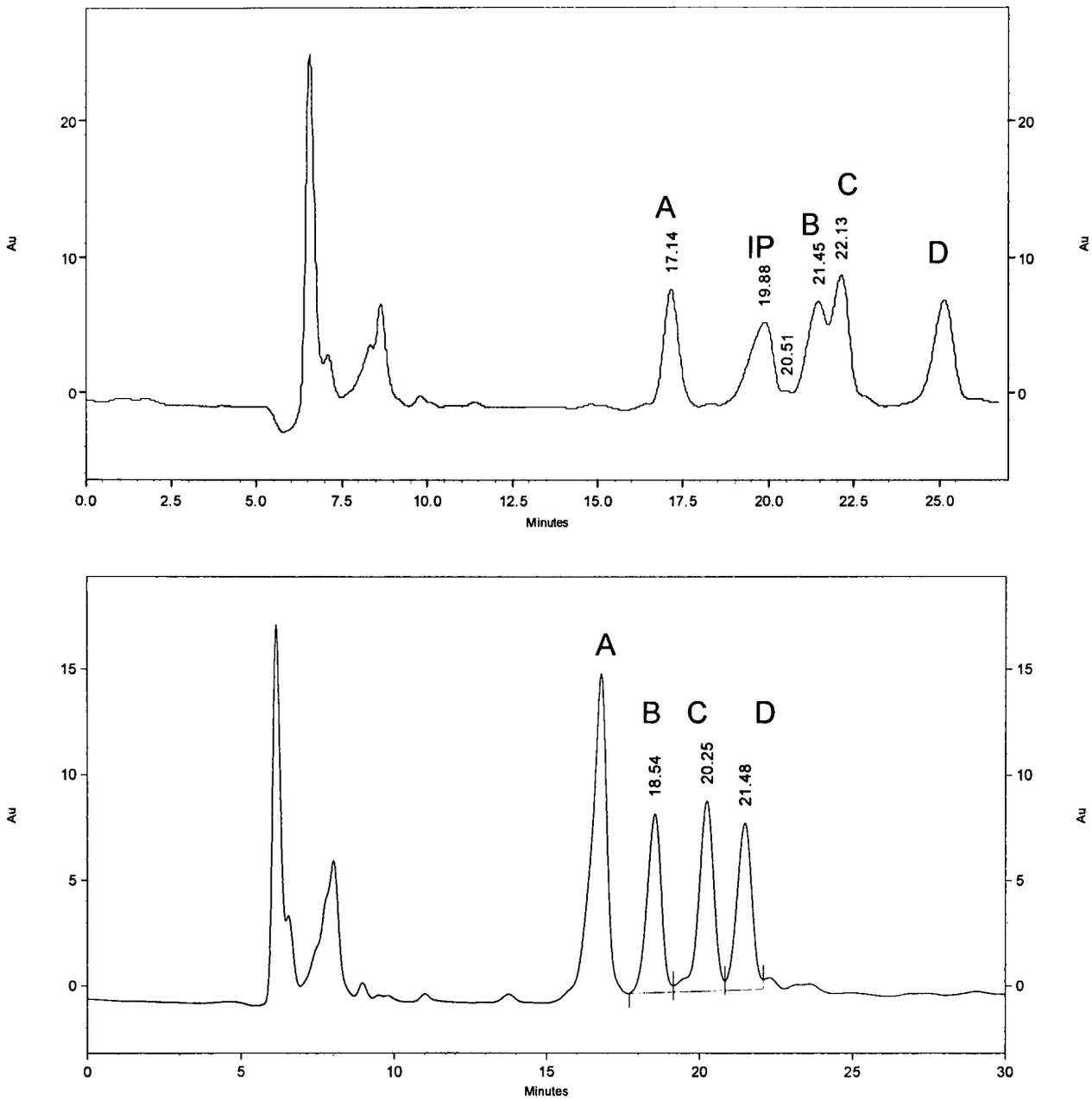


Figure 4.8. HPLC separation of the major component of *C. pyrivora* 2,7-diacetoxyundecane on Chiralpak AD-H column in 0.5% propan-2-ol/hexane at 0.3 ml/min and 210 nm on the UV detector. (upper: partially resolved B & C peaks and the impurity: IP; lower: all four stereoisomers were resolved)

Later it was found that regular conditioning of the column brought about the separation of the four stereoisomers of 2,7-diacetoxyundecane (Figure 4.8 lower). The two stereoisomers of 7-acetoxyundecane-2-one were also resolved by HPLC (Figure 4.9).

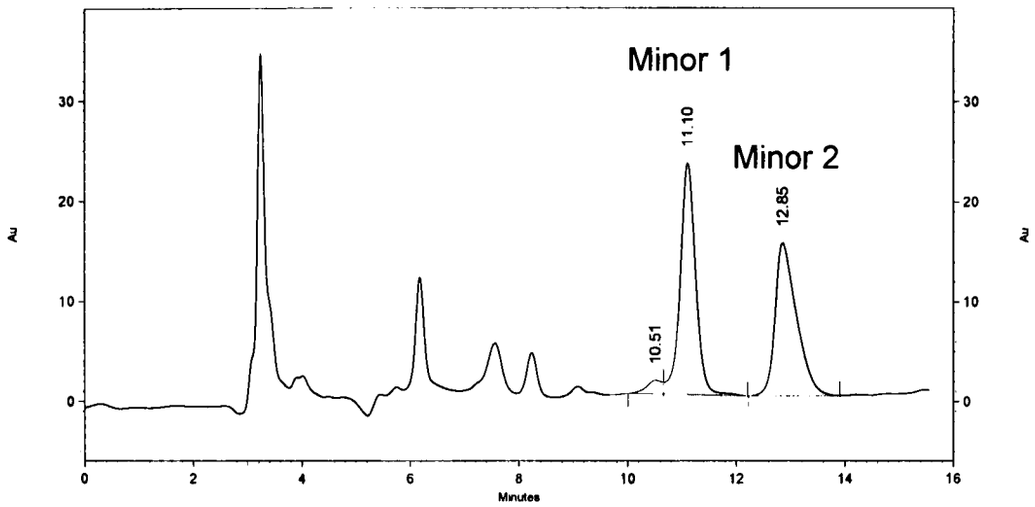


Figure 4.9. HPLC separation of 7-acetoxyundecane-2-one on Chiralpak AD-H column in 1% propan-2-ol/hexaneIsopropan-2-ol at 0.5 ml/min and detected at 210 nm on UV detector.

4.3.7. Release rate experiment

Release rates of the proposed pheromone components from rubber septa, 2,7-diacetoxyundecane and 7-acetoxyundecane-2-one showed first order release rates (Howse et al., 1998). The initial release rate of 7-acetoxyundecane-2-one was nearly 20 times higher than that of 2,7-diacetoxyundecane, the former 12.5 µg/day and the latter 0.7 µg/day after two days respectively (Figure 4.10 upper and lower). After three weeks, the release rates were lower at 0.74 and 0.65 µg/day at 27^0C respectively. The release rates of five weeks aged rubber dispensers were similar, 2,7-diacetoxyundecane and 7-acetoxyundecane-2-one showed a very similar release rate of 0.2 µg/day.

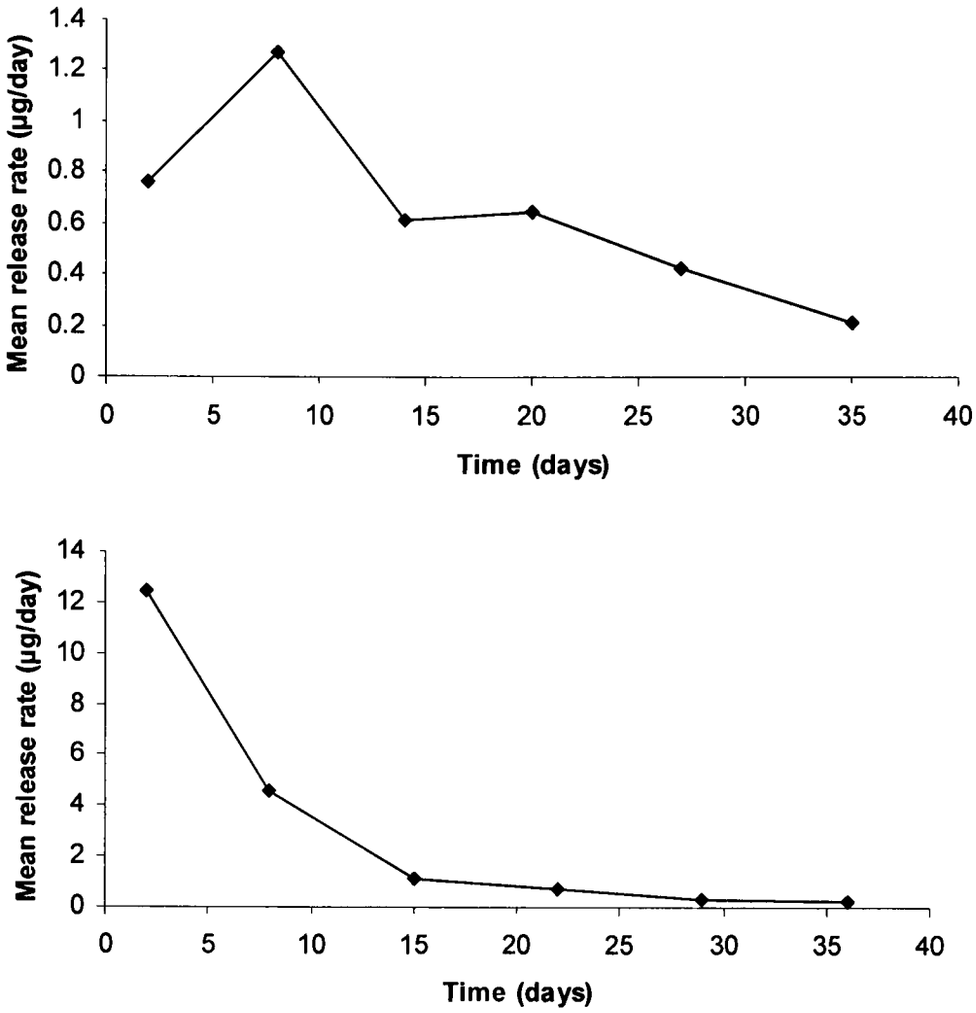


Figure 4.10. Release rate of 2,7-diacetoxyundecane (upper) and 7-acetoxyundecane-2-one (lower) from rubber septa dispensers with initial loadings of 100 µg in a wind tunnel (27°C; 8 km/hr wind speed; n=2)

4.3.8. Field tests

First field test

At Mole End Farm, males of *C. pyrivora* were significantly attracted to traps baited with the first and the last eluting stereoisomer of 2,7-diacetoxyundecane (A and D) from the HPLC (F=40.37, df= 7, 14 P<0.001; Figure 4.11). Although data were not analysed due to lower catches, similar results were observed at Elmstone Court field.

The catches of male midges in traps containing the second-eluting components B and C of 2,7-diacetoxyundecane were significantly less than in traps

containing A or D at both sites. Numbers of males caught with the mixture of stereoisomers B+C or the racemic mixture were not significantly different from those caught in unbaited traps ($P > 0.05$).

For the minor component, 7-acetoxyundecane-2-one, the racemic mixture and the enantiomer 1 were significantly attractive, but enantiomer 2 was not attractive. However, at Mole End Farm, no such activity of the minor component was seen due to a smaller population.

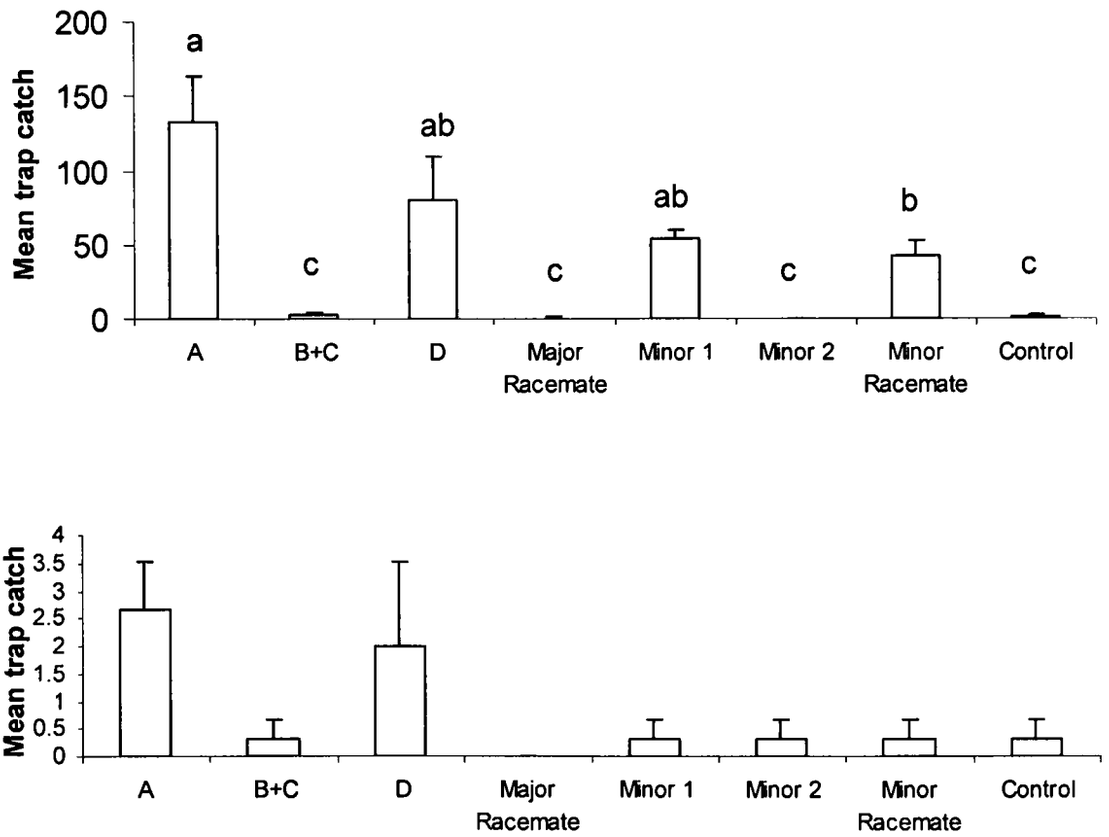


Figure 4.11. Mean number (±SE) of *C. pyrivora* males caught during three weeks at Mole End Farm (upper)and, Elmstone Court (lower), Maidstone; 3-23rd March 2008 with HPLC separated stereoisomers of 2,7-diacetoxyundecane (A, D and B+C), 7-acetoxyundecane-2-one (minor 1 and minor 2), racemates of the former and latter (Data from trap catches were transformed to log(x+1) and statistically evaluated by ANOVA followed by LSD test at $\alpha<0.05$. Means followed by the same letter are not significantly different).

Second field test

Stereoisomers A and C of 2,7-diacetoxyundecane attracted significant numbers of male midges (F=40.87, df=7, 21, P<0.001). In contrast to the first field test, D isomer was found to be significantly less attractive to male *C. pyrivora*. Traps baited with B caught fewer midges than unbaited control traps (P<0.05; Figure 4.12).

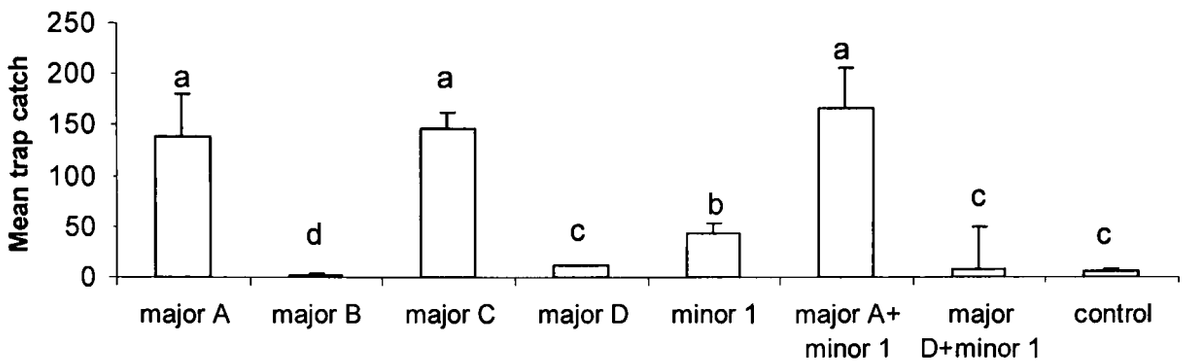


Figure 4.12. Mean number (±SE) of *C. pyrivora* males caught during one week at Elmstone Court, Maidstone (26th March – 1st April 2008 with HPLC separated stereoisomers of 2,7-diacetoxyundecane (A, B, C, D), 1st eluting isomer of 7-acetoxyundecane-2-one, racemates of the former and latter components, mixtures of A and D with minor 1 in 10:1 ratio. Data from trap catches were transformed to log(x+1) and statistically evaluated by ANOVA followed by LSD test at α<0.05 (means followed by the same letter are not significantly different).

The active isomer of 7-acetoxyundecane-2-one, minor 1 caught significantly less male midges than isomer A of the major component in this experiment. When both these components were combined numbers of males caught in traps increased, but, trap catches were not significantly higher (P>0.05). Addition of active stereoisomer, minor 1 did not show any effect on trap catches of D.

Third field test

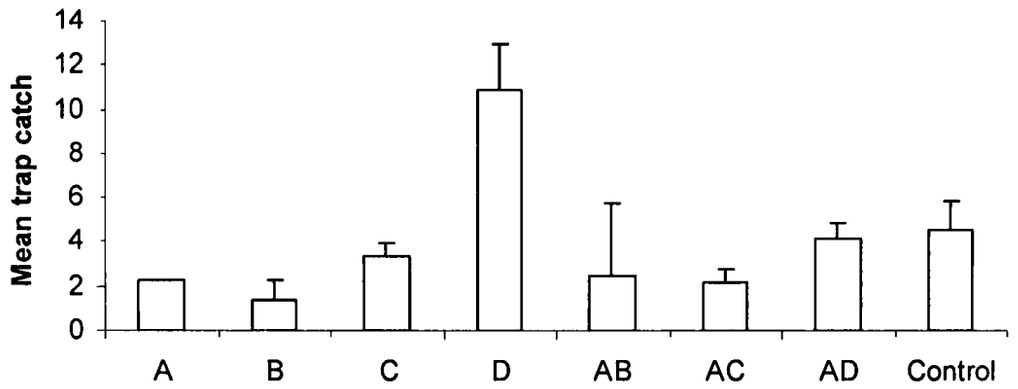
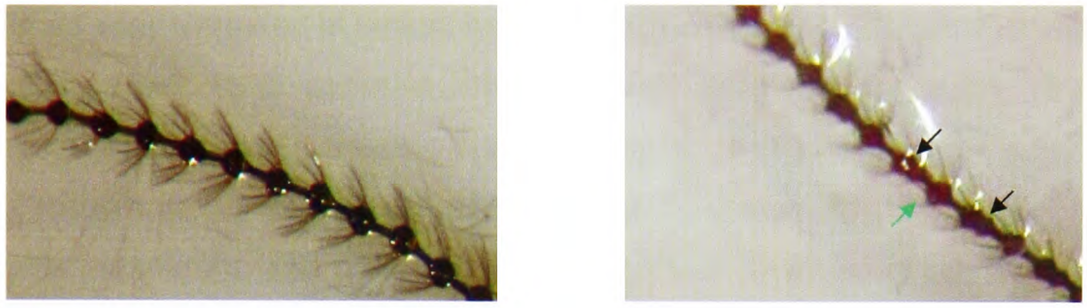


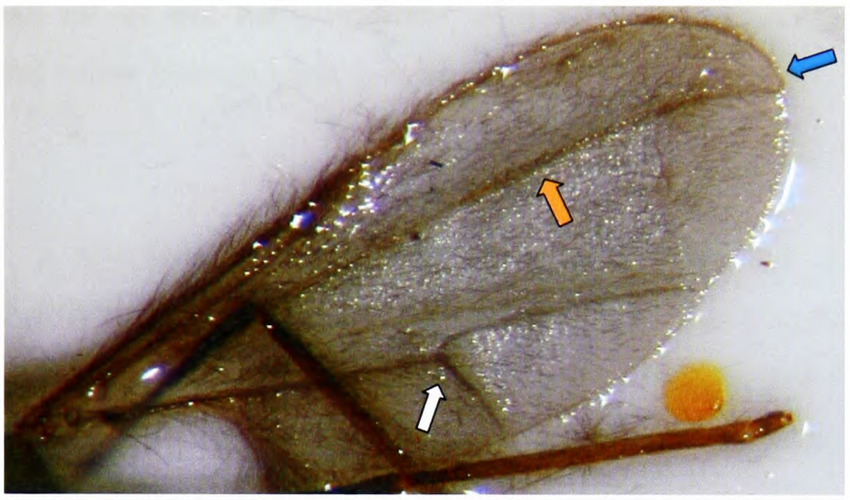
Figure 4.13. Mean number (±SE) of *C. pyrivora* males caught with HPLC separated stereoisomers of 2,7-diacetoxyundecane (A, B, C and D) and two way mixtures of A with B, C and D in 1:1 ratio carried out during 08-24 April 2008 at Court Lodge Farm, East Farleigh, Kent. Analysis of variance was performed on transformed data (log(x+1)) of trap catches.

In the third field trapping experiment all four stereoisomers of the major pheromone component and binary blends of isomer A with the other three isomers attracted midges, but ANOVA showed there were no significant differences between the catches in the baited and unbaited traps (Fig. 4.13; $P<0.076$). Further examination of the catches showed the presence of at least one other species as well as *C. pyrivora*.

Specimens of the two species were obtained from sticky bases and examined under a microscope. The antennal segments (nodes) of *C. pyrivora* are equal in size (Figure 4.14 a), but the unknown species had alternating small and large segments (arrows in black). The larger nodes seem to comprise two sub-units fused together (Figure 4.14 b). The inter-segmental sections connecting the nodes are the same length in *C. pyrivora* (Figure 4.14 a). However, in the unknown species some inter-segmental parts are slightly elongated (Figure 4.14 b).



(a) (b)



(c)

(d)

Figure 4.14. Morphology of antennae and wings of male *C. pyrivora* (a and c; magnification approximately x60) and unknown species (b and d; magnification approximately x35). Green and black arrows: alternating small and large segments of unidentified species; orange arrow: radial vain of *C. pyrivora*; blue arrow: radial vain where it meets the near the tip of the wing of *C. pyrivora*; white arrow: cubital fork present in wings of *C. pyrivora*.

Reduced wing venation is typical for midge species and this character was seen in both species. In *C. pyrivora*, the radial vein (arrow in orange) is straight or nearly so and turned downward just near the tip of the wing where it meets the wing margin (blue arrow), whereas in the unknown species it ran straight towards the margin. The presence of a cubital fork (white arrow) at the middle of the wing makes *C. pyrivora* distinguishable from the unknown species. The anal vein branches off at the halfway point, one turning downward, almost right angle, and the other branch runs in a straight line to the wing margin (Meade, 1888).

Keith Harris, a specialist in midge taxonomy, examined the specimens and suggested that it could be an undescribed species. The suggestion that the unknown species could be the raspberry cane midge, *Resseliella theobaldi,* was rejected by Harris due to the differences in size and the slight variations of features of male genitalia. Until a positive identification was made, Harris referred to the unknown species as *Resseliella* Type 2.

Fourth Field test

It was discovered that the inconsistent results obtained previously in trapping test were probably due to the present of at least one other midge species. A fourth field test was carried out during 2009 with careful examination of the trap catch. The first and the third eluting isomers from HPLC, A and C, of 2,7-diacetoxyundecane were significantly attractive to males midges (F=45.72, df= 4, 20 P<0.001; Figure 4.11) as observed in the second field test (Figure 4.21). Although traps baited with C caught half the numbers of males than of those traps baited with A, statistically A and C were not significantly different. For stereoisomers B, D and the racemate, R the numbers of males were not significantly different from that in unbaited control traps.

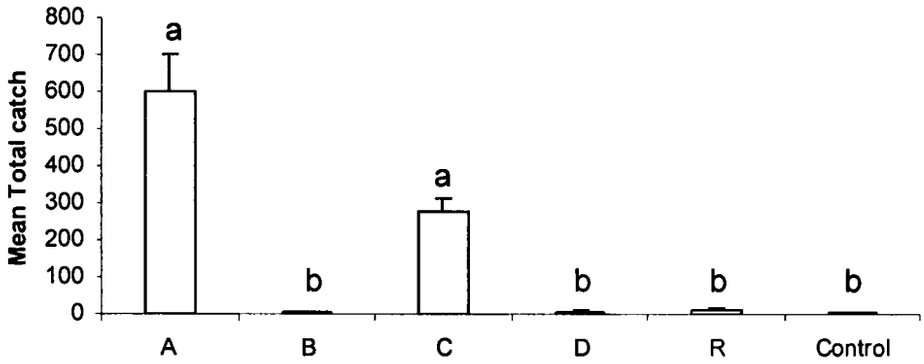


Figure 4.15. Mean number (±SE) of *C. pyrivora* males caught during two weeks at Mole End Farm Maidstone; 19-30 March 2009 with the racemate and HPLC separated stereoisomers of 2,7-diacetoxyundecane, A, B, C and D (Data from trap catches were transformed to log(x+1) and statistically evaluated by ANOVA followed by LSD test at $\alpha<0.05$. Means followed by the same letter are not significantly different).

4.4. DISCUSSION

4.4.1. Rearing

Rearing *C. pyrivora* larvae for pheromone collection was a huge challenge. This is because, *C. pyrivora* has only one generation per year and simulating the exact conditions required for completing its life cycle was not an easy task. It was found that wet paper towels were the best substratum for rearing, as it prevented desiccation of larvae and retained the moisture for the whole period from overwintering larval stage to emergence of adults. Although the larvae reared in the incubator survived, none pupated. Larvae only pupated under natural conditions in an outdoor insectary. As suggested by Passlow (1965) specific conditions are required for larvae of Cecidomyiidae to emerge. For example, sorghum midge, *C. sorghicola,* needed high relative humidity (98-100%) and temperature ranging from 16-32^{0}C. Unless these conditions are met larvae may diapuse for an extended period.

4.4.2. Identification of pheromone components

The sex pheromone produced by *C. pyrivora* females was identified as a blend of two closely related components, 2,7-diacetoxyundecane and 7-acetoxyundecan-2-one. The pheromone components of *C. pyrivora* are similar to known pheromone components of other Cecidomyiidae species. The major component which is a diacetate structurally resembles the pheromone components of the pea midge, *Contarinia pisi* (Hillbur et al, 1999) and the Swede midge, *Contarinia nasturtii* (Hillbur et al, 2005). The components in the *C. pisi* blend are (2*S*,11*S*)-2,11-diacetoxytridecane and (2*S*,12*S*)-2,12-diacetoxytridecane and in *C. nasturtii* are (2*S*,9*S*)-2,9-diacetoxyundecane and (2*S*,10*S*)-2,10-diacetoxyundecane. Although *C. pisi* and *C. pyrivora* are members of the same genera, their minor components belong to different classes. The former is mono acetate, (2*S*)-2-acetoxytridecane and the minor component of the latter species is a ketoacetates.

4.4.3. Field tests

Higher attractiveness to male midges was seen for individual stereoisomers A and D of the major component in the first field test. Results obtained from subsequent field tests contradicted the above results, and showed traps baited with stereoisomer D were less attractive to males of *C. pyrivora*. Also, the third eluting isomer from HPLC, C, was found to attract more male midges while mixture of B and C found to be less attractive, suggesting that B can be the inhibitory isomer. Interestingly, stereoisomer A showed the highest attractiveness in both field tests. The field test results obtained in March, 2009 clearly showed that A and C stereoisomers were active while isomers B and D were not attractive to males. Further, male midges were attracted to the racemic 7-acetoxyundecan-2-one and minor 1. Addition of the active stereoisomer minor 1 increased the attractiveness of A lures for males but not significantly. This suggests that stereoisomer A and minor 1 are most likely to be the naturally produced components in the *C. pyrivora* pheromone blend.

4.4.4. Unknown species

The results of the third field test were misleading and traps baited with individual isomers and two-component blends of 2,7-diacetoxyundecane with other isomers attracted another species with superficially similar features. It was suspected that the stereoisomer D of 2,7-diacetoxyundecane attracted the unknown species. Adults of *C. pyrivora* were present for only a period of two weeks per year in the field. It has only one generation per year, during which time their presence cannot be determine by any other means. Also, there was no record of the presence of another midge species in pear orchards early in the spring, other than *D. pyri,* which is smaller and whose pheromone component differs from that of *C. pyrivora* (Chapter 3).

Pheromone baited traps were deployed in the field well in advance when pear buds were green. Emergence of *C. pyrivora* coincided with the white bud stage of pear. However, it was not clear from the field data when the unknown midge began to appear in the traps. The confusion in the results may have been caused due to the overlapping of the life cycles of two species and attraction of males of the unknown midge species into our *C. pyrivora* traps. Although, sticky bases were examined using hand lens, distinguishing the two species was not possible in the field. Tests were conducted in three different sites. The presence of the unknown midge was confirmed at Court Lodge Farm, East Farleigh, Kent but it is difficult to speculate about their presence at Mole End Farm, Preston and at Elmstone Court, Maidstone. Nevertheless, trap catch data suggested that it may have present at Mole End Farm, Preston at the same time as *C. pyrivora,* during early March.

Examination of midge samples collected from sticky bases by midge taxonomist Keith Harris, indicated that the unknown species could be related to *Resseliella spp.* This could be the case as sex pheromone of *Resseliella theobaldi*, (2*S*)-2-acetoxyundecan-5-one, and the minor component of *C. pyrivora* pheromone are both ketoacetates with 11 carbon atoms.

4.4.5. Release of pheromone

In the wind tunnel at 27°C the pheromone was released at a reasonable rate during four weeks by the rubber septa. It is assumed that pheromone released from rubber septa could last more than four weeks as the temperature in the field during the time of the emergence of *C. pyrivora* is less than 15°C. In nature the amount of the major, 2,7-diacetoxyundecane and the minor, 7-acetoxyundecane-2-one components released by females of *C. pyrivora* was approximate 0.4 and 0.07 ng respectively. On the contrary, release of 7-acetoxyundecane-2-one from septa containing 100 µg was 20 times higher than that of 2,7-diacetoxyundecane. Therefore the initial release rate of 2,7-diacetoxyundecane : 7-acetoxyundecane-2-one from lures containing a 10:1 ratio, as used in field tests, would be 1: 2 which is different from that of the natural ratio. Towards the third week the release ratio got near to 10: 1 and release rates of both pheromone components continued to decrease and by week five pheromone releasing from rubber septa was at an equal rate, 0.2 µg/day. The traps of the blend experiment were kept in the field just one week. Therefore the experiment should be repeated with a blend closer to the natural ratio to see whether it can improve the trap catch significantly. Hillbur et al, (2000) demonstrated in wind tunnel experiments that release rate of the pheromone affected attraction to source. When release rate from the conspecific female gland extract was higher or lower than 10 pg/min attraction by *C. pisi* males was reduced. It is difficult make a conclusion about the efficiency of the release rate of pheromone of *C. pyrivora* from rubber septa lures as synthetic pheromone mixture was not tested against female gland extract.

CHAPTER 5

IDENTIFICATION OF FEMALE SEX PHEROMONE OF BLACKCURRANT LEAF MIDGE *Dasineura tetensi* (Rübsaamen)

5.1. INTRODUCTION

Dasineura tetensi is an important pest in blackcurrant plantations. It was first reported as a pest in Kent about 1931 and afterwards spread to nearby counties (Barnes, 1948). It can be easily found where blackcurrant is grown, mainly in UK, Europe and New Zealand.

Adults are brown to yellow bodied with dark bands across the abdomen. They emerge from soil in mid April, mate and eggs are laid on the upper or lower surfaces of folded young leaves. The whitish eggs hatch within a few days and larvae feed on rolled-up leaves. The number of eggs per leaf can vary from 4-5 or more. The larval period may last for 10-14 days and then the orange-coloured, fully-grown mature larvae drop to the soil for pupation. Larvae burrow through the surface soil a few centimetres below and form a silken cocoon. The mature larvae stay inside the cocoon for about two weeks before pupation. The larvae of the last generation overwinter in cocoons (Barnes, 1948; Alford, 1984).

In the UK there are typically three generations per year. However, the number of generations may vary according to climatic conditions and availability of new growth for oviposition (Barnes, 1948).

The larvae feed on the surface of the terminal leaves resulting in gall formation. The damaged leaves do not grow normally and attack can often be recognised by the twisted and curled leaves. Later, the damaged leaves become necrotic. In some instances damage caused by *D. tetensi* masks the symptoms of

reversion virus (Barnes, 1948; Alford, 1984). In times of severe infestation 90% of shoots can be damaged and as a result elongation of the canes is reduced (Hellqvist, 2005). *D. tetensi* is a serious pest in nursery stocks and young plantations. It is considered as less important in fruiting plantations. Hellqvist (2005) has shown that the damage to vegetation can significantly affect the shoot growth but not the berry production. However, Pedersen et al., (2002) revealed that the damage to foliage can reduce the berry production to some extent.

Normally *D. tetensi* is controlled by spraying broad spectrum insecticides and pest monitoring is important in order to prevent calendar-date based application of insecticides without knowing the exact level of damage or the population density. Female *D. tetensi* were shown to produce a sex pheromone which attracts males by Garthwaite et al. (1986). Identification and synthesis of the pheromone could provide the basis for the development of pheromone traps for monitoring the pest and also for development of new control methods.

5.2. METHODS AND MATERIALS

5.2.1. Insect Collection

Shoots infested with *D. tetensi* were collected from a field in Horsmonden, UK, in May 2007. The fully-grown larvae crawled out from the curled edges of damaged leaves and were picked up with a paint brush and introduced individually into small, clear plastic tubes containing a piece of wet filter paper as described in General Methods and Materials, Section 2.1.

5.2.2. Pheromone collection

Newly emerged adults were sexed based on the antennal morphology and volatiles were collected from males and females separately by means of air entrainment as described in Section 2.2 of General Methods and Materials.

5.2.3. Gas chromatography coupled to electroantennography (GC-EAG)

A HP6890 (Aglilent Technologies) GC linked to portable EAG instrument was used in the analysis of responses from male antennae to the female volatile collections from *D. tetensi* as reported in Chapter 2, Section 2.4.

5.2.4. Gas chromatography linked to mass spectrometry (GC-MS)

EAG active components were analysed by GC-MS on a Varian Saturn 2200 ion trap mass spectrometer as described in Chapter 2 Section 2.5.2.

5.2.5. Micro-hydrogenation of naturally occurring major pheromone component in the *D. tetensi* female volatile collections

Catalytic hydrogenation was performed on an aliquot (20 µl) of volatiles collected from females of *D. tetensi* as described in General Methods and Materials, Section 2.6.

5.2.6. Dimethyl disulphide (DMDS) reaction

The extracts of *D. tetensi* females (B-EMRF3, B-EMRF4 B-EMRF6 and B-EMRF7) with similar GC profiles were combined and concentrated to approximately 200 µl under a stream of nitrogen. An aliquot (100 µl) of the combined volatile extract was mixed with 20 µl of DMDS and iodine in ether (6 mg/ml; 10 µl). The reaction mixture in the sample vial was sealed and heated for 4 hr in an oven at 63° C. The reaction was quenched with equal volumes (100 µl) of an aqueous solution of sodium thiosulphate (5%) and hexane. The hexane layer was separated off and excess solvent was evaporated under a stream of nitrogen. The reaction was monitored by GC-MS on a non-polar column. The temperature programme of the mass spectrometer was slightly modified with initial temperature at 50°C for 2 min, then at 6°C/min till 250°C and held for 15

min in order to monitor the DMDS products as they appear late in the run due to addition of two thiomethyl groups.

5.2.7. HPLC separation of stereoisomers of (*Z*)-2,12-diacetoxy-8-heptadecene

The racemic (*Z*)-2,12-diacetoxy-8-heptadecene was separated by HPLC on a chiral phase (Chapter 2 Section 2.7) with solvent composition 0.4% propan-2-ol in hexane at 0.6 ml/min. Elution of peaks was monitored by UV detector at 200 nm. (*Z*)-2,12-Diacetoxy-8-heptadecene was baseline separated into three peaks and elutes were collected into sample vials by hand. The qualitative analysis of samples was done by GC.

The first eluting peak from the HPLC (peak A; Figure 4.7) was further resolved into two stereoisomers by using 0.15% propan-2-ol in hexane at 0.3 ml/min. Prior to injection samples were evaporated to dryness under a gentle stream of nitrogen and taken up in 100-200 µl of hexane (HPLC grade; Fisher Scientific). Eluates were collected by hand and quantified by GC.

5.2.8. Release rate experiment

Two rubber septa dispensers containing the racemate of (*Z*)-2,12-diacetoxy-8-heptadecene at loadings of 100 µg were placed in a wind tunnel (27°C; 8 km/hr) as reported in Chapter 4 Section 4.2.8. Pheromone dispensers were entrained using the apparatus used for entraining midges as explained in Chapter 2 Section 2.2. Air flow was maintained at 1.5 L/min and the pheromone from the dispensers was collected onto Porapak Q filters for 24 hrs. Porapak Q filters were eluted with 1 ml of dichloromethane (Pesticide Grade; Fisher Scientific) and 20 ng of 1-acetoxy hexadecane was added before analysis by GC-MS. Volatiles were collected for 24 hrs at 0, 7, 14, 15, 22 and 30 days after the start of the experiment.

Quantitative analysis of entrainment samples of (*Z*)-2,12-diacetoxy-8-heptadecene by GC or GC-MS was found to be difficult as release of the pheromone by the septa was too low to be detected.

Therefore a higher dispenser loading of the pheromone (1 mg) was used. In order to enhance the sensitivity of the analysis further, the GC-MS method was modified. Instead of normal splitless injection, programmed temperature vaporizing injection (PTV) was employed. This technique made it possible to analyse larger volumes at a time. Aliquots of 5 µl were analysed on a Varian CP 3800 gas chromatograph linked to a Varian Saturn 2200 ion trap mass spectrometer with a PTV injector initially held at 60°C for 0.2 min and then increased at 200°C/min to 220°C. Samples were analysed on a fused capillary Supelcowax-10 column (Supelco, USA; 30 m x 0.25 mm i.d.). Oven temperature was programmed at 50°C for 2 min, then at 6°C/min to 240°C and held for 5 min. Helium was used as the carrier gas (1.0 ml/min).

5.2.9. Field tests

Field tests were carried out with white delta traps and rubber septa pheromone dispensers as described in Chapter 2 Section 2.8.

Trap Height experiment

In a trial organised by EMR, traps baited with individual stereoisomers of (*Z*)-2,12-diacetoxy-8-heptadecene A, B, C and D (4 µg each) were deployed at seven different commercial blackcurrant plantations in Kent at the time of the flight of the first generation. The trap catches were found to be lower than anticipated and it was suspected that the height of the traps could affect the catches. Traps placed 0.5 m above the ground caught fewer males than those at 10 cm above the ground. It was also found that amongst the four stereoisomers, traps baited with C attracted more males than those baited with B, C, or D (unpublished data). The following experiment was designed on the basis of the above study.

White delta traps baited with rubber septa dispensers containing 5 µg of isomer C isolated from HPLC separation of the racemic (*Z*)-2,12-diacetoxy-8-heptadecene were deployed in a blackcurrant plantation (5 acres) in Stonebridge, Horsmonden, Kent, UK on 19th May 2008. Traps were placed 23 m apart in two blocks, one six rows in from the border and the other seven rows from the first block. Traps were hung at four different heights 3, 10, 30 cm and 1 m above the ground from bamboo cane supports. Two bamboo canes were inserted diagonally into the ground at a distance of ~20 cm apart. Where they crossed was tightened with sticky tape and traps were hung from the crossed joint using plastic ties. Trap catches were recorded at weekly intervals until 9th June 2008.

Blend experiment

HPLC separated isomers of (*Z*)-2,12-diacetoxy-8-heptadecene A, B, C and D (5 µg per septa) and binary mixtures of C with A, B and D in 1:1 ratio (5 µg each) were tested alongside the racemic mixture (20 µg) and unbaited traps at two sites in Kent, UK in May 2008. The first was the south end of the plantation in Stonebridge, Horsmonden where the trap height experiment was carried out. Traps were deployed in three blocks at seven row intervals. The first block was seven rows in from the edge. Nine traps were placed at 23 m intervals in each row 3 cm above the ground. The second site was a blackcurrant plantation at Wellbrook Farm, Faversham. Traps were placed as at the first site except that the distance between traps was 20 m. Traps were set on 19th May 2008 and catches recorded at weekly intervals until 9th June 2008.

Dose experiments

In the first test six different doses, 0,1, 3, 10, 30, 100, 300, 1000 and 3000 µg of the racemic (*Z*)-2,12-diacetoxy-8-heptadecene were loaded into rubber septa dispensers and these were tested at two sites in Kent, UK in June, 2008. Traps were placed 20 m apart, with nine traps per block at 3 cm above the ground. Three replicates were set out at the plantation in Stonebridge, Horsmonden, 23 m apart. The first block was seven rows in from the border row and the second and the third blocks were positioned at 16th and 24th rows from the border row,

respectively. A further three replicates were set out at blackcurrant plantation in Wellbrook Farm, Faversham, with traps 20 m apart at seven row intervals.

In a second test the activities of stereoisomer C and the racemate of (*Z*)-2,12-diacetoxy-8-heptadecene at lower doses were compared. The racemate lures were loaded with four times the amount used of C isomer, giving equivalent quantities of C in the racemate dispensers. Traps of the dose experiment test 1 were replaced with new delta traps containing lower doses of racemate (10, 1, 0.1, and 0.01 µg), stereoisomer C (2.5, 0.25, 0.025 and 0.0025 µg) and unbaited in the blackcurrant plantation at Stonebridge, Horsmonden, Kent, UK. The experiment was carried out in July, 2008 and trap catches were taken weekly for three weeks.

Trap colour experiment

It was observed that the trap bases were contaminated with another insect. The effectiveness of colour in reducing trapping of non-target insects was tested.

Delta traps and matching sticky bases were made in six different colours (white, blue, red, green, yellow and black). Traps and sticky bases were arranged to give traps with matching inserts and traps with white inserts (Table 5.1). Traps were made from coloured Correx sheets and coloured inserts were covered with a layer of glue (Barrier Glue: Agralan, UK). Rubber septa lures were impregnated with 100 µg of racemic (*Z*)-2,12-diacetoxy-8-heptadecene. Each colour combination was replicated twice and the test was carried out at Wellbrook Farm, Faversham in June, 2008. Trap inspection and Insect identification were carried out by Dr. Michelle Fountain and Mr. Adrian Harris at East Malling Research.

Table 5.1. Colour combinations used in traps

Treatment name	Trap colour	Insert colour
W-W	White	White
B-W	Blue	White
R-W	Red	White
G-W	Green	White
Y-W	Yellow	White
Blk-W	Black	White
W-B	White	Blue
B-B	Blue	Blue
R-R	Red	Red
G-G	Green	Green
Y-Y	Yellow	Yellow
Blk-Blk	Black	Black

5.3. RESULTS

5.3.1. Insect Collection

Approximately 12,300 mature larvae of *D. tetensi* midge were reared in plastic tubes under laboratory conditions. Most larvae became mouldy or shrivelled during the rearing and only 36% of larvae reached adulthood.

5.3.2. Pheromone collection

Volatiles from males and females were collected by air entrainment for 3-7 days. Thirteen volatile collections from female *D. tetensi* (Table 5.2) and four volatile collections from male *D. tetensi* (Table 5.3) were obtained for analysis.

Table 5.2. Volatile collections made from female *D. tetensi*

Filter ID	Number of female midges	Filter ID	Number of female midges
BF1	226	B-EMRF4	393
BF2	362	B-EMRF5	688
BF3	85	B-EMRF6	399
BF4	339	B-EMRF7	396
B-EMRF1	70	B-EMRF8	286
B-EMRF2	456	B-EMRF9	58
B-EMRF3	338		

Table 5.3. Volatile collections made from male *D. tetensi*

Filter ID	Number of male midges
BM1	97
BM2	58
BM4	128
B-EMRM9	90

5.3.3. GC-EAG Analysis

In GC-EAG analysis the antennae of the male midges consistently responded to two components in the volatile collections from female *D. tetensi* on both polar and non polar GC columns (Figure 5.1). The peak corresponding to the higher intensity response was considered as the major pheromone component and the one responsible for the lower intensity response was the minor pheromone component. The response for the major component appeared at 19.18 min and 20.21 min and the minor response at 18.86 min and 19.2 min on polar and non polar columns respectively (Table 5.4). Furthermore, GC traces of male and female volatile extracts were compared and a peak was observed for the major component only in female extracts, indicating that it was a component of the female sex pheromone. GC-EAG analyses of volatiles collected from males on female EAG preparations did not elicit any responses from female midges.

Table 5.4. Comparison of retention time (RT), retention index (RI) and inter-column differentials of major and minor components of *D. tetensi* with known midge pheromone components on polar and non polar columns relative to acetate standards

Compound	Non-polar GC column		Polar GC column		Δ*
	RT (min)	RI	RT (min)	RI	
GC-EAG					
Major compound	20.21	1835	19.18	2042	207
Minor compound	19.20	1720	18.86	1999	279
GC-MS					
Major compound	33.30	1835	34.75	2042	207
Hydrogenated major compound of *D. tetensi*	33.80	1871	34.74	2042	171
(*Z*)-2,13-diacetoxy-8-heptadecene	33.42	1845	34.98	2061	216
2,13 diacetoxyheptadecane	33.92	1878	34.86	2053	175
(*Z*)-13-acetoxy-8-heptadecen-2-one	19.22	1730	20.60	2054	324
7-acetoxyundecane-2-one	13.56	1161	14.34	1435	274

*The difference of retention index on polar and non polar GC columns

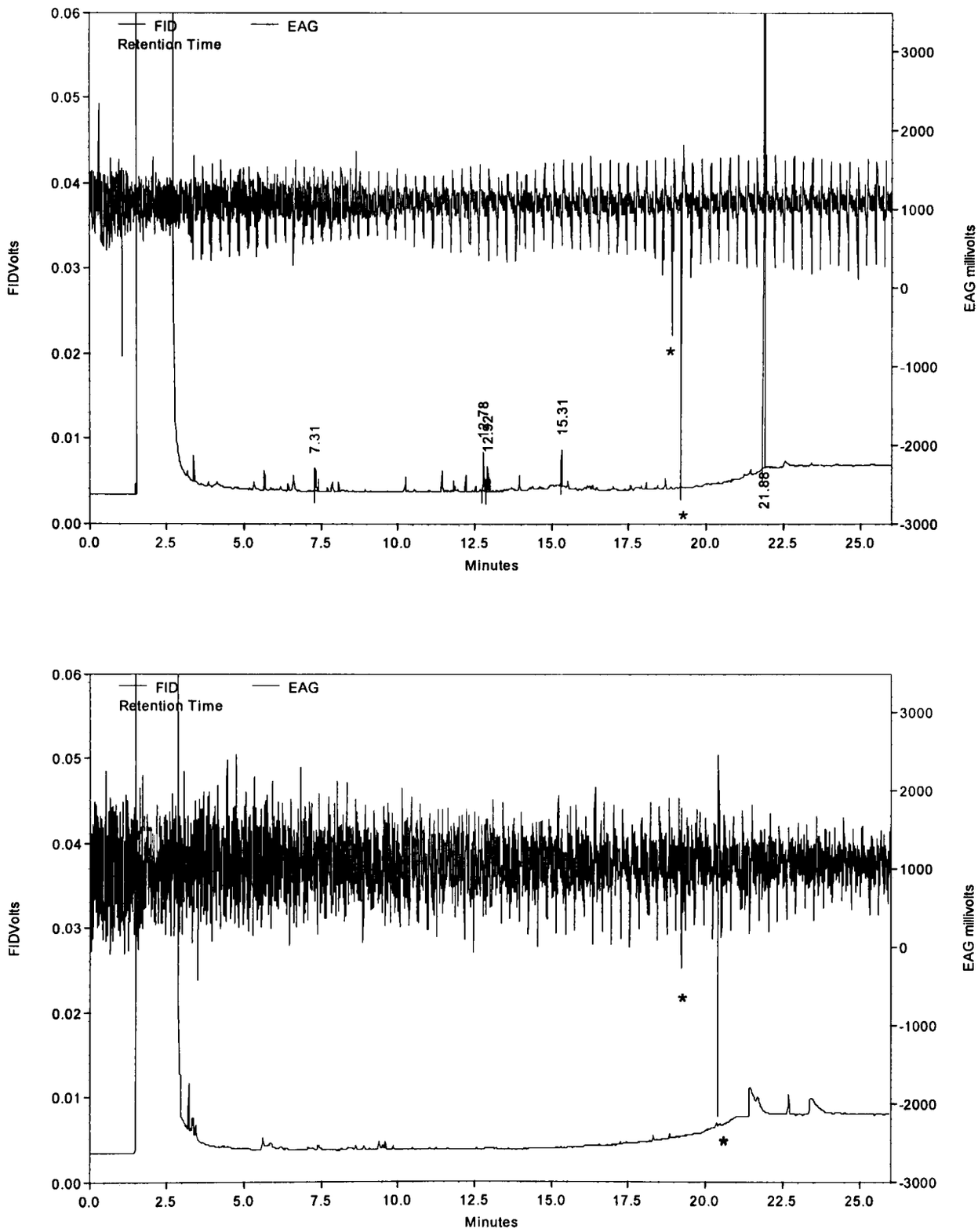


Figure 5.1. GC-EAG analysis of volatile collections from female *D. tetensi* with male EAG preparation (polar column: upper; non polar column: lower; EAG responses to pheromone components marked by *).

5.3.4. Identification of major pheromone component

The retention indices of the major EAG-active component from *D. tetensi* (Table 5.4) were compared with the previously identified female sex pheromone of the pear leaf midge, *D. pyri*. These were very similar and the inter-column differential was found to be fairly close. Inter-column differential is independent of the number of carbon atoms and depends on the chemical nature of the compound. This indicates that the major pheromone component of *D. tetensi* most probably has 17 carbon atoms with one double bond and two acetate groups.

GC-MS analyses

Analyses of individual volatile collections BF1-BF4 by GC-MS did not show a peak at the retention time for the major component. However, when four extracts were combined, a tiny peak was observed.

The mass spectra of the major pheromone components of *D. pyri* and *D. tetensi* are strikingly similar with many fragments in common of which*m/z* 234, 150, 121, 81, 67, 61, and 43 were the most distinguishable (Figure 5.3 and 5.4). Acylium ion *m/z* 43 (CH_3CO^+) was formed due to the cleavage of the C-O bond of the acetate moiety and *m/z* 61 was due to elimination of the acetic acid unit and are characteristic ions for acetate esters. Furthermore, the ion at *m/z* 234 probably arises from the elimination of two acetoxy groups from a 17-carbon diacetate with one double bond having the molecular weight of 354 (Figure 5.3). The presence of the tiny fragment at *m/z* 87 suggests that one acetate group was located on the second carbon atom as in other midge pheromones. It was considered that the marginal differences of retention index differential of *D. pyri* and *D. tetensi* components could be due to the second acetate group being on different carbons.

Cleavage of the α-bond at C_{11}-C_{12} and elimination of acetate group on C12 as an acetic acid unit resulting in *m/z* 150/149 were quite noticeable in the *D.*

tetensi spectrum.

Also the cleavage of the α-bond (C_{12}-C_{13}) followed by removal of two acetate groups from *m/z* 283 (354-71) gives *m/z* 163 (15%) indicated the location of the second acetate group (Figure 5.2).

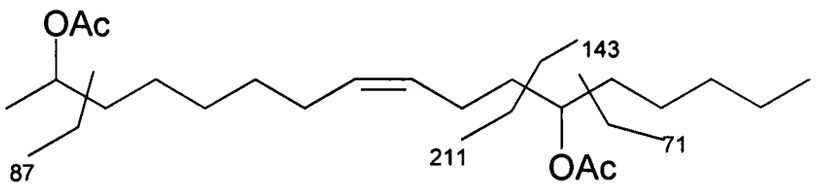


Figure 5.2. Mass fragmentation of candidate major component of *D. tetensi*

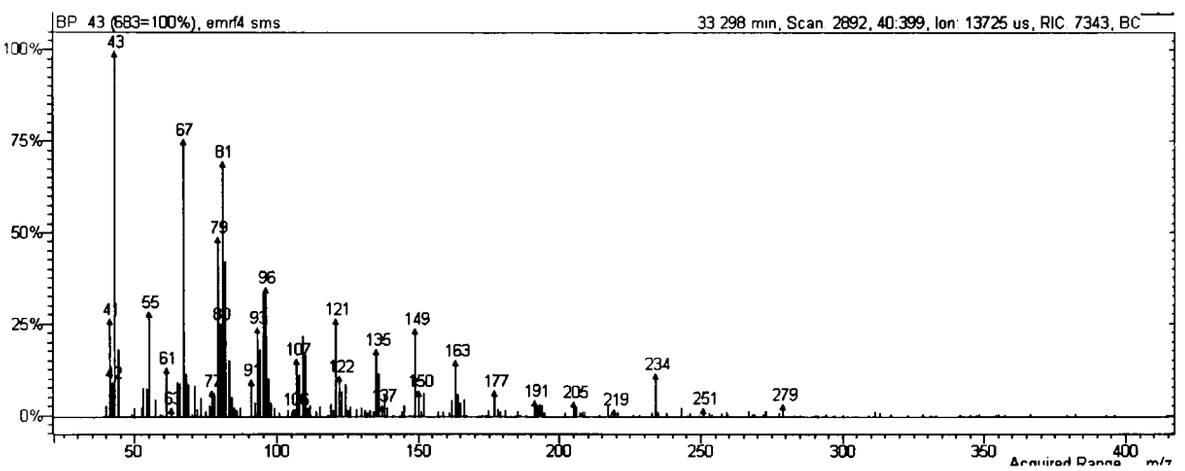


Figure 5.3. Mass spectrum of *D. tetensi* pheromone major component

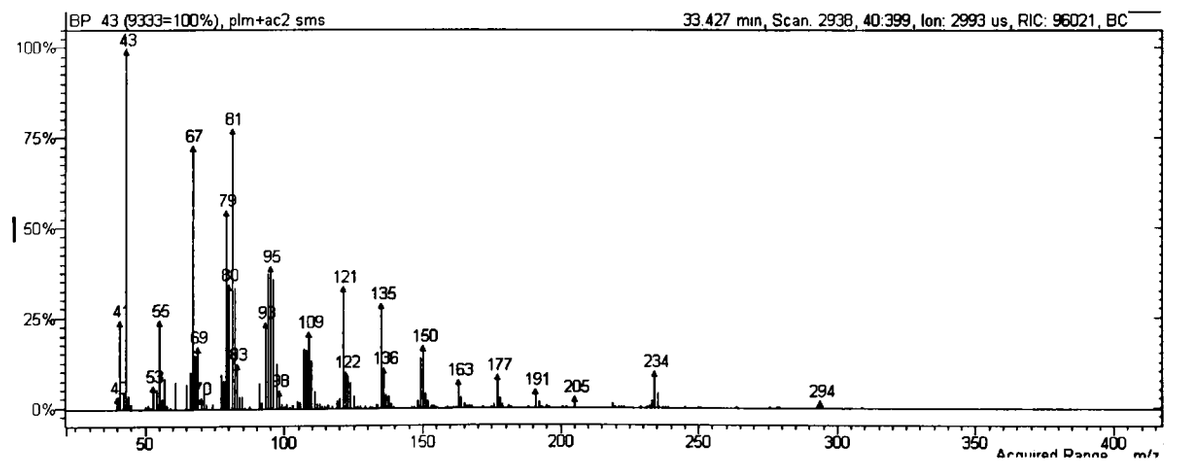


Figure 5.4. Mass spectrum of (*Z*)-2,13-diacetoxy-8-heptadecene, the major pheromone component of *D. pyri*.

The mass spectrum of the *D. pyri* major pheromone component, *(Z)*-2,13-diacetoxy-8-heptadecene, showed an ion at *m/z* 177 (15 %) formed due to the C_{13}-C_{14} cleavage from the mono unsaturated chain (*m/z* 234-57).

Micro-hydrogenation

GC-MS analysis after hydrogenation of the collection of volatiles from females of *D. tetensi* showed a peak considered to be the hydrogenated major EAG active component (Figure 5.5). Certain fragments such as *m/z* 234, 150, 251 and 83 have gone up by m/z 2 units, suggesting presence of a single double bond.

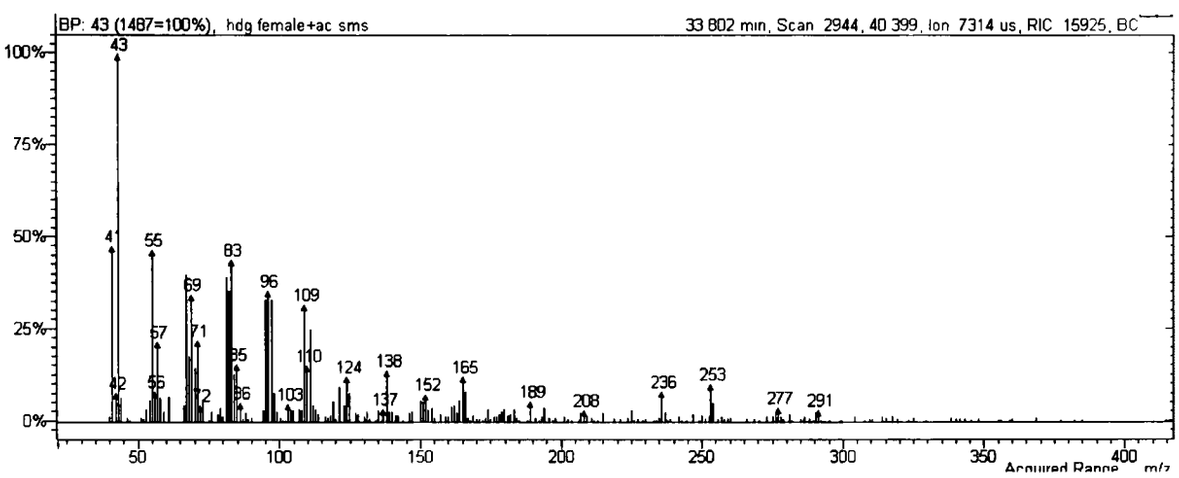


Figure 5.5. Mass spectrum of hydrogenated major component of *D. tetensi* pheromone.

Hydrogenated *D. pyri* major component (2,13-diacetoxyheptadecane) and the candidate *D. tetensi* major component (2,12-diacetoxyheptadecane) were identical to two components in the pheromone blend of red cedar cone midge, *Mayetiola thujae* (Table 5.5) (Gries et al., 2005).

The differences in retention indices of the hydrogenated major component of *D. pyri* vs. *D. tetensi* with two of *M. thujae* pheromone components (2,12-diacetoxyheptadecane and 2,13-diacetoxyheptadecane) and the synthetic analogue, 2,11-diacetoxyheptadecane were compared (Gries et al., 2005). It was understood that *D. tetensi* and *M. thujae* retention indices were obtained from similar columns (non-polar: DB-5 and polar: DB-210) and therefore can be compared. *M. thujae* retention indices were calculated relative to hydrocarbon

and therefore for comparison purposes *D. tetensi* retention indices which were calculated relative to series of acetate ester having even numbers of carbons had to be converted relative to hydrocarbon values. Therefore, 430 and 800 units were added to retention data of *D. tetensi* on non polar and polar GC columns respectively (Table 5.5).

The retention index differentials of *D. pyri* vs. *D. tetensi* were closer to *M. thujae* pheromone component, 2,13-diacetoxyheptadecane vs 2,12-diacetoxyheptadecane than 2,13 diacetoxyheptadecane vs. 2,11-diacetoxyheptadecane (Table 5.4). This suggests that the second acetoxy group of *D. tetensi* major component was likely to be positioned on the twelfth carbon of the alkane chain. Evidently, the fragment *m/z* 71 (25%) indicates that second acetoxy group was on C12 (Figure 5.2) and it was formed due to the cleavage of the 12-13 carbon – carbon bond. Also, mass spectra of hydrogenated *D. tetensi* major component when compared with that of Gries et al. (2005) data on 2,12-diacetoxyheptadecane showed that presence of *m/z* 165 is unique when acetoxy groups are located on C2 and C12. Further, the mass fragmentation of 2,13-diacetoxyheptadecane differs in this respect and occurrence of similar fragmentation yield *m/z* 179.

Table 5.5. The retention indices of pheromone components of *M. thujae* and and *D. tetensi* relative to *n*-alkanes.

Pheromone components	Non-polar	Polar
2,13-diacetoxyheptadecane*	2279	2853
2,12-diacetoxy heptadecane *	2273	2844
2,11-diacetoxy heptadecane *	2269	2839
D. tetensi major component	2265	2842

*Gries et al. (2005)

Micro DMDS reaction

A micro-scale DMDS reaction was employed in an attempt to confirm the position of the double bond. The reaction was first carried out with synthetic (*Z*)-2,13-diacetoxy-8-heptadecene in order to understand the fragmentation pattern

of the DMDS adduct. The retention time and a mass spectrum were obtained for the derivative. Incorporation of two SCH_3 groups on the carbon-carbon double bond increases the molecular weight of the parent molecule by 94. Cleavage between the 8- and 9-carbons carrying the methylthio groups yields two intense fragments at *m/z* 171 and 157 (Figure 5.6). The reaction was then carried out with a sample of volatiles collected from female *D. tetensi.* The GC-MS chromatogram was scanned for *m/z* 43 (100 %) and 109 (60 %) which were quite prominent ions. The former was formed due to the cleavage of C-O bond of the acetoxy group and the latter fragment was resulted from leaving of methylthio group from *m/z* 157. However, we didn't observe these distinctive fragments on the mass spectrum and no peak was detected for the DMDS derivative of the natural major component around the retention time where the peak for the derivative of the synthetic compound occurred. Scanning at *m/z* 171 and 157 or the corresponding ions for double bonds in different positions also failed to show any clear products.

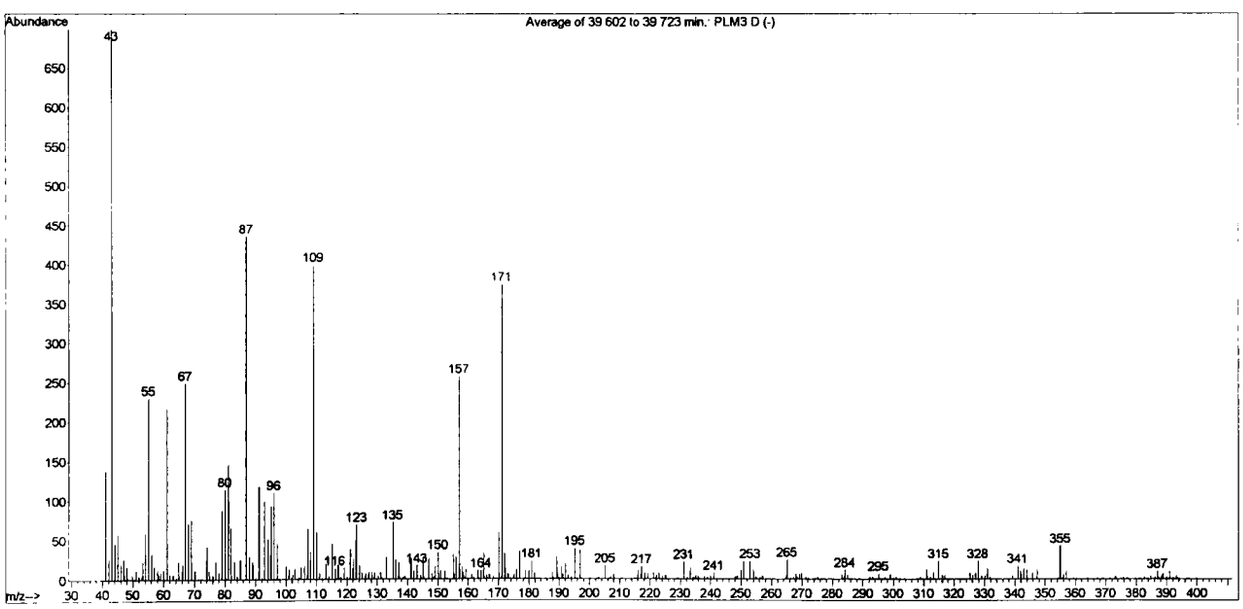


Figure 5.6 Mass spectrum of DMDS aduct of the major component of *D. pyri* pheromone (*Z*)-2,13-acetoxy-8-heptadecen.

Comparison of mass spectrum and GC retention indices suggested the possible structure for the major pheromone component of *D. tetensi* was most likely to be (*Z*)-2,12-diacetoxy-8-heptadecene. This compound was synthesised by Prof

Hall (Appendix 1) and found to have identical retention times and mass spectrum to those of the proposed major pheromone component of *D. tetensi*. Furthermore, hydrogenation of this compound gave 2,12-diacetoxyheptadecane which had the same retention times and mass spectrum as the product of microhydrogenation of the major pheromone component (Figure 5.5).

5.3.5. Attempted identification of minor component

Although the presence of the minor component was obvious from the GC-EAG analysis, it was difficult to obtain a good mass spectrum as it was only present in minute quantities.

The minor component was more polar than the major as indicated by the greater GC retention index difference between polar and non polar columns (Table 5.4). It was close to those of ketoacetates, less than for apple leaf midge, *D. mali,* pheromone (*Z*)-13-acetoxy-8-heptadecen-2-one (Table 5.4) and close to minor component of the pheromone of *C. pyrivora,* 7-acetoxyundecane-2-one. This suggested the minor component to be a acetoxy-ketone corresponding to the major component with no double bond, ie. 12-acetoxyheptadecane-2-one or 2-acetoxy heptadecane-12-one (Figure 5.7).

Figure 5.7. The proposed structures of the minor component of *D. tetensi* 12-acetoxyheptadecane-2-one (left) or 2-acetoxy heptadecane-12-one (right)

Chemical ionisation GC-MS analyses with methanol as a reagent were carried out in an attempt to confirm the possible proposed structure, 12-acetoxy heptadecane-2-one. Chemical ionisation with methanol yields (M+1) ion and the mass spectrum was scanned for *m/z* 313 (M+1) and 251 (due to removal of acetate group from 313), as these were strong ions in the CI mass spectrum of

(Z)-13-acetoxy-8-heptadecen-2-one. However, the presence of any of expected fragments was not clearly observed in the mass spectrum.

5.3.6. HPLC separation of stereoisomers of (*Z*)-2,12-diacetoxy-8-heptadecane

The sex pheromone of *D. tetensi*, (*Z*)-2,12-diacetoxy-8-heptadecene has two chiral centres and therefore consists of two enantiomeric pairs. The synthetic compound was resolved into two individual isomers (C and D) and the remaining two isomers (A&B) were eluted unresolved on the chiral phase (Figure 5.8).

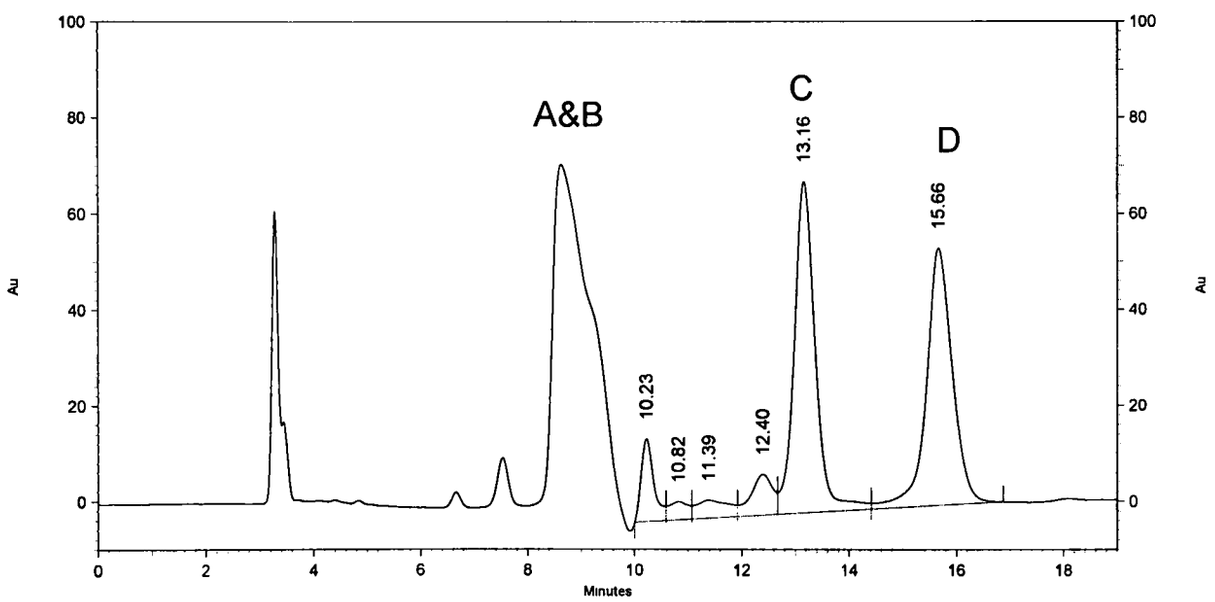


Figure 5.8.HPLC separation of racemate of (*Z*)-2,12-diacetoxy-8-heptadecene

Subsequently A & B were separated in a solvent system having lower polarity than the one used previously, although resolution of peaks was not complete due to slight overlapping at the baseline (Figure 5.9). However, the separation was adequate enough for make two collections of A and B stereoisomers. Analysis of the collections of A and B on chiral HPLC revealed that A was not contaminated with B. Similarly, collections of B were analysed and slight tailing was observed due to the presence of very small quantities of A in some

collections of B.

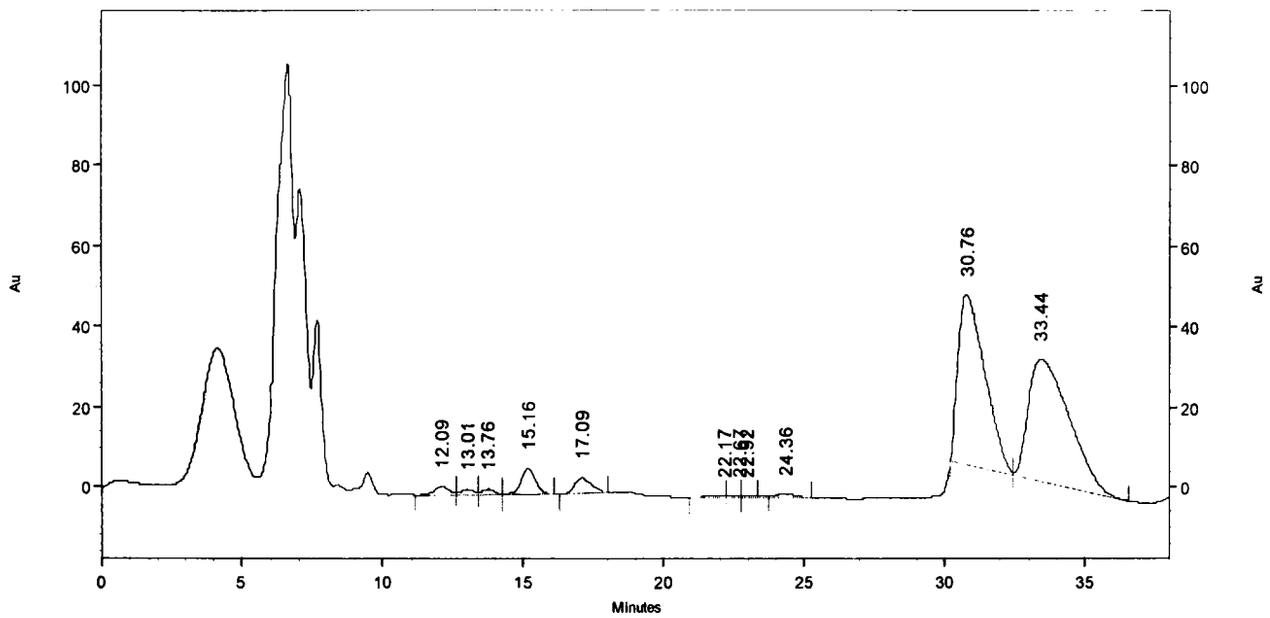


Figure 5.9. HPLC separation of the first eluting peak of (*Z*)-2,12-diacetoxy-8-heptadecene

5.3.7. Release rate experiment

Initially emission from rubber septa dispensers containing 1 mg of (Z)-2,12-diacetoxy-8-heptadecene was 20 ng/day at 27°C and 8 km/hr windspeed and thereafter release of pheromone became steady at 10 ng/day for at least 30 days (Figure 5.10).

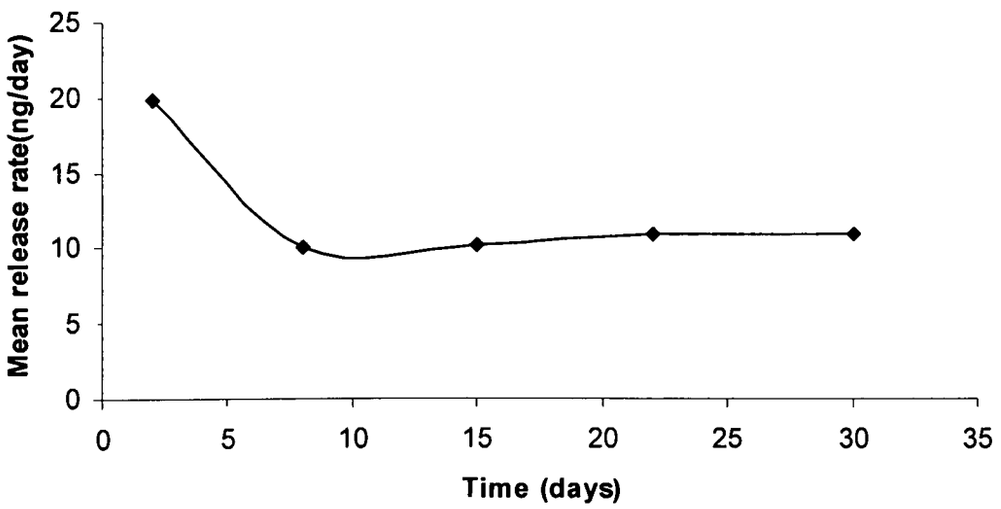


Figure 5.10. Release rate of (*Z*)-2,12-diacetoxy-8-heptadecene from rubber septa dispensers containing1 mg in a wind tunnel in the laboratory (27°C; wind speed: 8 km/hr; n=2)

5.3.8. Field tests

Trap height experiment

Higher numbers of males were caught in traps placed very close to the ground, at 3 and 10 cm (F=31.91, df=3, 9, P<0.001) (Figure 5.11). There was no significant difference between numbers caught at 3 or 10 cm (P>0.05). However, when height was increased logarithmically, trap catches were significantly decreased and at 1 m height trap catches were as low as 0.003% of numbers of males caught at 3 cm.

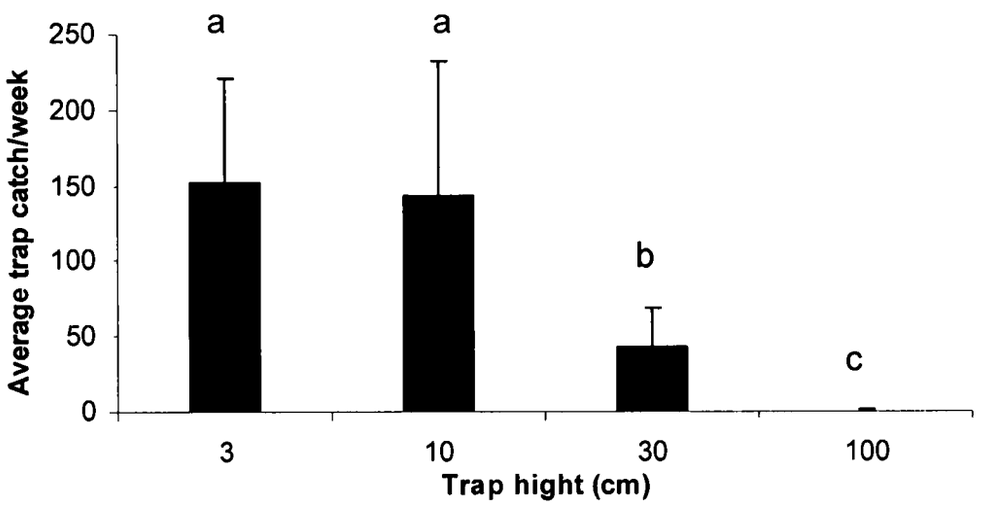


Figure 5.11. Mean total number (± SE) of *D. tetensi* males caught with third eluting stereoisomer of (*Z*)-2,12-diacetoxy-8-heptadecene from HPLC, C at different traps heights during 19th May-9th June 2008 at blackcurrant plantation at Stonebridge, Horsmonden, Kent. Means followed by the same letter are not significantly different (P>0.05) (analysis of variance ANOVA was performed on transformed (log(x+1)) data of trap catches).

Blend experiment

Traps baited with stereoisomer C attracted more *D. tetensi* males (F=49.40, df=8, 40, P<0.001) but those traps baited with isomers A, B and D did not catch significantly more males than the blank. Addition of B to C did not reduce the catches. Addition of D reduced the catches but not significantly so. Combining A with C significantly reduced the catches (P<0.05) but traps still caught significantly more than the unbaited traps. Therefore, A is a strong inhibitor as it lowered the activity of C when combined. The racemic mixture attracted

significantly more midges than unbaited traps, but the racemate was significantly less attractive to males than stereoisomer C (P<0.05). The low level of attraction of the racemate may have been caused by the presence of inhibitory stereoisomers in the racemic mixture. The traps containing racemic mixture caught 17% of that caught by C alone (Figure 5.12).

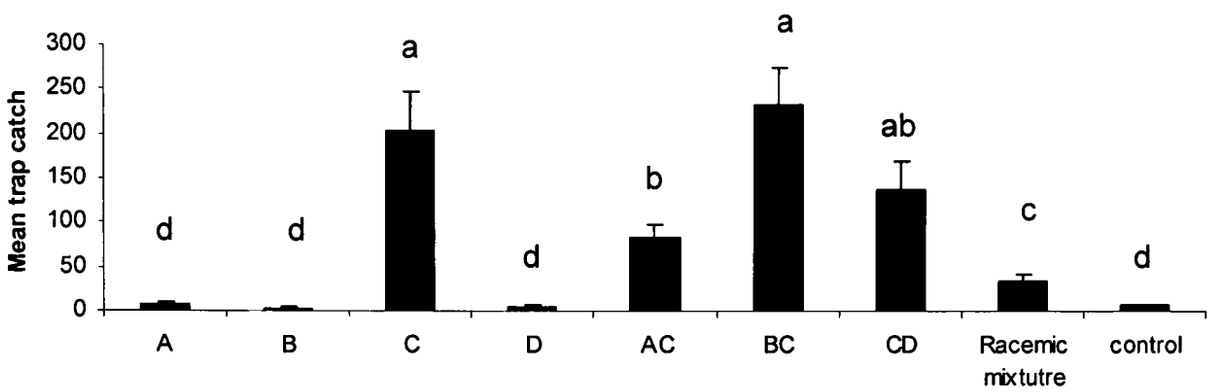


Figure 5.12. Mean total number (± SE) of *D. tetensi* males caught with HPLC separated stereoisomers of (*Z*)-2,12-diacetoxy-8-heptadecene (A, B, C, D) and combinations (CA, CB, and CD) and racemate during 19th May-9th June 2008 at blackcurrant plantation at Stonebridge, Horsmonden, and Wellbrook Farm, Faversham Kent. Means followed by the same letter are not significantly different (P>0.05) (analysis of variance ANOVA was performed on transformed (log(x+1)) data of trap catches).

Dose experiment

In the first dose experiment with different doses of racemic of (*Z*)-2,12-diacetoxy-8-heptadecene, lower doses attracted more midges than higher doses. Significant numbers of *D. tetensi* males were caught at 1 and 3 µg per dispenser (F=9.14, df=8,40 P<0.001). Higher lure loads (3000, 1000, 300, & 100 µg) attracted males to the same extent as the control. Numbers caught by traps containing 10 and 30 µg dispenser loads did not differ from lower or the higher doses (Figure 5.13).

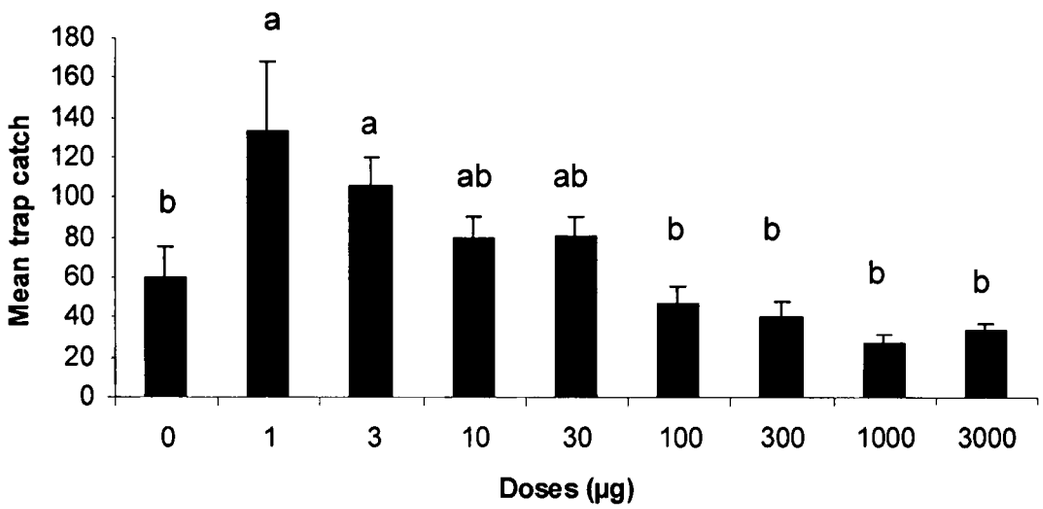


Figure 5.13. Mean total number (± SE) of *D. tetensi* males caught in traps baited with different doses of racemic (*Z*)-2,12-diacetoxy-8-heptadecene (1, 3, 10, 30, 100, 300, 1000 and 3000 µg) during 16th June- 23rd June, 2008 at Stonebridge, Horsmonden, and Wellbrook Farm, Faversham Kent. Means followed by the same letter are not significantly different ($P>0.05$) (analysis of variance ANOVA was performed on transformed (log(x+1)) data of trap catches).

In the second dose experiment with isomer C of (*Z*)-2,12-diacetoxy-8-heptadecene, higher numbers were attracted to dispensers containing 2.5 µg of C isomer ($F=18.56$, $df=8, 16$; $P<0.001$). However, 10 µg racemate dispensers had equivalent amounts of C as in dispensers containing 2.5 µg of C. Those traps baited with racemate dispensers attracted significantly fewer males than equivalent C dispensers. Lower doses of C (0.0025 and 0.025 µg) were as unattractive to males as untreated control (Figure 5.14). Racemate lures of 0.01 µg were attractive to males to the same extent as unbaited control traps. However, increasing lure load of racemate by ten fold gradually increased the attractancy and traps with 0.1, 1 and 10 µg lures trapped significantly more males than the control. Interestingly, trap catches of the racemate (R) and isomer C were compared. At R 0.1:C 0.025 the racemate was more attractive to males than C, at 1:0.25 attractiveness of R and C were statistically not significantly different, and at 10:2.5 isomer C attracted significantly more males than the racemate.

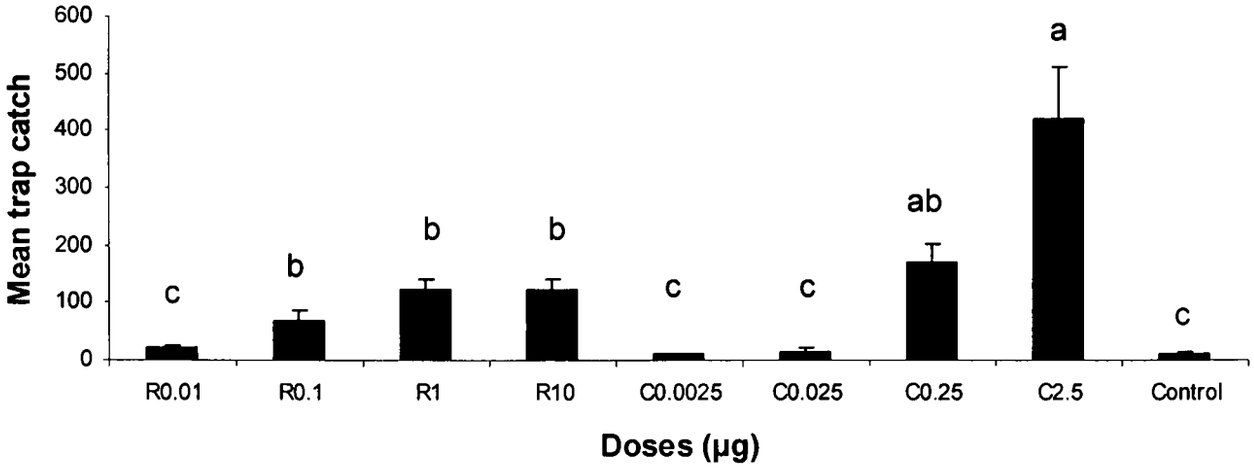


Figure 5.14. Mean total number (± SE) of *D. tetensi* males caught with different doses of racemate (0.01, 0.1, 1 and 10) and C isomer (0.0025, 0.025, 0.25 and 2.5) of (*Z*)-2,12-diacetoxy-8-heptadecene during 16th-23rd June 2008 at blackcurrant plantation at Stonebridge, Horsmonden, Kent. Means followed by the same letter are not significantly different (P>0.05). (analysis of variance ANOVA was performed on transformed (log(x+1) data of trap catches).

Trap colour experiment

Apart from males of *D. tetensi*, traps baited with the racemate of (*Z*)-2,12-diacetoxy-8-heptadecene attracted bumble bees, flies, thrips, hymenopterans, beetles, aphids, lacewings, syrphids, moths, spiders, earwigs and bugs. Trapping of males of *D. tetensi* was not affected by colour during a four week period (p>0.087). However, red-red, black-white and blue-white trap-insert combinations captured higher numbers of *D. tetensi* males (Table 5.6). Although trap catches were not significantly different, more flies were attracted to traps than any of the other non target species. Higher numbers of non-target species were attracted to yellow-yellow and yellow-white (Table 5.6.)

Table 5.6. Mean total number of *D. tetensi* males and other insects caught by each of trap-insert colour combination

	D. tetensi	Bumble Bees	Flies	Thrips	Hymen-optera	Beetles	Aphids	Lace wings	Syrphids	Moths	Spiders	Earwigs	Bugs
Y-Y	12.8	1.0	35.8	0.3	3.8	1.3	1.8	0.0	0.0	0.0	0.0	0.0	0.5
Y-W	28.0	0.0	42.5	0.0	1.0	0.8	1.5	0.0	0.0	0.0	0.8	0.3	0.5
G-G	14.0	0.0	27.0	0.0	2.5	0.3	2.0	0.0	0.0	0.0	0.0	0.0	0.5
G-W	16.5	0.0	18.5	0.0	0.5	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
R-R	37.3	0.0	20.0	0.0	1.8	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0
Blk-W	31.8	0.0	25.0	0.3	0.3	0.5	0.0	0.0	0.0	0.0	0.0	0.3	0.0
B-B	15.8	0.5	30.8	0.0	0.8	0.5	0.3	0.0	0.8	0.0	0.3	0.0	0.3
B-W	35.3	0.3	32.8	0.0	0.5	0.5	0.0	0.0	0.3	0.0	0.5	0.0	0.0
W-B	11.3	0.0	26.8	0.0	2.5	0.8	0.0	0.0	0.3	0.0	0.0	0.3	0.0
Blk-Blk	16.5	0.0	16.0	0.0	0.3	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.3
R-W	17.0	0.0	17.8	0.0	1.3	1.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0
W-W	31.0	0.0	29.5	0.0	0.3	1.3	0.8	0.0	0.0	0.0	0.0	0.0	0.3

5.4. DISCUSSION

Two consistent responses were observed during electroantennographic recording from male antennal preparation of *D. tetensi* for volatile samples collected from conspecific females. The GC peak of the candidate pheromone was compared with that of *D. pyri* (Chapter 3) as both species are *Dasineura*, and possessed similar mass spectrum and retention data. The major compound was identified as *(Z)*-2,12-diacetoxy-8-heptadecene and one of the corresponding acetoxy-ketones, 12-acetoxyheptadecane-2-one or 2-acetoxyheptadecane-12-one was proposed as the minor component. Retention and mass spectrum data of the synthetic *(Z)*-2,12-diacetoxy-8-heptadecene were compared with the natural candidate pheromone component and the structure of the pheromone component was confirmed.

HPLC separated four isomers (A, B, C and D) of *(Z)*-2,12-diacetoxy-8-heptadecene on a chiral phase and these were tested in the field. Traps baited with isomer C were attractive to males of *D. tetensi* but A, B, and D were as unattractive as unbaited traps when tested individually. When two-way mixtures of C with A, B or D in 1:1 ratio were tested, results indicated that B could be the inactive isomer as it did not affect the trap catch of males. D reduced the attraction of isomer C but the effect was not significant, thus D could be partially inhibitory. A mixture of A with C attracted significantly fewer males of *D. tetensi* suggesting that A is strongly inhibitory. As a result traps baited with the racemic 2,12-diacetoxy-8-heptadecene caught significantly fewer males than the isomer C. The racemate of *D. tetensi* major pheromone component was attractive to conspecific males unlike *D. pyri* (Chapter 3) or *C. pyrivora* (Chapter 4) major components.

Traps baited with lower doses of the racemate such as 1 and 3 µg were more attractive to male midges than higher doses. Attraction of males of *D. tetensi* weakened when dosage of the racemate was lower than 0.1 µg and higher than 100 µg. On the contrary traps containing the racemic female sex pheromone of *D. pyri* (Chapter 3) and *C. pyrivora* (Chapter 4) were unattractive to respective male midge species.

Catches of males were significantly higher in pheromone traps placed close to the ground, especially at 3 and 10 cm above the ground and the same effect was seen during trapping of *D. mali* by Heath et al. (2005). Garthwaite et al., (1986) described trapping male *D. tetensi* using delta traps with caged females and later it was also reported traps baited with female sex pheromone of *D. mali* hung at ground level captured more males compared to 0.5 m or above (Cross et al. 2009a; Suckling et al., 2007). This was mainly associated with the emergence pattern of adult Cecidomyiidae and their mating behaviour, i.e. males and females emerge from the soil and mate close to the eclosion site (Sharma and Franzmann, 2001; Harris and Foster, 1999). It was also reported by Condrashoff (1962) males of *Contarinia* spp. have a restricted vertical flight which contributed to the fact that more males are caught close to ground level. Upwind flight of males of *D. mali* was horizontal and described as a narrow zig-zag or straight followed by counterturning (Harris et al, 1996). Placement of trap influences the trap catch and can be optimised if life traits are considered. For instance, findings of Rothschild and Minks (1977) were later justified by Kovanci et al. (2006) that Oriental fruit moth, *Grapholita molesta* were caught in sex pheromone baited traps placed at upper canopy where mating activities take place. Similarly, traps placed close to the top and outside of the canopy recorded higher catches of codling moths (McNally and Barnes, 1981).

White delta traps attracted many other non-target species including beneficial species (bees) other than *D. tetensi* males. However, none of the colour combinations of trap and inserts affected the trap catches of *D. tetensi* males or the non target species significantly. A similar trap colour-inserts test was carried out by Cross et al. (2009a) to minimise the non target insects in traps baited with pheromone of *D. mali*. As in the case of *D. tetensi*, effect of trap colour on trapping *D. mali* did not show any significance. However, more non-target species were caught in white, yellow and blue traps while fewer non-target species were caught in red, green and black coloured traps.

The spectral reflectance from each of the coloured plastic traps is different. The range of reflectance is broad for white and yellow in comparison to other colours

(Clare et al., 2000). Therefore, more non target species are attracted to traps with those colour combinations. The peak colour reflectance is in the range of 500-580 nm for yellow which may act as a foliage simulant (Barker et al., 1997). Further work needs to be carried out for making the traps less attractive to non target species. Including minor component in the lure and modifying the conventional delta trap may help reducing non-target species.

Although determination of absolute configuration of *D. tetensi* was not completed, it is possible to compare the activities and the chirality relative to *D. pyri*, as *D. pyri* and *D. tetensi* pheromone components are identical in every aspect except the position of the second acetate group of *D. tetensi* that shifts by one carbon. Therefore, it was assumed the elution order of the stereoisomers of the pheromone of the *D. tetensi* from the HPLC on chiral column to be the same as in the case of *D. pyri*. The stereoisomers of both components (*Z*)-2,13-diacetoxy-8-heptadecene and (*Z*)-2,12-diacetoxy-8-heptadecene were carried out on the same HPLC chiral column with similar eluting solvent system. Bielejewska et al. (2005) showed that the order of elution from chiralpak-AD phase is unaltered if the components in the solvent mixture remain the same. If the assumption is correct, the third eluting isomer of *D. pyri* is of 2*S,12R* configuration which can be the configuration of the naturally occurring pheromone component of *D. tetensi*. The first eluting isomer of *D. tetensi* pheromone from HPLC is strongly inhibitory would have 2*R,2R* configuration. In evolutionary perspective, in an event of the receptor neurons of *D. tetensi* and *D. pyri* are not capable of differentiating (*Z*)-2,12-diacetoxy-8-heptadecene and (*Z*)-2,13-diacetoxy-8-heptadecene due to structural similarities the cross attraction between these two species is prevented as the *R,R* configuration inhibits males of *D. tetensi*. Traps baited with 5 µg C or 10 µg racemate at 3-10 cm above the ground can now be used to monitor this pest and red traps minimise catch of non-target insects

CHAPTER 6

IDENTIFICATION OF THE FEMALE SEX PHEROMONE OF THE BLACKBERRY LEAF MIDGE, *Dasineura plicatrix* (Loew)

6.1. INTRODUCTION

Dasineura plicatrix (Loew) is a pest of blackberry, raspberry and loganberry and is distributed throughout central and Western Europe. Larvae feed on the upper surfaces of folded young leaves and larval feeding causes discolouration of leaves which become twisted and eventually turn black. In severely attacked plants an inhibition of growth can be seen (Barnes, 1948).

It has been shown that females of *Dasineura spp.* produce sex pheromones to attract males for mating and that these pheromones can be used as a monitoring tool in integrated pest management (Cross and Hall, 2009). Work on identification of the female produced sex pheromone of *D. plicatrix* is described in this Chapter.

6.2. MATERIALS AND METHODS

6.2.1. Insect Collection

Mature larvae for this study were obtained from heavily-infested blackberry shoots collected from a blackberry plantation in the Netherlands (NL) by Herman Helsen and from Tuesley Farm, Godalming, UK in July-September, 2008. Larvae were introduced individually into small plastic tubes (1.5 cm i.d. x 2.3 cm: Sarstedt AG., Germany) with a piece of wet filter paper inside and tubes were stored in temperature (18- 23°C) and light regulated room in the insectary until adults emerged as described in Chapter 2 Section 2.1 (Table 6.1).

Table 6.1. *D. plicatrix* larvae reared in the laboratory for pheromone collection.

Date	Origin	Number of larvae
July, 2008	NL	5265
July, 2008	UK	3500
September, 2008	NL	1880

6.2.2. Pheromone collection

Charcoal purified air was passed over males and females separately in glass chambers and volatiles were collected onto Porapak Q. The exact procedure was described in Chapter 2 Section 2.2.

However, slight modifications were made to the original method. The amount of absorbent material in the filter was doubled (400 mg) and the rate of the air flow was increased from 300 ml/min to 400 ml/min. It was observed that volatile collections made with fewer females and filters containing more adsorbent material produced a detectable peak for the candidate pheromone component by GC-MS. In previous midge pheromone work with *D. pyri* and *D. tetensi*, entraining large numbers of midges did not indicate a detectable pheromone peak when analysed by GC. Therefore above technique was carried out for the first time on a trial-and-error basis using males of *D. plicatrix* and was found to be successful.

6.2.3. Gas chromatography linked to electroantennographic recording (GC-EAG)

GC-EAG analyses of samples of volatiles collected from virgin female *D. plicatrix* were carried out as described in Chapter 2 Section 2.4. Recordings of signals from the antennae from whole male preparations were carried out. Male midges were exposed to female volatile collections F1-F5 on polar and non-

polar GC columns.

6.2.4. Gas chromatography linked to mass spectrometry (GC-MS)

GC-EAG analyses of volatiles from virgin females and males of *D. plicatrix* were carried out with the Varian CP 3800 gas chromatograph linked to a Varian Saturn 2200 ion trap mass spectrometer fitted with fused capillary columns coated with polar and non-polar phases (Chapter 2 Section 2.5).

6.2.5. Micro-hydrogenation of natural pheromone component

An aliquot of 40 µl of a volatile sample (F3) from *D. plicatrix* females in hexane was mixed with 10% Palladium on carbon catalyst and hydrogen gas was bubbled through a piece of fused silica capillary tubing for 1 min as described in Chapter 2 Section 2.6. The reaction mixture was analyzed by GC-MS as above.

6.3. RESULTS

6.3.1. Insect Collection

Despite the large numbers of *D. plicatrix* larvae reared, in total 10,645, only 18.4% emerged as females and were used in the entrainment.

6.3.2. Pheromone collection

Two collections were made from male *D. plicatrix* (M1-M2) and 12 collections from females (F1-F12). Numbers entrained varied from 526 to 60 females (Table 6.2).

Table 6.2.. Numbers of *D. plicatrix* male and females used in the volatile collections (M1 & M2 are males and rest are females)

Filter	Number of midges	Filter	Number of midges
M1	268	F6	86
M2	367	F7	79
F1	212	F8	101
F2	526	F9	66
F3	365	F10	108
F4	107	F11	133
F5	117	F12	60

6.3.3. GC-EAG analysis

GC-EAG analysis of volatiles collected from females on male midge preparations elicited two responses from males on polar column (Figure 6.1) whereas only one EAG response was elicited from males on non-polar column (Figure 6.1). The responses on polar column were consistent and appeared at 17.05 min (major component) and 16.65 min (minor component).

A prominent response for the major component was seen when female volatile collections F2, F3 and F4 were tested. However, when F1 volatile collection was tested response for the minor component appeared with a higher intensity. Co-injection of equal volumes (1.5 µl) of F1 and F2 gave responses for both the minor and the major components (Table 6.3). Later it was found that the sensitivity of the GC-EAG system was not quite up to the standard at the time the analysis. The absence of the response for the minor component on non-polar column may have been due to this issue or the fact that the major and the minor components were of similar retention times.

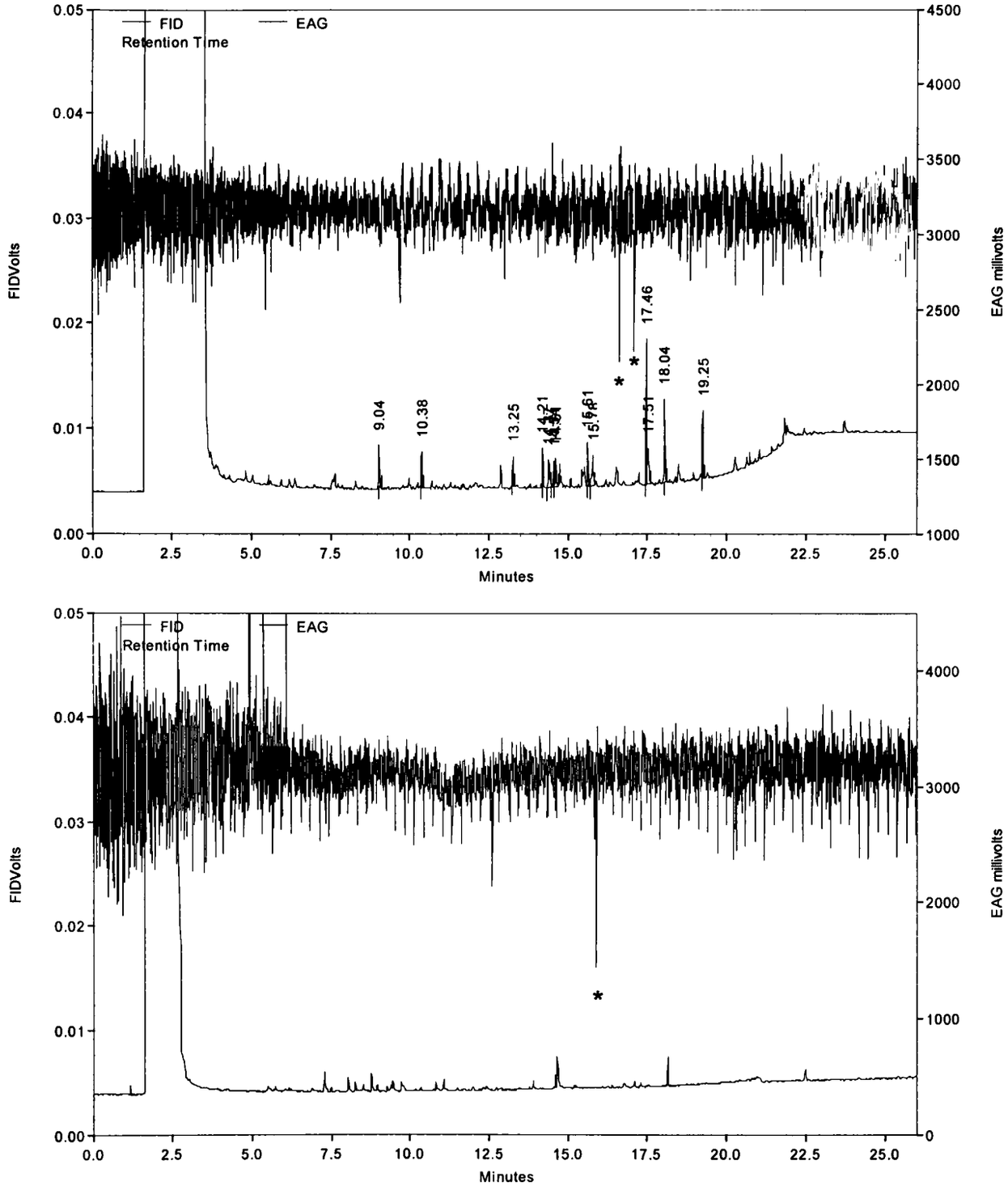


Figure 6.1. EAG responses from males of *D. plicatrix* to volatiles collected from conspecific females analysed on polar column (upper) and on non-polar GC column (lower)

Table 6.3. Retention times (RT) and retention indices (RI) of the natural major (**M**) and minor (**m**) pheromone component and other synthetic standards on non-polar and polar GC columns in GC-EAG and GC-MS

		Polar		Non polar		
		RT	RI	RT	RI	Δ^1
GC-EAG active components in F1	**m**	16.65	1394	15.95	1388	6
	M	17.08	1440	-		52
GC-EAG active components in F2	**M**	17.05	1437	15.88	1388	49
GC-EAG active components in F1+F2	**m**	16.65	1394	-		
	M	17.07	1439	-		
GC-EAG active component in F3	**M**	17.07	1439	-		
GC-EAG active components in F4	**m**	16.65	1394	15.95	1388	6
	M	17.07	1439	-		50
2-acetoxy pentadecane (GC-EAG)		16.51	1375	16.24	1422	-47
EAG active components on GC-MS	**M**	25.38	1438	26.13	1382	56
EAG active components on GC-MS	**m**	24.51	1384	-		
Hydrogenation of natural components (GC-MS)		24.46	1383	26.69	1410	-27
(Z)-2-acetoxy-8-heptadecene			1590		1588	2
2-acetoxy pentadecane (GC-MS)		24.23	1376	26.78	1416	-40

[1] Difference between RI on polar column and RI on non polar column.

6.3.4. GC-MS analysis

Comparison of male and female GC traces on the non-polar column of the GC-MS revealed that a peak for the candidate component exists only in female volatile collections (Figure 6.2). This was detected in collections F1, F3, F4, F6, F8 and F9. Identical mass spectra were obtained on GC-MS polar and non-polar columns for the major component (Figure 6.3).

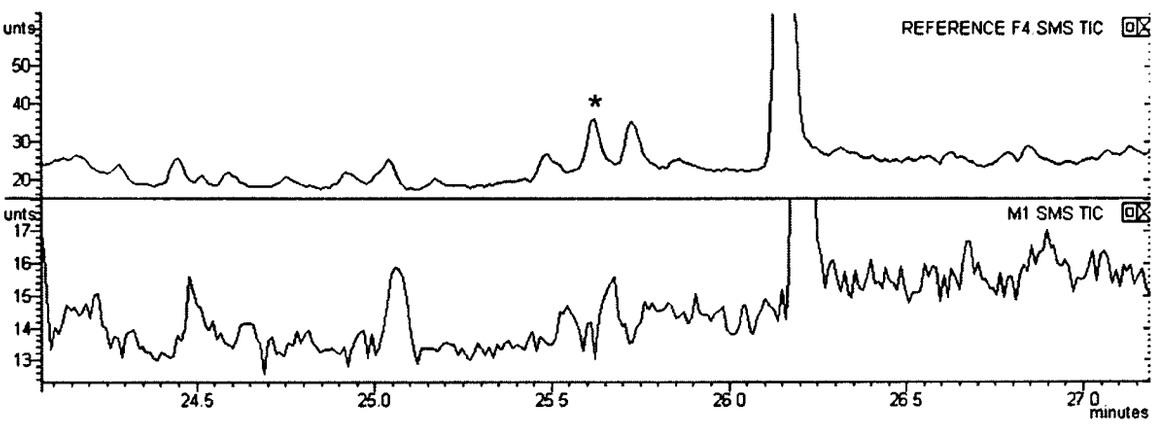


Figure 6.2. Comparison of GC traces of volatile collections of female (upper) and male (lower) of *D. plicatrix* on polar column (* denotes female specific peak).

GC retention times and the inter-column differentials showed that both the minor and the major components were two carbon atoms less than those of (*Z*)-2-acetoxy-8-heptadecene, a previously identified midge sex pheromone from *D. gleditchiae* (Table 6.3). The difference of the RI on GC polar and non polar column being smaller suggests that the pheromone components consist of a single acetate group. Therefore, the candidate pheromone components are proposed to have a linear chain with fifteen carbon atoms with a single acetoxy group.

The mass spectrum of the component corresponding to the major EAG response confirmed that it was an acetate ester with the diagnostic ions *m/z* 43 and 61. McLafferty rearrangement gave rise to *m/z* 207 which was of lower intensity (7%) formed due to the removal of acetate group from the suggested parent pheromone component *m/z* 266 (Figure 6.3).

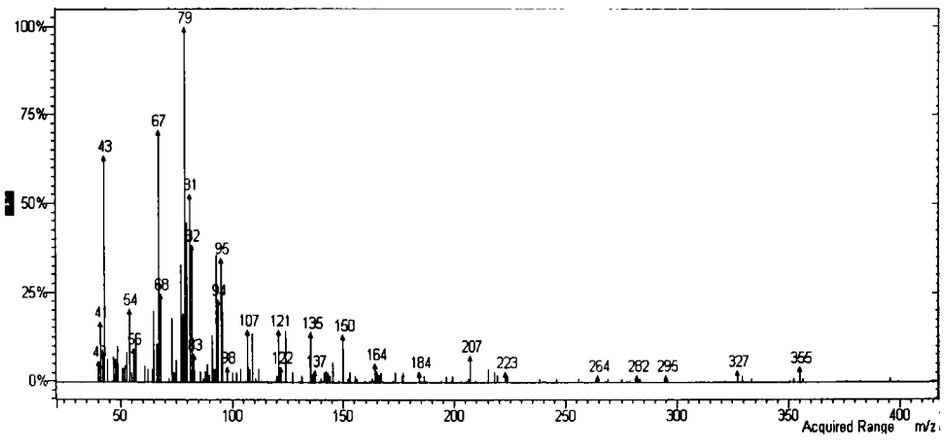


Figure 6.3. Mass spectrum of major pheromone component of *D. plicatrix*.

The presence of the *m/z* 79 (C_6H_7; 100%) was an indication of the diene fragment. Many moth pheromones contain two non conjugated double bonds and *m/z* 79 indicates the presence of this key fragment (Ando, 2004).

GC-EAG retention index data of the minor and the major components showed that the minor component was less polar than the major component. In fact minor component eluted 46 units (1440-1394) before the major component on the polar column suggesting that the minor component lacked one of the two double bonds present in the major component. The rationale is that the major and the minor components were structurally related as in many other pheromone blends. The elution of the major and the minor components close together on non-polar column of GC-EAG showed further evidence that these two components were not just related but also of similar polarity. Therefore the minor component could be a mono-acetate with a single double bond on a 15-carbon chain. A mass spectrum was acquired for the peak which was thought to be the minor component on polar column but the same was not found on non polar column (Figure 6.4).

Ion *m/z* 208 was formed due to the removal of acetoxy group from the parent molecule of weight of 268. The spectrum of the minor component of *D. plicatrix* was scanned for selected ions *m/z* 208 and 61 of which traces of these ions were observed. However, the total ion spectrum of the peak under consideration

found to be different from that from *D. gleditchiae* pheromone, (*Z*)-2-acetoxy-8-heptadecene in spite of the proposed similarities in their structures (Figure 6.4).

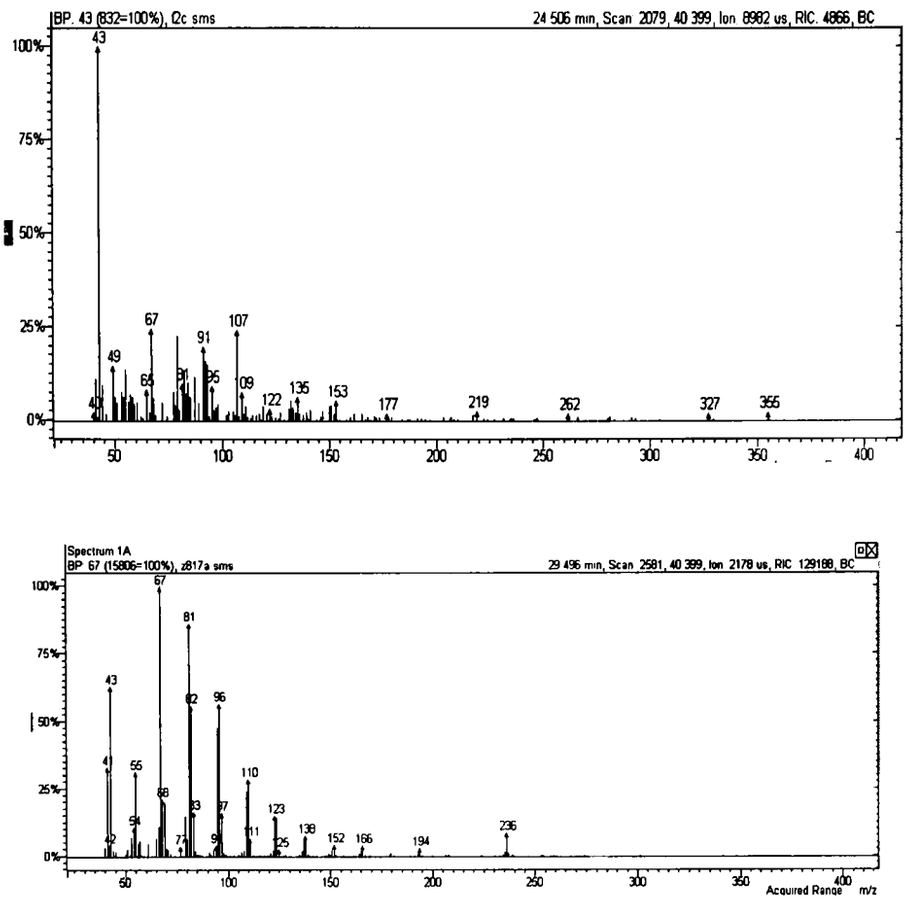


Figure 6.4. Mass spectrum of the minor component of *D. plicatrix* (upper) and *D. gleditchiae*, (*Z*)-2-acetoxy-8-heptadecene (lower)

6.3.5. Micro-hydrogenation of natural pheromone component of *D. plicatrix*

After microhydrogenation of a collection of volatiles from female *D. plicatrix*, a peak for the hydrogenated major component was difficult to find on GC-MS analysis. Scanning for ion *m/z* 43 and 61 for an acetoxy group and comparison of previous GC profiles with the hydrogenated trace revealed that the hydrogenated pheromone peak appeared as a shoulder of an existing peak at 24.27 min and 26.29 min on polar and non polar columns respectively (Figure 6.5). Matching retention index data of hydrogenated natural pheromone component and the synthetic 2-acetoxypentadecane (synthesised by Prof. Hall, NRI) on both the polar and the non polar columns confirmed that the pheromone component was an unsaturated component having 15 carbons and an acetate

group on the second carbon as in previously identified midge pheromones.

Although the mass spectrum obtained for the hydrogenated pheromone component on non polar column was relatively of poor quality in comparison to that on the polar column, ions at *m/z* 153, 125, 111 and 83 indicated an increase of *m/z* 4 units due to hydrogenation of two double bonds (Figure 6.6).

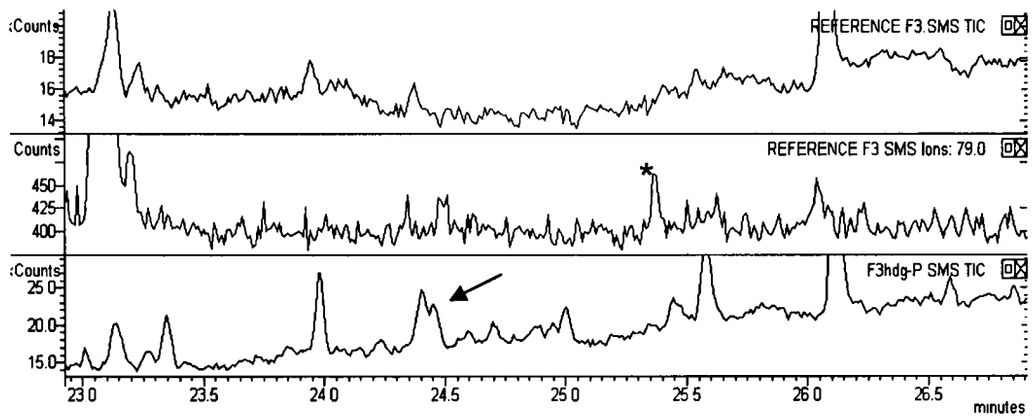


Figure 6.5. GC trace of hydrogenated pheromone peak (lower: arrow in black) and the pheromone peak of *D. plicatrix* before hydrogenation that was observed only after scanning the upper GC trace for *m/z* 79 (middle: *)..

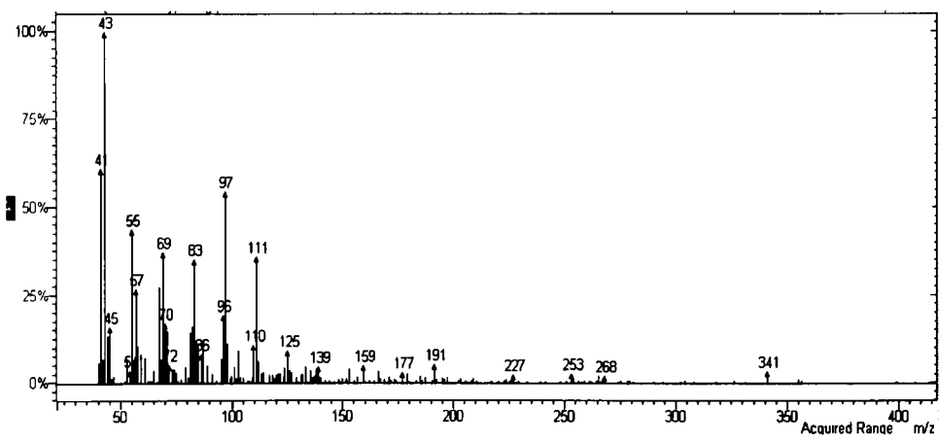


Figure 6.6. Mass spectrum of the hydrogenated product of the major natural pheromone component of *D. plicatrix* female volatile collection.

Due to the insufficient quantities of pheromone in the volatile collections, derivatisation of the natural pheromone components was not possible in order to study the exact position of the double bonds. Nevertheless, the position of the double bonds in the blackberry midge pheromone major component was proposed by taking structural features of the pheromones of *D. tetensi* and

D. pyri into consideration as all three species belong to the *Dasineura* genus.

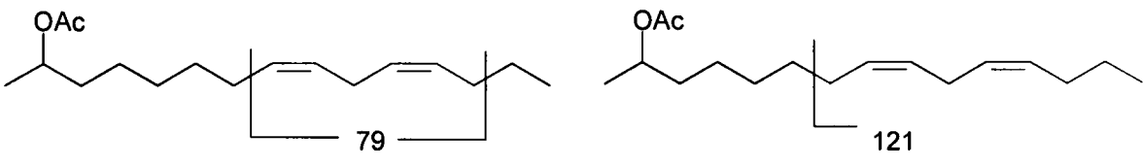


Figure 6.7. Mass fragmentation of (*Z*)-2-acetoxy-8,11-pentadiene

In a hypothetical context replacing acetoxy group on 12th or 13th carbons with a double bond and a removal of two terminal carbon atoms from (*Z*)-2,12-diacetoxy-8-heptadecene and (*Z*)-2,13-diacetoxy-8-heptadecene would form 2-acetoxy-8,11-pentadiene and 2-acetoxy-8,12-pentadiene respectively. However, only 2-acetoxy-8,11-pentadiene would give characteristic fragment for non conjugated diene *m/z* 79 (Figure 6.7). Therefore, the structure of the blackberry midge is likely to be (*Z*)-2-acetoxy-8,11-pentadiene not (*Z*)-2-acetoxy-8,12-pentadiene.

Further, *m/z* 121 formed due to allyl cleavage of C6-C7 bond and it is a strong evidence that the structure could be (*Z*)-2-acetoxy-8,11-pentadiene (Figure 6.7).

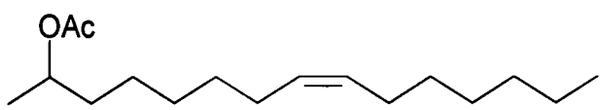


Figure 6.8. The proposed structure for the minor component of *D. plicatrix,* 2-acetoxy-8-pentadecene

The location of the double bond of the minor component could be 8th carbon as in many other *Dasineura* species. Therefore, the proposed structure for the minor component is 2-acetoxy-8-pentadecene (Figure 6.8).

6.4. DISCUSSION

Two EAG responses were elicited from male antennae of *D. plicatrix* in GC-EAG analyses of volatiles collected from conspecific females. The chemical structures of two EAG-active components were proposed as (*Z*)-2-acetoxy-8,11-pentadecadiene and (*Z*)-2-acetoxy-8-pentadecene taking retention index and the mass spectral data into account. These are the first midge pheromone components with fifteen carbon atoms, the missing link in the Cecidomyiidae pheromones comprised of varying carbon chain lengths from 9, 11,13 and 17.

From an evolutionary view point, pheromones of *D. mali,* (13*R*,8*Z*)-13-acetoxy-8-heptadecen-2-one, *D. pyri,* (8*Z*,2*R*,13*R*)-2,13-diacetoxy-8-heptadecene, *D. tetensi,* (*Z*)-2,12-diacetoxy-8-heptadecene, *D. gleditchiae* (*Z*)-2-acetoxy-8-heptadecene (Molnár et al., 2009) and the pheromone components of *D. plicatrix* may have evolved from the same biosynthetic pathway with a common substrate and enzymes. In order to avoid inter-breeding among close relatives alterations to the biosynthesis of sex pheromones has taken place. Changes such as shortening of carbon chain, positional changes of the double bond and modification of functionalities have taken place within the genus *Dasineura*.

Although inter-column differentials of the minor component, (*Z*)-2-acetoxy-8-pentadecene and (*Z*)-2-acetoxy-8-heptadecene (Molnár et al., 2009) were closely matched, the mass spectra were not exactly similar. Minute quantities of the minor component made it difficult to obtain a good mass spectrum as abundance of it was lower than the detectable range of the GC-MS. Therefore, the structure elucidation was mainly based on the retention index and also the fact that close similarites were seen within the family Cecidomyiidae and genus *Dasineura*.

The minor component could be either a bio-synthetic intermediate formed due to a less selective desaturation step occured in the biosynthesis or it may have produced via a different substrate–enzymatic system. The female-produced sex pheromone components of the light brown apple moth, *Epiphyas postvittana*

have been identified as (*E*)-11-tetradecenyl acetate and (*E,E*)-9,11-tetradecadienyl acetate (Bellas et al., 1983). Foster and Roelofs, 1990 showed biosynthesis of the above was initiated by 11-desaturation of myristic and palmitic acids. A single desaturase enzyme may also be multifunctional as suggested by Serra et al. (2007). In the biosynthesis of female sex pheromone of processionary moth, *Thaumetopoea pityocampa,* (*Z*)-13-hexadecen-11-ynyl acetate, a desaturase enzyme functions as 11-desaturase, 11-acetylenase, and 13-desaturase. Also (*Z,E*)-9,11- tetradecadienyl acetate, sex pheromone of *Spodoptera littoralis* was reported to be biosynthesised by action of specific (*E*)-11 and (*Z*)-9 desaturases on tetradecanoic acid (Martinez et al, 1990). Knipple et al (2002) has explained the reason behind the functional diversities observed in desaturases involved in sex pheromone bio-synthesis and stated that it can have taken place via similar routes. Further, desaturases evolved diverse substrate specificities and stereo-specificities to produce unsaturated fatty acid precursors of different chain lengths, location of the double bond, degree of unsaturation, and the geometry of the double bond, *Z*-cis or *E*-trans.

CHAPTER 7
SUMMARY AND DISCUSSION

7.1. SUMMARY

7.1.1. Pheromone identification

The hypothesis that the sex pheromones of Cecidomyiidae species are more chemically diverse than previously thought was based on the identification of a new class of compounds, the acetoxy-ketones. The results of this project, identification of sex pheromone from pear leaf midge, *Dasineura pyri,* pear midge, *Contarinia pyrivora*, blackcurrant midge, *Dasineura tetensi* and blackberry midge, *Dasineura plicatrix* showed that our hypothesis was false as no new classes of compounds were discovered. The pheromone components from the above species fall into classes such as monoacetates, diacetates and acetoxyketones identified previously.

However, there are about 5000 described Cecidomyiidae species in the literature and amongst them only 15 species have been investigated for their sex pheromones. Hence there is not enough evidence to prove that my hypothesis is false. Furthermore, an acetoxy-ketone was identified from *C. pyrivora* and the minor component of *D. tetensi* was proposed to be an acetoxy-ketone. Therefore the supposition that the acetoxy-ketones are widespread cannot be eliminated.

In summary, female-produced sex pheromones from three of the above species were identified and significant progress was made on identification of the pheromone components of the fourth. All the compounds are novel structures and have not been reported from any other Cecidomyiidae species (Table 7.1). Some components of this work were presented at conferences (See Appendix 3).

Table 7.1. Summary of identified female-produced sex pheromones of respective midge species

Species	Pheromone compound
D. pyri	(2*R*,13*R*,8*Z*)-2,13-diacetoxy-8-heptadecene
C. pyrivora	2, 7, diacetoxyundecane 7-acetoxyundecane-2-one
D.tetensi	(*Z*)-2,12-diacetoxy-8-heptadecene
Dasineura plicatrix	15 carbon, mono acetoxy diene

Dasineura pyri

Approximately 20,000 mature larvae of *D. pyri* were collected from infested plant materials and reared individually in plastic tubes to adulthood in the laboratory. Eighteen female and 9 male volatile samples were made by trapping volatiles from batches of up to 1,160 midges. An easy and reproducible technique for EAG recording was developed using whole insects with both antennae. GC-EAG analysis detected two active components, a major and a minor in terms of amount present and magnitude of the EAG response. Interpretation of the mass spectrum and comparison of GC retention times with a wide range of synthetic standards available at NRI lead to (*Z*)-2,13-diacetoxy-8-heptadecene as the probable structure. This was synthesised and found to have identical MS spectrum and GC retention indices to the natural compound. The minor component was present in too small amounts to be identified. Initial field tests revealed that the racemic (*Z*)-2,13-diacetoxy-8-heptadecene was not attractive to *D. pyri* males, so a method was developed to separate stereoisomers by HPLC in very pure form. Field tests in UK and New Zealand of the separated isomers showed the first eluting isomer was highly attractive while the rest of the isomers were completely unattractive. Testing two-way mixtures of the attractive isomer with other isomers showed that the second eluting isomer inhibited attraction, explaining the unattractiveness of the racemic mixture.

Synthesis of the individual stereoisomers of (*Z*)-2,13-diacetoxy-8-heptadecene was found to be very difficult for determining the absolute configuration. Syntheses of the stereoisomers were found to be easier in the absence of the double bond on the 8th carbon. The hydrogenated pheromone component showed that the attractive isomer had the 2*R*,13*R*-configuration.

Contarinia pyrivora

One difficulty come across during pheromone identification from *C. pyrivora* was the rearing of larvae as it has only one generation per year and adults appear for two weeks only in early spring. Previous attempts to rear larvae and terminate the long dipause under laboratory conditions were unsuccessful (unpublished data). For the present work, approximately 3,000 larvae were reared and from those that survived under outdoor conditions only a very small number, about 135, emerged as adults. Volatiles were collected from males and females and analysed by GC-EAG using techniques developed for the work on *D. pyri*. The presence of two active components was detected in female volatile collections. These were then identified by interpretation of GC-MS and GC retention data as 2,7-diacetoxyundecane for the major component, and 7-acetoxyundecane-2-one for the minor component. The diacetate was very similar to those compounds identified from other midge species previously and the acetoxy-ketone was a novel structure. Stereoisomers of both the major and the minor components were separated by HPLC on a chiral phase and used in the field tests. Field test data was confused by the presence of another species similar in appearance which was also being attracted to pheromone traps. Field test results in 2009 indicated that the first-eluting isomer A was the attractive isomer and the third-eluting C was also attractive. The second-eluting isomer B is likely to be the inhibitory isomer as it inhibited the activity of C when B and C were tested as a mixture.

Dasineura tetensi

Male *D. tetensi* showed two EAG responses to two components in the volatile collections from females. The major component, (*Z*)-2,12-diacetoxy-8-heptadecene, was very similar to the pheromone identified from *D. pyri*. Careful comparison of the MS and retention data indicated that the second acetoxy

group was moved one position along the carbon chain. Possible structures for the minor component were proposed as 12-acetoxyheptadecan-2-one or 2-acetoxyheptadecan-12-one. Extensive field testing of the racemate, four HPLC separated individual isomers of the major component, two component blends, doses, trap height and colour was done. The third eluting stereoisomer from HPLC was highly attractive to males of *D. tetensi* while the first and the last eluting stereoisomers inhibited the activity of the active stereoisomer. The second eluting stereoisomer did not affect the attractiveness of the active isomer. The racemic (*Z*)-2,12-diacetoxy-8-heptadecene was less attractive to midges at higher concentration while the racemate at lower concentration attracted comparatively more midges. On the other hand, traps baited with lower lure loads of the active isomer attracted higher numbers of male *D. tetensi*. Traps deployed close to the ground attracted significant numbers of males but the colour of the trap did not affect the catches of *D. tetensi* or the non target insects flying into traps.

Dasineura plicatrix

Work is at a very preliminary stage. Two responses were elicited from the male EAG antennal preparations of *D. plicatrix* by the volatiles collected from conspecific females. Retention indices and mass spectra were then compared with those of Cecidomyiidae pheromones already identified. The structure of the major component was proposed to be a 15 carbon, mono acetoxy diene. This will be the first recorded Cecidomyiidae pheromone component having fifteen carbons.

7.1.2. Development of New techniques

During the course of this work, several new techniques were used which will be of value in future work on insect pheromones in general and midge pheromones in particular.

Rearing gall midges

Mass rearing of midges in laboratory for pheromone collection and bioassays is a difficult task. It involves choosing the right substrate, rearing conditions and

approach for terminating the diapause of overwintering larvae. As the amount of pheromone produced by an insect is very small, in the pico-nanogram range, a large number of insects is needed for pheromone collection.

The widely practised method of obtaining midges is by separating the cocoons of overwintering larvae from a soil-water mixture by sieving (Doane et al., 1987; Cross and Crook, 1999). However, this method is very time-consuming when midges are needed in large numbers.

Rearing of late larvae collected from fresh plant material seems to be a less cumbersome technique. Mature larvae are individually reared on a piece of wet filter paper in closed plastic tubes so that pheromone can be collected from virgin females soon after they emerge. A rapid post-diapause development following termination of diapause is favoured by the moisture in the tube and high incubation temperature.

Pheromone collection

Collecting pheromones from midges is challenging owing to their small size, fragile nature and the short life span. Mostly the midge pheromone work is based on abdominal tip extractions of calling females. For example, in work on pea midge, *Contarinia pisi* (Hillbur et al., 1999), red cone cedar midge, *Mayetiola thujae* (Gries et al., 2005) and swede midge *Contarinia nasturtii* (Hillbur et al., 2005) solvent extracts of pheromone glands were used in the analysis. The incorporation of cuticular material and other impurities from various sources requires an intermediate step of purification of extracts by liquid chromatography for analysis. Collecting volatiles involves passing charcoal-filtered air over living insects and trapping volatiles onto a porous adsorbent. Collecting volatiles from midges was preferred over gland extraction, mainly because pheromone gland extracts contain substances other than pheromone components such as inhibitory precursors and also large numbers can easily be processed in collecting volatiles.

GC-EAG

Different techniques were used in GC-EAG analysis, for instance making the

EAG -preparation and the application of stimuli on them. EAG preparations were of whole insects suspended between electrodes, unlike in other cases of midge pheromone analysis reported where other methods were employed, such as Plexiglas electrode-well method (Hillbur et al, 2001) and recording from antennae attached to the head (Gries et al., 2002). This whole body preparation gave reproducible results. Furthermore, instead of humidified air (Hillbur et al, 2001), the GC column effluent was delivered onto insect preparations with nitrogen pulses (Cork et al., 1990).

Chiral HPLC separation of enantiomers

Successful separation of enantiomers/diastereomers using a polysaccharide chiral phase in combination with an organic mobile phase has been reported. Enantioseparation of drugs is widely used (Aboul-Enein, 2001; Grinberg et al., 2007) but the application of this technology to insect pheromones is relatively new (Pu, et al., 1999; Qin et al., 1997).

This technique was first used in separating stereoisomers of midge pheromones by Gries et al. (2000) and then by Hall et al. (2009). The isomers of the orange blossom wheat midge, *Sitodiplosis mosellana,* (2*S*,7*S*)-2,7-dibutyroxynonane and raspberry cane midge, *Resseliella theobaldi* female-produced sex pheromone, (2*S*)-2-acetoxyundecan-5-one were separated by chiral HPLC. In this work, the diastereomers of the sex pheromone of *D. pyri, (Z)*-2,13-diacetoxy-8-heptadecene, *D. tetensi*, (*Z*)-2,12-diacetoxy-8-heptadecene, *C. pyrivora,* 2,7-diacetoxyundecane and 7-acetoxyundecane-2-one were conveniently separated for testing attractiveness to males in the field.

7.2. DISCUSSION

7.2.1. Pheromones of Cecidomyiidae

One of the aims of this work was to explore the chemical diversity of midge pheromones. Following the discovery of the acetoxy-ketone structures in components of the pheromones of the apple leaf midge, *D. mali* (Suckling et al.,

2007; Cross and Hall, 2009) and the raspberry cane midge, *Ressseliella theobaldii* (Hall et al., 2009), it was proposed that the structures of midge pheromone components might be more diverse than previously thought.

In fact, although the pheromone components identified in this work are all novel structures, they follow the general patterns of structures found in midge pheromone components identified previously.

Similar structural features were seen amongst *D. pyri, C. pyrivora*, *D. tetensi, D. plicatrix* and previously identified sex pheromone from species of Cecidomyiidae. All are based on a linear chain with an odd number of carbons varying from 9-17. For example, the sex pheromone of the orange blossom wheat midge, *S. mosellana,* consists of 9 carbon atoms ((2*S*,7*S*)-2,7-dibutyroxynonane; Gries et al., 2000), swede midge, *C. nasturtii*, 11 carbons ((2*S*,9*S*)-2,9-diacetoxyundecane; Hillbur et al., 2005), pea midge, *C. pisi,* 13 carbons ((2*S*,10*S*)-2,10-diacetoxyundecane; Hillbur et al., 1999), and apple leaf midge, *D. mali* 17 carbons ((13*S*,8*Z*)-13-acetoxy-8-heptadecen-2-one; Cross and Hall, 2009) in the chain. All have an oxygenated functionality on C2 and it could be an acetoxy, butyroxy, alcohol or keto group (Table 7.2) and if the pheromone contains di-fuctionalities, the second functionality could be on C7 ((2*R*,7*S*)-2,7- diacetoxytridecane; Choi et al., 2004), C9 ((2*S*,9*S*)-2,9-diacetoxyundecane; Hillbur et al., 2005), C10 ((2*S*,10*S*)-2,10-diacetoxy-undecane; Hillbur et al., 2005), C11 ((2*S*,11*S*)-2,11-diacetoxytridecane; Hillbur et al., 2001), C12 ((2*S*,12*S*)-2,12-diacetoxyheptadecane; Gries et al., 2005), C13 ((2*S*,13*S*)-2,13-diacetoxyheptadecane; Gries et al., 2005) or C14 ((2*S*,14*S*)-2,14-diacetoxyheptadecane; Gries et al., 2005). The most commonly encountered pheromone components in Cecidomyiidae are acetate esters and the keto-acetate structures belong to a new class of compounds only found so far in the female sex pheromones of the apple leaf midge, *D. mali* (Cross and Hall, 2009), the raspberry cane midge, *R. theobaldi* (Hall et al., 2009) and the minor component of *C. pyrivora* (Chapter 4).

Table 7.2. Functionalities found in pheromones of Cecidomyiidae

Functionalities	Example	Reference
Acetates	(2*S*,10*E*)-2-acetoxy-10-tridecene	Foster et al., 1991
	(2*S*,4*Z*,7*Z*)-2-acetoxy-4,7-tridecadiene	Gries et al., 2002
	(2*R*,8*Z*)-2-acetoxy-8-heptadecene	Molnár et al., 2009
Butyrates	(2*S*,8*Z*)-2-butyroxy-8-heptadecene	Liu et al., 2009
	(2*S*,7*S*)-2,7- dibutyroxynonane	Gries et al., 2000
Ketones	(13S,8Z)-13-acetoxy-8-heptadecen-2-one	Cross and Hall, 2009a
	(2*S*)-2-acetoxyundecan-5-one	Hall et al., 2009
Alcohols	(2*S*,10*E*)-10-tridecen-2-ol	Andersson et al., (2009)

Pheromones of Cecidomyiidae are not very diverse. For example the chemical structures of *D. tetensi* closely resemble the *D. pyri* major component and also that of red cone cedar midge, *M. thujae* pheromone component, (2*S*,13*S*)-2,13-diacetoxyheptadecane (Gries et al., 2005). The suggested structure for the minor component of *D. tetensi*, 12-acetoxy heptadecane-2-one is related to that of *D. mali* (Cross and Hall, 2009) and *C. pyrivora* minor pheromone components (Chapter 4). Further, the close similarities of pheromone components are explained by either being *Contarinia* or *Dasineura*. In contrast the pheromone of the Douglas-fir cone midge, *C. oregonensis*, (2S,4Z,7Z)-2-acetoxy-4,7-tridecadiene (Gries et al., 2002) do not show striking similarities with the pheromones of other members of the same genus which comprise mostly diacetates. The slight variation in structural features may have evolved in sex pheromones so that species-specificity is maintained, preventing inter-sexual activities amongst closely related species of the same family. Apart from the diene component identified from *C. oregonensis,* two more pheromone components with two double bonds were identified as minor components from Hessian fly, *M. destructor*. Andersson et al., (2009) described the two components as (2*S*,8*Z*,10*E*)-2-acetoxy-8,10- tridecadiene and (2*S*,8*E*,10*E*)-2-

acetoxy-8,10-tridecadiene. Further, 2-acetoxy-8,11-pentadecadiene was suggested as the major pheromone component of *D. plicatrix* (Chapter 6). The biosynthetically preferred location of the double bond seems to be the eight position as seen in pheromones of *D. pyri* (chapter 3), *D tetensi* (chapter 4), *D. picatrix* (chapter 6) *D. mali* (Cross and Hall, 2009) and the minor components of Hessian fly Andersson et al., (2009).

Pheromones of Diptera

Sex pheromones, oviposition attractants and other close range attractants acting as sex stimulants from other families of Diptera such as Psychodidae (sand flies), Tephritidae (fruit flies), Muscidae (house flies) Glossinidae (tsetse flies) and Drosophillidae (flies) have been widely studied (Wicker-Thomas 2007; Luntz, 2003).

Unlike Cecidomyiidae species whose pheromone components are all female produced, the sex pheromone /attractant of the other families of Diperans could be either produced by males (sand flies) or females (house fly). As far as the chemistry of the Dipteran pheromones is concerned a clear pattern can't be seen among the known sex pheromone and other attractants involved in sexual behaviour. The chemistry of the sex pheromones varies from simple hydrocarbons, n-heptadecane, sex pheromone of farmyard midge, *Culicoides nubeculosus* (Mordue and Mordue, 2003), (*Z*)-9-tricosene and heneicosene, cuticular components of *Musca domestica* (Carlson et al, 1971) to complex homosesquiterpenes characterised as 3-methyl-α-himachalene (Hamilton, et al., 1996a), (*S*)-9-methylgermacrene-B (Hamilton, et al., 1996b) and mono cyclic di-terpene, cembrene-1 (Claudio et al, 2006) identified as male sex pheromones from *Lutzomyia longipalpis* from different localities in Brazil (Hamilton, et al., 2004). Also, spiroacetals were identified as the sex pheromone components of *Batrocera oleae* (Fletcher et al., 1992). Structural complexity also can be seen in cuticular components of tsetse flies, 15,19-dimethylheptatriacontane, 17,21-dimethylheptatriacontane, and 15,19,23-trimethylheptatriacontane, chain with 37 carbons with methyl branches. Laurence and Pickett, (1982) have identified an oviposition attractant which is a heterocyclic lactone, 5*R*,6*S*-6-acetoxy-5-hexadecanolide from *Culex quinquefasciatus* egg rafts. Mostly these

pheromone components are perceived by the respective sex either by contact or are only effective at a short distance due to lack of volatility.

Pheromones of Lepidoptera

Moth pheromones are highly diverse but structurally related. Two main types were described by Ando et al. (2004). Most compounds in Type I are either acetates, alcohols or aldehydes and the functional group positioned at the terminal carbon of the straight chain. In general, the length of the chain varies from 10-18 carbons having 0-3 degrees of unsaturation, for example, the major components of sex pheromones of *Ostrinia nubilalis,* (*Z*)-11-tetradecenyl acetate (Kochansky et al, 1975), *Heliothis zea,* (*Z*)-11- hexadecenal (Klun et al., 1980; Teal, et al., 1984) and *Cydia pomonella,* (*E,E*)-8,10-dodecadienol (McDonough and Moffitt, 1974). Type II compounds are polyunsaturated hydrocarbons and epoxy components (Miller, 2000: Ando et al., 2004). Hydrocarbons and epoxides are straight chain and containing 17-23 carbon atoms. The sex pheromone of gypsy moth *Lymantria dispar,* (7*R*,8*S*)-*cis*-2-methyl-7,8-epoxyoctadecane (Bierl, 1970) and tiger moth, *Holomelina lamae* 2-methylheptadecane represent epoxides and hydrocarbons. On the contrary the major sex pheromone of the Pistachio twig borer (Lepidoptera: Tineidae), *Kermania pistaciella,* (2*S*,12*Z*)-2-acetoxy-12-heptadecene (Gries et al., 2006) is very similar to Cecidomyiidae pheromones containing a straight chain with an odd number of carbon atoms and an oxygenated functionality on the second carbon.

7.2.2. Pheromone blends

In all four midge species investigated, GC-EAG analyses of volatiles from virgin females indicated the presence of two components eliciting EAG response from conspecific males and likely to be candidate pheromone component. In *D. pyri* and *D. tetensi* only the major components were identified and synthesised. In *C. pyrivora* both major and minor components were identified and synthesised. However, in all three species one isomer of the major component was highly attractive to conspecific males in field trapping test and in *C. pyrivora* addition of

the minor component did not significantly increase the catches of the major component.

Among the other midge species, a single component pheromone was identified in apple leaf midge, *D.mali* (Cross et al., 2009), orange blossom wheat midge, *S. mosellana* (Gries et al., 2000), aphidophagous gall midge, *A. aphidimyza* (Choi et al., 2004), and locust bean midge, *D. gleditchiae* (Molnár et al., 2009). Three components in the red cone cider midge, *M. thujae* (Gries et al., 2005), pea midge, *C. pisi* (Hillbur et al., 2001), four components in the pheromone blend of raspberry cane midge, *R. theobaldii* (Hall et al., 2009) and seven components in Hessian fly, *M. destructor* (Foster et al., 1991; Andersson et al., 2009) have been reported. In chrysanthemum midge, *Rhopalomyia* spp. (Liu et al., 2009) and *R. theobaldii* only one component was required for the attraction in the field while in *C. pisi* all three components were required to attract males to the traps (Hillbur et al., 2001). All three components of *M. thujae* (Gries et al., 2005) were equally active and no synergistic effect was seen at 1:1:1 ratio.

In moths, *Heliothis* zea, *H. virescens* and *H. armigera*, have similar pheromone blends and the main component in the blend is (*Z*)-11-hexadecenal. Additional components such as (*Z*)-9-hexadecenal are (*Z*)-11-hexadece-1-ol are also present in above species. However, (*Z*)-9-tetradecenal is reported only from *H. virescens* but not from *H.* zea or *H. armigera* (López Jr et al., 1990). The presence of a minor component plays an important role in species recognition. Baker et al., 2004 demonstrated that in related species *H. subflexa* and *H. virescens* the males have subtle differences in the tuning of antennal receptor neurons for distinct pheromone blends. Both species have majority of receptor neurons sensitive to their major pheromone component, (*Z*)-11-hexadecenal. However, sensitivity varies when receptor neurons are exposed to minor components. Further, *H. zea* showed electrophysiological responses to the interspecific repellent, (*Z*)-9-tetradecenal in the conspecific *H. virescens* pheromone blend and it is likely that this discrimination contributes to reproductive isolation between these two species (Christensed et al., 1990).

7.2.3. Stereospecificity

As with all Cecidomyiidae pheromone compounds identified to date (Table 1.1) the pheromone components have at least one chiral centre. Although the configuration of the naturally produced compounds was not determined for any of these species, in all cases one stereoisomer has the most attraction to conspecific males and this is assumed to be the one produced by females.

In *D. pyri*, (2*R*,13*R*)-2,13-diacetoxy-8-heptadecene was highly attractive to males. The 2*S*,13*S* and 2*S*,13*R*-isomers were unattractive and did not affect the attractiveness of the 2*R*,13*R*-isomer when mixed with these in 1:1 ratio. The 2*R*,13*S* isomer was unattractive to males and when mixed with the 2*R*,13*R* isomer completely inhibited the attractiveness of the males. When HPLC separated individual isomers were tested on male antennal preparations electrophysiological responses were elicited by the 2*R*,13*S* isomer as well as the most attractive, 2*R*,13*R* isomer. This suggests that a male *D. pyri* has neurone receptors for attractive as well as inhibitory pheromone components. Having specialized receptor neurons capable of detecting non-natural/antagonists or pheromone analogues avoids attraction between heterospecifics.

Field test data obtained in 2008 and 2009 suggested that a slightly different pheromone system exists in *C. pyrivora*. Two isomers of 2,7, diacetoxyundecane were attractive to males. The first eluting isomer of 2,7, diacetoxyundecane from HPLC attracted males more strongly than the third while field test data implied the second eluting isomers was the one that responsible for inhibition of the racemic mixture. In contrast the racemic mixture of the minor component 7-acetoxyundecane-2-one was as attractive to males of *C. pyrivora* as one of the two isomers.

The third eluting isomer of 2,12-diacetoxy-8-heptadecene from the HPLC was more strongly attractive to males of *D. tetensi* than other three isomers. The first

eluting isomer strongly inhibited the activity of the isomer which was highly attractive to males. The absolute configuration of *D. tetensi* was not determined, but it is possible to compare the activities and the chirality relative to *D. pyri*, as *D. pyri* and *D. tetensi* pheromone components are identical in every aspect except the position of the second acetate group of *D. tetensi* that shifts by one carbon. Therefore, it was assumed the elution order of the stereoisomers of pheromone of the *D. tetensi* from the HPLC on chiral column was the same as in the case of *D. pyri*. The stereoisomers of both components (2*R*,13*R*)-2,13-diacetoxy-8-heptadecene and (*Z*)-2,12-diacetoxy-8-heptadecene were carried out on the same HPLC chiral column with similar eluting solvent system. Bielejewska et al. (2005) showed that the order of elution from chiralpak-AD phase is unaltered if the components in the solvent mixture remain the same. The configuration of the third eluting isomer of *D. pyri* would then be 2*S,13R* which is assumed to be the configuration of the naturally occurring pheromone component of *D. tetensi (*2*S,* 12*R)*. The first eluting isomer of *D. tetensi* pheromone from HPLC is strongly inhibitory and has a 2*R,* 12*R* configuration. From an evolutionary perspective, in the event the receptor neurons of *D. tetensi* and *D. pyri* are not capable of differentiating (*Z*)-2,12-diacetoxy-8-heptadecene and (*Z*)-2,13-diacetoxy-8-heptadecene due to structural similarities, the cross attraction between these two species is prevented as (2*R*,12*R*)-2,12-diacetoxy-8-heptadecene inhibits males of *D. tetensi*.

The racemate of the major components of *Rhopalomyia longicauda* (Liu et al., 2009), *C. oregonensis* (Gries et al., 2002), *D. gleditchiae* (Molnár et al., 2009), *S. mosellana* (Gries et al., 2000), *R. theobaldi* (Hall et al., 2009) and *D. mali* (Cross et al., 2009) were found to attract males of respective species to the same extent as its naturally occurring isomer. The opposite isomer/s could be either unattractive as in *D. gleditchiae* (Molnár et al., 2009) or having slight attraction as in the case of *D. mali* (Cross et al., 2009). Thus the stereochemistry at the second carbon atom is important for attraction. For example the racemic mixture of the sex pheromone of *A. aphidimyza* was unattractive but the isomer (2*R*,7*S*)-2,7-diacetoxytridecane was found to be attractive to males both in the Y-tube assay and in the field (Choi et al., 2004). It was shown that 2*R,7R*–isomer was strongly inhibitory and 2*S,7R* was

relatively less inhibitory than 2*R*,7*R*-isomer. Hillbur, et al. (2001) demonstrated that two diacetates (2*S*,11*S*)-2,11-diacetoxytridecane and (2*S*,12*S*)-2,12-diacetoxytridecane of the three component blend of *C. pisi* needed to have the *SS* configuration in the chiral centres of both components for higher activity. Trap catches of males by the three component blend were significantly reduced when the active diacetates were substituted with isomers which did not occur naturally. (2*R*,11*S*) and (2*S*,11*R*)- 2,11-diacetoxytridecane strongly inhibited the activity of the active blend. Leal (1996) stated that in pheromone blends which consist of several chiral compounds, the antagonistic effect of the opposite enantiomer is better tolerated as the isolation of the species is supported by additional components in the specific pheromone blend.

Although there are many examples which describe the olfaction of insects at cellular level, there is every little work done with respect to Cecidomyiidae species. Work done by Boddum (2007) explained the sex pheromone receptors were located in sensilla which were found to be different morphologically in *C. nasturtii,* and *M. destructor*. For instance sensilla trichodea (long hair like sensilla) were sensitive to sex pheromone in hessian flies while it was sensilla circumfila (enlarged loops) showed activity for pheromone in swede midge, *C. nasturtii*. Eight types of responses were observed from sensilla circumfila. Amongst different types of sensory cell types, one type responded to the complete blend, the naturally occurring isomer, (2*S*,10*S*)-2,10-diacetoxyundecane and to its racemate. The other types were seen respond to only to the racemic 2,10-diacetoxyundecane suggesting that this particular cell type probably has receptors for stereoisomers which were behaviourally antagonistic. Sensilla trichodea of *M. destructor* housed two types of receptor neurons according to Boddum (2007) each of which respond to only one or a few of the tested pheromone components. Based on the response profile of receptors, these were categorised into eight types, many of which responded to two pheromone components.

Stereospecificity in other insect pheromones

Zhang et al. (2006) stated that olfactory discrimination is related to the structure at the asymmetric centre of the pheromone molecule. The pheromone, (*R*)-

maconelliyl-(*S*)-2-methylbutanoate and (*R*)-lavandulyl (*S*)-2-methylbutanoate, interacts with the olfactory receptors of male pink hibiscus mealybug, *M. hirsutus*. The chiral centre of the acid moiety with *S* configuration elicits attraction whereas the *R* configuration induced an inhibitory effect according to Zhang et al. (2006). However, the attraction showed some degree of tolerance toward the change of chirality in the alcohol portion of the pheromone molecules.

The presence of different olfactory receptor neurons for differentiating stereoisomers of the pheromone component has been demonstrated by cellular recording of many insect species (Ulland et al., 2006; Kalinová et al., 2001; Larsson et al.,2002). With the *Popillia japonica* female produced sex pheromone, (*Z*)-5-(1-decenyl) oxacyclopentane-2-one, (*R*)-japonilure demonstrated strong attraction for conspecific males (Tumlinson et al., 1977) whilst the opposite enantiomer, *S* attracted *Anomala osakana* males (Leal, 1996). However, the presence of any of the opposite enantiomer in traps of either *Popillia japonica* or *Anomala osakana* inhibited the male attraction to these traps (Leal, 1996; Tumlinson et al., 1977). It was later revealed by Leal and Mochizuki (1993) that *Popillia japonica* possess olfactory- receptor neurons, co-localized in one sensillum, extremely specific to (*R*)- and (*S*)-japonilure, the latter being antagonistic, whereas in *A. osakana* beetle sensilla contains only one receptor neuron which is tuned only to its pheromone and but not to the opposite enantiomer. In species such as bark beetles, receptor cells can discriminate enantiomers of their pheromone compounds from the opposite enantiomer. Light and Birch (1979) showed that *Ips paraconfusus* was inhibited by (*R*)-ipsdienol, the pheromone which is produced by boring males of *I. pini*. On the other hand the pheromone of *I. paraconfusus* predominantly contains (*S*)-ipsdienol and also (*S*)-ipsenol and both of these components are inhibitory to *I. pini*. Species of *Ips* have different receptor cells which can differentiate *R* from the *S* isomer of ipsdienol (Mustaparta et al., 1980). In sympatric species gypsy moth, *Lymantria dispar*, and nun moth, *L. monacha*, the sex pheromone of the former was identified as 100% (+)-dispalure (Bierl et al., 1970) and the gypsy moth antennae were sensitive to its pheromone and to the antipode (Klimetzek et al., 1976). In gypsy moth one type of receptor cells responded to

(+)-dispalure and the other type to (-)-dispalure, the opposite enantiomer of the sex pheromone. Although the sex pheromone of of nun moth is a mixture of 90% (-)-dispalure and 10% of (+)-dispalure, antennal receptors were only sensitive to (+)-dispalure of the male moth (Hansen, 1984).

7.2.4. Biosynthesis

The pheromone biosynthetic pathway is the key process that leads to synthesis of pheromones in insects. There are different pathways, for example, Lepidopteran pheromones are synthesised via the fatty acid pathway and in beetles pheromone is biosynthesised from either fatty acid, amino acid or isoprenoid pathways. In some cases dietary substances can be converted to pheromones (Jurenka, 2004).

Although the biosynthesis of pheromone in Cecidomyiidae species is yet to be elucidated fully, Foster et al. (1991) have investigated pheromone biosynthesis in Hessian fly, *M. destructor.* They showed sex pheromone was not produced when female Hessian flies were decapitated. However, upon injecting female head extracts into decapitated females, biosynthesis of sex pheromone resulted and thus initiation of biosynthesis is triggered by some factor in the female head. In a number of insects pheromone biosynthesis is activated by release of Pheromone Biosynthesis Activating Neuropeptide (PBAN) from the sub-oesophageal ganglion (Raina and Klun, 1984; Raina et al., 1989). Although precursors of the sex pheromone were not detected in *M. destructor*, (*E*)-9-dodecenoic acid was found in the gland. This was isolated and identified as methyl (*E*)-9-dodecenoate after methylation of the pheromone gland contents. It was suggested that the chain shortening of palmitate (16C) by four carbons followed by Δ9-desaturation, addition of acetyl-SCoA, α-decarboxylation and acetylation yielded the pheromone. One of the prominent characters of Cecidomyiidae species is that the pheromones consist of odd number of carbons and the decarboxylation is the key event contributing to this process. Reed et al. (1994) examined the biosynthesis of major sex pheromone component, (*Z*)-9-tricosene of house fly, *Musca domestica*. Incubation of (*Z*)-15-

tetracosenoyl-CoA with microsomal preparations gave (*Z*)-9-tricosene and (*Z*)-15-tetracosenal. The latter component was converted to (*Z*)-9-tricosene in the presence of both NADPH and O_2 catalysed by cytochrome P450.

The possible biosynthesis pathway of Cecidomyiidae pheromones of 17 carbon chain is shown in Figure 7.1. Desaturation at *Δ7* position (1) of palmitic acid followed by acetyl SCoA (2) and decarboxylation (3). Acetylation of *Z*8-heptadecen-1-ol (4) yields 2-acetoxy-8-heptadecene. However, biosynthesis of di-functional compounds was found difficult to predict.

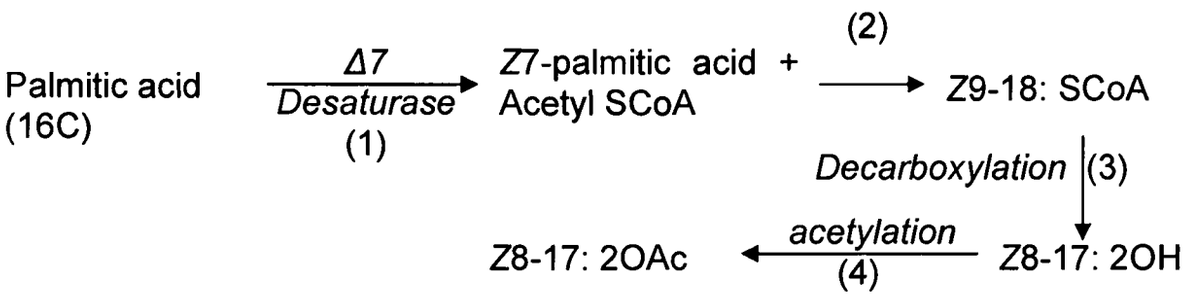


Figure 7.1. Possible biosynthesis of 17 carbon mono acetate components

A blend of compounds has evolved to make up the species-specific pheromone and a biosynthetic pathway regulates the biosynthesis of these components. The amount of enzymes present in related species may vary as Morse et al. (1990) showed in two related *Choristoneura* moths where the enzyme responsible for converting the acetate ester into aldehyde was significantly lower in one species than the other. The substrate specificity of enzymes may vary as in females of redbanded leafroller moth, *Argyrotaenia velutinana* (Roelofs and Jurenka, 1996). The major component, (*Z*)-11-tetradecenyl acetate (Roelofs and Arn, 1968) is produced due to lack of specificity of chain shortening enzymes and selective acetyltransferase action on the substrate14-carbon component (Roelofs and Jurenka, 1996).

7.3. POTENTIAL USE OF MIDGE PHEROMONES

Having sex pheromone traps deployed in orchards and plantations helps monitoring the emergence of the pest and the analysis of the trap data can be used in determining the time of application of insecticide. A considerable amount of work was done on developing sex pheromones of orange blossom wheat midge (Bruce et al., 2007), raspberry cane midge (Hall et al., 2009) and apple leaf curling midge (Cross and Hall 2009) into a monitoring trap or a control device. For example, Smith and Chapman (1996) demostrated that black bucket traps, one with a funnel and the other with a sticky surface were not effective in monitoring emergence of *D. mali* as those traps caught fewer midges. Due to lack of species specificity, these traps caught non-target species as well. Further they reported that the percentage of apple shoots infested with eggs of *D. mali* was not correlated with the number of adult females caught in traps. However, Cross et al. (2009) showed that sex pheromone trap catch of *D. mali* males was correlated with female egg laying and demonstrated that the pheromone traps were highly effective for monitoring flight activity of successive generations in the field and used for predicting the severity of the damage and thereby recommending insecticide applications. The sex pheromones might also be used for control of midges. Suckling et al. (2007) reported that mass trapping of apple leaf midge, *D. mali* could reduce the population by 99 % in the absence of immigration of mated females.

Pheromone traps for monitoring raspberry cane midge, *R. theobaldi* apple leaf midge, *D. mali* (East Malling Research) and orange blossom wheat midge, *S. mosellana* (Agrisense) are available for growers in UK.

7.4. FUTURE WORK

Although significant progress has been made in the identification and establishing the activity of the female-produced sex pheromones of *D. pyri, C. pyrivora*, *D. tetensi* and *D. plicatrix* further work is required before making these pheromone components available for growers commercially.

7.4.1. *D. pyri*

The minor component of *D. pyri* is still to be identified and the proposed structure needs to be confirmed.

7.4.2. *C. pyrivora*

The active isomers of the minor and the major components were identified from field tests, but the absolute configuration of these components are not yet known. The absolute configuration of the minor, 7-acetoxyundecane-2-one and the major components, 2,7-diacetoxyundecane components are to be established.

7.4.3. *D. tetensi*

The possible structure for the minor component of *D. tetensi* was proposed. It is to be chemically synthesised and the confirmation of the structures to be done. The activity of the minor component in the field is to be carried out.

The absolute configuration of the attractive isomer of the major component of *D. tetensi* needs to be established.

7.4.4. *D. plicatrix*

The possible structures for the minor and the major components of *D. plicatrix* are proposed. However, the positions of the double bonds were speculative. More pheromone needs to be collected and the positions of the two double bonds needed to be confirmed by DMDS reaction. Pheromone components should be tested in the field to identify the active and inhibitory isomers.

7.4.5. Biosynthesis

Foster et al. (1991) proposed a possible pathway for Hessian fly sex pheromone biosynthesis. In his work, presence of an unusual fatty acyl moiety, (*E*)-9-dodecenoate was detected and suspected that it could be an intermediate of the biosynthesis. However, the exact pathway was not determined and further work

is required to elucidate the pathways involved in midge pheromone biosynthesis.

7.4.6. Effect of plant volatiles on behaviour of midges

Host plant volatiles are important for phytophagous insects in finding a mate and oviposition after mating. It was shown that females of several midge species are attracted to volatiles from the host plant (Galanihe and Harris (1997); Birkett et al. (2004); Gordon and Williamson (1991); Hall et al., unpublished data). Crook et al (2001) demonstrated attraction of *D. tetensi* to host volatiles although there was no distinction of resistant and susceptible varities. Further work is required to identify the chemicals involved in attraction of these midge species to host volatiles, particularly those that attract females. Also it would be interesting to see the possibility of finding a host odour which can be used as a synergist or a compound that enhances the sex pheromone attraction of males.

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APPENDIX 1

SYNTHESES OF PHEROMONES

Pear leaf midge, *Dasineura pyri*

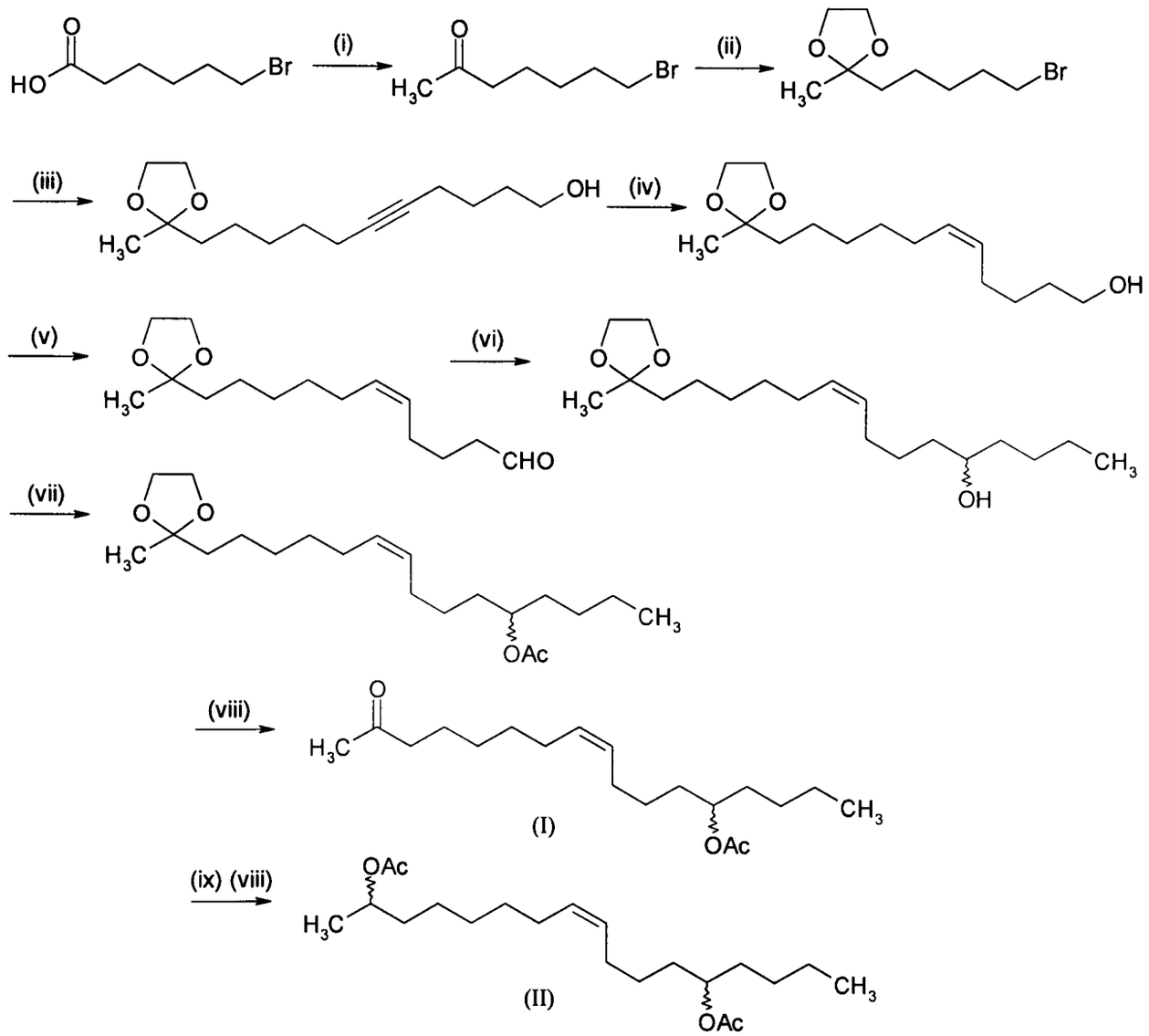

Figure 1. Synthesis of (*Z*)-13-acetoxy-8-heptadecene (I) and (*Z*)-2,13-diacetoxy-8-heptadecene (II) (reagents: (i) MeLi/ether; (ii) ethanediol/pTSA/toluene; (iii) 5-hexyn-1-ol/Li/liquid NH_3/THF; (iv) H_2/Lindlar catalyst/EtOAc; (v) oxalyl chloride/DMSO/Et_3N/ CH_2Cl_2; (vi) BuMgBr/ether; (vii) acetic anhydride/pyridine; (viii) acetone/pTSA; (ix) lithium aluminium hydride/ether).

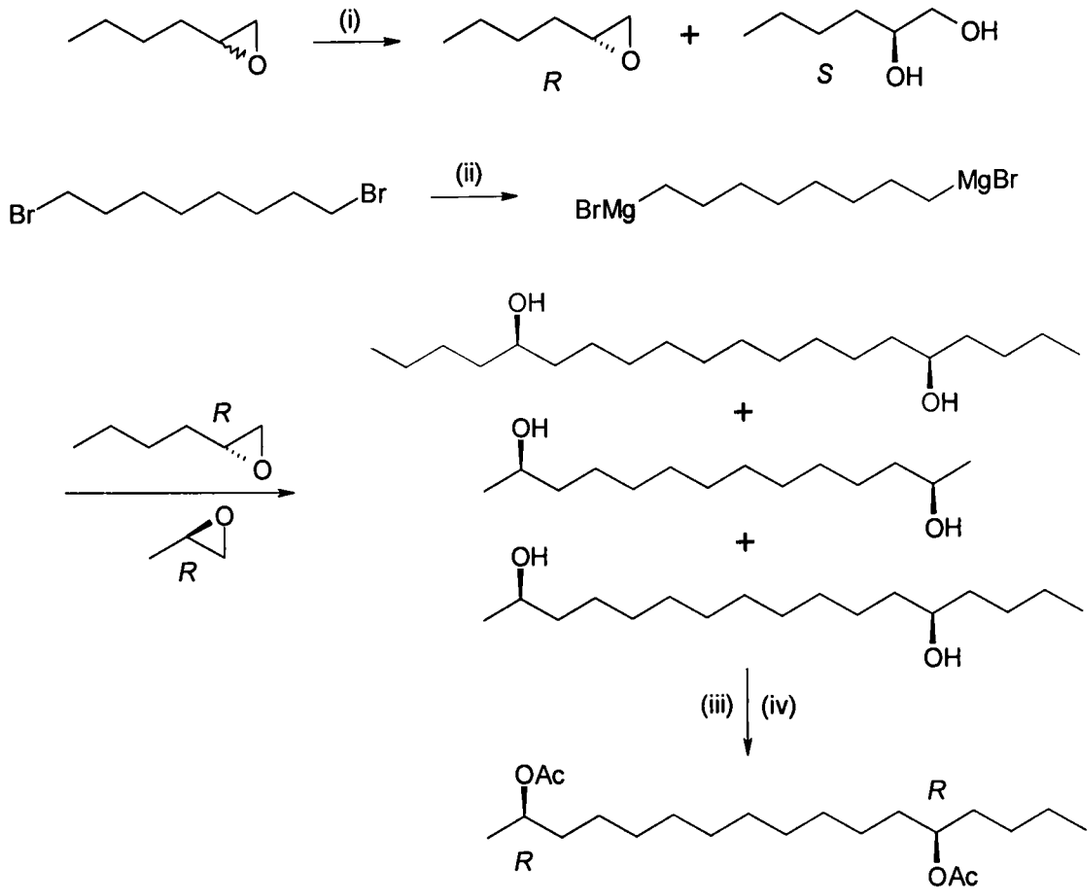

Figure. 2. Synthesis of (*R,R*)-2,13-diacetoxyheptadecane (reagents: (i) (*R,R*) Jacobsen reagent/THF/H_2O; (ii) Mg/THF; (iii) chromatographic separation; (iv) Ac_2O/pyridine).

Pear midge, *Contarinia pyrivora*

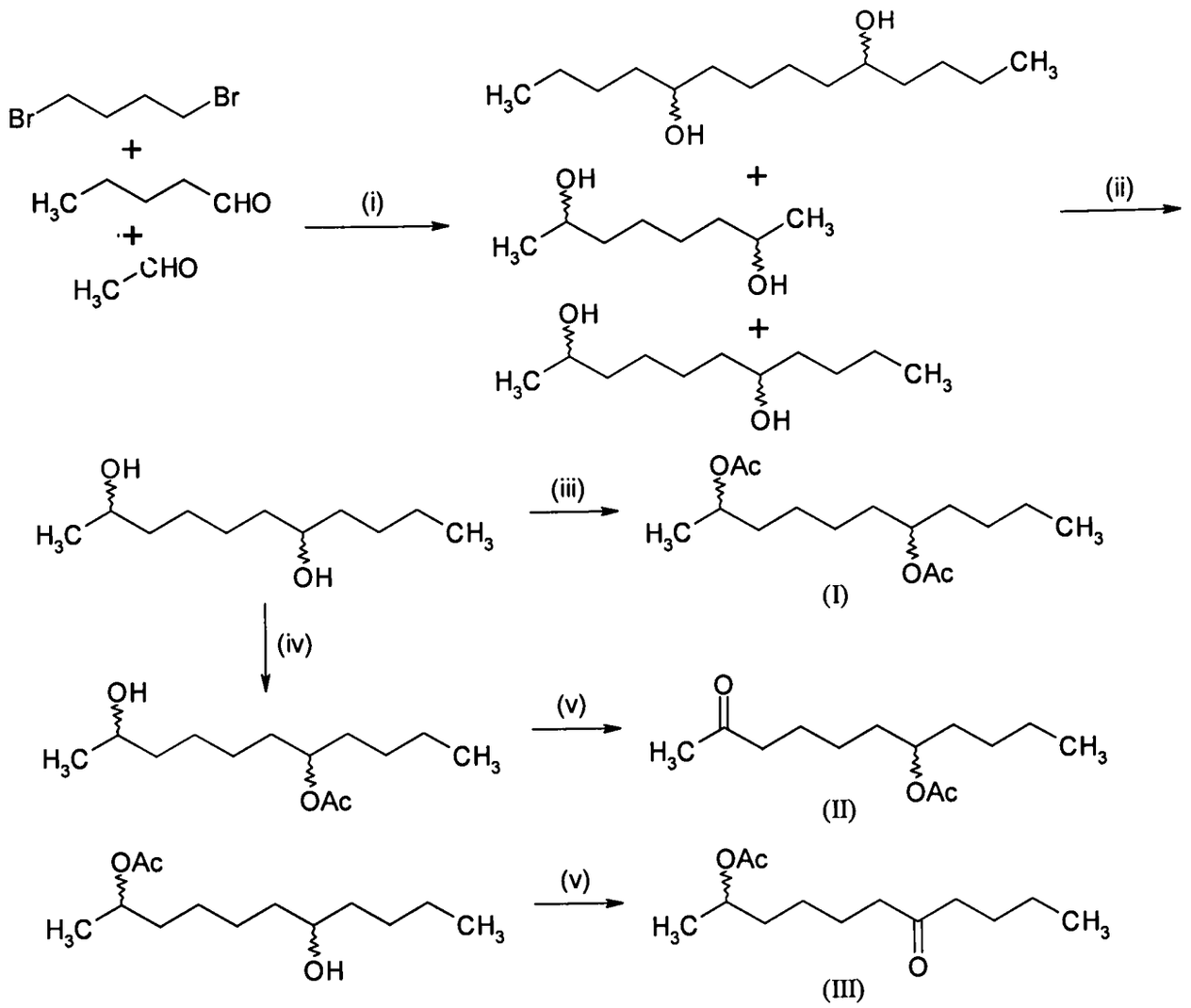

Figure 3. Synthesis of 2,7-diacetoxyundecane (I), 7-acetoxy-2-undecanone (II) and 2-acetoxy-7-undecanone (III) (reagents: (i) magnesium/THF; (ii) chromatography on silica gel; (iii) excess Ac_2O/pyridine; (iv) 1 equiv Ac_2O/pyridine; chromatography on silica gel; (v) pyridinium dichromate/dichloromethane; chromatography on silica gel).

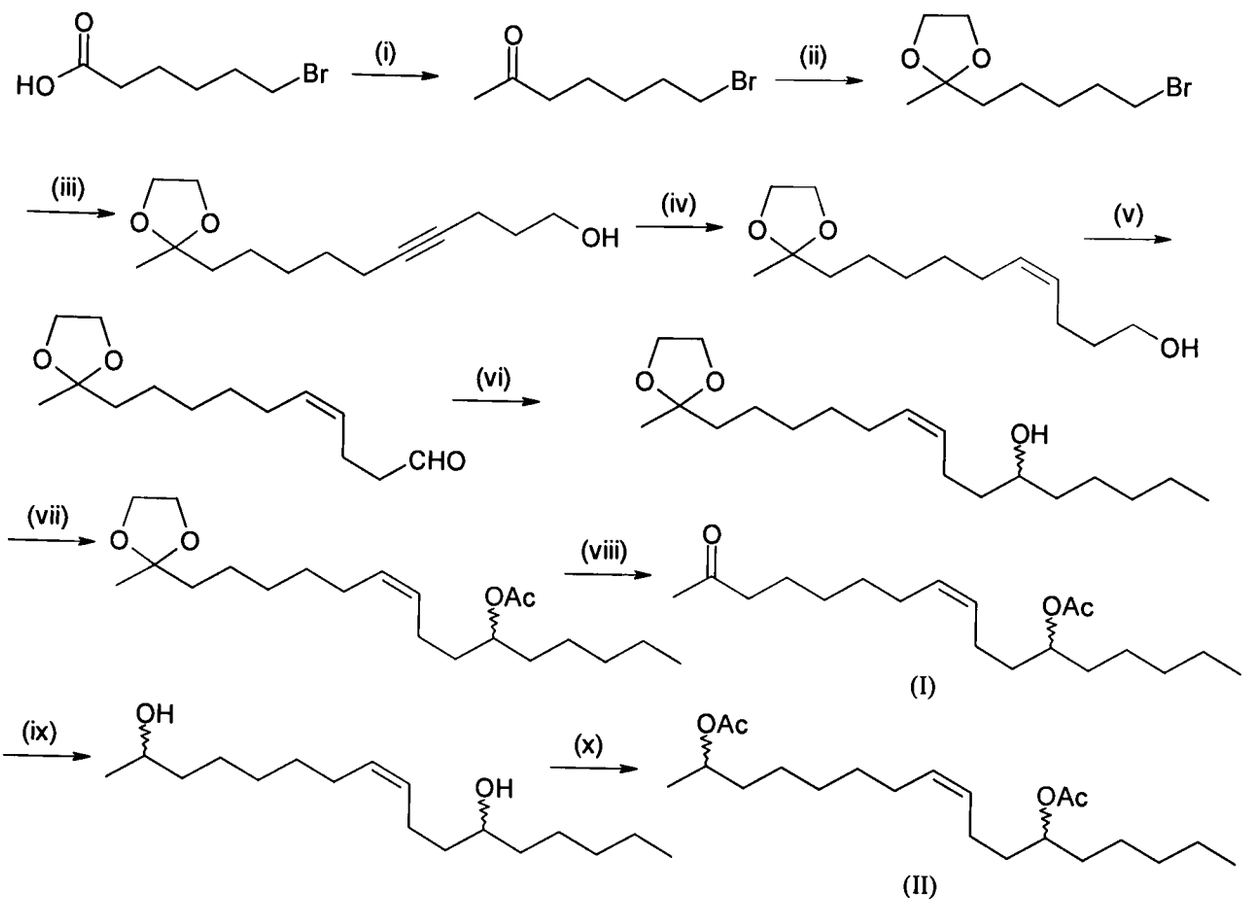

Figure 4. Synthesis of (*Z*)-12-Acetoxy-8-heptadecen-2-one (I) and (*Z*)-2,12-diacetoxy-8-heptadecene (II) (reagents: (i) MeLi/ether; (ii) ethanediol/pTSA/toluene; (iii) 4-pentyn-1-ol/$LiNH_2$/liq NH_3/THF; (iv) H_2/Lindlar catalyst/THF-hexane; (v) $(COCl)_2$/DMSO/CH_2Cl_2/ Et_3N; (vi) $C_5H_{11}MgBr$/ether; (vii) Ac_2O/pyr; (viii) acetone/pTSA; (ix) LAH/ether; (x) Ac_2O/pyr)

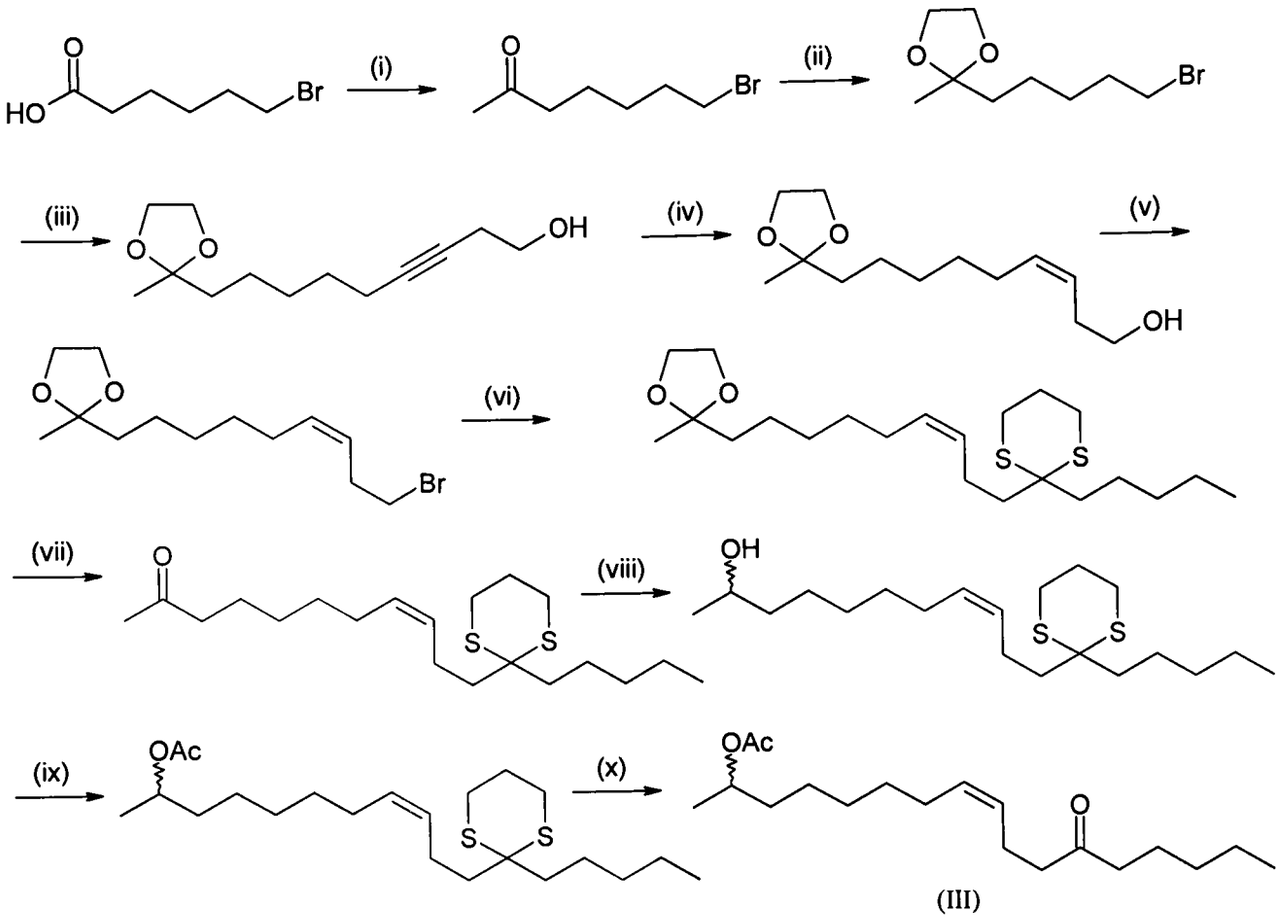

Figure 5. Synthesis of (*Z*)-2-acetoxy-8-heptadecen-12-one (III) (reagents: (i) MeLi/ether; (ii) ethanediol/pTSA/toluene; (iii) 3-butyn-1-ol/$LiNH_2$/liq NH_3/THF; (iv) H_2/Lindlar catalyst/THF-hexane; (v) $CBr_4/Ph_3P/CH_2Cl_2$; (vi) 2-pentyl-1,3-dithiane/BuLi/THF; (vii) acetone/pTSA; (viii) $NaBH_4$/EtOH; (ix) Ac_2O/pyr; (x) $CuCl_2$/CuO/aq. acetone)

APPENDIX 2

Data Analysis

Pear leaf midge, *D. pyri* (CHAPTER 3)

Second field test

	A	B	C	D	Untreated
week1	21	0	0	0	0
	6	0	0	0	1
	31	0	0	0	0
week2	22	0	1	0	0
	19	0	0	0	0
	16	0	0	0	0
total	115	0	1	0	1
Mean	38.33		0.167		0.167
SE	4.72	0.00	0.24	0.00	0.24

Analysis of variance

Variate: LOG10((Total+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Blocks stratum	2	0.01818	0.00909	0.56	
Blocks.*Units* stratum					
Treatments	4	5.25507	1.31377	81.58	<.001
Residual	8	0.12883	0.01610		
Total 14		5.40207			
l.s.d.		0.2389			

Third field test

	A	E	F	G	control
	1	0	2	1	0
	1	0	0	1	0
	0	0	0	1	0
Total	2	0	2	3	0
Mean	0.67	0	0.67	1	0

Fourth field test

Activity of single stereoisomers

	A	B	C	D	E	racemic mixture	Control
	106	3	0	2	284	27	0
	90	0	2	1	144	3	1
	210	0	0	1	343	7	0
	192	0	0	0	51	0	1
	340	4	8	4	190	6	0
Mean	187.6	1.4	2	1.6	202.4	8.6	0.4
SE	44.68	0.87	1.55	0.68	51.44	4.76	0.24

Analysis of variance

Variate: LOG10((Total+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	1.10097	0.27524	2.94	
Block.*Units* stratum					
TreatTrial1	6	26.18454	4.36409	46.56	<.001
Residual	24	2.24953	0.09373		
Total	34	29.53504			
l.s.d.		0.3996			

Activity of Blends

	A	(A+B)	(A+C)	(A+D)	Control
	612	3	1245	328	8
	780	6	820	432	1
	74	4	233	135	2
	195	3	220	191	1
	597	0	277	926	3
Mean	451.6	3.2	559	402.4	3
SE	134.75	0.97	204.87	140.85	1.30

Analysis of variance

Variate: LOG10((Total+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	0.89035	0.22259	2.43	
Block.*Units* stratum					
TreatTrial2	4	24.45561	6.11390	66.85	<.001
Residual	16	1.46334	0.09146		
Total	24	26.80929			
l.s.d.		0.4055			

Pear midge, *C. pyrivora* (CHAPTER 4)

Field test

First field test

Elmstone Court

	A	B+C	D	Racemate Major	Minor 1	Minor 2	Racemate Minor	Control
	1	1	5	0	0	0	0	0
	4	0	0	0	0	0	1	0
	3	0	1	0	1	1	0	1
Sum	8	1	6	0	1	1	1	1
Mean	2.67	0.33	2.00	0.00	0.33	0.33	0.33	0.33
SE	0.88	0.33	1.53	0.00	0.33	0.33	0.33	0.33

Mole End Frarm

	A	B+C	D	Racemate Major	Minor 1	Minor 2	Racemate Minor	Control
	70	1	137	1	63	0	22	1
	168	0	56	1	45	0	49	3
	160	6	49	0	56	0	56	1
Sum	398	7	242	2	164	0	127	5
Mean	132.67	2.33	80.67	0.67	54.67	0.00	42.33	1.67
SE	31.42	1.86	28.24	0.33	5.24	0.00	10.37	0.67

Analysis of variance

Variate: LOG10((Mole_End+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	0.01607	0.00803	0.14	
Block.*Units* stratum					
Treat_Mole	7	15.69883	2.24269	40.37	<.001
Residual	14	0.77767	0.05555		
Total	23	16.49257			
l.s.d.	0.4127				

Second field test

	major A	major B	major C	major D	minor 1	major A+ minor 1	major D+minor 1	control
	80	1	166	12	52	86	5	11
	84	1	102	9	37	246	14	5
	262	0	150	10	20	110	7	2
	130	6	168	14	66	222	9	2
Mean	139	2	146.5	11.25	43.75	166	8.75	5
SD	42.54	1.35	15.37	1.11	9.89	39.87	1.93	2.12

Analysis of variance

Variate: LOG10((Total+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	0.16756	0.05585	1.12	
Block.*Units* stratum					
treatment	7	14.27989	2.03998	40.87	<.001
Residual	21	1.04809	0.04991		
Total	31	15.49554			
l.s.d.	0.3285				

Third field test

	A	B	C	D	AB	AC	AD	Control
	3	3	7	45	7	7	9	13
	12	6	19	24	9	7	17	7
	2	0	1	18	2	1	5	3
	1	2	0	0	2	2	2	13
Mean	4.5	2.75	6.75	21.75	5	4.25	8.25	9
SE	2.53	1.25	4.36	9.27	1.77	1.60	3.25	2.44
	18	11	27	87	20	17	33	36

Analysis of variance

Variate: LOG10((Total+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
block stratum	3	0.24085	0.08028	0.84	
block.*Units* stratum					
treatment	7	1.47943	0.21135	2.21	0.076
Residual	21	2.00974	0.09570		
Total	31	3.73002			

Fourth Field test

	A	B	C	D	R	Control
	310	6	329	7	4	5
	431	3	178	11	1	4
	752	7	298	10	10	6
	671	2	369	9	34	3
	838	11	208	0	1	4
Total	3002	29	1382	37	50	22
Mean	600.4	5.8	276.4	7.4	10	4.4
SE	99.36	1.59	36.17	1.96	6.22	0.51

Analysis of variance

Variate: LOG((Total+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	2.4595	0.6149	1.19	
Block.*Units* stratum					
treatment	5	118.1983	23.6397	45.72	<.001
Residual	20	10.3402	0.5170		
Total	29	130.9980			
lsd				0.949	

Trap Height experiment

Height	**3cm**	**10cm**	**30cm**	**100cm**
	22.5	8	0.25	0
	84.25	70.5	15.75	0
	161.75	97.25	32.5	0.25
	341.5	401.75	121.25	1.75
Mean	152.50	117.50	42.44	0.50
SE	69.14	87.81	27.08	0.42

Analysis of variance

Variate: LOG10((Total+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	1.1696	0.3899	2.64	
Block.*Units* stratum					
height	3	14.1562	4.7187	31.91	<.001
Residual	9	1.3309	0.1479		
Total	15	16.6567			
l.s.d.			0.6151		

Blend experiment

	A	B	C	D	AC	BC	CD	R	Blank
	5	3	147	9	48	129	159	15	9
	5	0	97	8	43	85	140	40	10
	4	5	407	4	144	348	281	45	7
	7	3	255	2	103	329	84	58	1
	13	7	172	0	74	283	70	22	4
	10	3	140	6	87	222	96	33	6
Mean	7.33	3.50	203.00	4.83	83.17	232.67	138.33	35.50	6.17
SE	1.43	0.96	46.05	1.42	15.32	43.89	31.73	6.39	1.35

Analysis of variance

Variate: LOG10((total+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	5	0.38231	0.07646	1.22	
Block.*Units* stratum					
treatment	8	24.83174	3.10397	49.40	<.001
Residual	40	2.51315	0.06283		
Total	53	27.72720			
l.s.d.	0.2925				

Dose experiment- Test 1

Dosage (µg)	0	1	3	10	30	100	300	1000	3000
	23	61	64	97	61	41	52	24	41
	19	127	94	55	79	23	28	20	25
	71	283	138	105	67	46	74	47	44
	84	178	148	103	61	52	28	15	35
	117	81	64	81	112	86	31	38	17
	46	66	124	34	106	36	31	18	38
Mean	60.00	132.67	105.33	79.17	81.00	47.33	40.67	27.00	33.33
SE	15.48	35.09	15.03	11.81	9.28	8.72	7.63	5.18	4.22

Analysis of variance

Variate: LOG10((Total+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	5	0.48455	0.09691	2.93	
Block.*Units* stratum					
Treatment	8	2.41714	0.30214	9.14	<.001
Residual	40	1.32248	0.03306		
Total	53	4.22417			
l.s.d. 0.2122					

***Dose experiment*- Test 2**

	R0.01	R0.1	R1	R10	C0.0025	C0.025	C0.25	C2.5	Blank
	29	42	160	104	12	30	255	423	10
	19	45	76	176	8	6	162	198	2
	13	122	138	85	11	10	93	636	23
Means	20.33	69.67	124.67	121.67	10.33	15.33	170.00	419.00	11.67
SE	3.30	18.51	17.78	19.60	0.85	5.25	33.19	89.42	4.33

Analysis of variance

Variate: LOG10((Total+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	0.28345	0.14172	2.55	
Block.*Units* stratum					
Tretments	8	8.24308	1.03039	18.56	<.001
Residual	16	0.88824	0.05551		
Total	26	9.41477			

l.s.d. 0.4078

Trap colour experiment

Analysis of variance

Variate: LOG10((BCLM+1))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	1.73928	0.57976	6.23	
Block.*Units* stratum					
Colour	11	1.88334	0.17121	1.84	0.087
Residual	33	3.07011	0.09303		
Total	47	6.69273			

APPENDIX 3

Lakmali Amarawardana, David Hall, Jerry Cross, Michelle Fountain and Gunnhild Jåstad. 2008. Identification of the female sex pheromone of the pear midge, *Contarinia pyrivora*. IOBC Bulletin (*manuscript submitted*)

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