CHAPTER 1

INTRODUCTION

Crop production and storage generally has its attendant pest problems. Various ways have been proposed to define a pest (Clark, 1970; Bisplinghoff & Brooks, 1972; Bunting, 1972; Carlson, 1973) but from the agriculturalist's point of view the definition of an insect pest or vector is essentially economic, that is, whether or not an insect is causing sufficient damage to necessitate control measures (Kumar, 1984).

Pests of crops and their status are likely to vary according to the local environmental conditions. Where the same pest occurs widely, its pest status is not always the same. Damage inflicted varies according to the country, crop and season as well as many other factors, including farming practices (Kumar, 1984). Sometimes, an insect pest that used to be of minor pest status can become a major pest. A number of scenarios can be cited where such situations can arise:

1. accidental or intentional introduction of an insect to areas outside their native range where they escape from the controlling influence of their host plants;

2. when the insect becomes a vector of a plant or animal pathogen;

3. when an insect moves from native plants to introduced plants, which is common for polyphagous and oligophagous insects. For example, the polyphagous larvae of *Helicoverpa* and *Heliothis* (Lepidoptera: Noctuidae) have become serious pests of

cultivated cotton and other crops within the native range of the moths (Gullan & Cranston, 1994);

4. the creation of dense aggregations of predictably available resources that encourage the proliferation of specialist and generalist insects like the case of monocultural ecosystems in which food crops, forest trees and livestock are grown;

5. inappropriate or prolonged use of insecticides which eliminate natural enemies of the phytophagous insects while selecting for insecticide resistance in the latter (Gullan & Cranston, 1994);

6. the planting of a crop variety which is resistant to a particular major pest, hence reducing insecticide. Consequently, minor pests that used to be controlled by the spray for the major pest may now themselves become major pests. The introduction of transgenic cotton, resistant to lepidopteran larvae, provides an example of this (Fitt, 1998).

Some pests that occur in Australian cotton whose pest status might change as a result of the shift from broad-spectrum sprays to sprays targeting individual insects include the rough bollworm, *Earias huegeliana* (Graede) (Plate 1) and the cotton tipworm, *Crocidosema plebejana* (Zeller) (Plate 2). Likewise, the pest status of the green mirid, *Creontiades dilutus* (Stål) (Plate 3) might change due to the large scale cultivation of transgenic cotton in Australia. These three species are the subject insects of this thesis which will be described in more detail in Chapter 2.

Various methods are available to control and manage insect pests. These include chemical control (despite the hazards of conventional insecticides), biological control, cultural control, resistant varieties and the use of semiochemicals or behaviour-modifying chemicals such as

sex pheromones. One of the methods gaining prominence is the control of insects using pheromones (Pasqualini, 2000). According to Shorey (1977), a pheromone is a chemical or mixture of chemicals that is released to the exterior by an organism and causes one or more specific reactions in a receiving organism of the same species. The use of pheromones has some advantages, which include their non-toxicity, targeting of specific insect pests, and sometimes fewer applications per season. Practical applications of sex pheromones in pest management systems include monitoring, mass trapping, attract-and-kill and mating disruption. Pheromone monitoring traps provide useful information as detection, threshold and density estimation of pests, and can be used as early warning systems for possible outbreaks, to survey population trends or to improve timing of other sampling methods or control measures such as insecticides.

Mass trapping and attract-and-kill are used to remove males and prevent mating. These techniques differ in only one respect, that is, insects are killed by an insecticide incorporated into the lure in the attract-and-kill system while in mass trapping the insect is entrapped with an adhesive or other physical device. In mating disruption, successful mating is prevented by the high doses of pheromone used to prevent males locating females (Howse *et al.*, 1998). Preceding the application of a pheromone, however, an understanding of pest biology in the field, isolation, identification and correct formulation of the pheromone blend is needed.

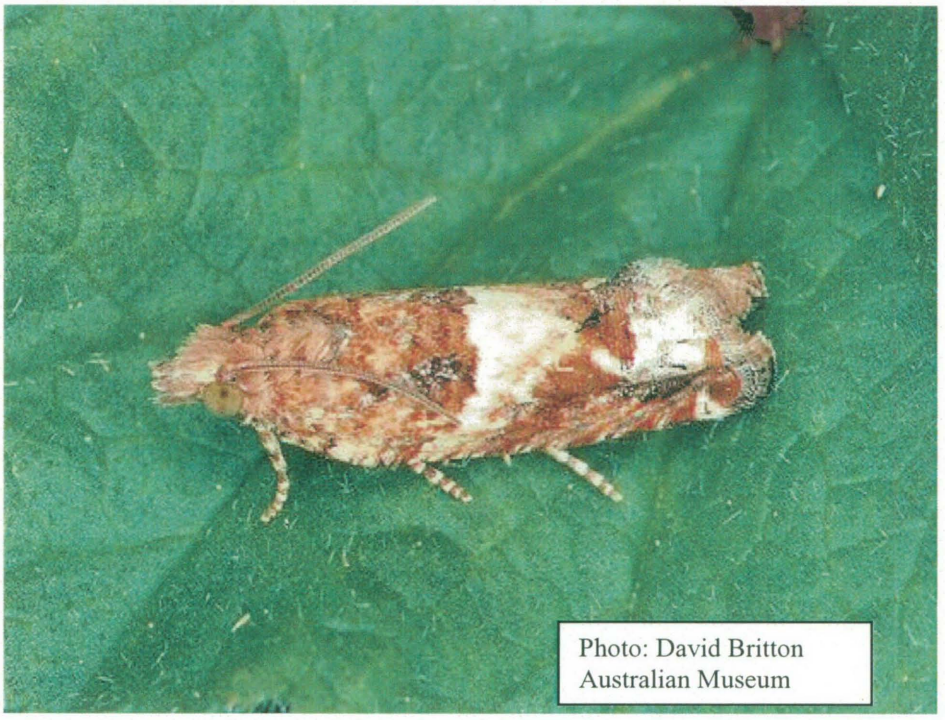
The late 1970's saw the availability and use of sex pheromones for the key pests of cotton in Australia, *Helicoverpa armigera* (Hübner) and *H. punctigera* (Wallengren) (Rothschild, 1978; Gregg & Wilson, 1991). However, very little information about the sex pheromones of other cotton pests exists. This particularly applies to occasional pest species such as the rough bollworm (*Earias* spp.), the cotton tipworm and the green mirids. The rough bollworm is likely to become more important with the expansion of the cotton industry to new areas,

especially in northern Australia, where seasonal conditions (eg. rainfall) could generate non-crop hosts and they move into conventional cotton managed with selective chemistry. In this

a

b

Plate 1. Adult (a) and larva (b) of the rough bollworm, *Earias huegeliana.*



a

b

Plate 2. Adult (a) and larva (b) of the cotton tipworm, *Crocidosema plebejana*.

a

b

Plate 3. Adult (a) and nymph (b) of the green mirid, *Creontiades dilutus*.

case the need to control this pest could lead to disruption of natural enemy populations increasing the risk of secondary pest outbreaks (mites, aphids, whitefly). Pheromones have been identified for some species in this genus in south Asia and Africa, and successfully trialled in mating disruption studies (Chamberlain *et al.*, 1993). There is, however, no information on the pheromones of the dominant Australian species, *E. huegeliana* (Plate 1). Similarly, the sex pheromone of the cotton tipworm, *C. plebejana* (Plate 2), has not been studied. Other pests, notably the true bugs (Hemiptera) such as the green mirids, *C. dilutus*, are becoming relatively more important due to the reduction in insecticide use associated with transgenic cotton. There is evidence that green mirids produce pheromones (Miles, 1995) but attempts at identifying pheromone components in this species have been unsuccessful, because of the low levels produced and the technical difficulties of identification (Khan, 1999). Monitoring methods for these occasional cotton pests would be very useful. Green mirids are also pests of legume crops, and monitoring methods would also be useful for the pulse industry.

Objectives of the study

This study aimed to investigate aspects of the reproductive biology of the rough bollworm (*E. huegeliana*), the cotton tipworm (*C. plebejana)* and the green mirid (*C. dilutus)*, including the isolation and identification of their sex pheromone components. The development of pheromone lures for these species might be useful as monitoring tools, in mass trapping and mating disruption or attract-and-kill applications.

Specifically, the objectives of the study were:

1. to collect and identify the components of the female sex pheromones from airborne volatiles and glands of the above three species;

2. to develop synthetic pheromone blends for these species; and

3. to test the attractiveness of these pheromone blends under laboratory and field conditions.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Insect communication

Insects communicate in various ways by touch, visual (sight), sound (auditory) and chemical (olfactory) means. Most touching involves the antennae and mouthparts but not much information is passed on by this form of communication (Chapman, 1998). Social insects such as ants have been reported to often stroke and groom each other with their antennae and mouthparts. Touching can be used for recognising nest mates, especially those that live in darkness as occurs in the mole cricket (Alexander, 1967). Visual communication in several kinds of flies and beetles is achieved by producing light. For example, both sexes of various fireflies (e.g., *Photuris versicolor* Quadrifulgens, *Photinus lucicrescens* Barber, *Photinus pyralis* Linn.) are known to use different codes of flashes to find and recognise each other (Forrest & Eubanks, 1995; Lloyd, 1969; Carlson & Copeland, 1982; 1985; Vencl *et al.*, 1994; Vencl & Carlson, 1997). Butterflies, flies and other insects use colours for visual communication. The males may have bright spots on their wings and communicate with females during courtship dances by displaying their colourful wings. Some butterfly species have patterns of ultraviolet colour on their wings essential for visual communication during courtship (Chapman, 1998). Visual cues are also used by some insect species to communicate with other species. For example, some insects have bright red or orange colours that are exposed only when they are threatened by predators. This is typically seen in the lubber

grasshopper, *Brachystola magna* (Girard) that flashes its bright red hind-wings when disturbed to threaten the predator and enhance the chance of escape (Joern, 1981).

Vibrations that pass through air, water and solid materials cause sounds in many orders of insects. These sounds either attract individuals of the same species or cause a response in another species, and can be made during normal activities such as flying or eating. Stridulation, the rubbing of body parts together to make sound, is known to occur in certain insects. Crickets and katydids make chirping sounds by rubbing a file of pegs on one forewing against a scraper on the other forewing. Similarly, grasshoppers rub their legs against their forewings to disclose their presence. In the Cicadidae family (O. Hemiptera, S.O. Auchenorrhyncha) and many other insects, drum-like membranes can vibrate to make sound (Alexander, 1960).

Chemical communication in many insects takes place with semiochemicals that are secreted by the insect's glands and released into the environment. Insects in search of food or mates rely on their sense of olfaction or smell. Very effective orientation mechanisms have been developed to localise odour sources over long or short distances and a high degree of performance has been achieved by insects in detecting and discriminating odours of different quantity and quality by means of olfactory receptor cells found in the antennae and mouthparts and sometimes other appendages (Boeckh, 1984 ; Seabrook, 1978).

Semiochemicals usually elicit certain specific responses. They act as attractants, repellents, arrestants, stimulants or deterrents. While attractants causes an organism to make oriented movements towards a source, repellents cause an organism to make oriented movements away from it. Chemical arrestants cause an organism to aggregate or stop when in contact with it. Those that act as stimulants cause insects to disperse rapidly, begin feeding, mating or

oviposition. Deterrents, on the other hand, inhibit feeding, mating or oviposition when in a place where an organism would in its absence, feed, mate or oviposit (Hamilton, 2001).

Generally, semiochemicals are divided into two broad types – pheromones and allelochemicals (Hamilton, 2001). Pheromones are intraspecific chemicals where both the emitter and receiver benefit. They are divided into five main subgroups: (i) sex pheromones to attract mates (e.g., in Lepidoptera and many other insects); (ii) alarm pheromones which signal danger (e.g., in aphids); (iii) egg marking pheromones which indicate when an egg has been parasitised (e.g., in parasitoids); (iv) territory and trail marking pheromones (e.g., in ants) and; (v) aggregation pheromones which attract other individuals for non-sexual purposes (e.g., in bark beetles). Other authors (e.g. Thornhill & Alcock, 1983) are of the view that since a calling insect is often trying to attract a mate, other individuals of the same sex may come to the vicinity of the calling individual to increase their likelihood of intercepting a mate while minimising the risk of discovery by natural enemies and avoiding the metabolic cost of producing a pheromone and hence the term aggregation pheromone is misused.

Allelochemicals, on the other hand, are interspecific chemicals and can be divided into four main groups based on the benefits derived by the emitter or the receiver. These groups are: (i) allomones (+,-), where these symbols indicate benefit or detriment to the emitter and receiver, respectively), e.g., in insectivorous plants (ii) kairomones (-,+) an accepted jargon even though nothing can evolve as a kairomone in and of itself (Blum, 1974) e.g., green leaf volatiles, fruit odours mainly made up of terpenes and esters, moth scale (waxy odour) extracts used to attract *Trichogramma*); (iii) synomones (+,+) e.g., insect- damaged leaf volatiles that attract the parasitoid of that insect); (iv) apneumones (+,+), chemicals emitted by non-living material that benefits the receiver but may be detrimental to other organisms

living in or on the non-living material (e.g., microbial and rotten fruit odours) (Hamilton, 2001).

2.2 Chemically based sexual communication in insects

Pheromones used in sexual communication by insects are usually mixtures of two or more compounds and because species require specificity of the communicating signals, the blends are in specific ratios. Closely related, sympatric species may produce pheromones with the same suite of chemicals but differing in the proportion of the components and sometimes release rate (Farrow, 1990; Roelofs, 1979; Roelofs & Cardé, 1974). These are used to maintain reproductive isolation between sympatric species. For example, the summer fruit tortrix moth, *Adoxophyes orana* (Fischer von Rösslerstamm) uses a 9:1 ratio of (Z)-9 and (Z)-11-tetradecenyl acetates (Meijer *et al.*, 1972) whilst *Clepsis spectrana* (Treitschke), which is sympatric and has the same flight period, uses a 1:9 ratio (Minks *et al.*, 1973). They are produced by one sex (usually females but there are exceptions) to attract the opposite sex. They are produced *de novo*, from materials obtained from food, in specific areas such as the pheromone glands of moths. Generally, they consist of blends of relatively simple, low molecular weight, volatile chemicals which are released only at specific times when mating is required. Detection by the opposite sex (usually males in lepidopteran insects) through specific sensory and neural mechanisms results in upwind orientation and eventually mate location. Although these pheromones elicit long distance anemotaxis and close range behaviour such as landing and fanning of wings, they may not always be sufficient for mating. Some insects may still need further close-range stimuli (chemical, auditory, visual) for mating to occur.

Pheromonal blends often determine the species-specificity of the emitted signal. The strict timing of sex pheromone production and response is very important for reproductive success and is one of the mechanisms employed by insects to achieve species specificity.

2.3 Factors affecting chemically mediated mating in insects

Chemically mediated mating in insects is known to be affected and influenced by various abiotic and biotic factors (McNeil, 1991). These factors include the age of the insect, temperature, humidity, photoperiod, light intensity, wind speed, host plants and hormonal factors.

Moth age: Moths that initiate calling generally have to be sexually mature. The age at which first calling occurs varies amongst insects. While females of *Leucania* (=*Mythimna*) *separata* Walker start calling on the third day (3^{rd} scotophase) (Kanda & Naito, 1979), those of *Helicoverpa (= Heliothis) armigera* (Hübner) began calling between days 2–5 at temperatures of 24 -26°C (Kou & Chow, 1987). Response of males to pheromones also depends on the age of the male. Three- to four-day old *Grapholitha molesta* (Busck) males were found to have the highest responses in terms of wing fanning (highly correlated to attraction) while walking towards the pheromone. This was followed by 2-day olds and then 1-day olds (Baker & Cardé, 1979). Females, however, did not call in the first 1-9 hours of emergence. Other tortricids that have been documented to call on the 1^{st} day of emergence include *Choristoneura fumiferana* (Clem.) and *Epiphyas postvittana* (Walker) (Lawrence & Bartell, 1972; Sanders & Lucuik, 1972).

Another aspect of mating behaviour affected by age is pheromone titre and duration of calling. Age-related changes in production and composition of pheromone in the boll weevil, *Anthonomus grandis* Boheman, showed an increase in pheromone production with a peak at 6

days for faecal samples and 9 days for headspace collections. The ratio of the boll weevil pheromone, made up of two alcohols (components I and II) and two aldehydes (components III and IV), changed with age. Component I was dominant for the first days of adult life, but the composition subsequently stabilised at 42.5:42.5:5:10 (I:II:III:IV) ratio. In addition, high levels of pheromone production were associated with well-developed accessory glands, while small, transparent glands produced little or no pheromone (Spurgeon, 2003).

The mean onset of calling time differed significantly with age for the arctiid moth, *Estigmene acrea* (Drury), where older females showed a tendency to call longer (Mazo-Cancino *et al.*, 2004). Glands of newly eclosed females contained a higher content of pheromone compared with that found in glands of 1-, 2-, 3- and 4-day-olds. About 45% of females of this species started calling during the first scotophase, with the highest numbers during the second, third and fourth scotophase. Mating in the females resulted in significantly less pheromone production than virgin females (Mazo-Cancino *et al.*, 2004).

Temperature: Temperature affects the onset and duration of calling, length of pre-calling time and frequency of calling in insects. Cardé and Roelofs (1973) reported higher ovipositor extrusion frequencies for *Holomelina immaculata* (Reakirt) at 24°C compared to 15°C. Conner *et al.* (1985) in their work on various arctiid species showed higher ovipositor frequency extrusions as the temperature increased from 15, 20 to 25°C. In another species, the potato aphid *Macrosiphum euphorbiae* (Thomas) oviparae, studies on the calling behaviour at three different temperatures in the laboratory and the field showed a decrease in mean age at first calling as temperature decreases. Mean age at first calling in the laboratory was found to decrease from 2.9 days at 20°C to 2.1 days at 10°C. In the field, with declining temperatures, mean age of first calling was 3.7 days in early September, reducing to 1.6 days at the end of September (Goldansaz & McNeil, 2003). In this aphid, mating to produce the

overwintering egg stages occurs late in the season when temperatures are low, therefore this observation was not surprising (Goldansaz & McNeil, 2003). Similarly, in most lepidopterans, the general trend is for a decrease in mating related activities as the temperature decreases. Roelofs *et al.* (1982), however, have reported an increase in pheromone related activities (e.g., precopulatory responses) of a geometrid, the winter moth, *Operophtera brumata* (L) as the temperature decreased.

Other insects have been known to initiate first calling at an earlier age at higher ambient temperatures (20–25°C) than at lower temperatures. These temperatures affected both the pupal and adult stages. First calling of *Pseudaletia unipuncta* (Haworth) females exposed to 15°C at both the pupal and adult stages were found to be much later than those exposed to 20 or 25°C (Turgeon & McNeil, 1982). A similar trend occurs even if only the adults or pupae are subjected to this temperature regime. Pre-calling period ranges of 3 to 9 days (median of 4-5) and 3 to11 days (median of 6) at 22-25°C and 19°C, respectively, have been reported for the moth, *Autographa gamma* (Linnaeus) (Hill & Gatehouse, 1992).

Temperature effects on duration and onset of calling for *Mamestra configurata* Walker were reported by Gerber and Howlader (1987). They observed longer calling times as well as early calling start times at temperatures between 15 and 25°C compared to 30-35°C in the night. In another species, *Choristoneura rosaceana* Harris, calling duration was not different at 15 and 20°C, but onset was significantly earlier at the former temperature than the latter (Delisle, 1992). Changes (increase or decrease) in prevailing temperature conditions also affect insect calling behaviour. *P. unipuncta* females transferred from 10 to 25°C had a similar mean onset time of calling as those already on 25°C on the first day (Delisle & McNeil, 1987). At 15 and 20°C, first calling in the Australian armyworm species, *Mythimna convecta* (Walker) was

found to be later compared to 25°C and the onset time of calling was also earlier at 15 and 20°C compared with 25 and 30°C (Del Socorro & Gregg, 1991; 1997).

Photoperiod: Photoperiod is one of the universal cues used by insects to predict changes in environmental quality. Its influence on the onset of reproduction and migration in insects is well documented. Photoperiod has been found to affect the age at first calling in some species, while it had no effect in others. Typical examples are illustrated in *M. configurata* and *P. unipuncta* females. Under three different photoperiodic regimes of 16:8, 12:12 and 8:16 (light:dark), females of *M. configurata* called for the first time during the 2nd scotophase after emergence (Gerber & Howlader, 1987). In *P. unipuncta*, longer scotophase in the cycles 18:6, 16:8, 14:10, 12:12 and 10:14 LD generally delayed age at first calling (Delisle & McNeil, 1986). A similar study in a North American population of the black cutworm moth, *Agrotis ipsilon* (Hufnagel) was conducted to determine effects of photoperiod on the calling behaviour, sex pheromone production and male response. As the length of the scotophase increased the onset time of calling occurred later with respect to the onset of the scotophase. Calling duration was similar across the photoperiods, (12:12, 14:10, 16:8 (L:D)) used. While young females reared under long-day photoregimes did not produce more pheromone than similar-aged females reared under short-day photoregimes, the quantity of pheromone was lower for 1- to 6-day old females than females of other ages (Gemeno & Haynes, 2001). Male response to pheromone was also significantly affected by the photoregime. The total number of males orienting to the pheromone source by upwind flight in the characteristic zigzagging pattern decreased as the length of the scotophase decreased in the cycles 12:12, 14:10 and 16:8. Total percentages orienting per photoregime were 61.7, 63.4 and 38.3%, respectively (Gemeno & Haynes, 2001). These results compared favourably with the French populations of *A. ipsilon*, where age of first calling was significantly earlier under a photoregime of 16:8 L:D (2.9 d) than under 12:12 L:D regime (3.5 d) (Gadenne, 1993). In *M.*

convecta, onset times advanced with longer daylength. At 20°C, age at first calling was increased by shorter day length (12 h and 14 h, compared with 16 h), with an interaction of daylength and moth age affecting duration of calling (Del Socorro & Gregg, 1991; 1997).

Light intensity: Light intensity tends to have variable effects on calling. Calling in the flour moth, *Anagasta kühniella* (Zell.) was unaffected by varying light intensities (Traynier, 1970). On the other hand, increasing light intensity from 0.3 to 300 lux in *Trichoplusia ni* (Hübner) inhibited calling under a 12 hour dark period (Sower *et al.*, 1970).

Wind speed: Wind speed influences on calling behaviour have been recorded in various species. Females of *T. ni* calling in wind speeds of 0.3 to 1.0 m/s spent longer calling, and speeds outside this range resulted in less calling or complete failure to call at 4 m/s (Kaae & Shorey, 1972). In the arctiid *Utethesia ornatrix* (L), frequency of ovipositor extrusion increased with increasing wind speeds up to 1.2 m/s, while no effect was noticed in five other arctiid species (Conner *et al.*, 1985).

Relative humidity and rainfall: Relative humidity can affect the onset of calling and age of first calling in some insects. For example, females of the European corn borer, *Ostrinia nubilalis* (Hübner) called earlier under high humidity conditions compared to drier conditions (Webster & Cardé, 1982). Influences of rainfall on calling behaviour of the aphid, *M. euphorbiae* oviparae were documented by Goldansaz and McNeil (2003). The aphids were noted to have stopped calling early in the afternoon coinciding with 10 mm of rainfall that lasted 1 hour. Calling however resumed immediately after the fall.

Host plants: Host plants not only attract insects for foraging, but also mediate sexual communication in areas such as pheromone production and release. Chemicals derived from the plant food are either used directly as pheromones or chemically modified to produce the specific pheromone needed. McNeil and Delisle (1989) reported that the presence of extracts

from pollen of sunflower, *Helianthus annuus* (L.) or the pollen itself increased ovarian development in the sunflower moth, *Homoeosoma electellum* (Hulst.) with associated early calling and longer calling duration in the female. Two host races of the larch budmoth, *Zeiraphera diniana* Guenée (Lepidoptera: Tortricidae) feed on larch (*Larix decidua* Miller) or Cembran pine (*Pinus cembra* L.). Females calling from their host trees cross-attract alien males with low probability (0.33-0.091) (Emelianov *et al.*, 2001). Host plant influences on calling behaviour of *Helicoverpa armigera* (Hübner) in Australia have been studied (Kvedaras, 2002). More females were found to commence calling at 2 days in the presence of pigeon pea volatiles than in its absence. Also more time was spent calling and more calling bouts were recorded in the presence of the volatiles than in its absence. In the presence of host volatiles, more pheromone was also produced in wild (F1) females, aged 2 and 3 days than in its absence. In an earlier work, Raina *et al.* (1991) found wild *Helicoverpa zea* females did not call unless host plant material such as an ear of maize with "silk" was present. A direct relationship was established between amount of pheromone produced by females and amounts of ethylene (volatiles from host plant) present (up to 1000 ppm). The Texas paint brush tree (*Castilleja indivisa*) is a host plant of *Heliothis phloxiphaga* and is known to greatly influence this insects calling behaviour and sex pheromone production. Not only are females more likely to call when exposed to the plant, but the amount of pheromone produced is about 50 times more in females reared from larvae collected in the field and exposed to the plant than those kept without it (Raina *et al.*,1986a).

2.4 Pheromone perception by insects

A downwind male detects, via sensory receptors on the antennae, the signal from an upwind pheromone source. The male then initiates a sequence of behavioural events which include upwind flight, movement directed along or through the chemical trail, arrestment of

locomotion and landing on the pheromone source (Farkas & Shorey, 1974). When a male arrives at the source of the pheromone, courtship and mating occurs. Pheromone-mediated flight mechanisms in insects have been reviewed (Cardé, 1984; Payne *et al.*, 1986). Two of these mechanisms, optomotor anemotaxis and self-steered counterturning or across-wind reversals, were reviewed by Baker (1989). 'Programmed' response to the pheromone which might be modulated by pheromone concentration or quality and response to losing the pheromone plume gives rise to self-steered counter-turning or across-wind reversals. In the case of optomotor anemotaxis, the flying insect locates the source by tracking the direction of the wind, usually with zigzags or lateral reversals.

2.5 Sex pheromones of Lepidoptera

Many insect species in the order Lepidoptera are known to employ sex pheromones, usually produced by the females to attract the males for mating. Males of nocturnal moths respond to sex pheromones disseminated by receptive females, sometimes over considerable distances (Nielsen & Common, 1994). The distance at which an insect first perceives and responds to a sex attractant source is however difficult to determine (Wall & Perry, 1987).

In noctuids, dermal glands located intersegmentally between abdominal segments 8 and 9 are known to produce these pheromones (Nielsen & Common, 1994; Hollander *et al.*, 1982; Jefferson *et al.*, 1968). The pheromones are synthesised *de novo* from smaller molecules or from precursors derived from food. Glands of *Trichoplusia ni* (Hübner) in which *(Z)*-7-dodecenyl acetate (Z7-12Ac) is the main pheromone component, have been shown to contain large amounts of (Z)-11-hexadecenoate and a fatty ester precursor, (Z)-7-dodecenaote (Bjostad & Roelofs, 1983). Chain shortening from (Z)-11-hexadecenoate to (Z)-7-dodecenoate and subsequent reduction and acetylation to (Z)-7-dodecenyl acetate has been

proven through labelling experiments. The scheme as proposed by Bjostad *et al.* (1985) is shown below:

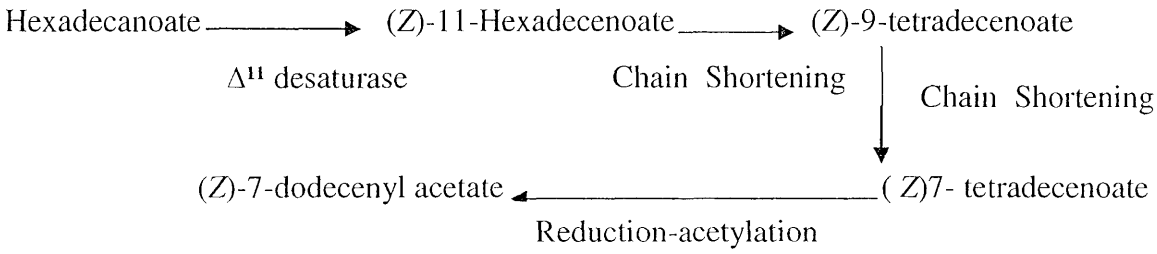
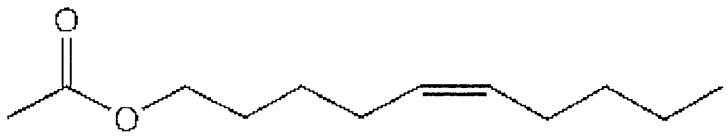
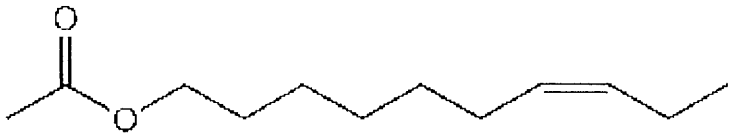


Figure 2.1. Proposed scheme for *de novo* synthesis of (Z)-7-dodecenyl acetate in *Trichoplusia ni* (Bjostad *et al.*, 1985).

Diverse pheromones with different chemical structures have been identified in many insects. In Lepidoptera, sex pheromone secretions in females, with few exceptions, have been found to consist of unsaturated, even-numbered, straight chain acetates, alcohols or aldehydes, (Shorey, 1976; Howse *et al.*, 1998). They are produced as a precise mixture of (*E*)– and (*Z*)-isomers (Brand *et al.*, 1979) and structurally similar nonisomers (Silverstein, 1982). The diversity amongst them is created by the position and number of the double bonds, the chain length, functional group and stereochemistry (Howse *et al.*, 1998; Chapman, 1998). A typical example of the position of the double bond is illustrated in the turnip moth, *Agrotis fucosa* (Butler). The pheromone components of this species are both acetates with the same carbon chain length but different in position of the one double bond present (Figure 2.2, Wakamura, 1980).



(Z)-5-decenyl acetate



(Z)-7-decenyl acetate

Figure 2.2. Sex pheromone structures of *Agrotis fucosa* showing different positions of the double bo
(Wakamura, 1980).

2.5.1 Sexual behaviour in Lepidoptera

Reproductive events like pre-mating, courtship and mating in lepidopterans have mostly been observed to occur during the night and also during the day in a few species. In most cases, females exhibit a "calling posture" when releasing pheromones which is characterised by a full extrusion of the ovipositor accompanied by raising of the wings above the abdomen and curving of the ovipositor downwards at about 45° (Itagaki & Conner, 1988; Noldus & Potting, 1990; West *et al.*, 1984). This behaviour is prominent in noctuid species such as the bertha armyworm, *M. configurata* (Howlader & Gerber, 1986). Extended legs, raised wings and

raised abdomen above substrate is the calling posture of the oriental fruit moth, *G. molesta* (Baker & Cardé, 1979).

Males that perceive pheromone in the air are first guided by their antennae to the female. In *T. ni*, for example, the resting moth has its antennae posteriorly and ventrally directed. Upon perceiving a female pheromone, they are raised and brought slightly forward, perpendicular to the body axis (Shorey, 1964). The moth then extends and vibrates the wings, the vibration increasing with time before flying towards the source of the pheromone. In the vicinity of the female, the male hovers in the air stream directly posterior to the female abdomen. The male uses its antennae to touch the extruded genital segment of the female several times before touching it with one of its fore tarsi. The male then extrudes its genital claspers while still hovering with vibrating wings directly above the female, curves its abdomen towards the female abdomen, and with proper contact, copulates with the female. The pair, once copulating, moves to a position with heads facing opposite directions. In the bollworm moth, *Heliothis zea*, the male also vibrates its wings during courtship, but wipes the scales of its claspers on the supporting surface between periods of vibration. Receptive females also vibrate their wings and have their terminal abdominal segment extended. The male then approaches from behind, taps the ovipositor of female with his antennae, crawls to a position parallel to the female, and snaps his claspers around the female genitalia to copulate. A receptive female does not move when approached and tapped on the ovipositor by the aroused male. Unreceptive females, even though demonstrate some small calling, tend to escape from the claspers of the male when attempting to copulate. Copulation time in *H. zea* lasts between ¾ to 1½ hours (Agee, 1969).

2.6 Sex pheromones of Hemiptera

In comparison with most lepidopteran insects, information on the sex pheromones in hemipteran insects has been limited. This has been attributed to the difficulty in collecting, isolating and identifying the pheromones as well as limited knowledge of the biology of bugs, including the ways they use pheromones (McBrien & Millar, 1999).

Appropriate physiological state has been shown to be important for bugs to produce or respond to pheromones (McBrien & Millar, 1999). For example, adults of long-lived species may take a number of days to mature and begin producing or responding to pheromones. In species that overwinter, bugs enter reproductive diapause in response to seasonal conditions and become unresponsive or do not produce pheromones (Strong *et al.*, 1970; Aldrich *et al.*, 1991). In addition, pheromone components are usually masked by large quantities of defensive secretions produced (McBrien & Millar, 1999). These secretions sometimes have alternative roles as pheromone components at lower concentrations (Millar *et al.*, 1997). Variations also occur between families and genera in relation to the sexes producing and responding to the pheromones. For example, Millar *et al.*, (1997) and Millar and Rice (1998) have reported sex specific compounds from extracts of males and females from the mirids *Phytocoris* spp. but no such compounds were found in another mirid species, the *Lygus* bug (Aldrich *et al.*, 1988; Gueldner & Parrott, 1978) and the alydid bug *Leptocorisa chinensis* (Dallas) (Leal *et al.*, 1996).

Compounds for communication and defence in bugs are produced by various glands (McBrien & Millar, 1999). Often the scent glands are readily located in characteristic sites where they can be found and dissected for study of their contents (Johansson, 1957; Staddon, 1979; 1986). Some bugs produce these compounds in discrete multicellular glands while others produce them in patches of unicellular glands (Evans *et al.*, 1990). *Campylomma*

verbasci (Meyer) males, for example, have been observed to be attracted to extracts of head and thorax but not the abdomen (Thistlewood *et al.*, 1989), while in *Lygus hesperus* (Knight), the area around the ovipositor is important in mate attraction (Graham, 1988). An emerging pattern for some bugs is that the lateral accessory glands of the metathoracic scent gland sometimes produce the pheromones (Aldrich *et al.*, 1999; 2000).

Detection of female-produced sex pheromone by adult males has been proposed as involving a number of antennal-specific proteins associated with olfactory sensilla and other sensilla (Dickens *et al.*, 1995; Chinta *et al.*, 1997; Dickens & Callahan, 1996). In *Lygus lineolaris* Palisot de Beauvois, olfactory sensilla have been noted to be absent in last stage nymphs but develop during the last moult to adult. Entire antennal flagellum removal in males of *L. hesperus* prevented them from responding to pheromone while removal of the last two segments did not alter their ability to locate females (Graham, 1988). Graham (1988) concluded that pheromone reception in mirids is concentrated on the second antennal segment, with the third and fourth segment being diminutive in nature (Aldrich *et al.*, 1988).

In contrast to other members of the Heteropteran sub-order where males attract females by chemical means (Aldrich, 1988; 1995), literature indicates that female mirid bugs are the attractive sex, producing pheromones that attract only adult males (Table 2.1).

Pheromonal compounds in the Miridae may be female-specific or produced by both sexes. Despite many compounds having been identified from bugs, the number of studies in which bug pheromone blends have worked is very small. Of the 10,000 species of the family Miridae worldwide (Aldrich, 1996), synthetic pheromone blends of only a few species, including *C. verbasci* (Smith *et al.*, 1991), *Phytocoris relativus* Knight (Millar *et al.*, 1997), *Phytocoris californicus* Knight (Millar & Rice, 1998), *Trigonotylus caelestialium* Kirkaldy (Kakizaki & Sugie, 2001) and recently *Distantiella theobroma* Distant and *Sahlbergella*

singularis Haglund (Padi *et al.*, 2002) have been developed to the extent where males can be caught. Work by various researchers (e.g., Wardle & Borden, 2003; Ho & Millar, 2002) for over ten years has not resulted in any pheromones for the *Lygus* bug. Numerous identified compounds are shared between various *Lygus* spp. and other heteropteran species (Wardle *et al.*, 2003), but no single compound or blend has been found to be attractive to males of any *Lygus* spp. in the field (Hedin *et al.*, 1985; Ho & Millar, 2002). This is against the background that quantitative differences exist in antennally active compounds. These quantitative differences in the occurrence or ratio of compounds have also been observed in chemicals emitted from calm and agitated *L. lineolaris* (Wardle *et al.*, 2003).

Species	Authors
Sahlbergella singularis	(Padi *et al.*, 2000)
Distantiella theobroma	(King, 1973; Padi *et al.*, 2000)
Creontiades dilutus (Stål)	(Miles, 1995)
Atractotomus mali (Meyer)	(Smith *et al.*, 1994)
Lygus hesperus	(Strong *et al.*, 1970; Graham, 1988)
Campylomma verbasci	(Smith *et al.*, 1991)
Lygus lineolaris	(Scales, 1968; Slaymaker & Tugwell, 1984)
Lygocoris pabulinus (Linnaeus)	(Blommers *et al.*, 1988; Groot *et al.*, 1998)
Helopeltis clavifer (Walker)	(Griffith & Smith, 1977)
Lygocori communis (Knight)	(Boivin & Stewart, 1982)

Table 2.1. Cited Miridae species with female-mediated sex attractant.

The class of pheromonal compounds identified for the Miridae has mainly been simple saturated and unsaturated esters of relatively short chain length as well as simple alcohols like octanol and 2-hexenol. In other bugs where the males are known to produce the attractant pheromones, the structure of the pheromones is complex, ranging from bisabolene oxide (Z-(1′S,3′R,4′S)-(-)-2-(3′4′-epoxy-4′methylcyclohexyl)-6-methylhepta-2,5-diene) to long chain

alkanes like nonadecane as in the green vegetable bug, *Nezara viridula* Linnaeus (Pentatomidae) (Borges, 1995; Aldrich *et al.*, 1987).

2.6.1 Sexual behaviour in Hemiptera

Most of the reproductive events like pre-mating, courtship and mating in mirids have been observed to occur during the day (Wheeler, 2001). The pre-mating period or the duration between last moult and first copulation, is generally short, temperature dependent and may range from a few hours to seven days (Groot *et al.*, 1998). For example, El-Dessouki *et al.* (1976) reported a pre-mating period of two to three days in summer and three to four days in winter for *Nesidiocoris tenuis* (Reuter). *L. hesperus* (Strong *et al.*, 1970), *L. pabulinus* (Groot *et al.*, 1996; Blommers *et al.*, 1997) and *Labops hesperius* Uhler (Coombs, 1985) have premating periods of five, six and seven days, respectively. Females of *D. theobroma* usually mate 4 to 5 days after the final moult (King, 1973) while in *Helopeltis antonii* (Sign.), mating occurs one day after the final moult (Ambika & Abraham, 1979).

Mating in mirids has been observed to occur during the day, although in some species, mating takes place at night time. As an exception, no distinct period has been found for *Lygus elisus* (Van Duzee), which tend to mate both at night and day (Graham, 1987). For some species, courtship behaviour has been observed to precede mating. Most of these courtship behaviours involve touching with the antennae as occurs in *N. tenuis* (El-Dessouki *et al.*, 1976). Chatterjee (1984) reported several antennal touches of the female abdomen by the male *N. caesar* before mating. Receptive females become immobile, allowing the males to mount. Wing flapping as part of courtship behaviour has been reported in *Cyrtorhinus lividipennis* Reuter (Liquido & Nishida, 1985). Both male and females flap wings in a coordinated movement, while the male moves around the female, finally grasping the female abdomen

with the forelegs. The male then positions his abdomen below the female before inserting the aedeagus.

Available literature on the duration of mating in mirids seems to suggest a short copulation time. It ranges from 7 seconds in *Neurocolpus nubilus* Say (Lipsey, 1970) through to 10 seconds in *C. lividipennis* to between 30 and 120 seconds in *Calocoris angustatus* Lethierry (Liquido & Nishida, 1985). *Pachypeltis maesarum* Kirkaldy, however, have been observed to mate for up to nine hours in the laboratory (Trehan & Phatak, 1946).

Mirid bugs like most other insects, face opposite directions after initial coupling. Multiple mating is common in mirids reared under laboratory conditions (Wheeler, 2001). Males of *L. hesperus* can mate up to seven times while the females mate up to three times (Strong *et al.*,1970; Strong, 1971). This is despite the fact that single mating results in viable egg production over the entire female egg laying life (Wheeler, 2001). Copulation in *L. pabulinus* can occur everyday (Groot *et al.*, 1998). Even though a single mating allows the female to maintain egg production for 2–3 weeks at 20°C (Blommers *et al.*, 1997), it has been suggested as "being insufficient for deposition of the entire complement of fertilised eggs" (Wheeler, 2001).

A number of studies have been done on the length of time from emergence of the adult female to deposition of the first egg (pre-oviposition) in mirids. Knowledge on the pre-oviposition period, for some insects, might be important in implementing attract-and-kill management programmes. The pre-oviposition period in mirids ranges from less than a day to about 20 days (Wheeler, 2001). Khan (1999) reported a range of 8 to 21 days for the Australian green mirid, *C. dilutus*. A 12-month field study indicated a pre-oviposition range of 1.2 to 3.0 days for *C. angustatus* (Hiremath & Viraktamath, 1992). Environmental factors like temperature, host plants and other stimulatory factors influence the length of the pre-oviposition period.

Stimulatory effects of males accelerating rate of egg maturation in *S. singularis* were suggested when the pre-oviposition period was found to be shorter in paired than unpaired females (Eguagie, 1977). A typical temperature effect has been demonstrated in *L. hesperus*. At 26.7°C, 15.6°C and 12.8°C, pre-oviposition periods were 7, 17 and 27 days, respectively (Strong & Sheldahl, 1970). Slightly longer periods have been observed during winter compared to summer in *N. tenuis* (El-Dessouki *et al.* 1976). Hori and Hanada (1970) and Hori and Kuramochi (1984) found pre-oviposition times varied according to generations but not on different host plants in *L. rugulipennis*.

Literature suggests evidence of reproduction from gametes without fertilisation (parthenogenesis) in a few mirid species with rare males. Some of these species include the European populations of *Campyloneura virgula* Herrich-Schaeffer (Carayon, 1989) and Greenland populations of *Chlamydatus pullus* Reuter (Bocher, 1971).

2.7 Sex pheromones in pest management

Sex pheromones form an integral part of a number of control programs instituted against a broad array of insect pests ranging from pests of horticultural crops through forest crops to stored product insect pests. This has been made possible by knowledge that pheromones are particularly important chemicals used for signalling between members of the same species. Another important reason is that sex pheromones are relatively long-lived semiochemicals, and they act over long distances, therefore they can be used to manipulate insects on reasonable spatial and temporal scales. They have been employed in various ways in integrated pest management (IPM) schemes.

Monitoring. One of the concepts on which IPM is based is the idea of the economic threshold. This is the pest population identified through a valid sampling method beyond which limiting measures are required to prevent economic losses (Pasqualini, 2000).

Information from pheromone trap catches, notably for lepidopteran species has been used to detect the presence of a particular pest and provide a measure of its abundance to recognise an impending outbreak. As well as providing an early warning of emergence, migration and density estimation of a pest, this information if related to a threshold catch is used to optimise the timing of insecticide spray. For example, in the Australian cotton industry, pheromones for the key pests of cotton, *H. armigera* and *H. punctigera,* made available since the late 1970's (Rothschild, 1978; Gregg & Wilson, 1991) have been widely used in research and for monitoring these pests. Though the traps do not accurately predict oviposition on a field-by-field basis (Daly & Fitt, 1993), they give useful indications of the overall abundance of *Helicoverpa* spp. Monitoring of the codling moth, *Cydia pomonella* Linnaeus and the pea moth, *Cydia nigricana* Fabricius was done in Britain using pheromone traps (Cammell & Way, 1988). Two traps placed in each pea field were examined every two days. When 10 or more moths were caught in either trap on two consecutive occasions, then calculations based on egg development rates were used to predict larval hatch and hence, optimum time for treatment (Lewis & Sturgeon, 1978).

Pheromone traps for monitoring have been cited as having five main strengths (Wall, 1990). They are the most sensitive sampling technique known. They require no power and little maintenance as well as being species specific. They can be operated by non-entomologists and are not labour-intensive. Their limitations, however, include lack of knowledge on their range of action, especially sampling range and how it changes with time (Wall & Perry, 1987), changes in trap efficiency as catches accumulate (Riedl, 1980), climatic effects on trap catch and also may not relate to pest damage or potential damage.

Mating disruption: Mating disruption (MD) is an insect management technique that prevents male insects from finding females. It relies on the broadcast of a synthetic copy of the

pheromones of an insect in large amounts to confuse males and limit their ability to locate calling females in an area. It was first thought that, to be an effective disruptant of mating, the synthetic pheromone blend had to be virtually indistinguishable from the natural pheromone blend. However, it is now clear from various MD work done, that it is sometimes possible to disrupt mating using only the main chemical component(s) in a pheromone blend (Fitzpatrick, 2001). The synthetic pheromone is injected into or enclosed in controlled-release devices, which are distributed in the field. These devices may be small plastic or polyvinyl chloride (PVC) tubes or spirals, flakes, "twist-tie ropes" (small plastic tubes with a twist-tie wire inside), clips similar to bread ties, microscopic polyurea capsules, or timed-release spray canisters. The tubes and flakes are designed to be scattered by hand or from an airplane. Spirals, twist-tie ropes and clips are attached by hand to branches. Microscopic capsules can be sprayed from a helicopter or conventional sprayer, and timed-release spray canisters are attached to stakes and placed in the field by hand. All of these controlled-release devices allow small amounts of pheromone to escape gradually into the air around the crop (Fitzpatrick, 2001). Sufficient flooding of the sex pheromone into the crop environment interferes with mate location by masking the natural pheromone plumes produced by the female. This affects the males' ability to respond to calling females, by mechanisms which are not fully understood, and which may vary between different species. One mechanism is by causing the male to follow false pheromone trails at the expense of finding mates (Carter & Fraser, 2003; Campion *et al.*,1989). Complete blends, which fully mimic the natural female-produced pheromone, are required when this mechanism is operative. In situations where an incomplete blend is used in MD, it is argued that an imbalance of sensory input occurs in the males' sensory cells (Fitzpatrick, 2001). Some of the males' sensory cells become adapted while the others continue to function. Thus the males are not able to accurately perceive the natural pheromone blend released by a female. For example, the

blackheaded fireworm, *Rhopobota naevana* (Hübner) pheromone contains at least three components, but only the main one is used as a mating disruptant. The sensory cells that are designed to perceive the main component receive so many odour molecules that those cells become adapted and stop responding. A male downwind from a calling female would then perceive only part of her chemical signal, that is, the part containing the second and third pheromone components. He would not recognize this as a pheromone, and would not fly upwind toward the female. Another possibility is that all his sensory cells might continue to receive information, but he would not recognize female pheromone because the ratio of odour molecules from the three components would be skewed (Fitzpatrick, 2001). Other proposed mechanisms include trail masking. This has been proposed to occur when the natural pheromone plume is obliterated by synthetic pheromone, making trail following impossible (Jones, 1998; Campion *et al.*, 1989).

Mating disruptions are usually successful when large areas are treated with the pheromones (Jones, 1998). This area wide MD reduces the likelihood of mated females flying in from neighbouring sites and laying eggs. The target pest population level at the time of treatment should be low to moderate. High pest population levels at sites of treatment with mating disruption usually result in failure because of chance encounters (males may not have to follow pheromone plumes to locate females) and mating occurring despite the pheromones from the controlled-release devices. Alternatively, chances of males following a pheromone plume to a female rather than the MD devices are very high with so many females emitting pheromones in a high population situation. MD is highly successful in insects that have one kind of host plant. In such insects there is no chance of mated females flying from nearby fields containing other host plants into field containing their host plant where the MD has been applied. MD has been successfully trialed and now used in cotton to control the pink bollworm, *Pectinophora gossypiella* Saunders in the U.S.A (Shorey *et al.*, 1974; 1976;

Kydonieus & Beroza, 1981), in Egypt (Critchley *et al.*, 1983; 1985; 1986) and in Pakistan (Critchley *et al.*, 1987).

Mating disruption achieves a reduction in the number of males locating females, which means fewer matings and a lowered population in subsequent generations. Jenkins *et al.* (1990) developed a mating disruption technique for managing the tomato pinworm, *Keiferia lycopersicella* (Walshingham) in Florida. Disruption traps are put in place when pheromone baited sticky traps, used to monitor the pinworm, capture five or more adult moths per night. Another successful application of mating disruption has been the control of *Earias insulana* Boisduval and *E. vittella* using pheromones in Pakistan (Critchley *et al.*, 1987). There has also been research on the use of pheromones for mating disruption in *Helicoverpa* spp. in cotton in Australia (Betts *et al.*, 1993) and in Pakistan (Chamberlain *et al.*, 1993), though in both cases immigration by mated females from outside the treated region has been a major problem.

Mating disruption has many advantages and these include: (i) reduction in insecticide use; (ii) presents lower hazards to farm workers since pheromones are non-toxic; (iii) pest resurgence is rarely seen when mating disruption is used as a pest control tool (iv) it reduces pressure for development of resistance to insecticides; (v) it enhances biological control methods since pheromones do not adversely affect parasites and predators. The disadvantages include: (i) the inability of MD to provide protection from many other pests which may have been kept below damaging levels by insecticide control against the key pest because of their specificity; (ii) initial cost often being very high compared to insecticidal control; (iii) at high pest population levels, an initial pesticide application may be required to bring pest population to a manageable level for mating disruption; (iv) not effective when windy conditions prevent a

continuous high concentration being present in the treated area, as sometimes occurs in orchards (Pfeiffer, 1995).

Mass trapping: Mass trapping is used primarily where the insects are attracted into containers, sticky traps, an electrocutor grid or surface treated with toxic chemicals or pathogens and annihilated. The underlying assumption of this approach is that scattering traps in the field ensures that many individuals will be trapped, significantly hindering fertilisation and the production of a new generation of pests. As a result, chemical treatments are reduced, and in some cases, the need for chemical treatment is eliminated. Successful mass trapping as a control method in the field has been reported in Israel for the Egyptian cotton leafworm, *Spodoptera littoralis* Boisd., which blights cotton, alfalfa, and vegetables, the processionary pine moth, *Thaumetopoea pityocampa* (Den. & Schiff.) in Spain, a severe pest of pine and the bark beetle, and *Ips typographus* (Linnaeus) in Scandinavia, which affects forest trees (Weslien & Lindelö, 1990; Weslien, 1992).

Mass trapping studies have been successful in some cases at removing large numbers of target pests from an area in a short time. Pierce (1994) reported a 96% season-long decrease of trapped moths and no moth infestations in food packages for the Indian meal moths in a food warehouse with densities of about 1 trap per 210 m^3. Work done by Pierce (1999) on long-term mass-trapping (over a nine year period) of cigarette beetle, *Lasioderma serricorne* (Fabricius) with its sex pheromone in a commercial bakery attributed the population suppression from reduced trap catch in later years of the study period to the effectiveness of the mass trapping. These successful applications have all been reported in closed systems. In most open field applications, little success has been achieved with mass trapping, especially in cotton. Campion and Nesbitt (1981) concluded this method was not suitable for pest management of *S. littoralis* in Egypt and Crete. In Israel however, some success has been

reported with *S. littoralis*. Egg clusters were reduced by 40 – 50% and insecticide treatment reduced by 20 – 25% with pheromone traps at one per 1.7 hectares (Teich *et al.*, 1979).

Mass trapping, however, has some practical problems. For aggregation pheromones that attract females, mass trapping may have direct impact on a population if substantial numbers of females are removed. However, for sex pheromones that attract only males a critical number of males must be removed to ensure that an effective number of females go unmated. For example, since an Indian meal moth male can mate up to 10 females in his lifetime (Brower, 1975), then over 90% of males need to be removed by trapping before an effect on female reproduction is realised (Phillips *et al.*, 2000).

Lure-and-kill : "Lure-and-kill", also described as "attracticide", "attract-and-kill," "male annihilation," or "attraction-annihilation" (Lanier, 1990; Jones, 1998) is a modified form of mass trapping in which moths lured by a synthetic pheromone are not caught in the traps, but are subjected to a killing or sterilising agent that effectively eliminates them from the population (Phillips *et al.*, 2000). An example of a successful approach is with the oriental fruit fly, *Bactrocera dorsalis* (Hendel), in which a lethal insecticide is mixed with the strong male attractant methyl eugenol. Male *B. dorsalis* readily feed on methyl eugenol once they encounter it. Male flies are active and responding to the lure-and-kill formulation during the day, and hence many males are dead by the time females are active and calling for mates during a brief time period at dusk (Phillips *et al.*, 2000). The lure-and-kill method is considered useful by some researchers because, unlike mating disruption, males are actually killed rather than "confused", and unlike trapping, traps do not have to be deployed and serviced, and many more "killing stations" can be set up or broadcast. Thus the lure-and-kill method is finding application for moth pests of various crops and some beetle pests such as the cotton boll weevil and the corn rootworm (Jones, 1998). This insect management practice

has been found to be a highly efficacious method of controlling the codling moth (*Cydia pomonella* L) in orchards all over the world (Hofer & Brassel, 1992; Charmillot *et al.*, 2000; Dickler *et al.*, 1998; Lösel *et al.*, 1998a; 1998b; 2000).

Gullan and Cranston (1994) have reported the successful use of the first three methods – monitoring, mating disruption and mass trapping - for certain moths, beetles and fruit fly pests. These three pheromone-based methods as suggested by Gullan and Cranston (1994) can be used to effectively control species which (a) are highly dependent on chemical (rather than visual) cues for locating dispersed mates or food sources, (b) have a limited host range, and (c) are resident and relatively sedentary so that locally controlled populations are not constantly supplemented by immigration.

2.8 Australian *Earias huegeliana* and related species

Earias huegeliana Gaede (Plate 1) is one of eight small Australian species included in the 47 described species within the genus *Earias* Hübner (Lepidoptera: Noctuidae) (Common, 1990). Also known as the rough bollworm, it is found throughout northern Australia, in the west south to Perth and in the east to northern South Australia and New South Wales. It also occurs widely on malvaceous plants in many South Pacific islands. Variations occur in the adult species, sometimes having green or dull ochreous longitudinal markings on the forewing, while sometimes-uniform dull ochreous cream colour (Common, 1990).

The larva feeds on the young shoots, flowers and seed capsules of cotton, *Gossypium hirsutum* L. as well as on other malvacaeous plants such as the native *Gossypium australe* (F. Muell) and *Gossypium populifolium* (Benth.) Tod., *Abelmoschus ficulneus* (L) Wight & Arn., *Hibiscus trionum* (L), *Hibiscus panduriformis* (Burm. F), *Abutilon otocarpum* (Schönmalve) *and Alyogyne hakeifolia* (Giord.) and on the boab tree, *Adansonia gregorii* (F. Muell.)

(Common, 1990). Closely related bollworms attack cotton in Africa and Asia. In Australia, two species, the northern rough bollworm, *Earias vittella* (Fabricius) and the spotted bollworm, *Earias fabia* (Stol), occur in northern Australia but not in the major cotton growing areas of southeast Queensland and northern New South Wales. Other species found in Australia include the endemic species *Earias parallela* Luc. and *Earias smaragdina* Butl., recorded in the Northern Territory and eastern Australia and New South Wales in the south.

Literature on *E. huegeliana* is very limited. Closely related to *E. huegeliana* is *E. vittella* (Pearson, 1958) which occurs in India through southeast Asia to New Guinea, the Solomons, Fiji and north-western Australia, the Northern Territory and north-eastern Queensland as far as Yeppoon. *E. vittela* is a pest of cotton and has also been reported to have some alternative hosts in the Malvaceae family.

Other *Earias* species include *E. bipalga, E. insulana* and *E. cupreoviridis. E. biplaga* is found from the Cape province of South Africa northwards as far as the arid sub-Saharan region. Characteristic of *Earias* species, some degree of adult polymorphism is very pronounced in *E. biplaga.* Typical colour patterns are restricted to the female. Covering most of Africa and Madagascar, Mauritius and Canary Isles is *E. insulana* which "extends northwards to the Mediterranean islands and southern Europe, and eastwards through the near and Middle East, including southern Arabia to India, China and South East Asia" (Reed, 1994). It is represented in Australia by a form *E. smaragdina* Butl., now regarded as synonymous with *E. insulana* (Reed, 1994). This species completely overlaps *E. biplaga* in Africa and *E. vittella* in Asia and Australasia, predominating over other species in the drier parts of their range.

Extremely widespread but ranging discontinuously is *E. cupreoviridis* which is found in southern China, Japan, Taiwan, the Philippines and the Palau Islands, India and south of the

Sahara in Africa. It has not been found to attack cotton in Africa and India, but reported to be of some importance in China where it attacks cotton (Li & Chou, 1937).

2.9 *Earias* as pests of cotton

Earias spp. are pests of considerable importance and are widely distributed in the old world and Australasia. Generally, the pest status of *Earias* on cotton varies from place to place. In Australia, it is considered as an occasional pest of cotton. It is regarded as little more than a nuisance in most rain grown-cotton areas of Africa while in several areas of the Indian subcontinent, North Africa and the Middle East, it is often the most important component of the pest complex (Reed, 1994). Shoot boring in the early stage of the crop and diversion to the flower buds as they appear is the pattern of infestation in *Earias*. Attack on the flower buds sometimes causes retardation of the bolls and a very prolonged but inefficient flowering curve. Build up of infestation in the crop is gradual with no sudden influx of egg-laying moths at a particular stage in the crop as with *Helicoverpa armigera*.

Bhugio *et al.*, (1987) reported 23-53% of *Earias*-damaged bolls in Pakistan in a three year study, while Pearson (1958) indicated a maximum level of 5% damage as typical in Egypt although there are occasional reports of up to 50% or more boll damage. Patel (1949) reported *Earias* caused 20% of loss of seed yield in India and 57-80% stained lint (Sidhu & Sandhu, 1977).

The life history, host plants and their seasonal history, the timing and length of the cotton season, rainfall, and natural enemies are factors that affect the status of *Earias* as pest. Laboratory studies on fecundity indicate that moths lay several hundred eggs with as many as 732 eggs from one female *E. insulana* (Megahed *et al.*, 1972 as cited by Reed, 1994). With no diapause, development depends primarily on availability of food supplies and temperature (Reed, 1994).

The moths are active at night, feeding on nectar but normally quiescent through the day, with wings flat and snugly folded, with an overall length of 12mm and wing expanse of 20–22mm. They will however fly when disturbed. *Earias* moths tend to be hardy and long-lived. *E. insulana* individuals have been cited as surviving for more than 80 days in the laboratory when adequately fed (Reed, 1994).

2.10 *Crocidosema plebejana* as a pest of cotton

The cotton tipworm, *Crocidosema plebejana* (Zeller) infests a range of malvaceous plants including cotton in southeast and central Queensland as well as the cotton producing areas of New South Wales (Pyke & Brown, 1996). It has been classified as a minor to occasionally serious pest of seedling cotton in Queensland and New South Wales (Evenson & Basinski 1973; Bishop & Blood, 1978). The terminal tunnelling of the larvae destroys the single stem habit of the cotton seedling. This results in vegetative growth at the expense of early reproductive growth (Pyke & Brown, 1996). Once larvae tunnel into the stem, control with insecticides becomes inadequate. The timing of insecticide use is therefore very important and most effective when sprays are timed on egg densities to coincide with peak abundance of early instars (Gage & Haynes, 1975).

Major insect pest outbreaks are reportedly influenced by weather, a factor affecting host plant abundance and quality (Wolda, 1978; Dempster & Lakhani, 1979; Tucker, 1984). The tipworm is no exception and its infestation levels on cotton vary greatly between seasons due to the availability of the malvaceous weed, *Malva parviflora* (L.). This weed, as well as another weed species, *Anoda cristata* (L.), tend to serve as sites for oviposition and larval development for this insect when cotton is out of season. Hamilton (1985) contended that tipworm population size could only be determined by accounting for changes in the availability of *M. parviflora*. He concluded that tipworm infestations were heavier after cool

and wet winters. Both plant abundance and the timing of senescence are crucial in determining the numbers of tipworm available to initiate infestation on cotton each year (Hamilton, 1985). In 1983, most Australian cotton growing areas experienced a serious outbreak. Hamilton (1985) reported a maximum tipworm density at Gatton of 2.2 larvae/plant on November 14, causing most plants to be "tipped out" (i.e., main terminal destroyed), thereby delaying crop development by several weeks. Most growers were reported to have used 3-4 insecticide applications specifically to control this pest.

2.11 *Creontiades dilutus* as a pest of cotton

The green mirid (GM), *Creontiades dilutus* (Stål) is a true bug (O. Hemiptera, S.O. Heteroptera). Ranked fourth among economically important insect orders (Arnett, 2000), the true bugs are characterised by piercing and sucking mouthparts. *C. dilutus*, identified in the early 1970's as a pest of cotton, is an endemic Australian species. Cassis and Gross (1995) described it as widely distributed from the southwest coastal to northwest coastal and from the northeast coastal to the southeast coastal regions. Apart from cotton it is found in many other crops such as lucerne, potatoes, soybeans, mung beans, pigeon pea, stone fruits, sunflower and grapes (Hori & Miles, 1993; Malipatil & Cassis, 1997; Carver *et al.*, 1991). Mirid damage results in premature abortion or deformation of fruits, leaf wilt and disease transmission. Other mirids found in cotton include the apple dimpling bug (yellow mirid), *Campylomma liebknechti* (Girault), the brokenbacked bug, *Taylorilygus pallidulus* (Blanchard) and the brown mirid, *Creontiades pacificus* (Stål) (Pyke &Brown, 1996). The brown mirid is considered equally damaging as the green mirid in cotton (Malipatil & Cassis, 1997; Khan, pers. comm. 2002).

The ultimate economic cost of GM damage on crops, especially cotton is uncertain (Bishop, 1980; Adams & Pyke, 1982; Forrester & Wilson, 1988). Perceptions of mirid status in cotton

have changed over the years. The introduction of large scale cotton farming saw the recognition of GM as a pest in the early 1970's (Room, 1979; Bishop, 1980). Prior to this period, various work in New South Wales by Wright and Nikitin (1964) and Wright (1970) did not include GM in insects damaging to cotton. For example, Room and Wardhaugh (1977) found mirids in cotton fields but attributed no damage to them because of their low density. A similar report by Sterling (1976) on mirids in Queensland did not include GM as a pest.

Experimentations on sap sucking bugs later found GM to be the most damaging of these bugs (Adams & Pyke, 1982; Adams *et al.*, 1984) which led to GM inclusion in the Cotton Pesticide Guide in the 1983-1984 season (Colton & Cutting, 1983). Blackened and shed squares, delay in maturity and tip damage to pre-squaring plants were some of the damage types recorded. In a separate work, Von Mengersen (1982) reported early season damage on cotton in the Namoi Valley with GM infestations. The damage was mainly poor fruit set and inside browning of young and small bolls.

GM these days is likened in its economic importance to its taxonomic counterparts in North America, *Lygus spp*. GM is a pest of the vegetative seedling stage until bolls are no longer vulnerable to attack (> 20 d old). They preferentially feed on the actively growing terminal of seedling plants and the squares and bolls on fruiting plants. The piercing and subsequent injection of saliva containing pectinase causes destruction of cells at the feeding site. Bolls less than ten days old attacked by GM die and fall off the plant. Older squares will be retained but damage to anthers or ovules may lead to distortion of the bolls known as "parrot beaking". Damage to bolls 10 – 20 days old generally results in lint staining and damage to seeds which, depending on the severity of damage, will result in losses in lint due to malformed locules (Khan, 1999).

The cotton industry in Australia has successfully reduced the use of insecticides. This has been the result of using insecticides targeting individual species and the planting of genetically modified cotton (Bollgard®, or Bt cotton, expressing genes from the bacterium *Bacillus thuringiensis*) which is resistant to Lepidopteran larvae (Fitt, 1998). The decline in use of broad-spectrum sprays is helping to boost beneficial insect numbers needed in the integrated pest management strategies (Fitt, 1994). Other minor pests, originally suppressed by these broad-spectrum insecticides are however becoming a problem. One such pest is the green mirid (GM) and it is now recognised as a very serious potential emerging pest. Due to the reduction in broad-spectrum pesticide use in cotton, mirids are now reported to be lingering longer into the season (Wilson, 2004).

The problem is that it is difficult to sample well – this has led growers to use low thresholds. In view of its emerging pest status, current mirid thresholds are being revised (Wilson, 2004). Both adults and nymphs damage the crop. The current threshold in cotton IPM systems is 0.5 per metre in cool regions and 1 per metre (adult and nymphs in warm season areas in Australia) (Johnson & Farrell, 2003).

CHAPTER 3

GENERAL MATERIALS AND METHODS

This chapter provides a review of pheromone isolation and identification procedures and the general materials and methods used in the experimental work presented in the subsequent chapters. Specific detailed methods related to particular sections are discussed in the relevant chapters.

3.1 A review of pheromone isolation and identification procedures

According to Tumlinson (1990), identification of insect pheromonal blends involves four essential components: (1) analytical chemical methods, (2) bioassays, (3) organic synthesis and (4) formulation.

Analytical chemical methods: These are methods that isolate, purify, identify and estimate the amounts and ratios of the pheromonal components in the insect, distinguishing them from all other insect compounds. These methods have been designed to be very sensitive because of the very minute quantities of pheromones produced by insects.

Identification of insect pheromonal compounds from less material than was required in early studies is now possible due to the development of highly sensitive instruments and microanalytical techniques. For example, identification of Bombykol, (*E,Z*)-10,12-hexadecadie-1-ol, from the pheromone glands of domestic silkworm moth, *Bombyx mori* (Linnaeus) was achieved over a 30-year period and involved over a million moths (Butenandt

et al., 1959). Now however, pheromone components can be identified from individual insects (Ma *et al.*, 1980).

Storage of female produced lepidopteran sex pheromones in glands anterior to the ovipositor lobes on the abdominal tip enables solvent extracts or rinses of the abdominal tips or excised glands to be prepared and analysed. The technique of solid sample gas chromatography (SS/GC) has been used to directly analyse the glands. The sex pheromone gland contents of individual *Symmetrischema tangolias* Gyen were analysed using this technique (Griepink, *et al.*, 2000). Excised glands from the abdomen were placed individually in special glass GC liners for the temperature programmable injector in the GC. The high desorption temperature desorbed all the pheromone components and enabled the liners to be reused immediately after the analysis, which was done using two-dimensional gas chromatography. This technique has the advantage of avoiding solvents and concentration steps that involve losses. Online thermal desorption of intact insects in gas chromatography has also been used to confirm the composition of the sex pheromone of *Adoxophyes orana* Fischer von Rösslerstamm (Lepidoptera: Tortricidae), *C. verbasci* (Heteroptera: Miridae) and *L. pabulinus* (Heteroptera: Miridae) (Drijfhout *et al.*, 2000). This method involves placing intact females/males or pheromone glands in the oven part of the thermodesorption system (TDS) and applying heat. Released compounds are transferred to the programmable temperature vaporiser (PTV) injector, cooled to -150°C. The sample is injected onto the GC column by rapidly heating the PTV to 250°C.

There are some complications in the pheromone identification process in bugs. Though the emerging pattern for some bugs is that the lateral accessory glands of the metathoracic scent gland sometimes produce pheromones (Aldrich *et al.*, 1999; 2000), other bugs unlike other insects such as moths, often do not have characteristic sites where the pheromone glands are

located. This makes dissection or study of the contents of the glands difficult (McBrien & Millar, 1999). Bugs tend to have a plethora of glands producing compounds for communication and defence, with some species producing pheromones in discrete, multicellular glands or in patches of unicellular glands (Evans *et al.*, 1990). Extraction methods employed on pheromone analysis in bugs have involved either soaking glands or collecting gland contents with a glass capillary (Aldrich *et al.*, 1993). Other approaches have been to separate the various parts like wings, legs, head, abdomen and thorax before extracting them separately (Millar *et al.*, 1997).

Collection and analysis of emitted material becomes very important if the chemical composition of the emitted pheromone blend may not be the same as that stored in the glands, as occurs in many species. For example, a comparison of the gland wash and effluvia of the western spruce budworm, *Christoneura occidentalis* Freeman shows differences in the ratio of the chemical compositions (Table 3.1).

Compound	Gland ratio	Effluvia ratio
(Z)-11-tetradecenal	1	10
(Z)-11-tetradecenyl acetate	7	3
(Z)-11-tetradecenol	0.73	8

Table 3.1. Differences in ratios of the chemical composition of *C. occidentalis* pheromone (Silk, 1982).

In field tests, only the aldehyde (Z)-11-tetradecenal, was required for long range attraction in *C. occidentalis* (Silk, 1982).

Aeration (Browne *et al.*, 1974) or static-air glass adsorption methods can be used to collect the emitted pheromone components. Aeration involves the trapping of volatile components from a stream of air passed over insects during the time of pheromone production. This is achieved by cold trapping (Browne *et al.*, 1974) or adsorbent trapping. Porous polymers like

Super Q, Tenax GC (a polymer of 2,6-diphenyl p-phenylene oxide) and Porapak Q (a polymer of styrene and divinylbenzene) have been used extensively. Glasswool (Weatherson *et al.*, 1981), glass beads and charcoal have also been used as absorbents. Trapped materials on these porous polymers can easily be thermally desorbed onto gas chromatographic columns or extracted into solvents for analysis. Methods of trapping airborne volatile compounds on capillary tubes and extracting the capillaries with only 2 – 3 µl of solvent have been described by Tóth and Buser (1992). These methods eliminate the stages of evaporation and concentration, which can result in contamination and loss of components.

Gas chromatography linked to a mass spectrometer (GC-MS) is the basic and most sensitive technique used in both purification and identification of volatile pheromones. Functional, geometric and optical isomers can be separated on a whole range of commercial columns ranging from low polarity (OV-101, a methyl silicone) through very polar (cyanosilicone) and liquid-crystal phases (cholesteryl p-chlorocinnamate) to chiral columns. Separation on the column is achieved by passing a carrier gas such as nitrogen or helium over the column. Detectors, especially flame ionisation detectors then detect the separated components. In a GC-MS, separated compounds are transferred directly into the ionisation chamber of the mass spectrometer. A total ion current (TIC) chromatogram, similar to a GC trace is obtained as well as spectra of the fragment ions as a result of the ionisation chemical (CI) or electron (EI). The structure of the unknown compound is determined by comparison of the unknown spectra with a library of reference spectra of known compounds that come with the MS data analysis program software. The fragmentation pattern of the ions generally gives an indication of the class of compound one is dealing with (Fleming & Williams, 1980; MacLafferty & Turecek, 1993; Budzikiewicz *et al.*, 1964).

High performance liquid chromatography (HPLC), a process where a liquid is used as the carrier phase as opposed to a gas in the GC is sometimes used in semiochemical research. It is particularly suited towards fractionating crude extracts with less volatile compounds.

Pheromone structure identifications involving complex and novel compounds occasionally call for the use of other spectroscopic techniques like ultraviolet (UV), infrared (IR) and Fourier transform nuclear magnetic resonance (FT-NMR) spectral analyses. The IR spectra is able to provide information about the functional group on the compound while the proton and carbon NMR provides information on the carbon atoms and the number of hydrogens on the carbon and those in its immediate vicinity.

Most insect pheromones contain some form of unsaturation. The presence and location of these points of unsaturation are sometimes determined by microchemical reactions. Microhydrogenation in methanol or pentane using 5% palladium/calcium carbonate as a catalyst is one way of determining the presence of unsaturation. Three basic methods - micro-ozonolysis (Beroza & Bierl, 1966; 1967), epoxidation and methoxymercuration-demercuration (Baker *et al.*, 1982) followed by GC-MS, have been used in the location of double bond positions. In the latter method, methoxylated products obtained from treatment of pheromone with mercuric acetate in methanol followed by reduction with sodium borohydride are analysed by GC-MS. Fragments in the MS are identifiable because of intense fragment ions from cleavage α to the methoxy group. This actually indicates the position of the methoxy group and hence, the double bond. Two pairs of fragment ions, each separated by fourteen mass units, allow unambiguous assignment of the double bond position for a monoene. The position of unsaturation in the pine beauty moth, *Panolis flammea* Denis & Schiffermüller pheromone was established using this technique (Baker *et al.*, 1982). In the former method, cleavage of the ozonide produced from the ozonolysis gives two aldehydes

that can be identified by comparison to authentic standards by their retention times on the GC. Bierl-Leonhardt, *et al.* (1980) identified the position of unsaturation in the aldehydes isolated from the tobacco budworm, *Heliothis virescens* Fabricius and corn earworm, *H. zea* by microepoxidation. This procedure involved the GC-MS of test compound after epoxidation with meta-chloro perbenzoic acid (MCPBA). Characteristic cleavages alpha (α) to the epoxide group produce fragment ions with relatively high abundance, an indication of the position of the epoxide and hence, the location of the double bond.

Organic synthesis and formulation: In order to use the pheromone on a practical basis as well as synthesise the compound to a high degree of purity, an organic chemical synthesis method has to be developed. These methods are selected and developed to construct specific molecules in exactly the same configuration as the natural material (Tumlinson, 1990). Synthesis therefore is relied upon to determine which enantiomer(s) or stereoisomer is active when the chirality of a molecule cannot be determined because of insufficient availability of the natural material (Tumlinson, 1990). For commercial scale applications it may be necessary to develop procedures for synthesis of the required compounds, but many new pheromones turn out to be blends of chemicals which are already produced in commercial quantities, either as components of the pheromones for other insect pests, or for other purposes. All the compounds used in this thesis were obtained from such sources, which are detailed in the appropriate Methods and Materials sections in subsequent chapters.

The final stages of the identification involve the formulation of the blends to provide a slow-release rate of pheromone over time. Sometimes formulations mimic the rate and ratio as emitted by the insect of interest. This step has been found to be very difficult. It has been achieved in most cases by loading pheromone into rubber septa, plastic matrices or the use of a semi-permeable membrane through which the pheromone passes at a desired rate. Products

developed based on the above mentioned principles include: (a) Hollow fibres - these are short fibres made of impermeable thermoplastic tubings sealed at one end and filled with liquid pheromone. Pheromone is released by evaporation at the liquid air interface (Jutsum & Gordon, 1989). Fibre length is used to control the effective life of the formulation; (b) Laminate flakes - made up of a porous central layer of pheromone sandwiched by two layers of vinyl (plastic laminate flakes). Regulating the thickness of the layers, concentration of the pheromone in the middle layer, stiffness of the plastic membranes and additives in the formulation control the emission rate (Kydonieus, 1977); (c) Twist-tie ropes - these are plastic fibres sealed at both ends and containing a hollow channel with pheromone and a soft wire to enable attachment to objects of all shapes (Jutsum & Gordon, 1989); (d) Microcapsules - this unique formulation consists of small droplets or particles of pheromone (protected by an outer shell of polymer such as polyamide). This formulation is known to provide a constant, sustained release over time. It also protects pheromones that are light sensitive and tend to degrade. Methods for determining release rates and ratios from some of these formulations have been developed. Heath *et al.* (1986) and Heath and Tumlinson (1986) have described methods of predicting release ratios in lepidopteran pheromones with similar volatility from rubber septa. Due to instability of some insect pheromones, antioxidants like vitamin E and butylated hydroxy toluene (BHT) are used in some of these formulations.

Bioassays: Bioassay methods are designed to enable evaluation of the pheromone components, which comprise only a small portion of the total chemical constituents of the insect. These processes are critical as they enable the separation and differentiation of active components from the inactive ones. Various methods have been developed based on behaviour or biological activity. For behavioural assays, various olfactometers have been used (Baker & Cardé, 1984). These usually involve sealed chambers connected to a pump that sucks air through the system. The test insect put in the middle of the chamber then has the

option of moving or flying to one or more of the test components in one of the inlets of the enclosed system. The shape, size and type of olfactometer used depends on the type of test insect.

Wind tunnel bioassay (Baker & Linn, 1984) is one of the most common methods to test behavioural responses of insects to isolated pheromonal compounds. Test insects are placed in the downwind end and the test compound, loaded onto a dispenser, is placed at the upwind end of the tunnel. A fan either blows or sucks air through the system at a set rate. Test insects are then released in the tunnel and their behavioural responses within a predetermined time are noted or recorded with a computerised program. Some of these behaviours include wing fanning, excitation, upwind flight, source contact and attempt at copulation. These behaviours help distinguish which compounds are used by insects for long or short range attraction and those which may influence entry into traps. Wind tunnels have been used successfully in determining the roles of compounds in many insects including *H. virescens* (Teal *et al.*, 1986), *Trichoplusia ni* (Hübner) (Bjostad *et al.*, 1984; 1985), *Spodoptera frugiperda* (Smith) (Malo *et al.*, 1972) and *Diatraea saccharalis* (Fabricius) (Batista-Pereira *et al.*, 2002). The size of the wind tunnel and whether the test insects flies or crawls to the source depends on the insect being tested and the overall goal of the research.

Field bioassay using traps is an effective option for testing an isolated component where sufficient material is available. Traps are baited with the isolated compound and left in the field to test activity towards the target insect. This type of bioassay is typically suited for lures that are developed for traps. Compounds identified through this process are effective in pheromone traps. A pheromone identified as effective in a particular trap may however, not be suited for other types of traps (Cardé & Elkinton, 1984). Trap design including colour and shape is therefore an important consideration in field bioassays. Trap designs include those

that make use of a sticky surface such as Delta, Panel, Diamond, Rhagoletis, Wing trap and Vapor Tape, and those designed for bigger insects such as the Unitraps and Texas traps (Phero Tech Inc., British Columbia, Canada). In cases where the complete blend is not required to lure an insect into a trap, components that elicit full range behaviours like close-range courtship, could be missed in this bioassay. For example, it is common knowledge that a single blend in a trap sometimes captures males of more than one species, while the females of these only attract conspecific males. The blend identified through field bioassays, therefore, may lack components important to the reproductive behaviour of the insect critical for control application methods like mating disruption (Tumlinson, 1990).

Electrophysiological assays that test fractions for biological activity are also used. This technique involves the use of the electroantennogram (EAG) which measures electrical depolarisation across the antenna of an insect. The depolarisation arising as a result of stimulation by the pheromone components passes over the antenna. Thousands of olfactory hairs located on the antennae filter and detect the pheromone in the air stream. Glass microelectrodes, usually made from capillary glass tubing are filled with saline solution to make electrical continuity between the haemolymph in the antennae and the silver/silver chloride junction of the recording system (Cork *et al.*, 1990). Signals from the electrodes, inserted into the tip and base of the antenna are then amplified and received on an oscilloscope. This effectively screens the biologically active compounds (Roelofs, 1984). This instrument is either used on its own or coupled to a GC (Struble & Arn, 1984). Moorhouse *et al.* (1969) first described EAG recordings from a GC effluent. Components eluting from the GC are separated into two fractions. One fraction goes into the GC detector and the other is usually puffed over the antennae of the insect in the EAG. Biologically active compounds on the antennae as reflected in the EAG recording are matched to a particular peak with same retention time on the GC. This peak is then identified by mass spectrometry

or other means and further analysed. Insects whose pheromones were identified using this method include *E. insulana* (Hall *et al.*, 1980), *H. armigera* (Nesbitt *et al.*, 1979) and *Corcyra cephalonica* Stainton (Hall *et al.*, 1987).

Nerve impulses from single cells as in single cell recording instead of the whole antennae hooked to the GC have been developed and used as a refinement of the EAG technique (Wadhams, 1984). Single olfactory cells respond to a limited spectrum of odours and hence, single cell electroantennography detection accurately identifies odours. Recordings are taken from single olfactory receptor neurones linked to gas chromatography and mass spectroscopy. In addition to being used in sex pheromone research, this technique has also been applied to numerous volatiles involved in insect-plant interactions to determine those compounds involved in host finding behaviour (Stranden *et al.*, 2003).

The disadvantage of all electrophysiological methods is that the method only measures antennal and not behavioural response. Hence, many compounds that are biologically active on the antennae do not necessarily work in the pheromone blends to attract insects. The response only gives an indication of the biological activity of the compound but not the type activity. These compounds could be alarm, aggregation or defence chemicals and not sex attractants.

All of the above bioassays are used in one way or the other and also in combinations during the isolation and identification processes of pheromone components.

3.2 Insect cultures

Initial cultures of the rough bollworm and the cotton tipworm came from field-collected larvae from Cecil Plains, Qld and Tamworth, NSW that were reared through to adult in the University of New England insectary on an artificial diet based on that of Forrester *et al.*

(1993), in 35-ml plastic cups (Solo, P101M, Urbana, Illinois, USA). The rearing conditions in the insectary were 25 ± 1°C and 13:11 light:dark (L:D) period with the dark period or scotophase during 1830-0530 h Australian Eastern Standard Time (AEST).

Moths emerged from these initial cultures were held in mating cages made of transparent plastic cylinders, 30 cm tall and 21.5 cm diameter, provided with dental wicks soaked in 5% sucrose solution as food. Moths were provided with paper towels as oviposition sites. Rearing from the initial eggs through to the adult stages was carried out in a reverse cycle controlled environment room with 16:8 light:dark (L:D) period, with the dark period during 0930-1730 h AEST. Adults emerging were then sexed and held individually in 150-ml plastic containers (Polarcup (Australia) Ltd., Bankstown, NSW) and fed with 5% sucrose in cotton wicks until ready for use in the experiments.

In the case of the green mirid, insects were collected from lucerne in Armidale, NSW using a sweep net. Mirids caught in the sweep net were aspirated into vials containing lucerne materials until they were transported to the insectary where they were separated into adults and nymphs. Rearing methods for green mirids generally followed those of Khan (1999). Adult females and males were transferred into mating cages containing fresh beans as food, oviposition sites and shelter. The mating cages consisted of clear 1 litre plastic containers with meshed lids to allow airflow. Beans were changed every other day and checked for eggs. Those with eggs were transferred into fresh containers with cotton dental wicks soaked in distilled water until egg hatching. Newly emerged nymphs were transferred into fresh containers with fresh beans. This process was continued until the nymphs developed into adults which were then held in individual containers until ready for use. Conditions of rearing were the same as those for the two other species described above. It was difficult to maintain a green mirid culture continuously in the insectary. Hence, fresh insects had to be collected

from the field when required. Late instar nymphs were therefore the preferred stage of collection for a quick source of virgin males and females for the experiments.

3.3 Pheromone collection procedures

Two methods of pheromone collection were adopted – glands or whole body extracts and air trapping.

3.3.1 Glands or whole body extracts

For the rough bollworm and the cotton tipworm, calling females that were observed with pheromone gland extrusions (abdominal tips) during the scotophase were used for extraction. The abdominal tips of calling females were removed by applying gentle pressure to the abdomen to extrude the ovipositor and then cut using a pair of spring-loaded fine scissors (Australian Entomological Supplies, Bangalow, NSW, Australia). Glands were then extracted in a small volume of dichloromethane or hexane following the reviewed methods of Golub and Weatherston (1984), Gries and Gries (1999), Witzgall *et al.* (2001) and Millar *et al.* (2002).

For the green mirids, whole body extractions involved immobilising the adult insects in a freezer for about 1-2 minutes and soaking in the desired solvent for 5 minutes. The solvent was then siphoned off into a clean 1.5 ml amber vial (Alltech Associates Australia, Baulkham Hills NSW, Australia) with a pasteur pipette.

3.2.2 Air trapping

Entrapment of volatiles was done using an all glass apparatus shown in Figure 3.1. Volatiles were collected from 3-4 virgin females or males housed in the glass chamber. Air was drawn into the flask through a filter of activated charcoal (10 cm x 2 cm; 10 – 18 mesh) at 50 ml/min

by means of a pump (Capex L2C, Charles Austen Pump Ltd, Surrey, England). The volatiles from the housed insects were then trapped on a 200 mg filter of Super Q (80/100 mesh; Alltech Associates Inc, U.S.A) held in place by silinised glasswool in a pasteur pipette. Trapped volatiles were eluted from the filter with dichloromethane or hexane before analysis.

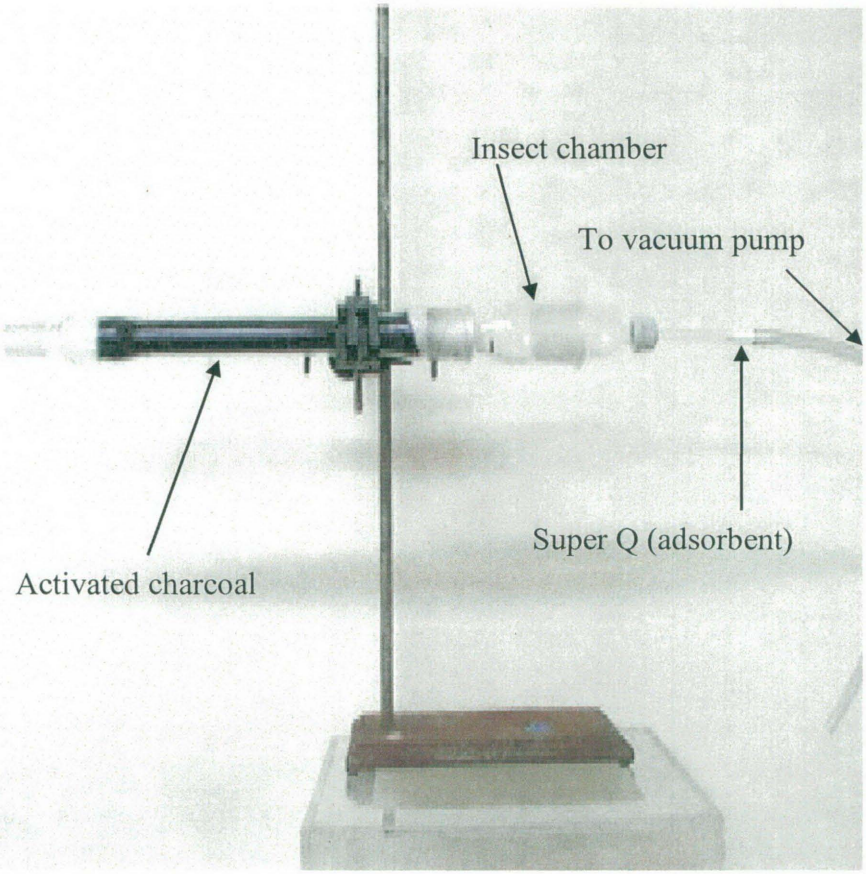


Figure 3.1. Air collection apparatus for pheromones.

3.4 Gas chromatographic (GC) methods

To analyse and identify pheromone components, two types of gas chromatographs were used. These were the gas chromatograph linked to a mass spectrometer (GC-MS) and the gas chromatograph with a flame ionisation detector (FID).

(a) Gas chromatograph linked to a mass spectrometer (GC-MS)

Gas chromatographic-mass spectrometric (GC-MS) analyses were done using a Hewlett Packard 6890 series gas chromatograph and HP 5973 mass selective detector (Hewlett-Packard, Palo Alto, U.S.A). The columns used on this GC were an AT 35 capillary column (30 m x 0.25 mm i.d x 0.25 μm) and a HP-5MS (5% Phenyl Methyl Siloxane, 30 m x 0.25 mm i.d., 0.25 μm film thickness; J & W Scientific, Folsom, USA) fused capillary column. The carrier gas was ultrapure helium set at a flow rate of 0.8 m/s. The column temperature was programmed from 40°C (0.50 min hold) to 250°C at 20°C min^{-1}. Temperatures of the splitless injector and the GC-MS interface were set at 280°C and 300°C, respectively. Total run time was 30 minutes. A mass spectrum was scanned from m/z 30 to 300 and acquired data were collected and analysed on a Hewlett-Packard workstation using HP Chem/Station software. Mass spectra obtained were matched with thousands of spectra stored in the Library of the HP Chem/Station software. Matches were then examined for molecular ions ($M^{\cdot+}$), M^+ minus recognisable fragments and other fragment ions consistent with the structure proposed. For example, a molecular ion (M^+) would have an associated M + 1 peak with intensity consistent with the number of carbon atoms present. These were then confirmed with spectra obtained from standard spectra run with retention times.

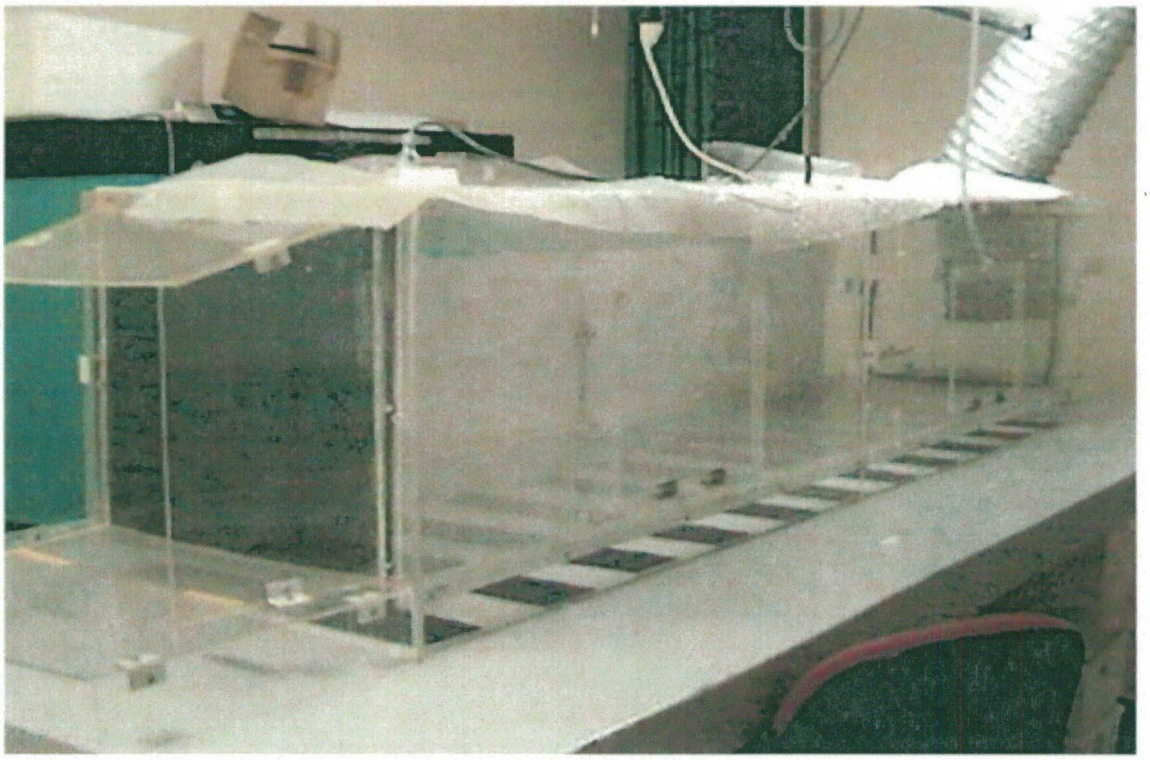
(b) Gas chromatograph with FID detector

A Varian Star 34000 gas chromatograph (Varian Associates, Texas, USA) fitted with a FID as a detector was used. This GC was equipped with an AT 35 capillary column (30 m x 0.25 mm i.d. x 0.25 μm). Conditions of run were the same as that of the Hewlett Packard 6890 GC, except the gas flow rate was set at 1.0 m/s. This column was more polar than the HP-5 MS. Data were collected by means of the Varian Star workstation 5.51 linked to the GC.

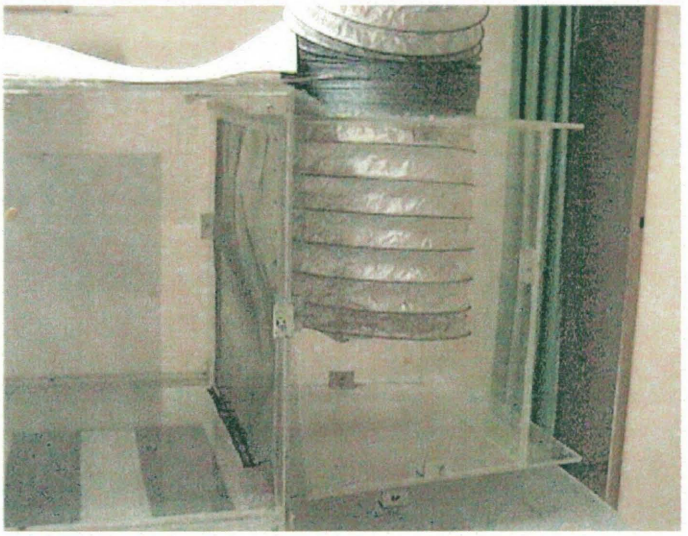
3.5 Wind tunnel bioassays

For the two lepidopteran species (rough bollworm and cotton tipworm), male response to pheromone and conspecific live females was studied in a 260 x 60 x 60 cm Plexiglas® wind tunnel (Figure 3.2a) similar to that described by Cardé and Hagaman (1979). Airflow of 30-40 cm/s was maintained by means of a fan which pulled air through the tunnel using a 30 cm-diameter exhaust tube leading to the outside of the building (Figure 3.2b). The temperature of the air stream in the middle of the tunnel was maintained at 24-26°C throughout the experiments. Moths used for the experiment were transferred to the wind tunnel room at least two hours before the onset of the scotophase to acclimatise to room conditions.

The test pheromone source was loaded on a 13 mm diameter glass fibre filter disc (type A/E, Pall Corporation, Michigan, U. S.A) outside the testing room. The impregnated filter paper, suspended on a white stand (30 cm high) was then placed at the upwind end of the wind tunnel. Test males were placed individually in screen cages. They were released at the downwind end of the tunnel and observed for 5 minutes.



a



b

Figure 3.2. Wind tunnel used in experiments to observe male behaviour (a); Exhaust tubing downwind of the tunnel (b).

A continuous red light source of intensity 1–1.5 lux in the wind tunnel was provided by continuous red photographic safelights (Encapsulite, Type R10, EncapSulite® International Inc., Rosenberg, Texas USA) suspended above the wind tunnel. To diffuse the light a plastic packaging material was placed between the tubes and the top of the wind tunnel. Observations were made in the second half of the scotophase when females are known to call, and recorded on a computer using The Observer software (V. 3.0) (Noldus Information Technology b.v, Costerweg 5, 6702 AA Wageningen, The Netherlands).

3.6 Field bioassays

3.6.1 Study sites

Figure 3.3 shows the sites where field experiments were conducted. Field trials were generally conducted in cotton growing areas in south-east Queensland and New South Wales (NSW). In Queensland, these sites include Cecil Plains (27° 32' S, 151° 12' E), Oakey (27° 26' S, 151° 43' E), Warra (26° 56' S, 150° 55' E), Goondiwindi (28° 33' S, 150° 18' E) and Mondure (26° 11' S, 151° 47' E). In NSW, field trials were conducted at Boggabri (30° 42' S, 150° 03' E), Gunnedah (30° 59' S, 150° 15' E), and Mullaley (31° 06' S, 149° 55' E) in the Upper Namoi Valley and Narrabri (30° 20' S, 149° 47' E) in the Lower Namoi Valley, as well as Dungowan (31° 13' S, 151° 07' E) and Armidale (30° 31' S, 151° 40' E). Most of the study sites experience mild winters (-5° to 25°C) and hot summers (18° to 45°C). Though cotton was the main crop in the study sites, some of the experiments were carried out in mung beans, soybeans and lucerne.

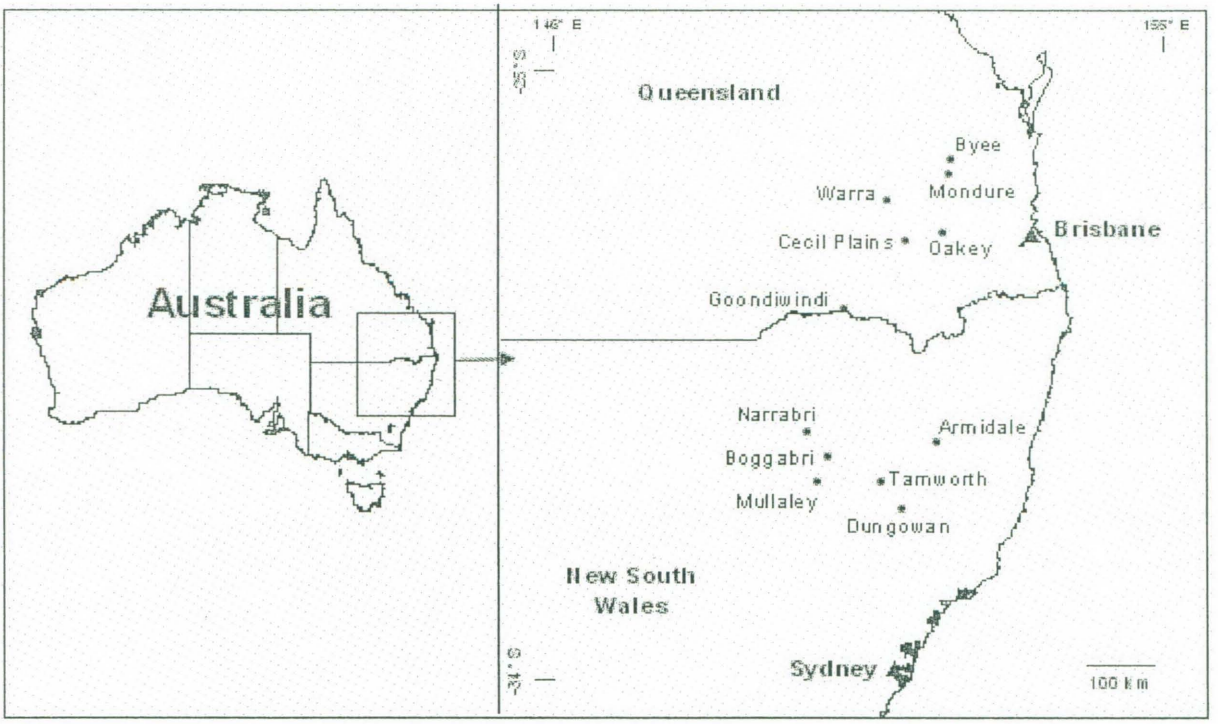


Figure 3.3. Map showing sites of field studies in Queensland and New South Wales relative to the major cities of Brisbane and Sydney.

3.6.2 Pheromone trapping

Pheromone blends prepared in hexane were loaded onto red rubber septa (Pherobank, Wageningen, The Netherlands) suspended by means of a paper clip in the trap. Pheromone traps used in the field to test the attractiveness of pheromone blends were of the Delta trap design (Phero Tech Inc, Delta, British Columbia, Canada V4G 1E9). Delta traps (Figure 3.4) were made by the author, and were moulded from white plastic Corflute® (Signwave, Parramatta, Australia). The sticky bases of the traps were made of 0.5 mm thick poster cardboard cut to 194 x 183 mm covered with white greaseproof paper on the surface and coated with Tangle-Trap Insect Trap Coating® (The Tanglefoot Company, Grand Rapids, Michigan, USA). Greaseproof paper was used to prevent drying out of the coatings in the field. The traps were held in place by a thin metal wire suspended on curtain rod stakes driven into the soil.

AgriSense® funnel traps (Entosol Australia Pty Ltd, Roselands, NSW, Australia) were used in the experiments to compare trap designs for the green mirid and rough bollworm pheromones and to investigate the feasibility of "attract-and-kill" and mating disruption using green mirid pheromones. The AgriSense® trap (Figure 3.5) consists of a lid where the pheromone lure is suspended and a canister provided with a dry killing agent (Dichlorvos, Sureguard, Sara Lee Household & Body Care (Australia) Pty Ltd., Heatherton Rd. Clayton, South Victoria, Australia) where insects are caught.

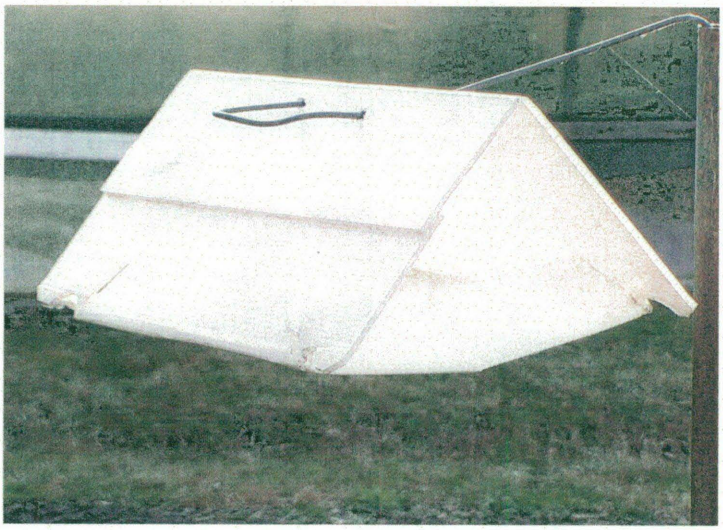


Figure 3.4. Delta trap.

Figure 3.5. AgriSense® trap.

3.6.3 Experimental designs

Most experiments were designed as Latin Squares, with three factors, location, treatment (trap type or pheromone blend), and time (trap rotation interval). It is recognised that within an experimental field, there would be insect density trends running both across the field and up and down the field. The Latin Square design therefore enabled random assignment of treatments within rows and columns, with each treatment once per row and once per column (i.e. equal numbers of trapping locations, trapping occasions, and treatments). It allowed treatments to be rotated through the locations so that each treatment occurred once at each location and on each occasion (Figure 3.6). Advantages of Latin Square designs in pheromone research include their ability to control variation in two different directions. The use and advantages of Latin Square designs have been reviewed by Perry *et al.*, (1980).

	Site				
Occasion	**I**	**II**	**III**	**IV**	**V**
1	**A**	**B**	**C**	**D**	**E**
2	**B**	**A**	**E**	**C**	**D**
3	**C**	**D**	**A**	**E**	**B**
4	**D**	**E**	**B**	**A**	**C**
5	**E**	**C**	**D**	**B**	**A**

Figure 3.6. A 5 x 5 Latin Square experimental design. A, B, C, D and E are different treatments (eg. pheromone blends), (Modified from Gomez & Gomez, 1984).

3.7 Statistical analyses

Statistical analyses of data were done using the R statistical package version 1.9.0 (R Development Core Team, 2004). Data were summarised using means and standard errors. Relationships between variables were determined using one way analysis of variance on log (x + 1) of the data followed by contrast to determine the least significant differences between means. Data that were not normally distributed after the transformation were analysed using the Generalised Linear Model (GLM) for logistic regression of binomial response variables or Poisson regression of multinomial response variable. Results of the GLM analysis are displayed in an Analysis of Deviance Table with a chi-square test for significance of variables. A P-value of 0.05 or less was taken as significant. Data on the frequency of observations in the wind tunnel bioassay and trap catches from the field trapping experiments were analysed using chi-square and generalised linear models in the R statistical program.

CHAPTER 4

SEX PHEROMONES OF THE ROUGH BOLLWORM, *EARIAS HUEGELIANA* (GAEDE) (LEPIDOPTERA: NOCTUIDAE)

4.1 Introduction

The rough bollworm (RBW), *Earias huegeliana* (Gaede) is considered an occasional pest of cotton in Australia. Rough bollworm populations in cotton are usually controlled by conventional insecticides sprayed to control the major pests of cotton such as *Helicoverpa* spp. Insecticide control of *E. huegeliana* is difficult once the larvae have burrowed into the plant. Recent trends in the cotton industry indicate a reduction in insecticide use and the adoption of integrated pest management (IPM) approaches that rely on less chemical use and are more environmentally friendly. Also, it is likely that more Australian cotton will be grown in northern (tropical) areas in future (Yeates, 2001). In these areas, irrigation water is more abundant, but there is also a greater diversity of Malvaceae (to which *Earias* spp. are specific), and knowledge of the distribution of other *Earias* species suggests that the climate may be more favourable for *E. huegeliana* in such regions. Bladder ketmia, a key host of rough bollworm is less well controlled by glyphosate so may become more of a weed problem in Roundup-ready (transgenic glyphosate-resistant cotton) systems, hence there may be more hosts for RBW. While transgenic (Bollgard II®) cotton will control RBW, the potential for resistance to this method in RBW is high because it is a Malvaceae specialist and at some times of the year it will be concentrated in cotton. Because of these factors, it is considered

that rough bollworm might become economically more important in the future, hence, there is a need to put in place improved control measures for this species. One of the options is the development of a good detection tool based on sex pheromones to monitor pest population and thus determine whether other control measures are necessary.

Research on the sex pheromones of the rough bollworm will further contribute to a better understanding of the ecology of this pest. A properly identified and formulated sex pheromone blend for *E. huegeliana* would be a good monitoring tool if trap catches reflect field populations. It would also be another tool in research to shed some light on the distribution, phenology and host relationships, and has a potential for use in mating disruption.

The female sex pheromones of the related species, *E. vittella,* and *E. insulana* have been previously identified (Hall *et al.*, 1980; Cork *et al.*, 1988) and were found to be highly attractive to conspecific male moths. *E. insulana* produces (*E,E*)-10,12-hexadecadienal as the main sex pheromone component. In addition to (*E,E*)-10,12-hexadecadienal, *E. vittella* produces hexadecanal, (*Z*)-11-hexadecenal, (*E,E*)-10,12-hexadecadienol, octadecanal and (*Z*)-11-octadecenal in a ratio of 2:1:10:1:2:4, respectively. Field testing, however, showed that a 2:10:2 mixture of (*Z*)-11-hexadecenal, (*E,E*)-10,12-hexadecadienal and (*Z*)-11-octadecenal (Figure 4.1) was equally attractive to male *E. vittella* as the six component mixture and equal in attractiveness to a virgin female moth (Cork *et al.*, 1988). The chemical structures of the pheromone compounds identified in *Earias* spp. are shown in Figure 4.1.

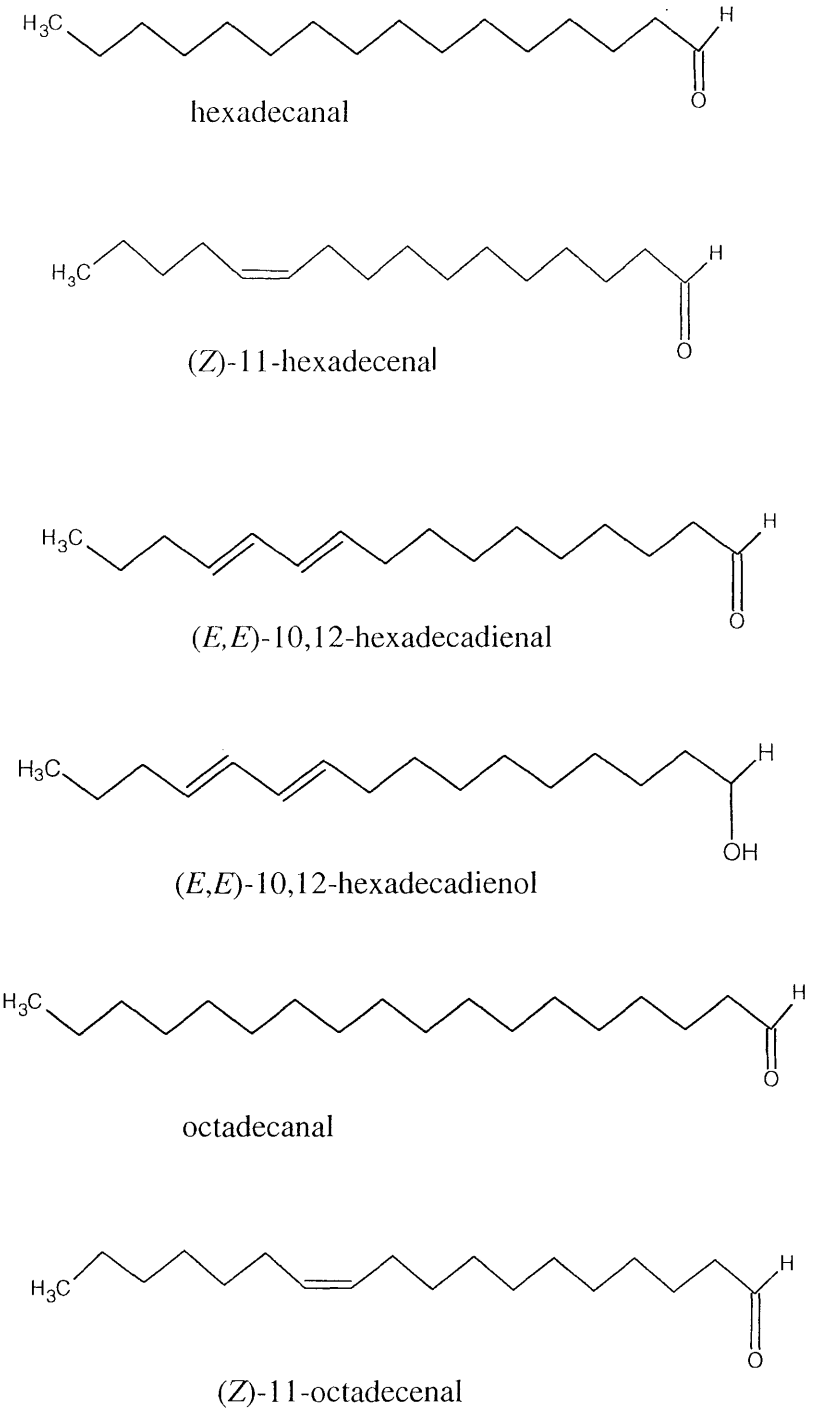


Figure 4.1. Pheromonal compounds and their chemical structures found in *Earias* spp. (Cork *et al.*, 1988)

In this chapter, studies on the isolation and analysis of the sex pheromone components of *E. huegeliana,* including laboratory and field bioassays are presented.

4.2 Materials and methods

4.2.1 Insect culture

Larvae of *E. huegeliana* were collected from Cecil Plains, Queensland on a malvaceous weed species, *Hibiscus trionum* (bladder ketmia). Larvae were carefully extracted from the bolls of the bladder ketmia using a scalpel and forceps. They were then transferred onto an artificial diet and reared under the conditions described in Chapter 3.2.

4.2.2 Pheromone extraction and analysis

Air trapping. Trapping of volatiles was done using 3-to 6-day old unmated females or males. Volatiles were collected from 3-4 insects held in an all glass apparatus (Figure 3.1), as described in Chapter 3. Insects were transferred into the experimental room at least four hours before collection. Volatile collection was done for the whole scotophase. Initial collection involved purging the system for the first two hours using a pasteur pipette without the adsorbent (Super Q). After the purging time, the empty pasteur pipette was replaced by a pipette with the adsorbent. Volatiles trapped on the 200 mg of Super Q were eluted with 3 ml of dichloromethane or hexane in 0.5 ml portions. Eluate was collected in amber vials for concentration and analysis. For quantification purposes, (*E,Z*)-10,12-hexadecadienal was used as an internal standard.

Gland extracts. Gland extracts were done when females were calling (Figure 4.2) which was observed to be between 1530 and 1730h AEST (that is, approximately 2 h before sunrise in

the reverse-cycle experimental photoperiod). The extracts were prepared in batches, with each batch containing 3 or 4 excised abdominal tips of calling females soaked in 20-30 µl of hexane in an amber vial. Extracts were allowed to stand at room temperature for 10 minutes before transferring the supernatant into 1.5 ml amber sample vials with 100 µl limited volume inserts. Sample vials were stored in the freezer until analysis (about 24 h).

Gas chromatographic-mass spectrometric (GC-MS) analyses were conducted on gland and air extracts using a Hewlett Packard 6890 series gas chromatograph and HP 5973 mass selective detector. The column used and conditions of run were as described in Chapter 3.

4.2.3 Field bioassays

A preliminary field trial was first conducted in cotton (cutout stage) at the "Glen Shee" property in Oakey, Qld, to test if the 4-component blend A and two blends with 2 components, B and C (Table 4.1) would attract *E. huegeliana* males in the field. Four Delta traps were placed about 40 m from each other: 1 for blend A, 2 for blend B and 1 for blend C. Trap catches were collected for 2 days only because the farmer sprayed a pyrethroid insecticide on the cotton two days after the traps were set up. Trap catches over this 2-day period were 42 (blend A), 48 and 44 (blend B) and 2 (blend C).

The major field trapping experiments were conducted at two sites, near Cecil Plains in the Darling Downs and near Mondure, Queensland. The design used was a Latin Square with three factors - treatment (trap type or pheromone blend), location and time (trap rotation interval). Blends used were coded as shown in the Table 4.1.

	BLEND / RATIO											
Chemicals	A	B	C	D	E	F	G	H	I	J	K	L
(E,E)-10,12-hexadecadienal	4	4	4	4	4	-	-	1	10	5	4	1
(E,E)-10,12-hexadecadienol	1	-	-	4	1	-	1	-	-	-	-	-
(Z)-11-hexadecenal	1	1	-	1	-	1	1	1	1	1	1	1
(Z)-11-octadecenal	1	-	1	1	-	1	1	-	1	1	1	-

Table 4.1. Code for pheromone blends of *E. huegeliana* used in field trapping experiments. Ratios used based on levels quantified from the air and gland collections in the GC-MS.

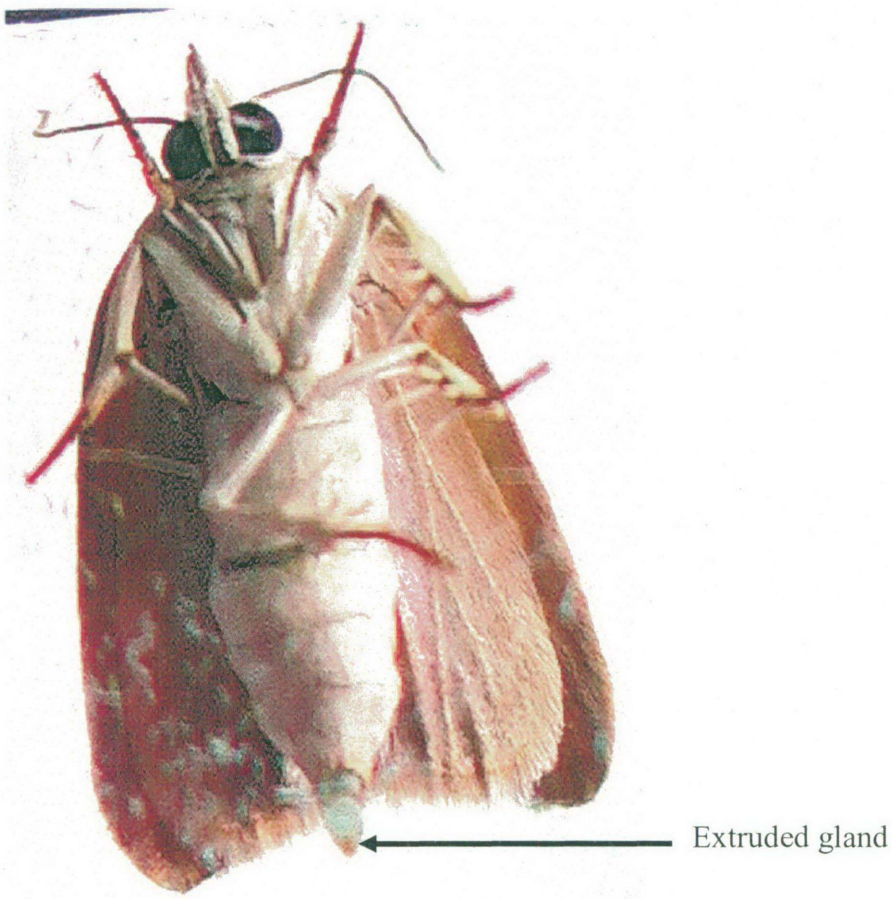


Figure 4.2. Calling female of *Earias huegeliana.*

Experiment 1a - Comparison of blends A, B and C

The experiment aimed to compare the attractiveness of the full blend consisting of 4 components (A) with that of two partial blends (B and C) in a ratio as determined in the GC analysis and a blank control (CT). Field tests were carried out in February 2003 on flowering cotton at "Glen Shee" in Oakey, Qld. The experimental design is shown in Figure 4.3. There were 3 different blends plus a control (CT), 4 rotation periods and 4 trap locations. Delta traps were located 100 m from each other, and cleared each day before rotation.

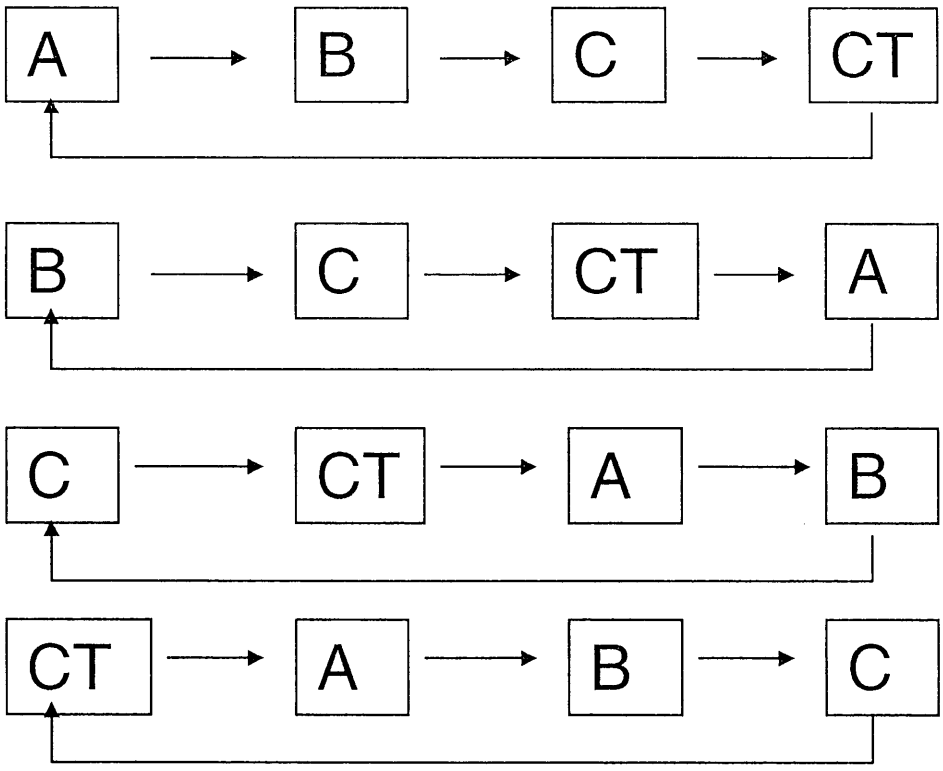


Figure 4.3. Experiment 1a. Layout of Delta traps at "Glen Shee", Oakey, Qld. This experiment was a 4 x 4 Latin Square design. Traps were rotated daily in the direction of the arrows. Letters refer to blends (Table 4.1; CT is Control)

Experiment 1b - Comparison of blends A, E and F

This experiment was a 3 x 3 Latin Square with 3 blends, 3 rotations and 3 trap locations aimed at further testing two other partial blends (E and F) compared with the full blend (A) using Delta traps. The experiment was also carried out in the flowering cotton at "Glen Shee", Oakey, Qld in February 2003.

During this experiment, observations of male response towards the pheromone lures in the traps were also done every night (for 3 nights) using night vision goggles (Litton Precision Products International, Rosebery, Sydney, Australia) and a torch fitted with red filter. Observations were done for 5 minutes every two hours from 2000 to 0600 h. A male response to the pheromone was scored as "approach". An insect was said to have approached the trap when it flew in the characteristic zigzag manner and was about 5 cm from the mouth of the trap. Observations were recorded on a cassette tape and later transcribed. Numbers of male insects caught in the trap were counted at the end of each observation session.

Experiment 2 – Comparison of other partial blends

In this experiment, unreplicated Delta traps for blends B, C, D, E, F, G and H were set up in a straight line, spaced 30 metres from each other on a cotton field (cutout stage) at "Yanco" in Cecil Plains, Qld. Traps were rotated in the direction shown on Figure 4.4. Trap catches, however, were only collected for four days in February 2003.

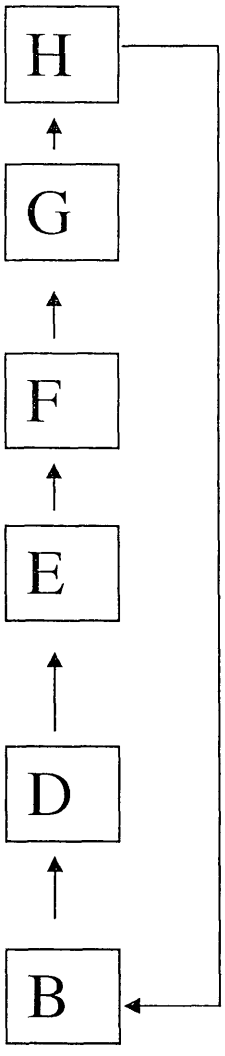


Figure 4.4. Experiment 2. Layout of Delta traps at "Yanco", Cecil Plains, Qld. Traps were rotated daily in the direction of the arrows.

Experiment 3 – Comparison of two types of traps using blend B

This experiment aimed to test the suitability of the AgriSense® funnel traps for trapping *E. huegeliana* compared with the Delta traps. The experimental design is shown in Figure 4.5 with 3 rotation periods, 5 trap locations and 2 trap types. The experiment was conducted on a harvested wheat field containing many bladder ketmia (*Hibiscus trionum* L.) weeds at

Mondure, Qld in January 2004. Traps were located 40m from each other within rows and 100 m between rows, and were cleared daily before rotation.

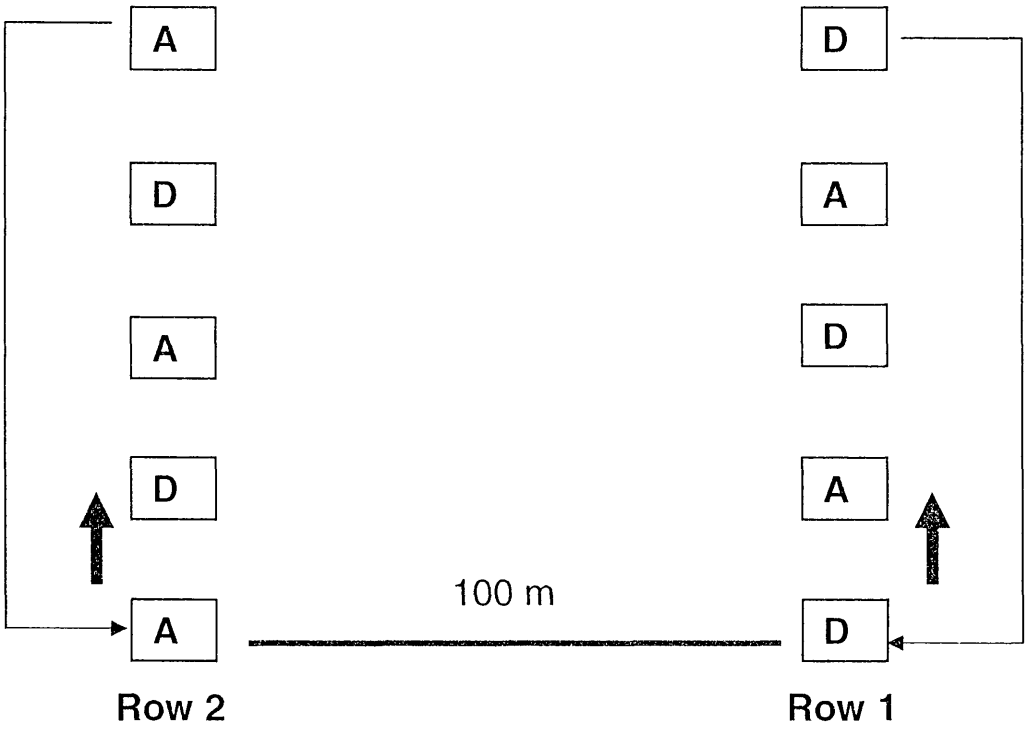


Figure 4.5. Experiment 3. Layout of traps at Mondure, Qld. Traps were rotated daily in the direction of the arrows. D – Delta trap ; A – AgriSense® trap.

Experiment 4 - Comparison of the sex pheromones of *E. vitella and E. huegeliana*

This experiment primarily aimed to compare the pheromone blend of *E. vittella* with that of *E. huegeliana* at different ratios. The *E. huegeliana* blend was tested at different ratios to determine an optimum blend. The experiment was a 5 x 5 Latin Square, with 5 rotation periods, 5 locations and 5 treatments. Delta traps were located 100 m from each other and 50 m in between rows, and were cleared and rotated daily. The experiment was conducted on cotton (cutout stage) at "Yanco", Cecil Plains, Qld in February 2004. The ratios of the *E. huegeliana* and *E. vittela* blends tested in this experiment are shown in Table 4.2.

	BLEND / RATIO				
Chemicals	EH1	EH2	EH3	EH4	EV
(*E,E*)-10,12-hexadecadienal	4	3	2	4	10
(Z)-11-hexadecenal	1	1	1	1	2
(Z)-11-octadecenal	-	-		1	2

Table 4.2. Experiment 4. Ratios of *E. huegeliana* (EH) and *E. vittela* (EV) blends.

Experiment 5 – Weathering experiment on blend components

This experiment was conducted to determine the rate of weathering from the rubber septa loaded with the pheromone components of *E. huegeliana.* Gas chromatographic analyses of extracts from pheromone septa kept in the freezer were compared with those from septa left in Delta traps exposed to normal weather conditions.

A total of 32 rubber septa, pre-cleaned by soaking in hexane for 2 days and air dried, were each loaded with a 200 µl pheromone blend containing 1.6 mg (*E,E*)-10,12-hexadecadienal, 0.4 mg (Z)-11-hexadecenal and 0.02 mg BHT in hexane. Half of the septa (16) were held in a glass container and kept in a freezer at –21°C and the other half (16) were used as baits in Delta traps set up outside the insectary in Armidale, NSW, from late February to early March 2004 (autumn). Three septa were taken every week from each of the two batches (i.e., freezer and traps) and soaked in 8 ml of hexane overnight. Samples were then spiked with 10 µl of

200 ppm (*E,Z*) 10,12-hexadecadienal, concentrated to 1000 µl by passing pure nitrogen over them before analysis on the GC-MS with conditions previously described in Chapter 3.4.

4.2.4 Wind tunnel experiment

Experiment 6 - Male response to sex pheromones in a wind tunnel

This experiment was designed to test the behaviour of males in the wind tunnel in the absence and presence of pheromones. Males were tested using an empty cage (blank), a 4:1 blend of (*E,E*)-10,12:16Al and (Z)-11:16Al (Blend B) and a 4:1:1 blend of (*E,E*)-10,12:16Al, (Z)-11:16Al and (Z)-11:18Al (Blend K). For each blend, a 13 mm diameter glass fibre filter paper disc was loaded with 4 µg of the mixture.

A total of thirty 3-day old unmated males were used for each treatment. Individual males held in 150-ml meshed plastic cages were transferred to the wind tunnel room at least 7 hours before the experiment to acclimatise. Either the empty cage or the pheromone blend was placed at the upwind end and each male was released from the cage at the downwind end of the tunnel. Males were allowed to respond for 5 minutes after one end of the cage was opened, and were scored for the following behaviours: (1) take off (2) upwind flight (3) downwind flight (4) approach to the source (5) contact with source and (6) attempt at clasper extrusion/copulating. All observations were recorded using The Observer (version 3.0) programme.

4.3 Results and discussions

4.3.1 Pheromone extraction and analysis

Gas chromatographic traces showed the presence of three identifiable pheromonal compounds from the concentrated extracts of air collection from calling females (Figure 4.6) and four

compounds from the gland extracts (Figure 4.7). Compounds I, II and IV were common to both the gland and air collections while compound III was only found in the gland extract.

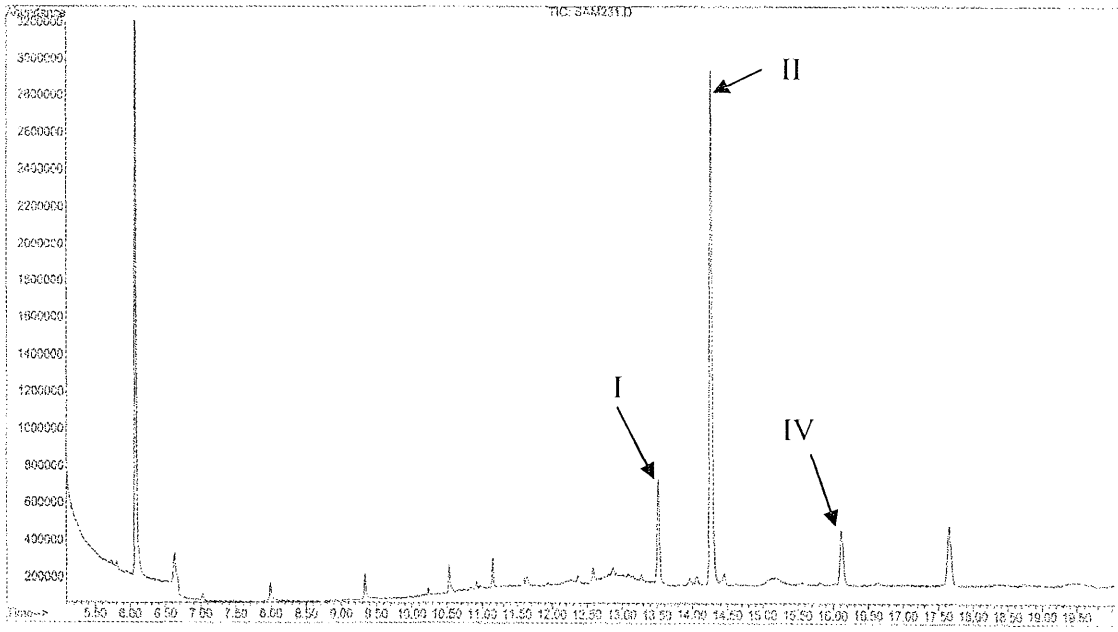


Figure 4.6. Gas chromatogram of *E. huegeliana* female air collection.

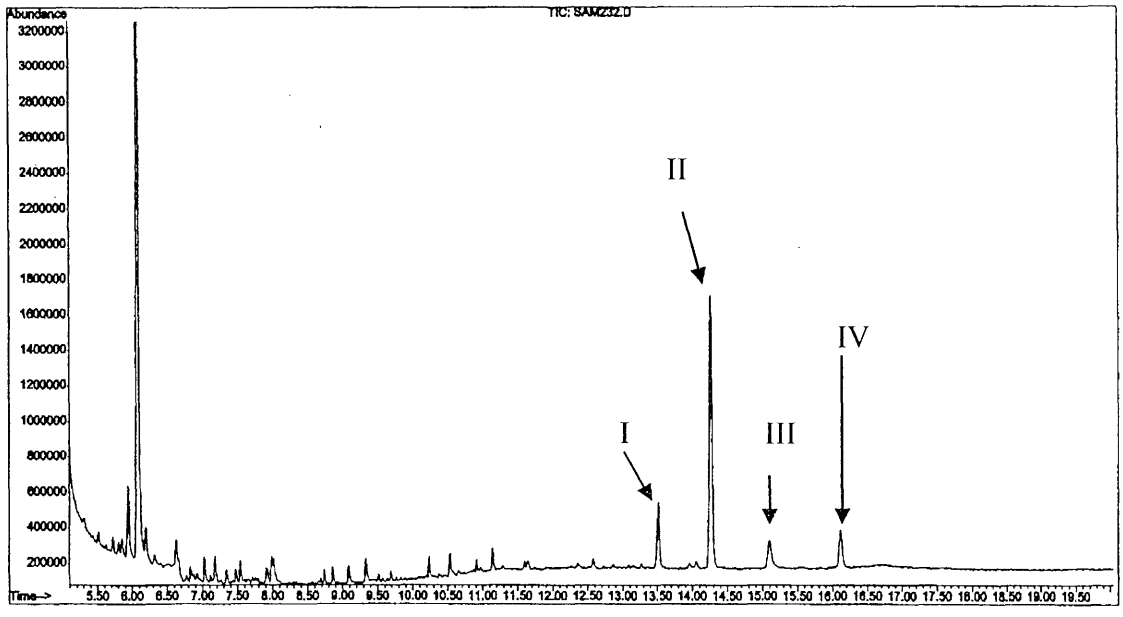


Figure 4.7. Gas chromatogram of *E. huegeliana* female gland extract.

The retention times of the four pheromonal compounds found in *E. huegeliana* females and compounds used as standards are given in Table 4.3.

Compound	Retention time (min)
Compound I	13.519
Compound II	14.266
Compound III	15.096
Compound IV	16.118
(*Z*)-11:16Al	13.518
(*Z*)-9:16Al	13.385
(*Z*)-7:16Al	13.292
16Al	13.610
(*E*,*E*)-10,12:16Al	14.262
(*E*,*Z*)-10,12:16Al	14.018
(*E*,*E*)10,12:16OH	15.054
(*Z*)-7:18Al	16.001
(*Z*)-9:18Al	16.103
(*Z*)-11:18Al	16.121

Table 4.3. Retention times of pheromonal compounds found in *E. huegeliana* females and compounds used as standards.

Figure 4.8 shows the spectra of compound II found in both the air collection and gland extracts, which show a relatively strong molecular ion at m/z 236 (14%) corresponding to a possible molecular formula of $C_{16}H_{28}O$, with other large fragments at m/z 67(100%), 81(48%) and 95 (24%). These data were consistent with a conjugated C_{16} aldehyde. The position of unsaturation of the insect-produced compound was predicted by comparison of its mass spectral data and retention time with those of the synthetic standards (*E*,*Z*)-10,12, and (*E*,*E*)-10,12-hexadecadienal (Figures 4.9 and 4.10, Table 4.3), two compounds that have been identified in other *Earias* spp. (Hall *et al.*, 1980; Cork *et al.*, 1988). The same retention time

and co-elution with (*E,E*)-10,12-hexadecadienal revealed its identity. The mass spectrum of the insect-produced material also matched that of (*E,E*)-10,12-hexadecadienal.

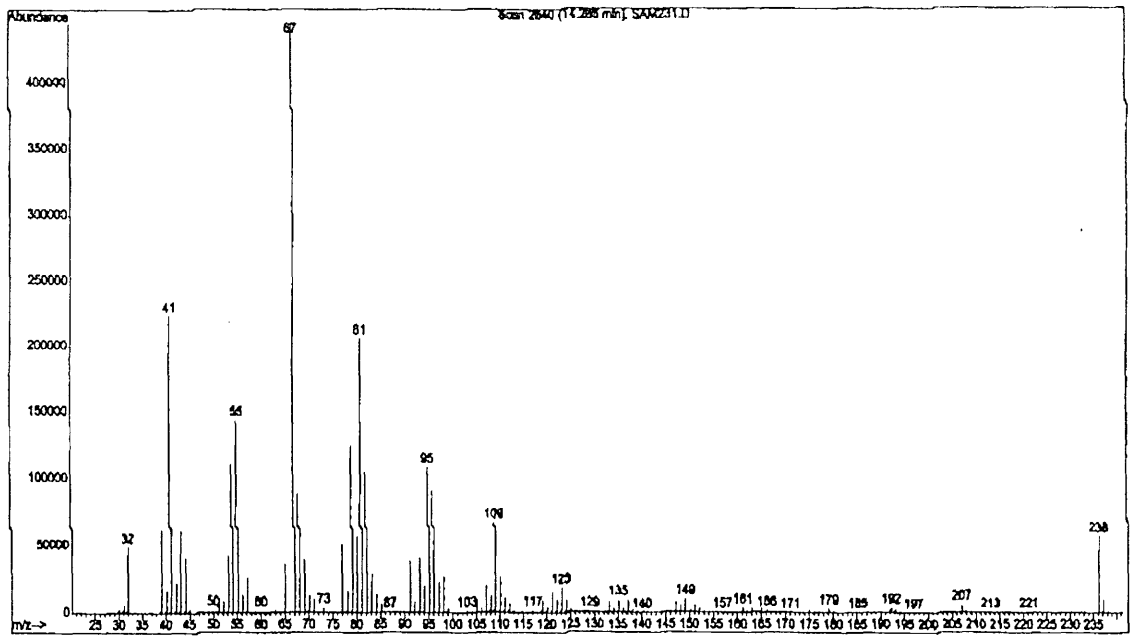


Figure 4.8. Mass spectra of compound II found in *E. huegeliana* female air collection and gland extracts.

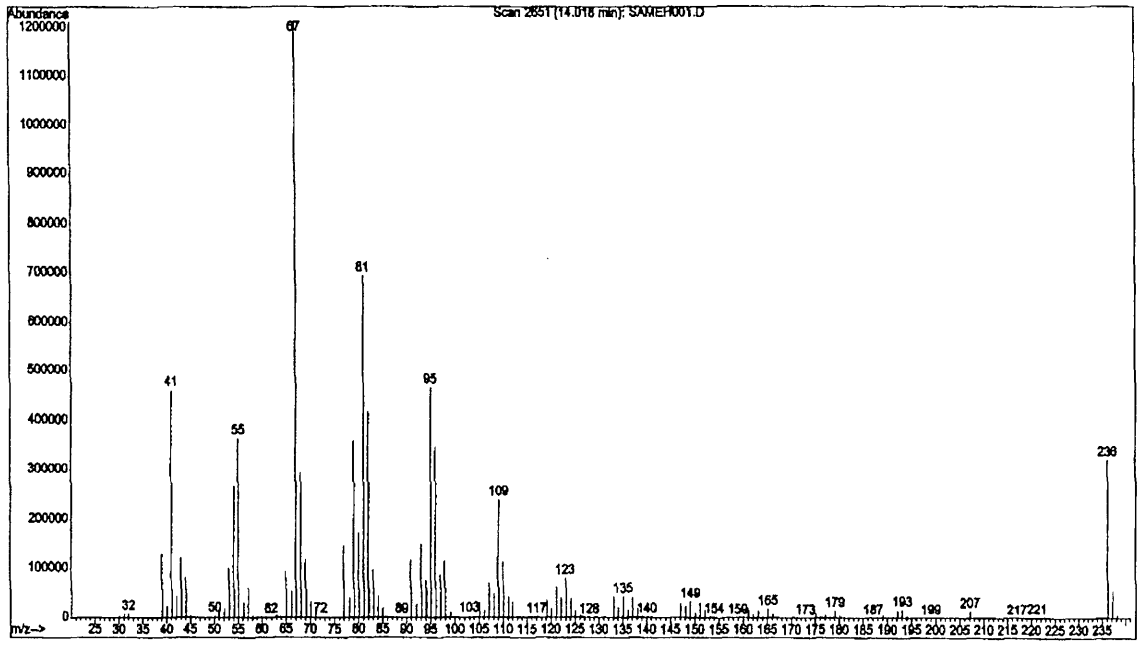


Figure 4.9. Mass spectra of standard (*E*,Z)-10,12-hexadecadienal.

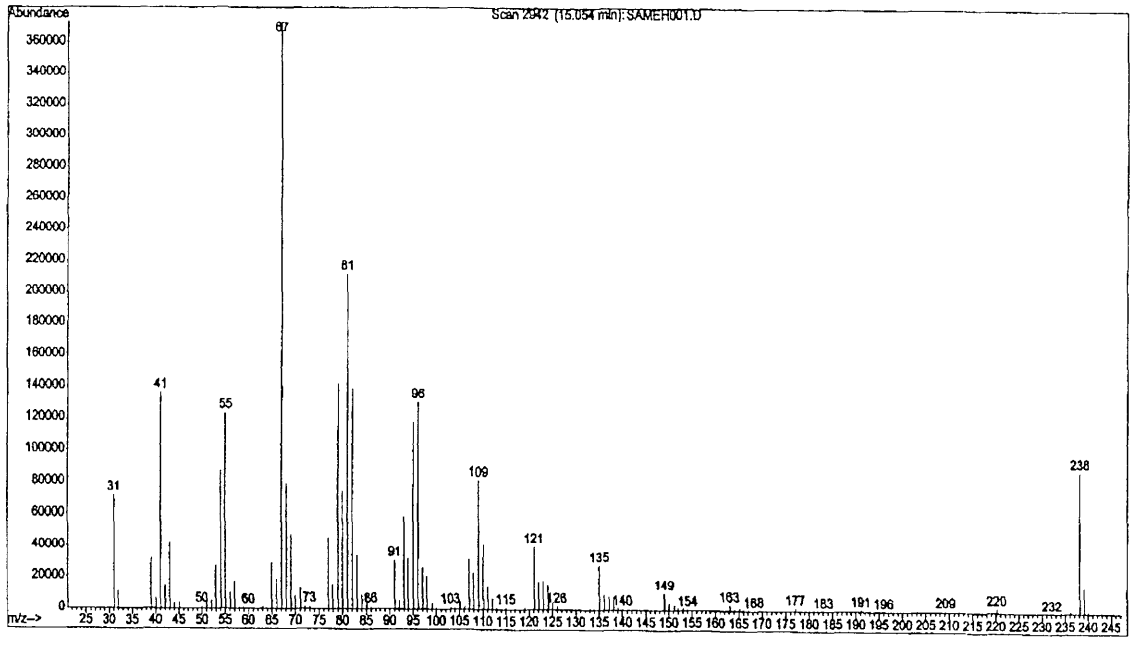


Figure 4.10. Mass spectra of (*E*,*E*)-10,12-hexadecadienal.

Similarly, spectra of compound III (Figure 4.11) gave a relatively strong molecular ion at m/z 238 (11%), suggesting a possible molecular formula of $C_{16}H_{30}O$. Ions at m/z 67 (100%), 81 (54%), 96 (30%) and 31 (29%) were consistent with a conjugated C_{16} alcohol. The retention time and mass spectrum of this compound matched that of (*E*,*E*)-10,12-hexadecadienol.

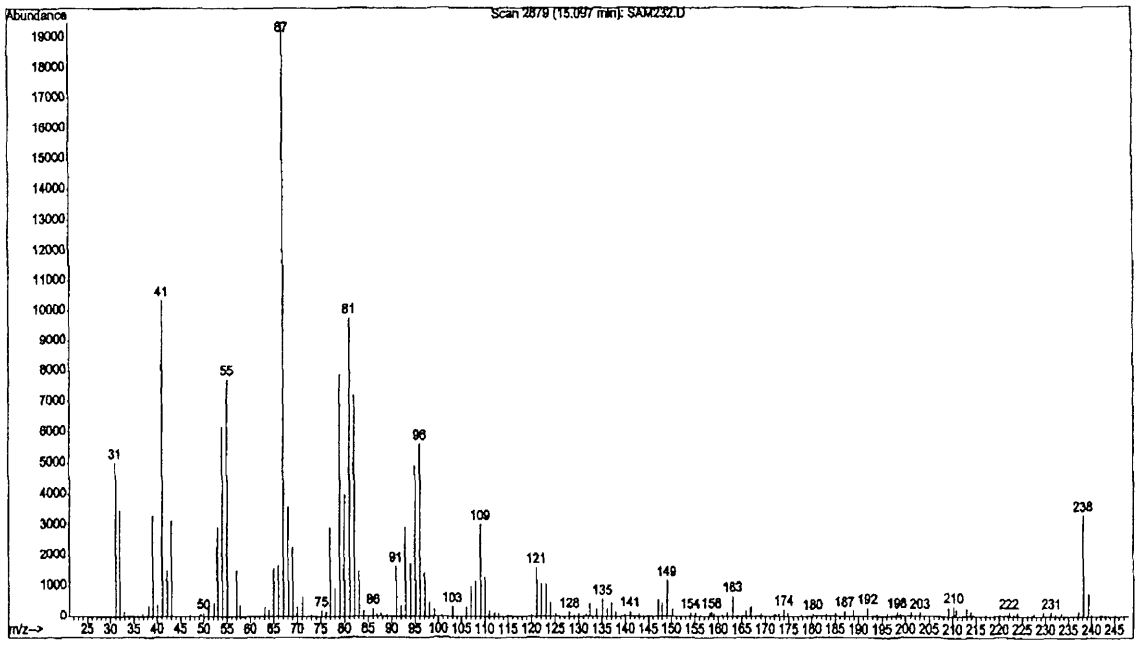


Figure 4.11. Mass spectra of compound III.

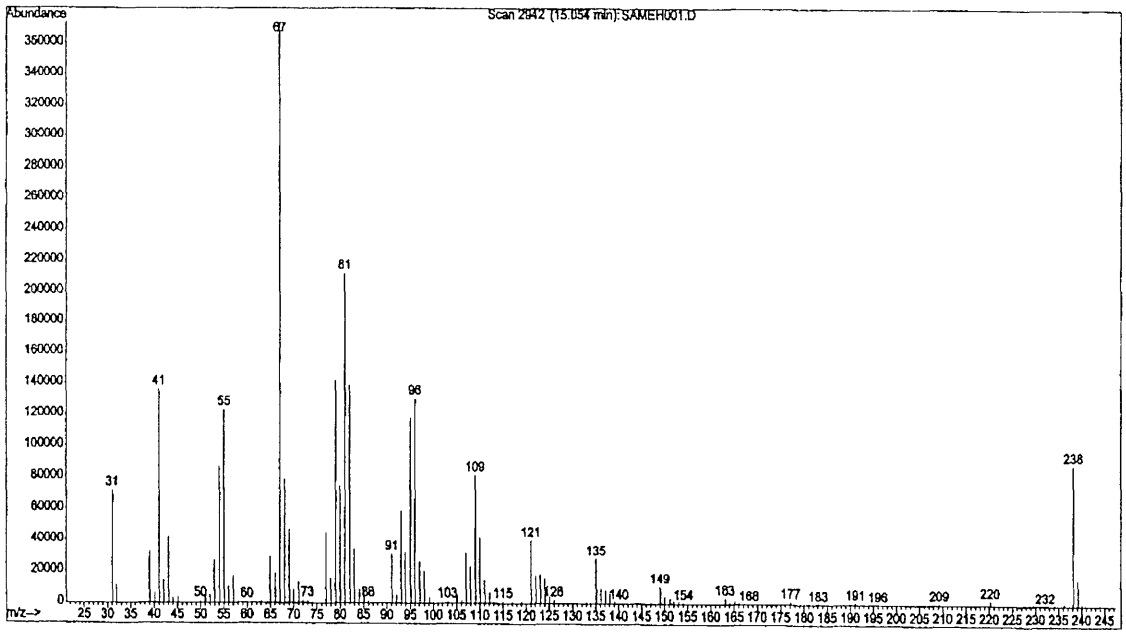


Figure 4.12. Mass spectra of standard (*E*,*E*)-10,12-hexadecadienol.

Compounds I (Figure 4.13) and IV (Figure 4.15) were identified from the spectra and retention times to be identical to authentic standards (Z)-11-hexadecenal (Figure 4.14) and (Z)-11-octadecenal respectively (Figure 4.16).

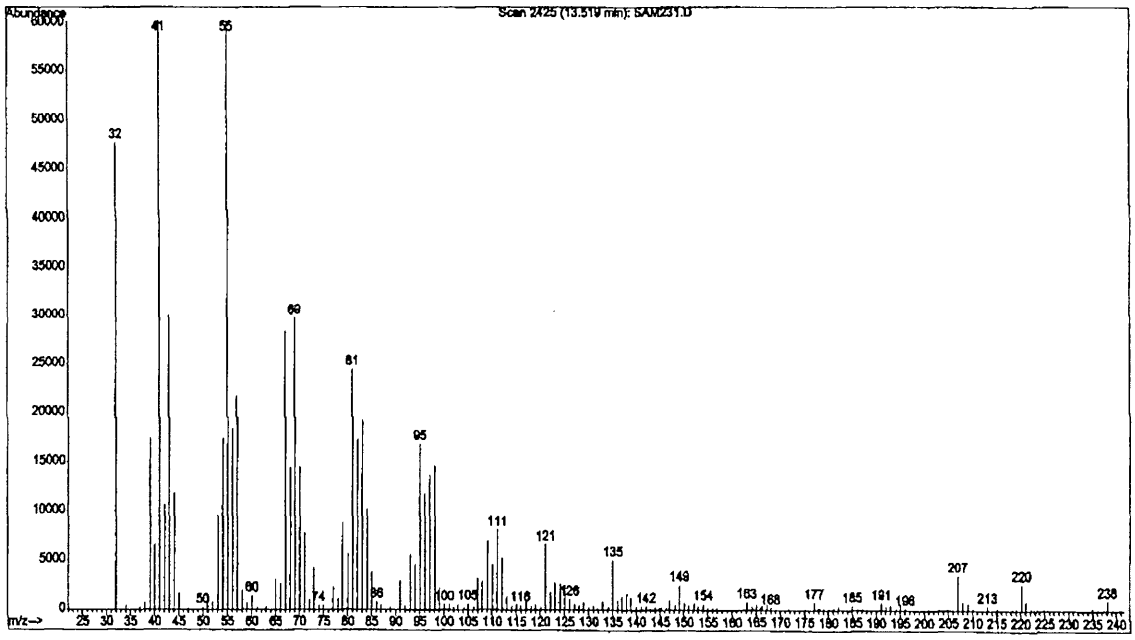


Figure 4.13. Mass spectra of compound I.

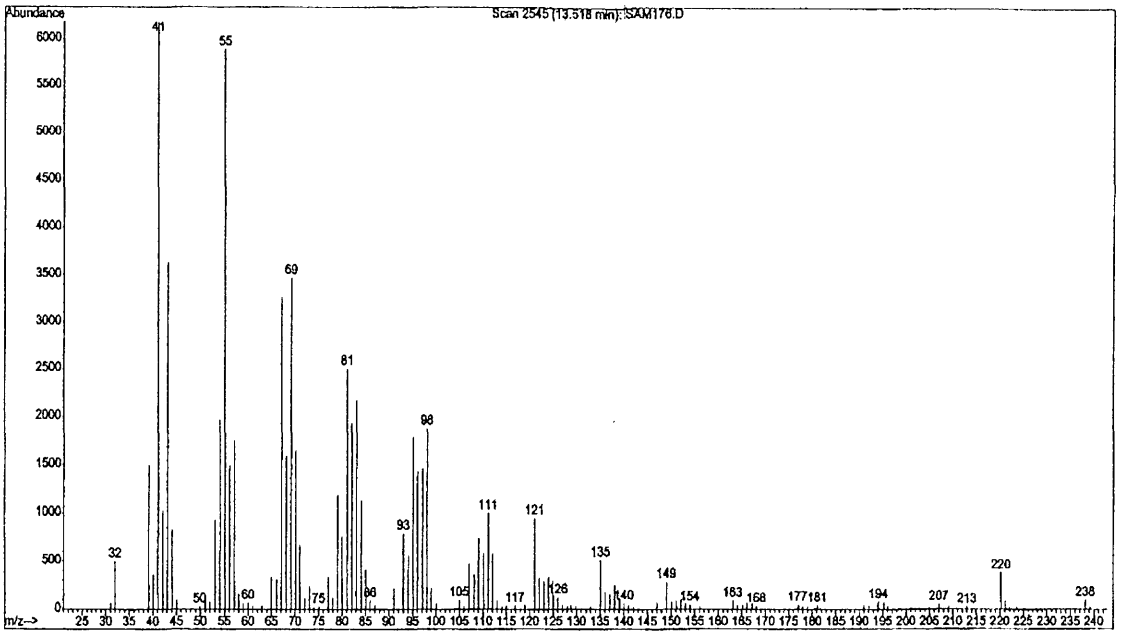


Figure 4.14. Mass spectra of standard (Z)-11-hexadecenal.

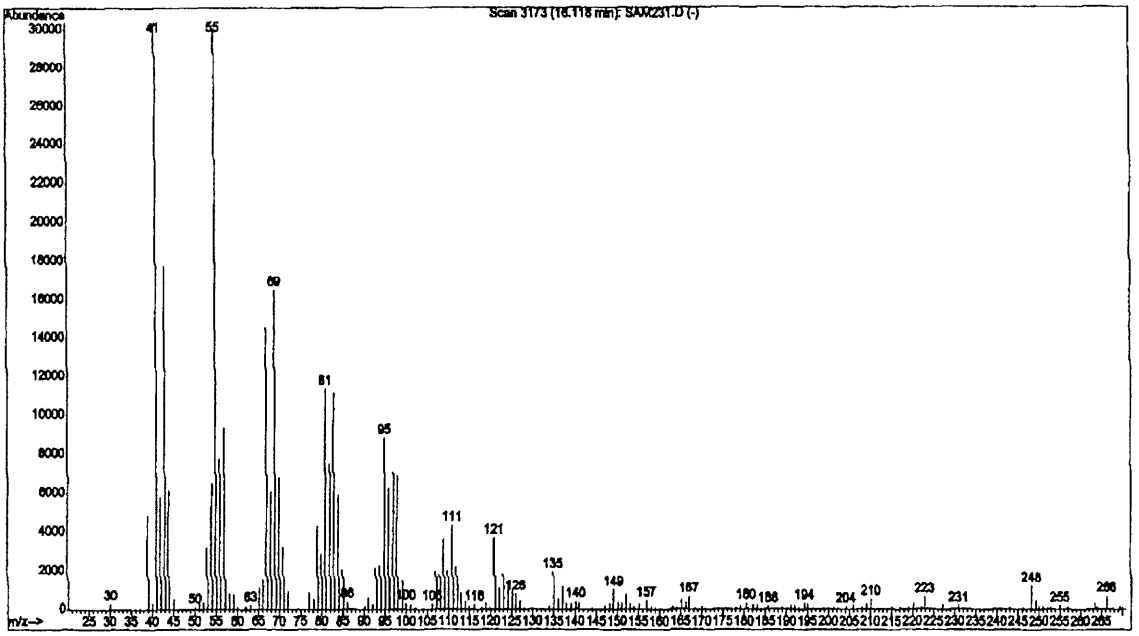


Figure 4.15. Mass spectrum of compound IV.

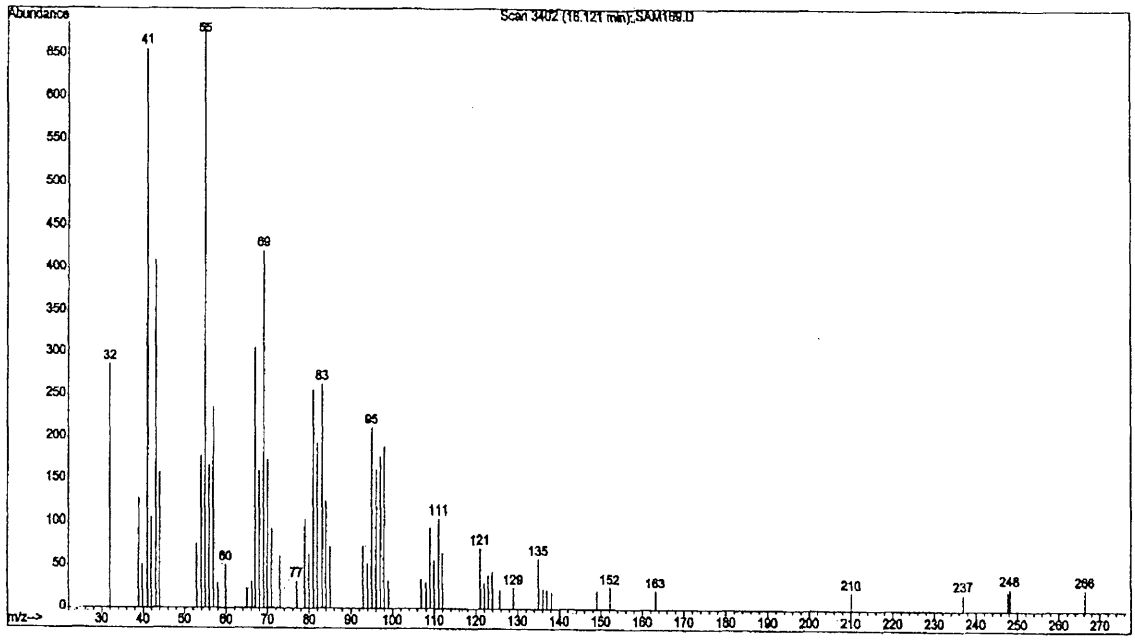


Figure 4.16. Mass spectra of standard (Z)-11-octadecenal.

4.3.2 Field bioassays

Experiment 1a – Comparison of blends A, B and C

Experiment 1a tested two partial blends B and C against the full blend A. Trap catches for this experiment were low due to pyrethroid spray on cotton by the farmer a few days before starting this experiment.

The results indicated highly significant effects of blend ($P < 0.001$) and time factors ($P < 0.001$). Mean trap catches per night are shown in Figure 4.17. Comparison of the means showed no significant difference between blends A and B ($P = 0.105$) but highly significant differences ($P < 0.001$) between blends A and C, between blend A and control (CTRL) and between blend B and control. Blends B and C were only significantly different at $P = 0.018$, and there were no significant differences between blend C and control. These results suggest that for any meaningful attraction of males to the pheromone to occur, both the major

component (*E*,*E*)-10,12-hexadecadienal and a minor component (*Z*)-11-hexadecenal, should be present in the blend.

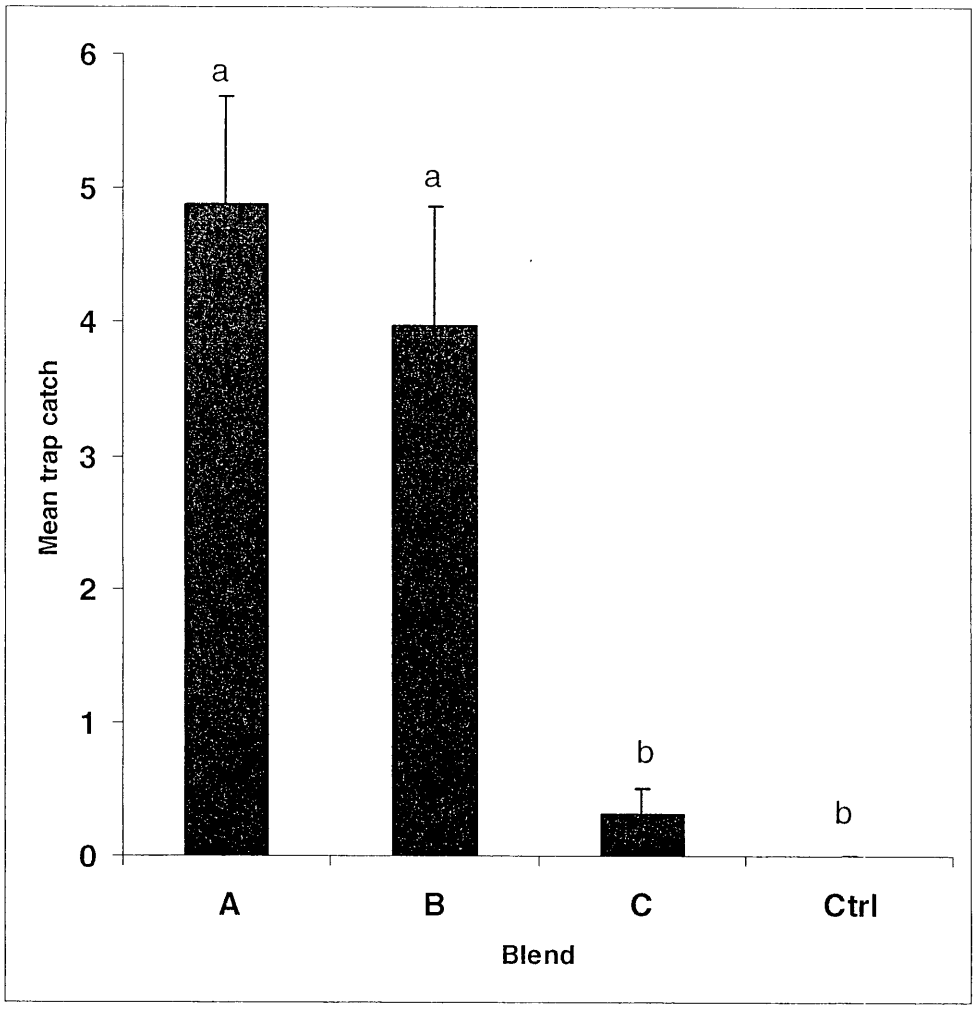


Figure 4.17. Experiment 1a. Mean (± s.e) catches of *E. huegeliana* males in traps baited with blends A, B, C and CTRL in cotton, "Glen Shee", Oakey, Qld. Columns with common letters are not significantly different (P > 0.05).

Experiment 1b - Comparison of blends A, E and F

This experiment compared two other partial blends (E and F) with the full pheromone blend (A). Only Blend A caught moths during the 3-day period with mean trap catches of 21 moths per night whilst blends E and F did not catch any moths (Figure 4.18). Blend E was made up

of a 4:1 mix of (*E,E*)-10,12-hexadecadienal and (*E,E*)-10,12-16OH, while blend F consisted of a 1:1 mix of (*Z*)-11: 16Al and (*Z*)-11:18Al.

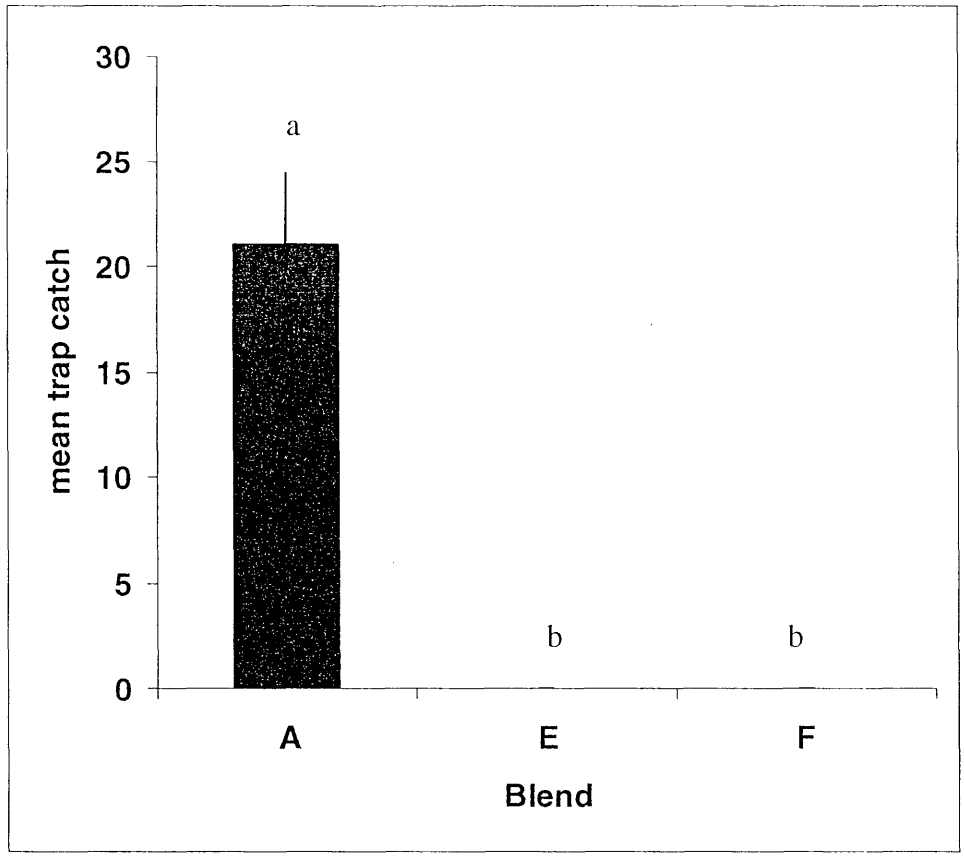


Figure 4.18. Experiment 1b. Mean Delta trap catches of blends A, E and F in cotton, "Glen Shee", Oakey, Qld. Columns with common letters are not significantly different ($P > 0.05$).

The analysis of variance yielded highly significant effect of blend type ($P < 0.001$) but no significant effects of trap rotation, location and day. Again, these results suggest that (Z)-11: 16Al and (*E,E*)-10,12:16Al are essential for the formulated pheromone blend to attract males, as observed in Experiment 1a.

Male response to sex pheromones in the field

E. huegeliana moths were observed to fly in the characteristic zigzag manner towards the full component blend (A) but not to the partial blends (E and F). On no occasion was a single insect seen flying around these partial blends, which did not catch any moths during this experiment (Figure 4.18).

With blend A, moths were observed to approach the traps during the second half of the night (Figure 4.19). Less than 1% approached between 2000–2200 h, with approaches increasing from 11.2% at 2400 h to 51.3% at 0400-0600 h. This period was also observed in the laboratory to be the time period when the female exhibited calling behaviour. These results suggest that pheromone production in *E. huegeliana* females appeared to be synchronised with male response during the second half of the night. Peak periods of approaches at particular times of the night correlated with trap catches. Peak trap catches (68%) occurred between 0400-0600 h, the time when peak approaches were also observed (Figure 4.20).

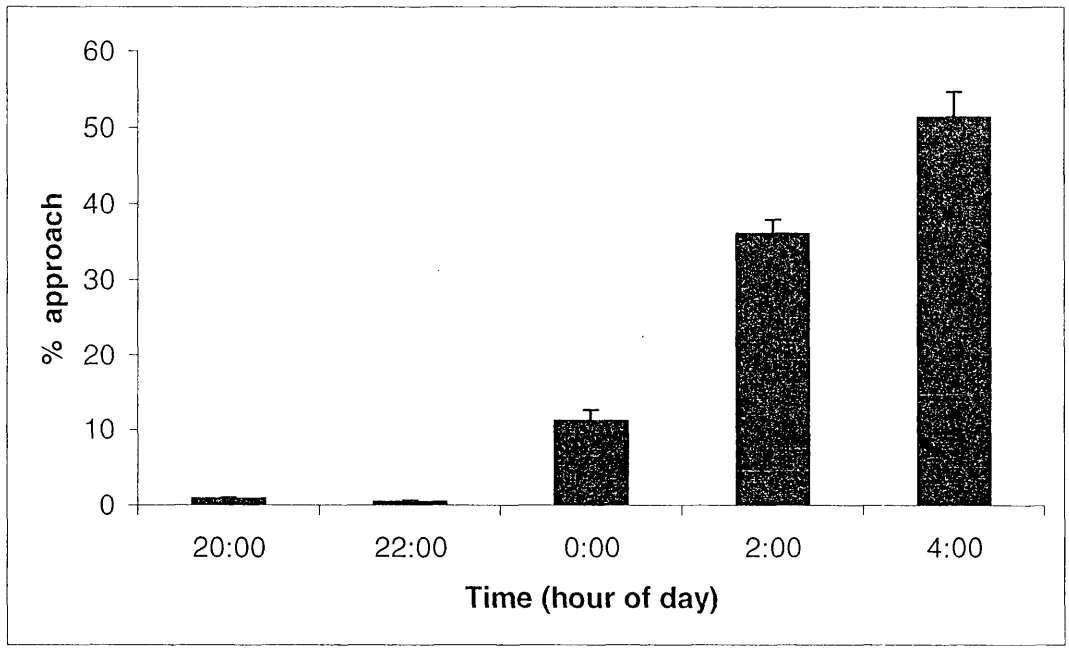


Figure 4.19. Percentages of *E. huegeliana* males approaching the pheromone trap (baited with blend A) per night at different times of the night over the three day period in cotton, "Glen Shee", Oakey, Qld.

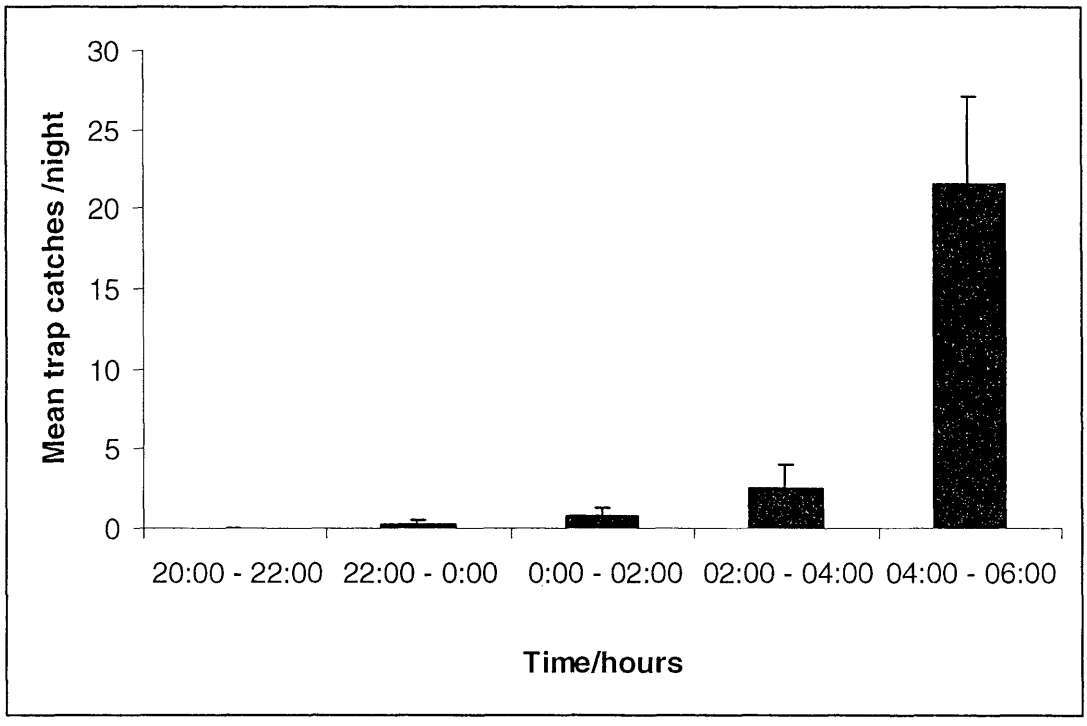


Figure 4.20. Mean catches of *E. huegeliana* males in Delta traps baited with blend A at different times of the night in cotton, "Glen Shee", Oakey, Qld.

Experiment 2 – Comparison of other partial blends

Mean catches of *E. huegeliana* males with blends B, D, E, F, G and H are shown in Figure 4.21.

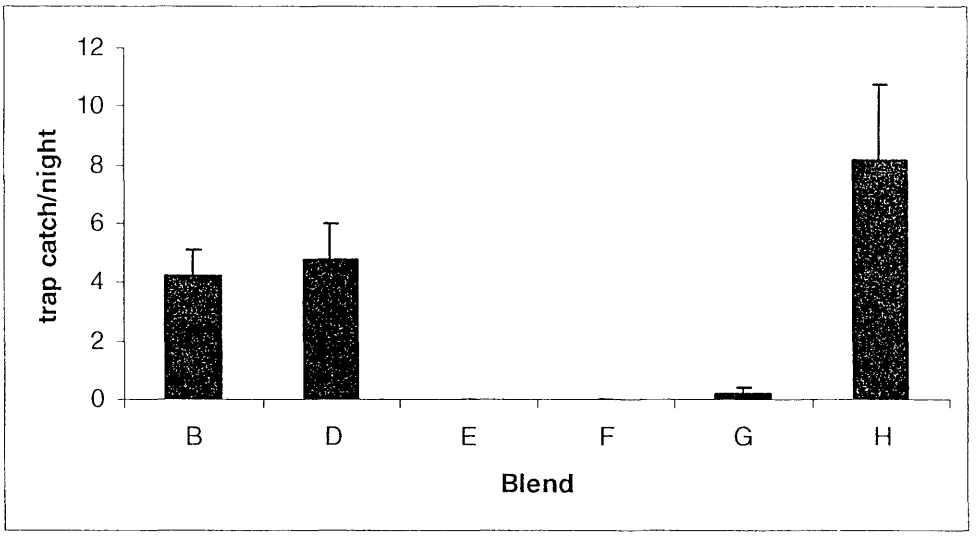


Figure 4.21. Experiment 2. Mean (± s.e) trap catches of blends B, D, E, F, G and H in cotton, "Yanco", Cecil Plains, Qld.

As in Experiment 1b, blends E and F did not catch any *E. huegeliana* males confirming that these blends were not attractive. Blend E did not contain (Z)-11:16Al and (Z)-11:18Al while blend F did not have (*E,E*)-10,12:16Al and (*E,E*)-10,12:16OH. Blend G caught only 1 male on one night. Blend G consisted of all the three minor components and not the major component (*E,E*)-10,12:16Al. Blend H also caught significant numbers of males. This blend was made up of a 1:1 mix of (*E,E*)-10,12:16Al and (*Z*)-11:16Al. These results suggest that a blend ratio range of 1:1 to 4:1 of (*E,E*)-10,12:16Al to (Z)-11:16Al would be effective in attracting male *E. huegeliana,* as found with blends B and H.

It can be deduced from the above blend combination trials that the only essential components of the pheromone blend that are needed for effective attraction of *E. huegeliana* males are the major component (*E,E*)-10,12-16Al and the minor component (Z)-11:16Al, as has been observed in Experiments 1a and 1b.

Experiment 3 – Comparison of two types of traps using blend B

The AgriSense® trap was compared with the Delta trap using blend B as the pheromone lure. Only the Delta traps caught male rough bollworm moths while the AgriSense® trap did not catch any moths during the 4 days of the trial (Table 4.3). GLM analysis showed no significant effect of day ($P = 0.100$) but a highly significant effect of the trap type ($P < 0.001$).

	AgriSense® Trap	Delta Trap
Day 1	0 ± 0	0.4 ± 0.2
Day 2	0 ± 0	1.2 ± 0.6
Day 3	0 ± 0	0.4 ± 0.2
Day 4	0 ± 0	1.6 ± 0.8
Mean (± s.e)	0 ± 0	0.9 ± 0.3

Table 4.3. Experiment 3. Mean (± s.e) catches of *E. huegeliana* per rotation interval in AgriSense® and Delta traps in bladder ketmia, Mondure, Qld.

The numbers of adult rough bollworms during this experiment were low. Nevertheless, the results clearly suggest that Delta traps are likely to be more efficient traps for trapping *E. huegeliana* than AgriSense® traps.

Experiment 4 – Comparison of the sex pheromones of *E. vitella* and *E. huegeliana*

The numbers of *E. huegeliana* moths in the field were low when this experiment was done. Mean trap catches for the four *E. huegeliana* blends (EH1, EH2, EH3, EH4) and the *E. vitella* blend (EV) ranged between 0.1 and 0.8 per night (Table 4.4). The significant factors affecting trap catches in this experiment were blend, day, row and position ($P = 0.010$, $P < 0.001$, $P < 0.01$, and $P = 0.050$, respectively). The attractiveness of the *E. vitella* blend was not significantly different from any of the *E. huegeliana* blends except blend EH1.

Blend	Mean Trap catches/night
EH1	0.1 ± 0.1^{a}
EH2	0.3 ± 0.1^{b}
EH3	0.3 ± 0.1^{b}
EH4	0.6 ± 0.2^{b}
EV	0.8 ± 0.3^{b}

Table 4.4. Mean (± s.e) trap catches of four *E. huegeliana* blends (EH1, EH2, EH3 and EH4) and *E. vitella* blend (EV) in cotton, "Yanco", Cecil Plains, Qld. Means in the same column followed by common letters are not significantly different ($P > 0.05$).

Despite the low numbers in the traps, the results suggest that the addition of (*Z*)-11:18Al in blend EH4 increased trap catches. Blends EH1, EH2 and EH3 consisted of (*E,E*)-10,12:16Al and (*Z*)11:16Al at different ratios but not (*Z*)-11:18Al, whereas, blend EH4 had all these 3 compounds. The *E. vittella* blend (EV) contained the same chemical blend as EH4 but in a

different ratio. There were no significant differences between the mean catches with EH4 and EV. On the other hand, blends EH2 and EH3 which had no (*Z*)-11:18Al, were not significantly different from EH4 and EV, suggesting the suitability of using a two-component blend of (*E,E*)-10,12:16Al and (*Z*)11:16Al in a ratio of 2:1 or 3:1 for *E. huegeliana*.

Experiment 5 – Weathering of pheromone components

This experiment gave an indication as to how long *E. huegeliana* pheromone lures may last under field conditions. The average temperature recordings during the weathering experiment are presented in Figure 4.22, which ranged between 19.3 and 23.3°C. This period covered late summer and early autumn in Armidale, NSW, which may be slightly lower compared to the temperatures in the cotton areas.

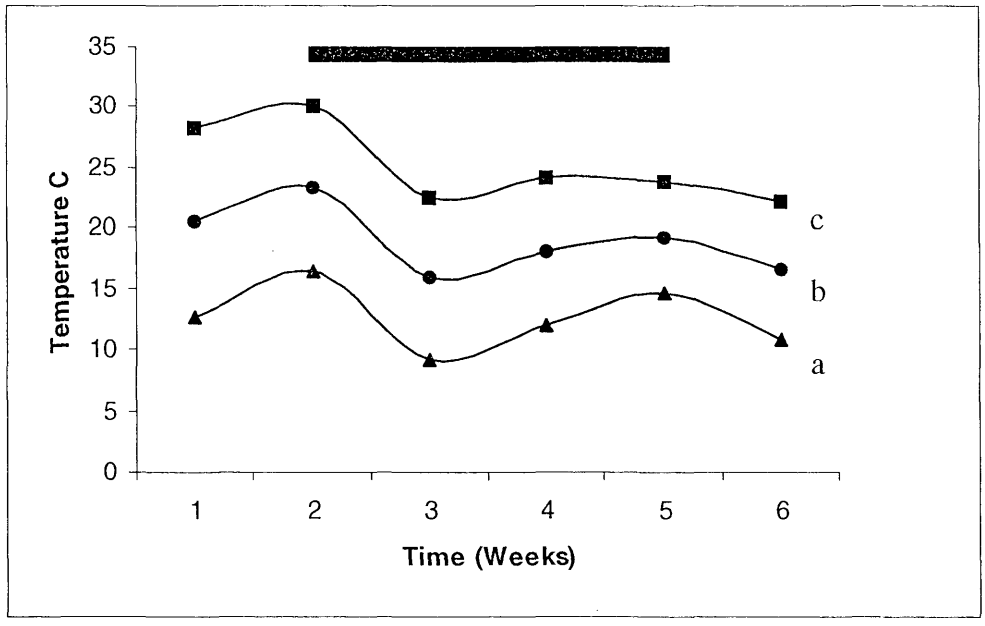


Figure 4.22. Mean weekly temperatures between February and March 2004 at Armidale, NSW (a – minimum; b – average of a and c; c- maximum). Dark bar indicates duration of the weathering experiment (18th Feb to 16th March). (Source: UNE weather records, http://fehps.une.edu.au/x/weather/news .html).

Changes in the amounts of the major component, (*E,E*)-10,12-16Al and the minor component, (Z)-11-16Al are shown in Figure 4.23. Septa loaded with individual components kept in the freezer and exposed to normal weather conditions in Delta traps were compared.

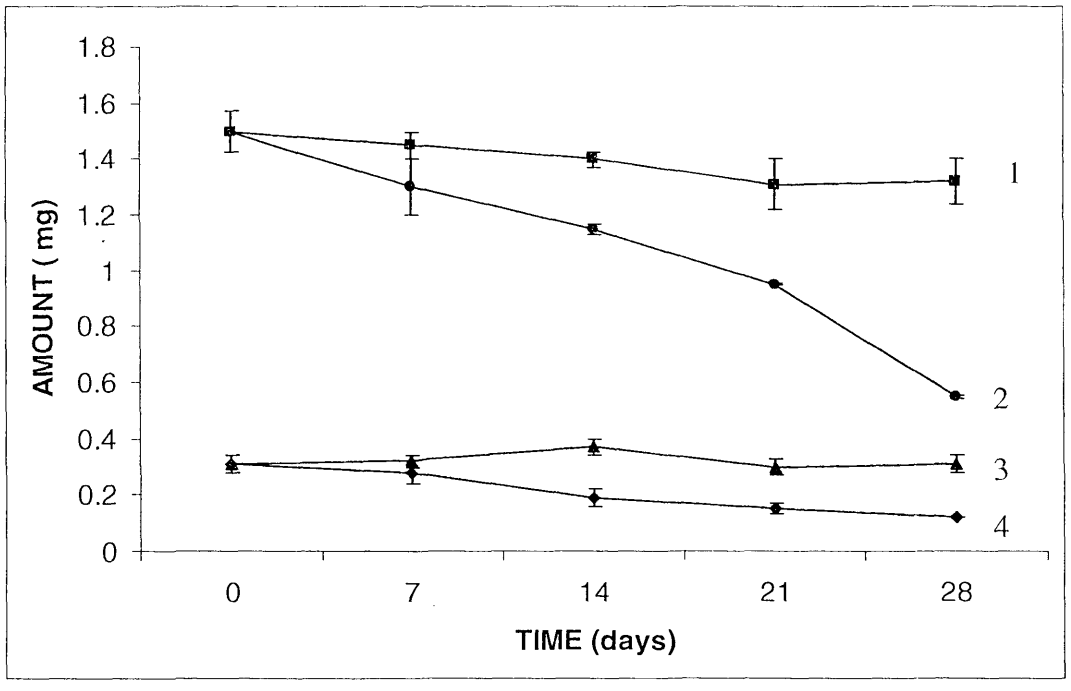


Figure 4.23. Degradation of the sex pheromone components of *E. huegeliana*. 1 and 2 = (*E,E*)-10,12-16Al stored in freezer and exposed to weather, respectively; 3 and 4 = (Z)11-16Al stored in freezer and exposed to weather, respectively.

Figure 4.23 shows that the active components of the blend were reduced to less than half the original amount when exposed to normal weather conditions, whereas, the freezer samples stayed relatively constant over the 4 week period. The rate of loss of the active components in the septa exposed to weather was approximately linear throughout the experiment. It is evident from the results that lures prepared on rubber septa with 2 mg loadings would last close to a month between a temperature range of 19.3 and 23.3°C before needing replacement in the field.

4.3.3 Wind tunnel bioassays

Figure 4.24 shows the various observations made on the male behaviour in the absence of a pheromone (blank or empty cage) and in the presence of two blends, B and K.

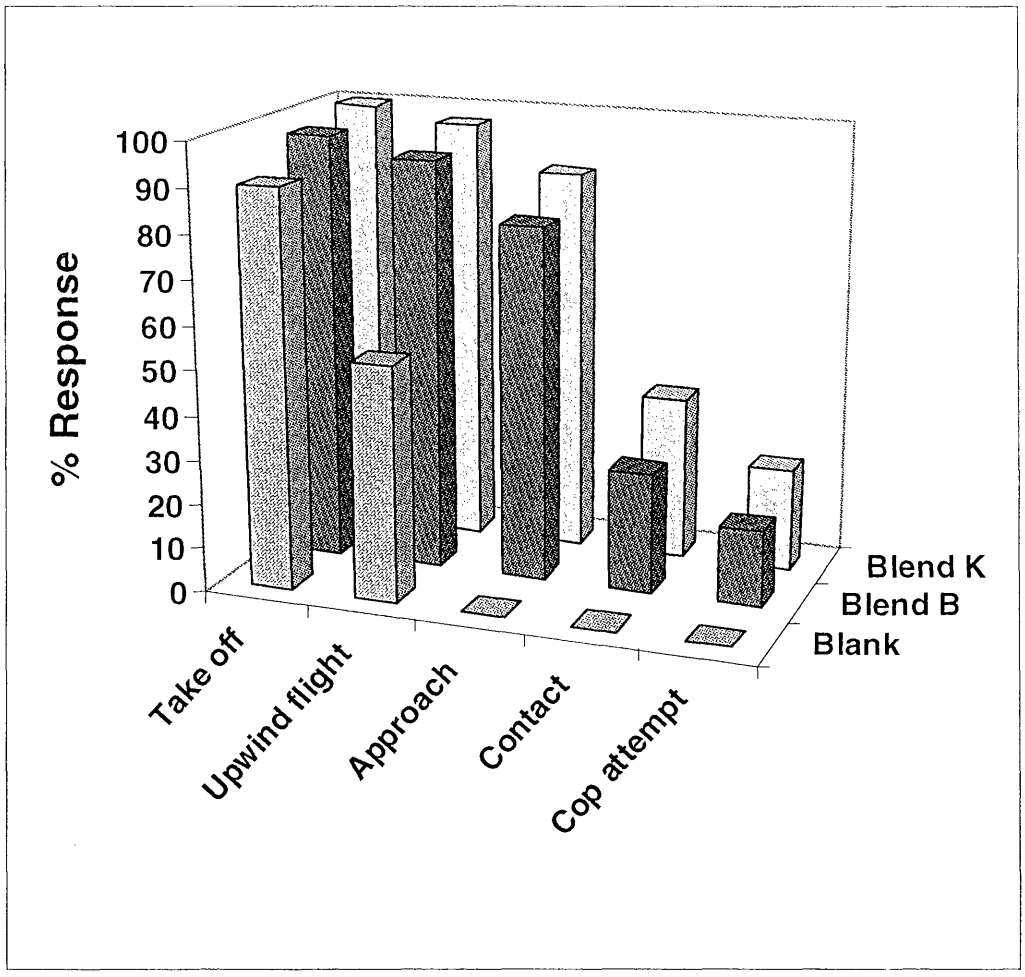


Figure 4.24. Experiment 6. Percentages of *E. huegeliana* males exhibiting different behaviours in the absence of a pheromone source (blank or empty cage) and in the presence of two blends, B (4:1 ratio of (*E*,*E*)-10,12:16Al and (*Z*)-11:16Al) and K (4:1:1 ratio of (*E*,*E*)-10,12:16Al, (*Z*)-11:16Al and (*Z*)-11:18Al). Males used were 4 days old.

In all cases where males were presented with empty cages (blank, ie, no pheromone source), 90% of them took off but only 50% flew upwind. Males usually took off and either spent sometime upwind before coming to rest or moved downwind, and there were no approaches

or contact in the absence of pheromone source. On the other hand, male response behaviours such as approach, contact and copulatory attempt, were observed in males tested with blends B and K.

The analysis of deviance from the GLM indicated that takeoff was not significantly different for the three treatments ($P = 0.108$). Many more male moths approached, made contact and attempted to copulate with the two pheromone blends than the empty cages (blank). The analysis of deviance indicated highly significant differences in treatments for upwind flight, approach, contact ($P < 0.001$) and copulatory attempt ($P = 0.004$).

Comparison of the mean response of upwind flight using contrast in R indicated no significant difference between blends B and K ($P = 0.741$) but highly significant differences between the two blends B and K, and the blank ($P < 0.001$). Similar trends were observed for approach, contact and copulatory attempt. These results are summarised in Table 4.9 below:

Behaviour	Comparison	P- Value
Approach	Blend B vs K	0.603
	Blend B vs blank	< 0.001
	Blend K vs blank	< 0.001
Contact	Blend B vs K	0.341
	Blend B vs blank	0.010
	Blend K vs blank	< 0.001
Copulatory attempt	Blend B vs K	0.446
	Blend B vs blank	0.053
	Blend K vs blank	0.006

Table 4.9. P-values showing the level of significance in the different treatments for *E. huegeliana* male response behaviours in a wind tunnel.

The results show no significant differences between blends B and K, supporting the previous conclusions that the three blend mix of (Z) –11:16Al, (Z)-11:18Al and (*E*,*E*)-10,12-16Al would be equally effective in trapping rough bollworms as the two blend mix of (Z)–11:16Al and (*E*,*E*)-10,12-16Al. In qualitative terms however, more contacts were made with the 3-component blend (K) compared with the 2-component blend (B).

4.4 Summary and implications for management

The sex pheromone of female *E. huegeliana* has been identified as a mixture of the major component (*E*,*E*)-10,12-16Al and the minor components (Z)–11:16Al and (Z)-11:18Al in a ratio of 4:1:1, respectively. Most lepidopteran sex pheromone systems are multi-components and the relative composition may be critical to be effective attractants. Field trapping studies not only indicated that a 4:1 ratio of (*E*,*E*)-10,12-16Al and (Z)–11:16Al was effective in attracting male moths, but also, that these two compounds were essential for activity of the blend.

The pheromone components identified in this species, (*E*,*E*)-10,12-16Al, (Z) –11:16Al and (Z)-11:18Al, are similar to the blend of the closely related species, *E. vittella*. Similarities in morphology, pheromone components and time of release of pheromones by females raise questions about their reproductive isolation and whether they are really different species. Electrophoretic or DNA studies to determine genetic differences might help resolve this. An experiment to determine the level of cross mating and hybridisation between males and females of the two species in the laboratory might also provide some useful information.

The weathering experiment indicated that pheromone lures at 2 mg loadings would last for one month in the field. The length of time that the lures remain attractive may, however, decline with higher average temperatures and exposure to direct sunlight since the major

component (*E*,*E*)-10,12-16Al is known to photochemically isomerise to the (*E*,*Z*) and (*Z*,*E*) isomers (Cork *et al.*, 1988). Cork *et al.* (1988) recommended protection of lures with sleeves of aluminium tubing or foil until dispensers that completely prevent isomerisation are developed.

In two other related species, *E. vittella* and *E. insulana* where communication is via the use of these same chemical components, the addition of (*E*,*Z*)-10,12-16Al reduced catch drastically while the (*Z*,*E*)-10,12-16Al did not have any effect (Cork *et al.*, 1988). Work remains to be done in this area with respect to *E. huegeliana*. The outcome of such a research could importantly influence the cost and how long one can use lures in pheromone traps in the field.

The time of peak male response to the pheromone as indicated by peak catches in traps appeared to be synchronised with the time at which females were observed to be calling or releasing the pheromone in the laboratory. It was found to be restricted to the second half of the night, especially between 0400 and 0600 h AEST in the field.

The pheromone lures developed in this work could be used as an effective management tool for *E. huegeliana*. However, for monitoring purposes, further data need to be collected to determine the correlation between trap catches and field infestations. Mating disruption applications might also be possible with this pest but a lot of data need to be collected before any recommendations can be made. This technique has been used successfully in Pakistan (Critchley *et al.*, 1987) for the closely related species *E. vittella*. It may not be economical to use mating disruption in areas where *E. huegeliana* is still a minor pest as occurs in southern Australia, but may be useful in northern Australia.

CHAPTER 5

SEX PHEROMONES OF THE COTTON TIPWORM, *CROCIDOSEMA PLEBEJANA* (ZELLER) (LEPIDOPTERA: TORTRICIDAE)

5.1 Introduction

The cotton tipworm, *Crocidosema plebejana* (Zeller) is a minor to occasionally serious pest of seedling cotton in Australia (Evenson & Basinski, 1973; Bishop & Blood, 1978). The terminal tunnelling of the larvae destroys the single stem habit of the cotton seedling.

Hamilton (1985) asserted that natural enemies are unlikely to have a major influence on the population dynamics of the cotton tipworm. Identification of the sex pheromone would therefore be useful for monitoring the population density of adults and perhaps as an environmentally safe method of controlling them.

Sex pheromones have been identified in many tortricids to date. A restricted range of sex attractants, comprising mainly C_{12} and C_{14} alkenyl acetates, with few C_{10} and C_{16} compounds have been reported to be in use by tortricids (Roelofs & Brown, 1982; Arn *et al.*, 1986). The major sex pheromone gland components of 30 species of Australian tortricid moths have been studied (Horak *et al.*, 1988). More than half of the species have (*Z*)-11-, (*E*)-11-, and/or (*Z*)-9-tetradecenyl acetates as the principal components, but these compounds were not detected

in some plesiomorphic taxa where combinations of (Z)-5 and (Z)-7 or (Z)-8 and (Z)-10 isomers were present.

No study of cotton tipworm pheromones has been published to date. The present work represents the first attempt to verify the use of long distance pheromones in mate attraction and to identify the components of the sex pheromones in this species. In this thesis, the sex pheromone components of *C. plebejana* have been isolated and identified as a result of studies utilising gas chromatography, mass spectroscopy, and laboratory and field bioassays. The synthetic blend was found to be attractive to *C. plebejana* males in the field.

5.2 Materials and methods

5.2.1 Insect culture

Initial cultures were established from larvae collected from *Malva parviflora* (marshmallow) plants in Tamworth (NSW) and the Australian Cotton Research Centre (ACRI, Narrabri, NSW). Larvae were carefully extracted from the fruiting structures or stems of the marshmallows using a scalpel and forceps. They were then transferred into individual containers with an artificial diet and reared under the conditions described in Chapter 3.2. Eggs harvested from mating cages were transferred into a 20 x 10 cm plastic bag with a moist dental wick and allowed to hatch in a reverse cycle controlled environment cabinets at 25°C under a 16:8 L:D regime with the scotophase during 0930–1730 h AEST. Hatched larvae were transferred individually using a fine small brush to individual cups with artificial diet, and covered with a small amount of organically grown barley green powder (Excel Botanicals, Coolangatta, Qld., Australia). Emerging adults were sexed and transferred to fresh cups and fed with 5% sucrose solution from a dental roll.

5.2.2 Pheromone extraction and analyses

Air trapping. Trapping of volatiles was done from 3-to 6-day old unmated females or males. Volatiles were collected from 3-4 insects held in an all glass apparatus (Figure 3.1), as described in Chapter 3.3. Insects were transferred into the collection room at least four hours before air trapping. Volatile collection was done for the whole scotophase. Initial collection involved purging the system for the first two hours using a pasteur pipette without the adsorbent (Super Q). After the purging time, the empty pasteur pipette was replaced by a pipette with the adsorbent. Volatiles trapped on the 200 mg of Super Q were eluted with 3 ml of dichloromethane or hexane in 0.5 ml portions. Eluate was collected in amber vials for concentration and analysis. Concentration was achieved by passing a gentle stream of pure nitrogen over the extract in the amber vial. For quantification purposes, samples were spiked with 60 ng each of (*Z*)-11: 18AC and *(E,E)*-10,12-hexadecadienol before concentration.

Gland extracts. Gland extracts were done when females were calling (Figure 5.1) which was observed to be between 1430 and 1730 h AEST, that is, in the last three hours of the scotophase in the reverse-cycle regime. The extracts were prepared in batches, with each batch containing 3 – 6 excised abdominal tips of calling females soaked in 20-30 µl of hexane in an amber vial. Extracts were allowed to stand at room temperature for 10 minutes before transferring the supernatant into 1.5 ml amber sample vials with 100 µl limited volume inserts.

Gas chromatographic-mass spectrometric (GC-MS) analyses were conducted on gland and air extracts using a Hewlett Packard 6890 series gas chromatograph and HP 5973 mass selective detector. The column used and conditions of run were as described in Chapter 3.4.

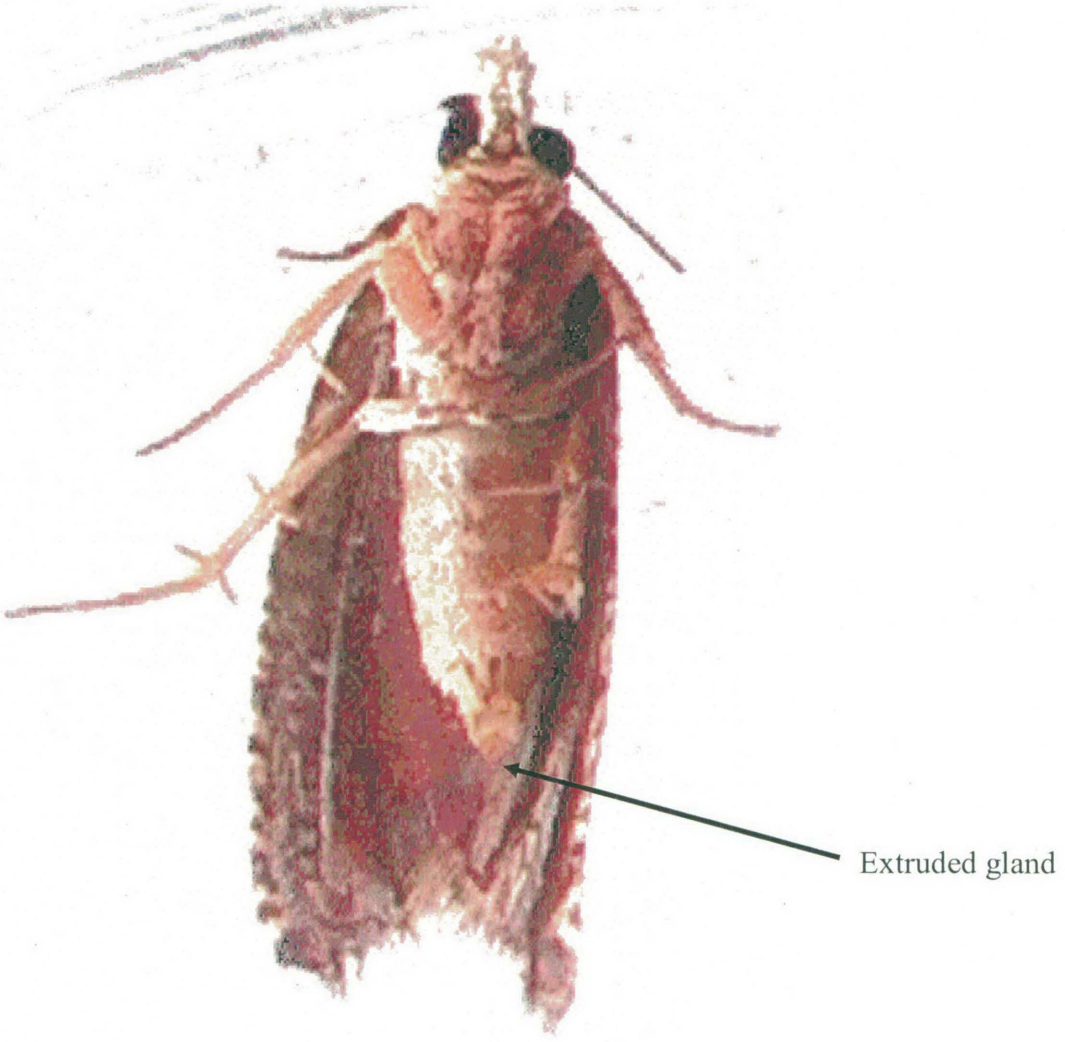


Figure 5.1. Calling female of *Crocidosema plebejana*.

5.2.3 Observations on female calling behaviour

Female behavioural observations were done in a reverse-cycle controlled environment cabinet (Model No. E&H, Controlled Environments, Pembina, N.D., USA) under the experimental conditions of 25°C and 16:8 L:D.

A total of sixteen females were used in the experiment. Newly emerged females were caged individually in 7.5 x 7.5 x 5 cm plastic cages with meshed tops and provided with dental wicks soaked in 5% sucrose solution as food. A small branch of marshmallow plant was provided for the insects to perch on. The calling behaviour of the females was observed continuously during the scotophase using a torch with a red filter, from day 1 to day 13. Dental wicks in the female cages were changed daily. All observations were recorded on a portable computer using The Observer (V. 3.0) program. Insects were scored for the following behaviours - calling, feeding, active and resting, but only the calling behaviour data were analysed.

5.2.4 Wind tunnel bioassays

Behavioural bioassays were conducted in the wind tunnel (Chapter 3.4) to test male responses to conspecific females, individual sex pheromone components or pheromone blends. Fifty 5-day old unmated males were used in the experiments. Males were held in groups of five in 15 cm x 8 cm diameter cylindrical meshed plastic cages and were transferred to the wind tunnel room at least 7 hours before the experiment to acclimatise. Either an empty cage or the pheromone was placed at the upwind end, and the males were released at the downwind end of the tunnel. Males were allowed to respond for 5 minutes after one end of the cage was opened, and were scored for the following behaviours: (1) take off (2) upwind flight (3)

downwind flight (4) approach to the source (5) contact with source and (6) clasper extrusion/ attempt at copulating. All observations were recorded using The Observer (V. 3.0) program.

Blends as coded in Table 5.1 were used in wind tunnel and field bioassays. The compounds, octadecanol (18OH) and 2-nonadecanone (2:19Kt) were sourced from Sigma-Aldrich, Castle Hill, NSW, Australia, and octadecyl acetate (18Ac) and octadecanal (18Al) from Pherobank, Wageningen, The Netherlands.

Experiment 1 - Male response to individual sex pheromone components

This experiment was designed to test the behaviour of males in the wind tunnel (Chapter 3.4) in the absence and presence of the individual pheromonal compounds identified (Figure 5.3). Males were tested using an empty cage (blank), 18Ac (compound IV), 18Al (compound I), 2:19Kt (compound III) and18OH (compound II). For each test compound, a 13 mm-diameter glass fibre filter paper disc was loaded with 4 µg of the compound.

Experiment 2 - Male response to conspecific females and sex pheromone blends

This experiment was designed to test the behaviour of males in the wind tunnel in the presence of conspecific females, a single component and five multi-component synthetic blends. Males were tested using 3 live females (FEM), to the blends listed in Table 5.1. For each blend, a 13 mm-diameter glass fibre filter paper disc (type A/E, Gelman Sciences, U.S.A) was loaded with 4 µg of the mixture.

5.2.5 Field bioassays

Preliminary field trials to test the attractiveness of *C. plebejana* pheromones to wild males were conducted at three sites at which there were established populations of marshmallow (seeded stage) at the "Kilmarnock" property in Boggabri, and one around the Gunnedah airport in NSW. These studies were hampered because few adult cotton tipworms were present, but no more favourable sites were located. Blends used were the 4-component blend T13, the 3-component blend T12 and the single component 18Ac (T11) (Table 5.1). Rubber septa were loaded with 2 mg of the blend and suspended in Delta traps. Three Delta traps, 1 for each blend were placed in a straight line, about 20 m from each other. Traps were left for one week at sites 1, 2 (Boggabri) and 3 (Gunnedah) before servicing. This trial ran from September 20 to September 27, 2003. Another set of traps (site 4) were set up in Boggabri on October 15, 2003 and serviced on October 16.

Further field trials to test the attractiveness of tipworm pheromones were not done due to the scarcity of tipworm populations around the cotton growing areas in NSW and Queensland during the remainder of the time available for the project. This meant that it was not considered worthwhile to conduct replicated Latin Square field experiments of the type described for the other two insects in this thesis.

	Blend composition (%)			
Blend	Octadecanal	2-nonadecanone	0ctadecyl acetate	Octadecanol
T11	-	-	1	
T12	2	2	1	-
T13		2	1	2
T18	1	-	-	-
T20	-	1	-	-
T21	1	1	-	-
T22	2	0	1	-
T24	-	2	1	2

Table 5.1 Pheromone blend composition for *C. plebejana* tested in the field. Source of chemicals; octadecanol and 2-nonadecanone from Sigma-Aldrich (Castle Hill, NSW, Australia) and octadecyl acetate and octadecanal from Pherobank, (Wageningen, The Netherlands). Blend ratios were based on proportions from pheromone extraction ratios from glands and air (Table 5.3).

5.3 Results and discussion

5.3.1 Pheromone extraction and analyses

GC-MS analyses of the concentrated extracts from the gland and air collections revealed various compounds, with two identified from the air collections and four from the gland extracts (Figures 5.2 and 5.3). The gas chromatogram of four compounds, octadecanal (18Al), octadecanol (18OH), 2-nonadecanone (2:19Kt) and octadecyl acetate (18Ac) used as standards to compare with those found in the air collections and gland extracts is shown in Figure 5.4.

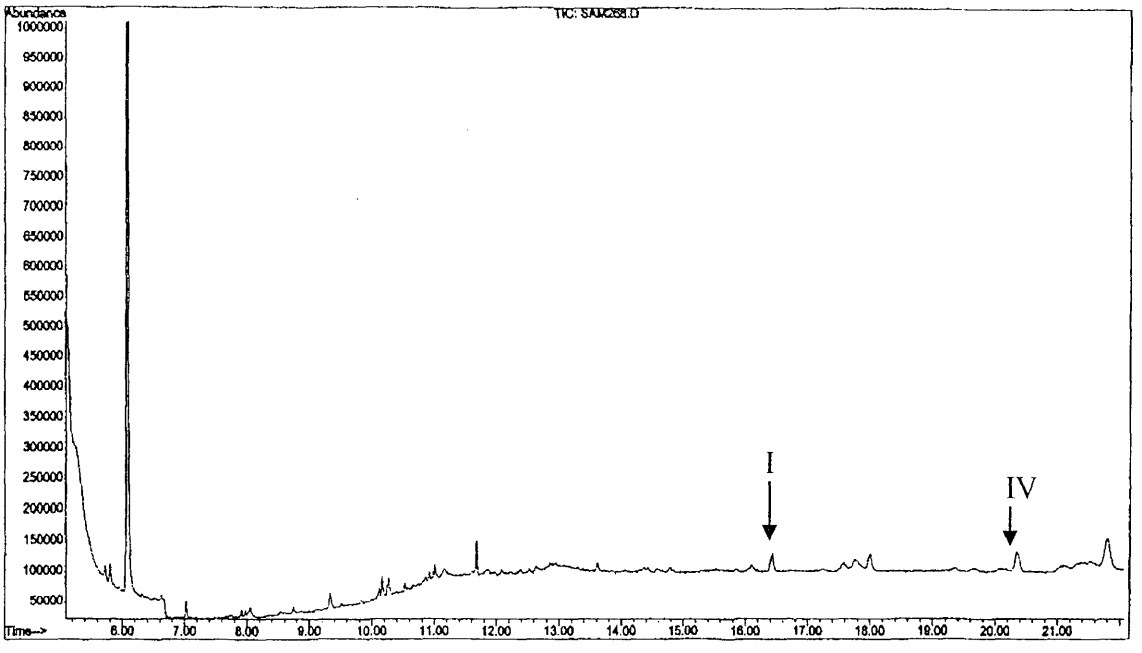


Figure 5.2. Gas chromatogram of air collections from *C. plebejana* females.

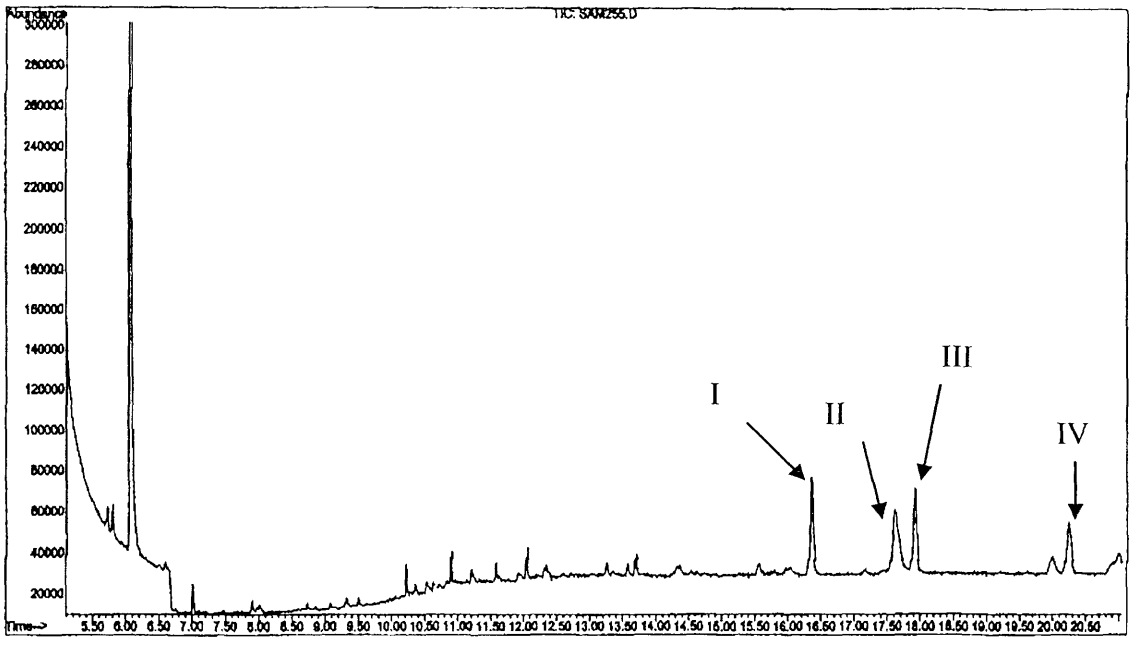


Figure 5.3. Gas chromatogram of gland extracts of *C. plebejana* females.

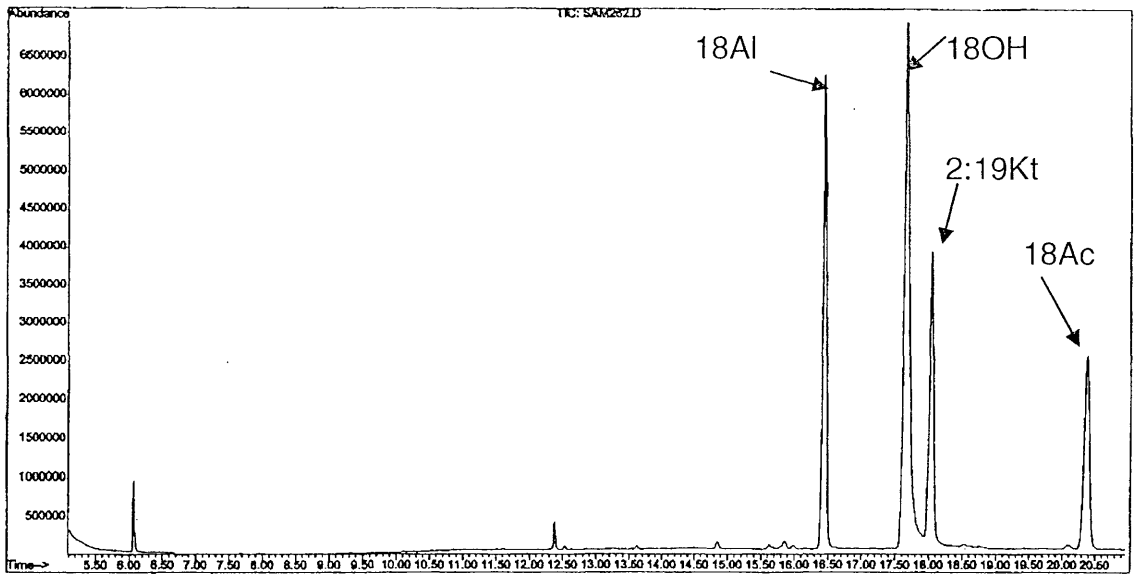


Figure 5.4. Gas chromatogram of standards octadecanal (18Al), octadecanol (18OH), 2-nonadecanone (2:19Kt) and octadecyl acetate (18Ac).

The retention times of the four compounds found in *C. plebejana* females and compounds used as standards are given in Table 5.2.

Figure 5.5 shows the spectra of compound IV found in both the air collections and gland extracts. The intense base peak m/z 43 (100%) (fragment ion (CH_3CO+) and the fragmentation pattern of compound IV indicated an aliphatic acetate. Cleavage alpha (α) to the carbonyl group of the acetate would produce the relatively stable m/z 43 fragment ion (Scheme 5.1). The pseudomolecular ion m/z 252 (2%)(M^+-60) is indicative of an 18-carbon acetate. The molecular weight of the compound is therefore 312. This was confirmed by comparison of the mass spectral data and the retention time with those of synthetic octadecyl acetate (Figure 5.4) and (*Z*)-11-octadecenyl acetate (Figure 5.6). Co-injection of the natural compound with octadecylacetate was conducted. The intensity of the GC peak increased, indicating co-elution.

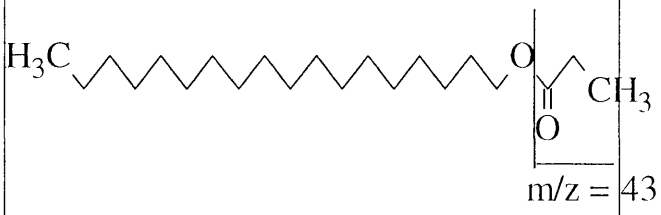
Compound	Retention time (minutes)
 I	16.353
II	17.608
III	17.910
IV	20.239
Hexadecanol (16OH)	14.413
Methyl hexadecanoate	14.854
Hexadecyl acetate	15.943
Octadecanal (18Al)	16.367
Octadecanol (18OH)	17.610
2-nonadecanone	18.050
Methyl cis,cis-9, 12 octadecadienoate	17.881
Methyl trans-9-octadecenoate	18.104
Z 11-octadecenyl acetate	19.833
Octadecyl acetate	20.246

Table 5.2. GC retention times for pheromone components I, II, III, IV and standards.

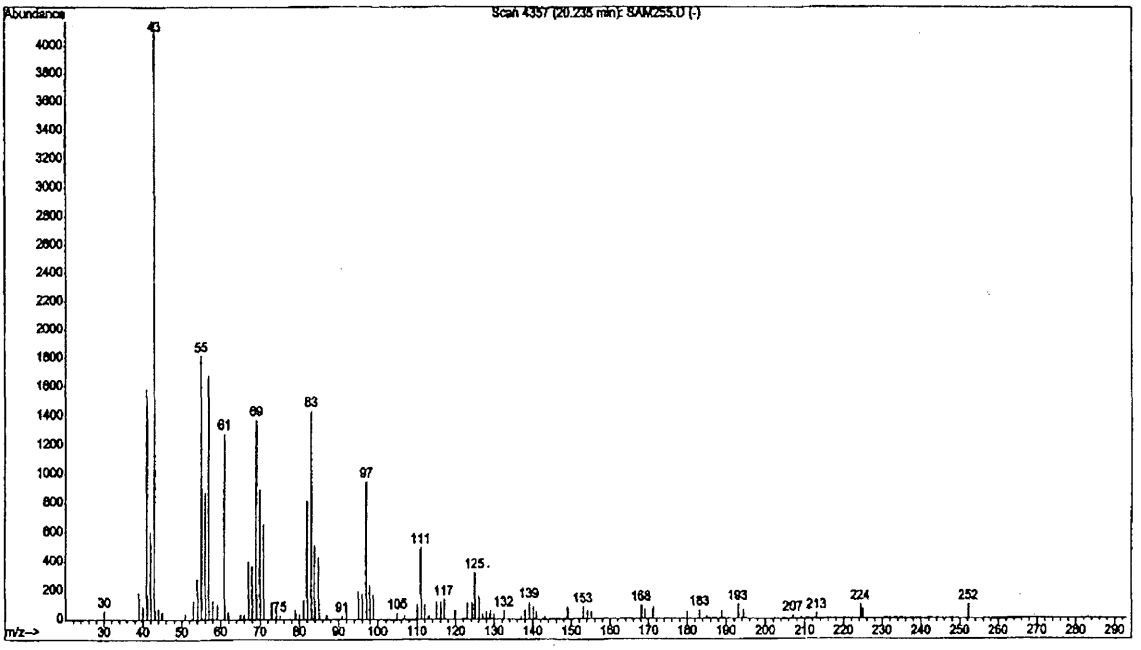
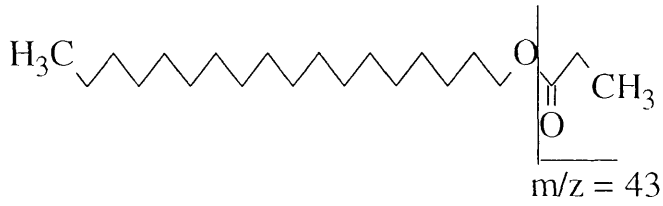


Figure 5.5. Mass spectrum of compound IV.



Scheme 5-1. Cleavage alpha (α) to the carbonyl group of the acetate leading to m/z 43.

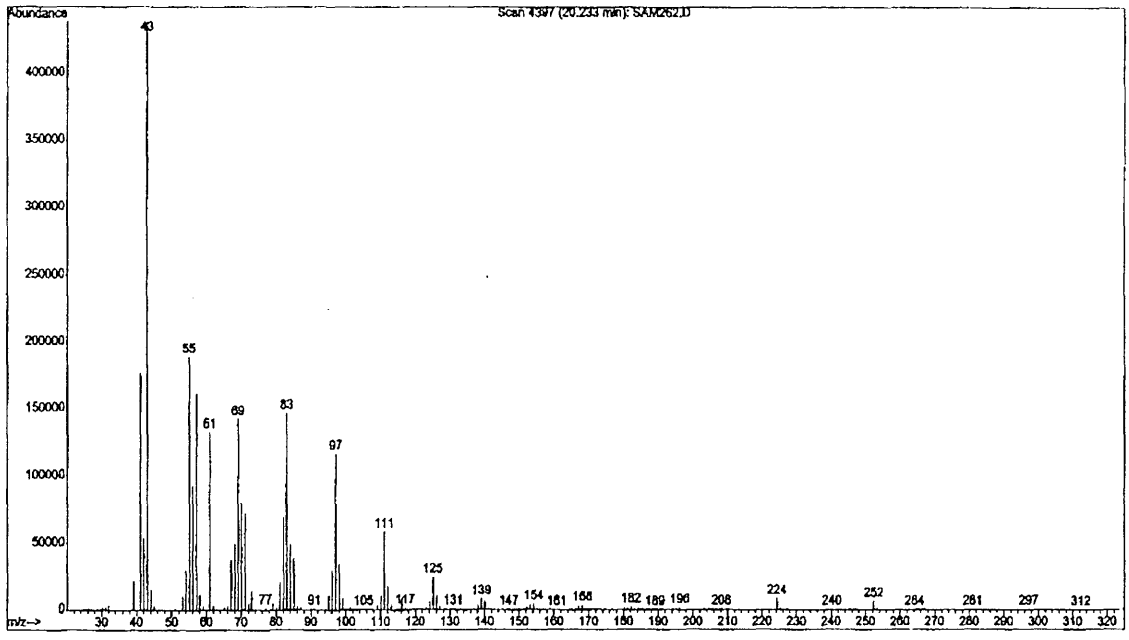


Figure 5.6. Mass spectrum of standard octadecyl acetate.

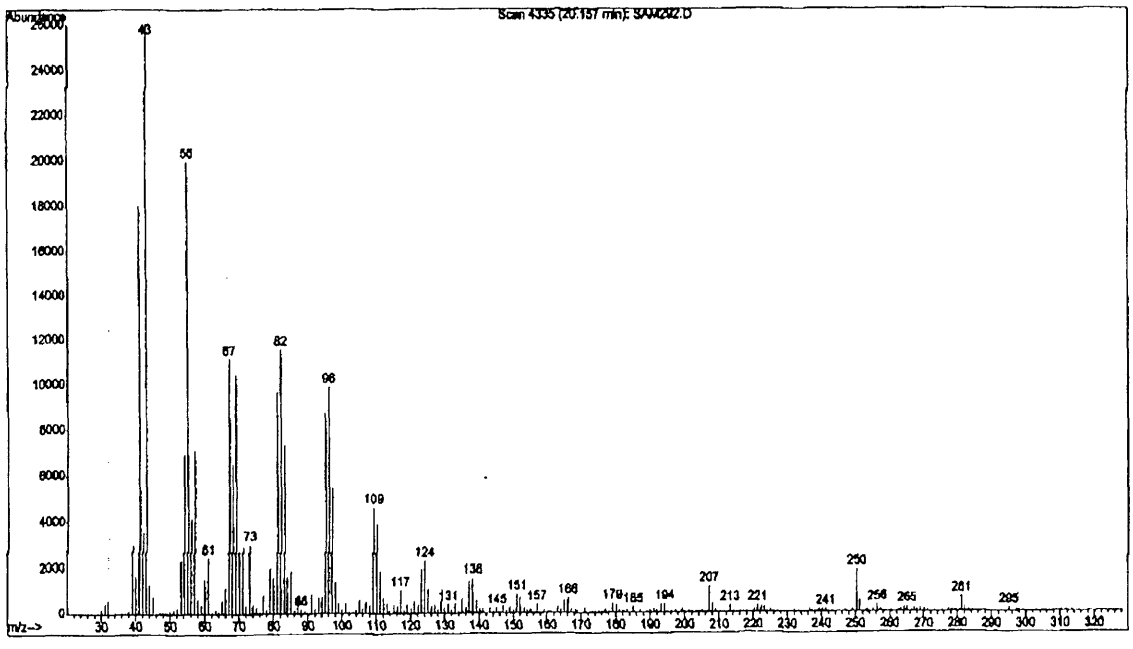
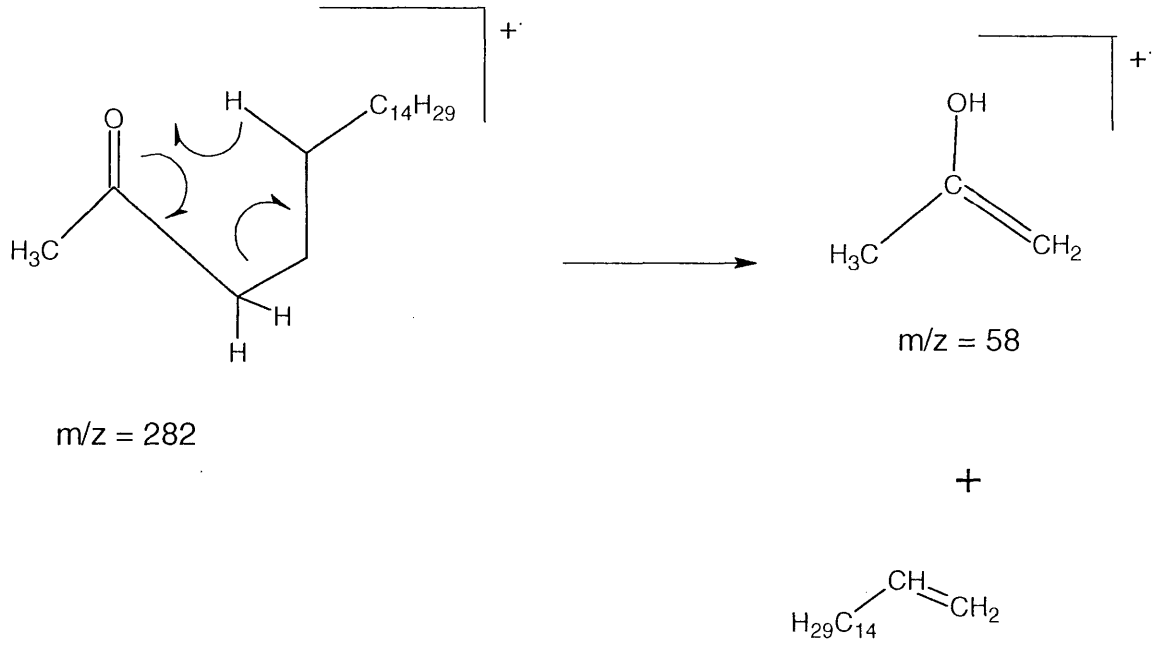


Figure 5.7. Mass spectrum of (*Z*)-11-Octadecenyl acetate.

Comparative GC retention behaviour of compounds I, II and III and some standard straight chain aldehydes, ketones, alcohols and enoates (Figure 5.4) indicated the presence of an 18 carbon aldehyde for compound I, 18 carbon alcohol for compound II and a 19 carbon ketone as compound III. The prominent molecular ion peak at m/z 282 (4%) in compound III (Figure 5.8) is indicative of an aliphatic ketone. Fragmentation in ketones occurs by cleavage of the C-C bond attached to the carbonyl carbon atom, and results in a stabilised R-C≡ O+ and R'-C≡ O+ ions in an asymmetric ketone. The m/z peak at 58 (73%) however suggests that the ketone is not asymmetric and that the pathway leading to its formation is the McLafferty cleavage (Scheme 5-2) which is characteristic of non-asymmetric ketones. Comparison of the mass spectra and the retention time to synthetic 2-nonadecanone indicated compound III was 2-nonadecanone.



Scheme 5-2. McLafferty rearrangement for the ketone (2-nonadecanone).

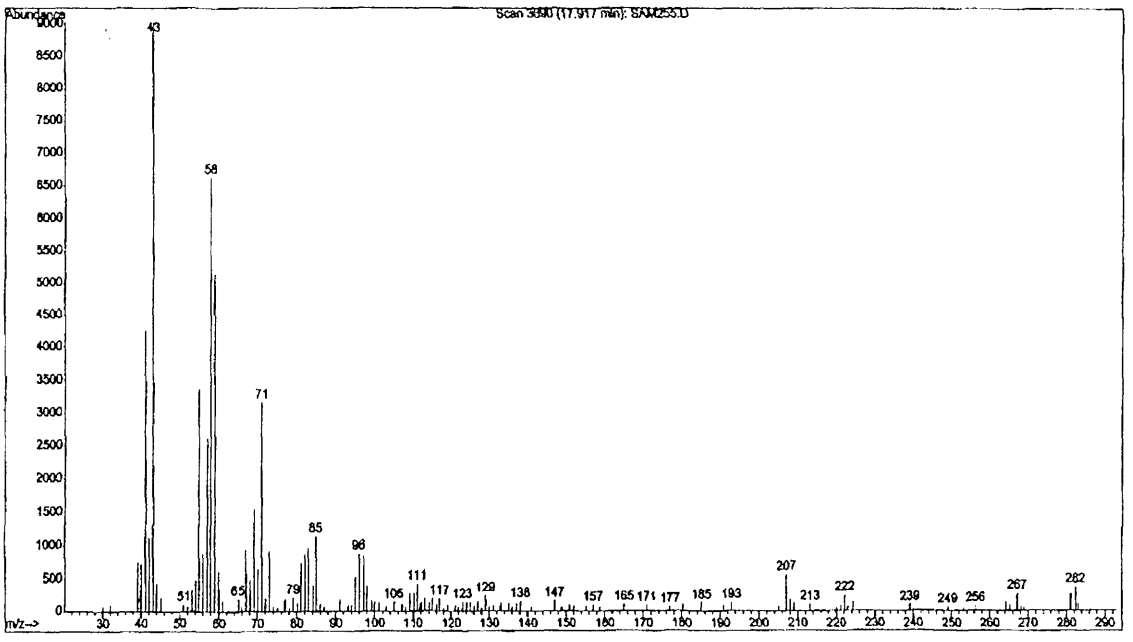


Figure 5.8. Mass spectrum of compound III.

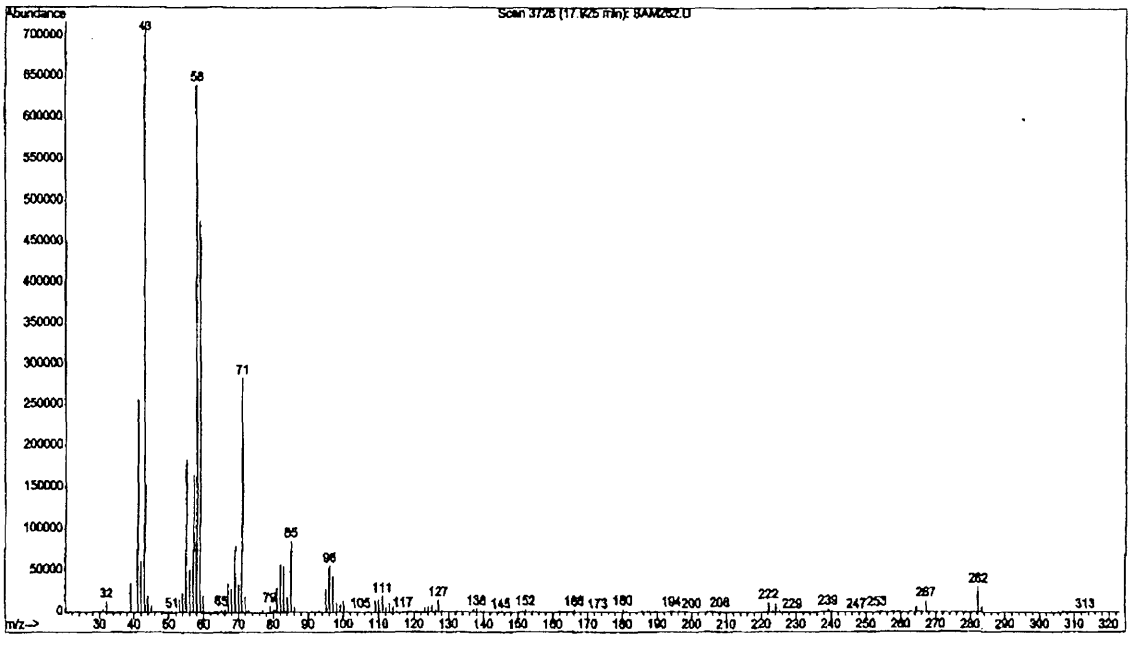
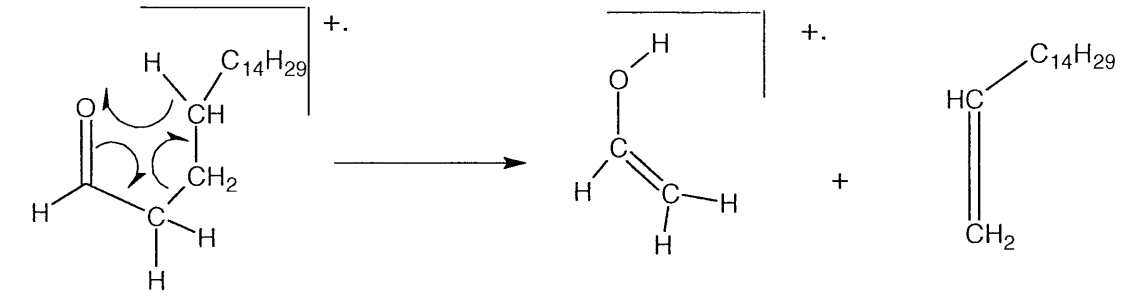


Figure 5.9. Mass spectrum of standard 2-nonadecanone.

Compound I was identified as octadecanal. Its mass spectrum and retention time (Figure 5.10, Table 5.2) were similar to those of the authentic standard (Figure 5.11) and co-eluted with it when run together with an increase in the peak height and area. The mass spectrum of compound I shows a weak molecular ion peak at m/z 268, typical of aliphatic aldehyde. Strong evidence for the aldehyde structure is further suggested by the presence of peaks at mass 39, 45, etc. indicating the presence of oxygen in the molecule and therefore distinguishing the strong peak at m/z 44 from that often formed in secondary amines. This loss of m/z 44 further suggest McLafferty rearrangement (Scheme 5-3) typical of aldehydes heavier than propanal with no substitution on the carbon alpha (α) to the CHO. As in most hydroxy compounds, the peak at m/z 250 is due to loss of water $(M - H_2O)^{+\cdot}$, but the complementary loss of mass 28 (CO) and 44 (CH_3CHO) is quite characteristic of aldehydes.



m/z 268 m/z 44

Scheme 5-3. McLafferty rearrangement for aldehyde accounting for peak at m/z 44.

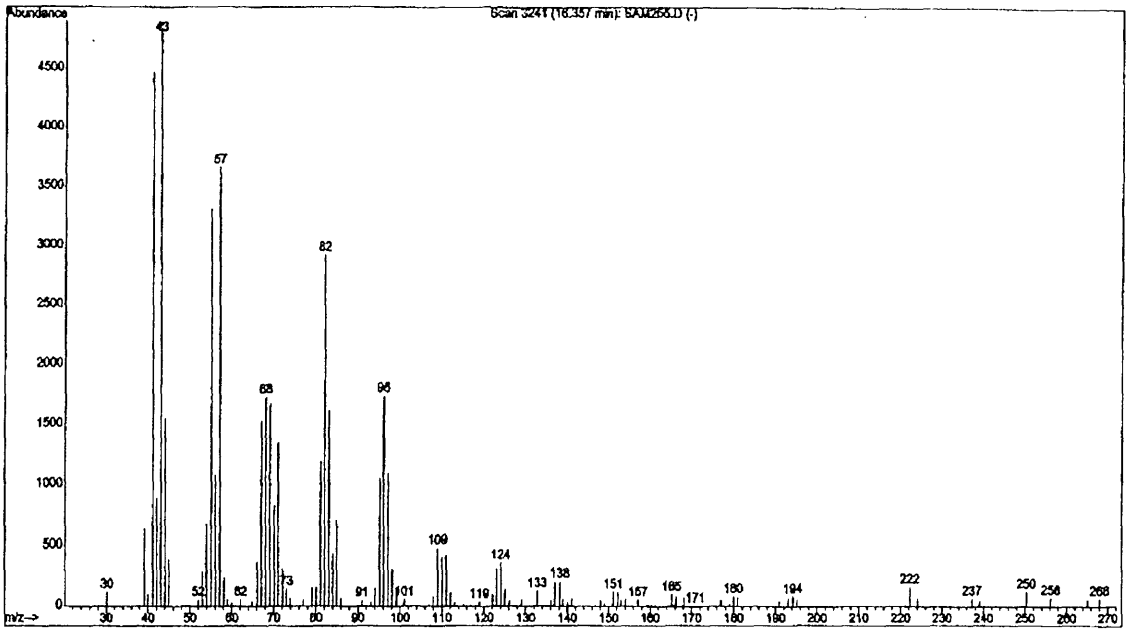


Figure 5.10. Mass spectrum of compound I.

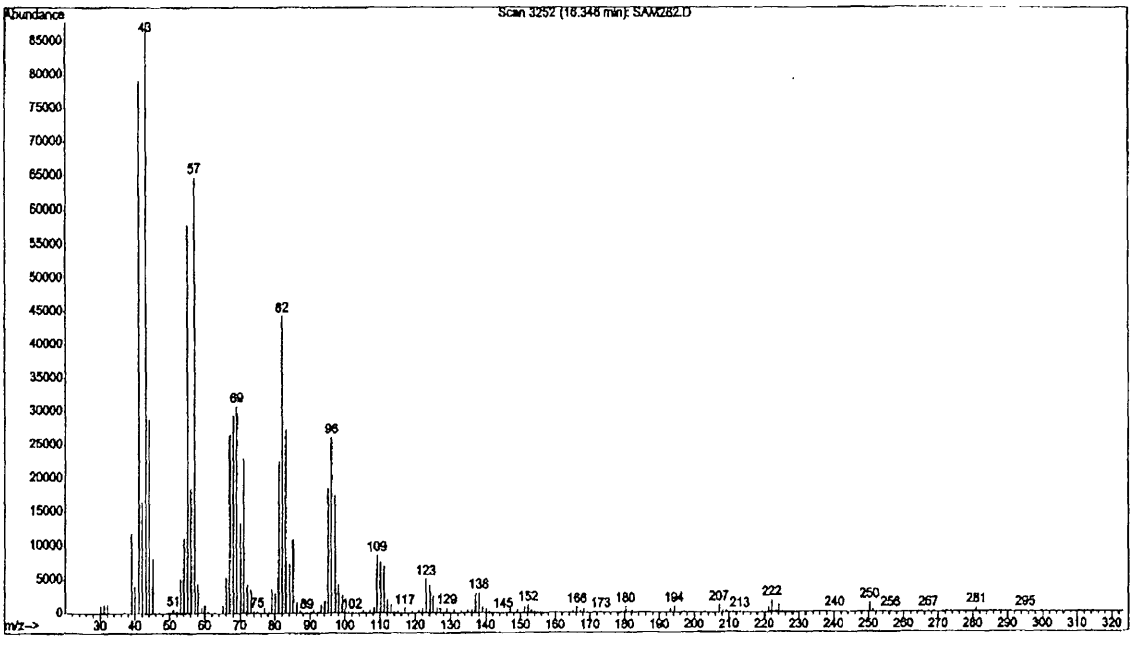
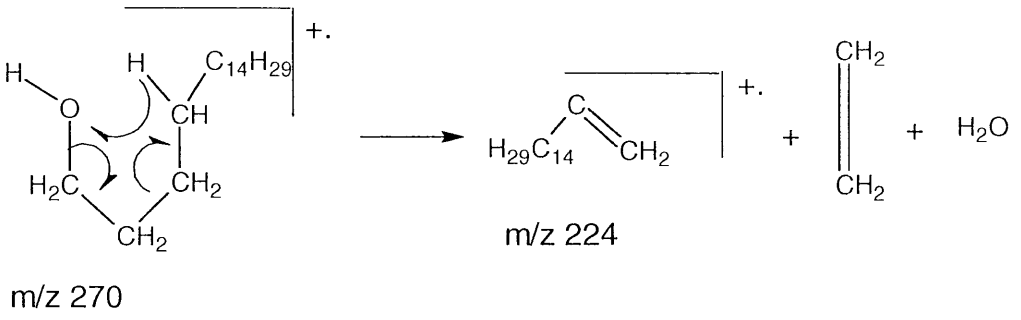


Figure 5.11. Mass spectrum of standard octadecanal.

Compound II had the same retention time as authentic octadecanol. Examination of the mass spectra (Figure 5.12) shows a prominent peak at m/z 31 suggesting the presence of oxygen. This m/z 31 results from the fragmentation of the C-C bond alpha (α) to the oxygen atom for primary alcohol. The pseudomolecular ion peak at m/z 269 (M- 1) indicates that the mass of compound is 270, corresponding to octadecanol. The peak at m/z 252 (M-H_20) further confirms this. The rest of the fragmentation pattern in the spectra mimics the pattern of the corresponding hydrocarbon except that there are peaks at 45, 73, etc because oxygen has a mass of 16 compared to 14 for a CH_2. Peaks resulting from the elimination of both water and alkene (M- 46 = 224; M-74 = 196, M – 102 = 168, etc.) were also observed. (Scheme 5-4).



Scheme 5-4. Re-arrangement in alcohol showing formation of m/z 224.

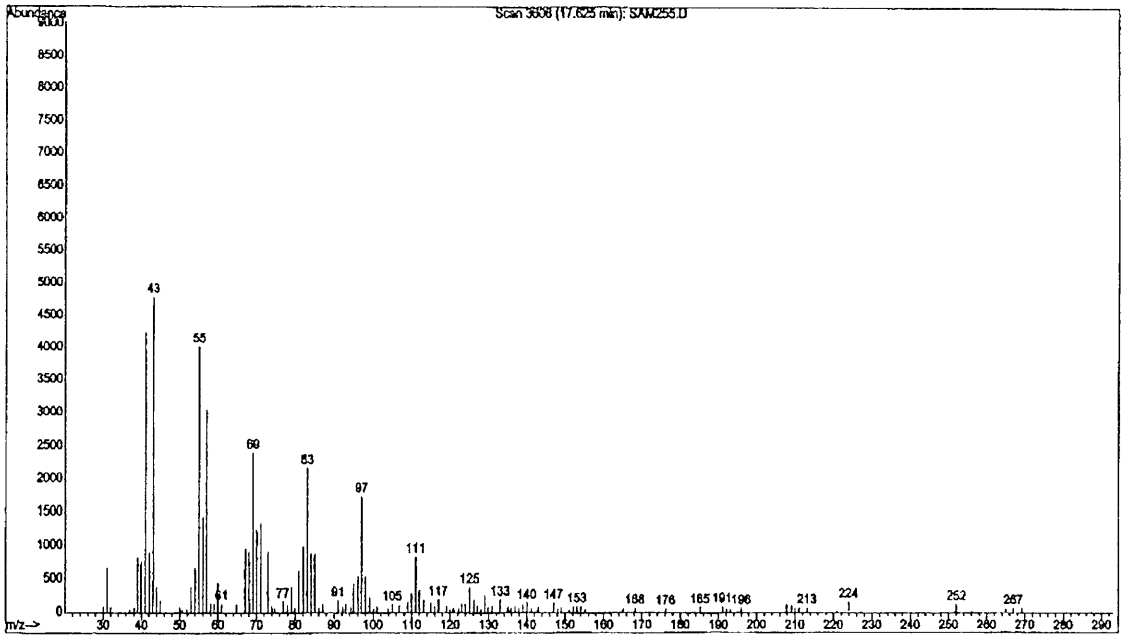


Figure 5.12. Mass spectrum of compound II.

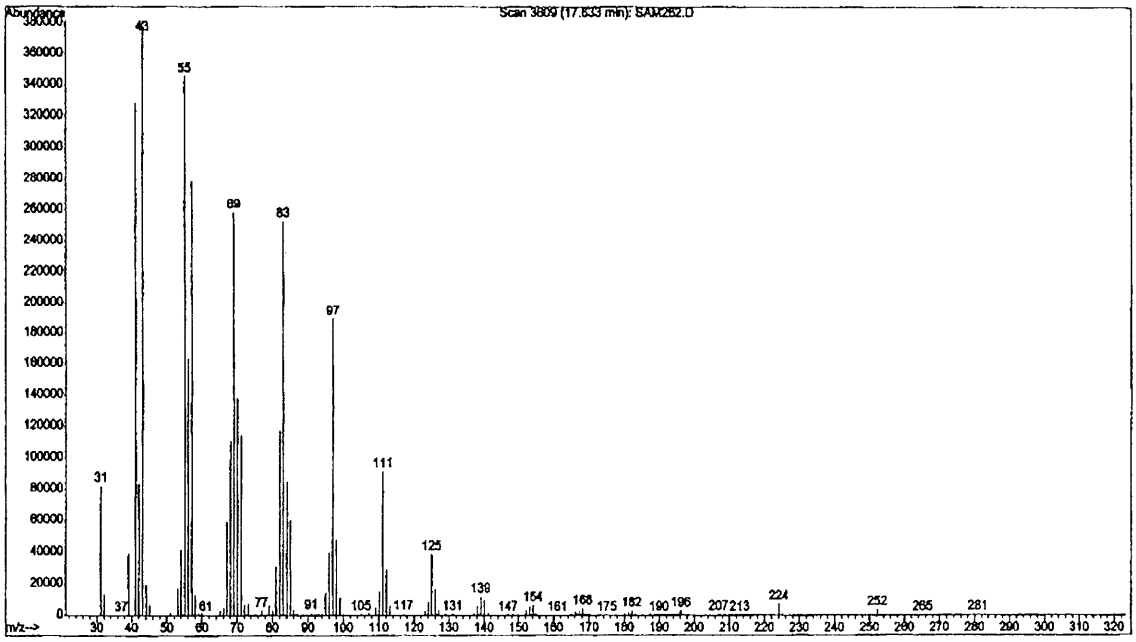


Figure 5.13. Mass spectrum of standard octadecanol.

Table 5.3 compares the amount of pheromonal compounds extracted from the glands with that in the air collections.

	Quantity per female equivalent/ng n = 3	
Compound	Gland extracts	Air collections
Octadecanal	4.0 ± 0.5	1.4 ± 0.4
Octadecanol	4.2 ± 0.8	-
2-nonadecanone	3.8 ± 0.4	-
Octadecyl acetate	1.9 ± 0.4	0.6 ± 0.2

Table 5.3. Estimated quantity of pheromone compounds in gland extracts and air collections of *C. plebejana* females.

Only two components, octadecanal and octadecyl acetate, were found in the air collections. The amounts of these compounds found in the gland extracts were generally higher than those in the air collections. The ratios however, were not different.

5.3.2 Studies on female calling behaviour

C. plebejana female had wings slightly raised above the abdomen with full protrusion of the ovipositor when calling (Figure 5.1). Figure 5.14 shows the pattern of first calling in *C. plebejana* females. None of the 16 females initiated calling until they were 3 days old, with 44% of them calling for the first time on the 3rd day. A few did not begin calling until they were much older, ie, 6-7 days old. Peak female calling (93.7%) occurred during the eighth scotophase, and the percentage of females calling decreased as they got older (Figure 5.15). Some of the moths (6.3%) never called throughout the experiment.

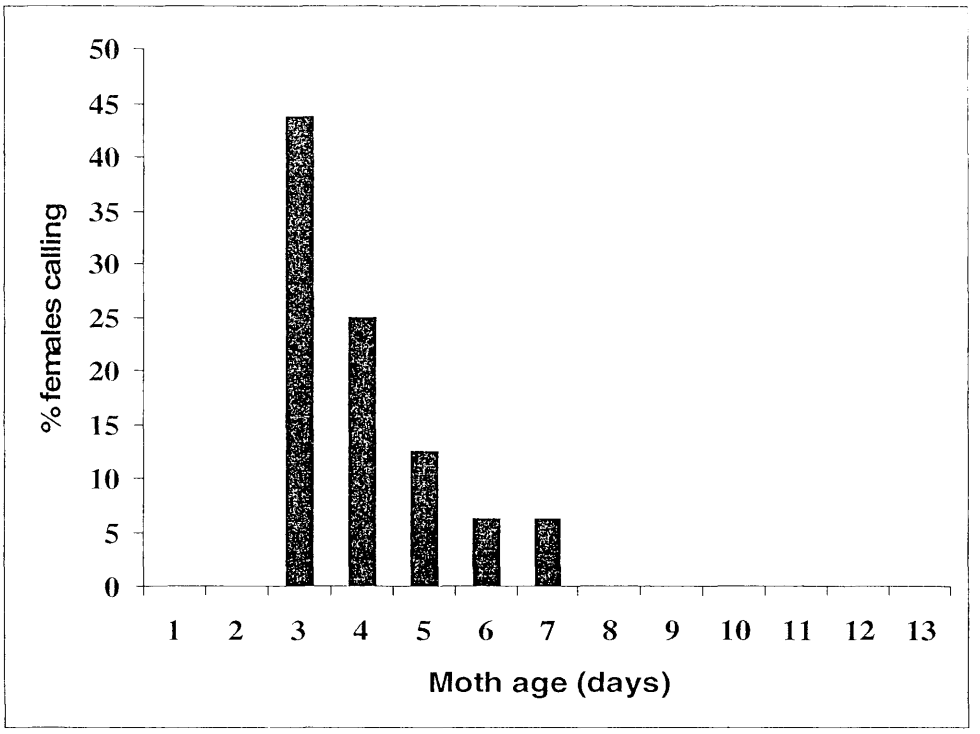


Figure 5.14. Percentages of *C. plebejana* females that first called at each age N=16).

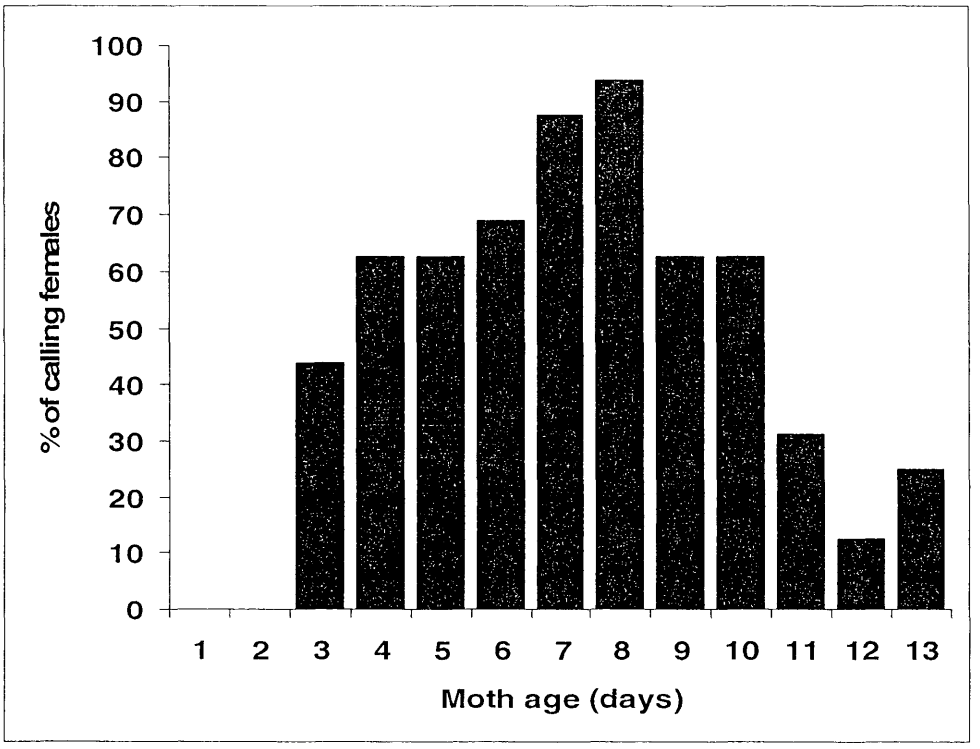


Figure 5.15. Percentages of calling *C. plebejana* females at different ages under 25°C and 16:8 L:D conditions (N=16).

Mean onset time of calling at different ages

C. plebejana females mainly initiated calling during the second half of the scotophase. Figure 5.16 shows the mean onset time of calling at different ages. Calling activity generally was initiated during the 5th to 6th hours after lights off and terminated during the 7th to 8th hours of the scotophase.

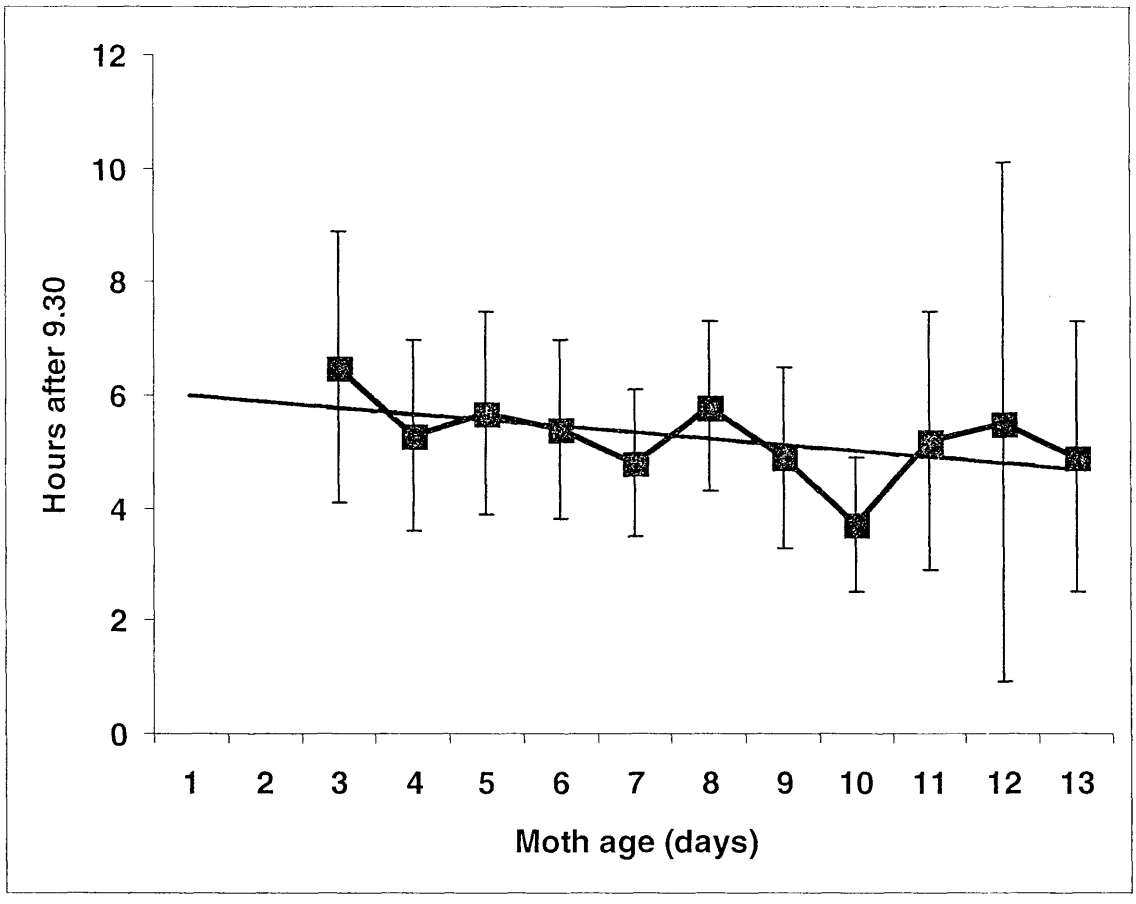


Figure 5.16. Mean onset time of calling in *C. plebejana* females at different ages under 25°C and 16:8 L:D conditions (N=16). Bars are (± SEM).

Linear regression indicated that although there was a trend for earlier onset of calling during the scotophase with increasing age, it was not statistically significant ($P = 0.11$, $R^2 = 0.26$).

Mean duration of calling with age

Figure 5.17 shows the mean duration of calling in *C. plebejana* females at different ages. Data were analysed using GLM with a Chi-square test for significance of age on calling duration. Mean duration of calling was highly significantly correlated with age ($P < 0.001$). *C. plebejana* females tended to call longer as they grew older with a maximum of 77 min occurring on the 7th day and then a gradual decrease to 4 min on the 12th day. The R^2 value of 0.76 indicates that 76% of the variation in calling duration could be explained by the polynomial function of the variable age.

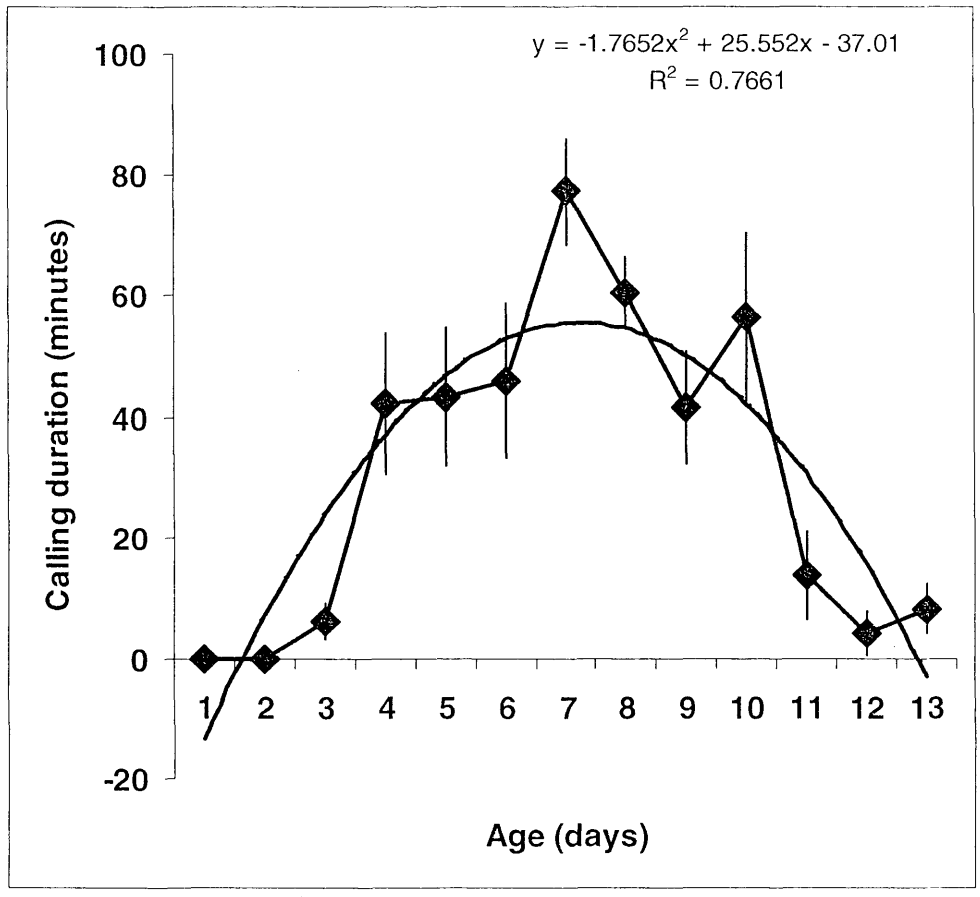


Figure 5.17. Mean duration of calling in *C. plebejana* females at different ages under 25°C and 16:8 L:D conditions (N=16). Bars are (± SEM).

Number of calling bouts with age

The numbers of calling bouts per night at different ages are shown in Figure 5.18. The highest number of bouts occurred when moths were between 5 and 10 days old.

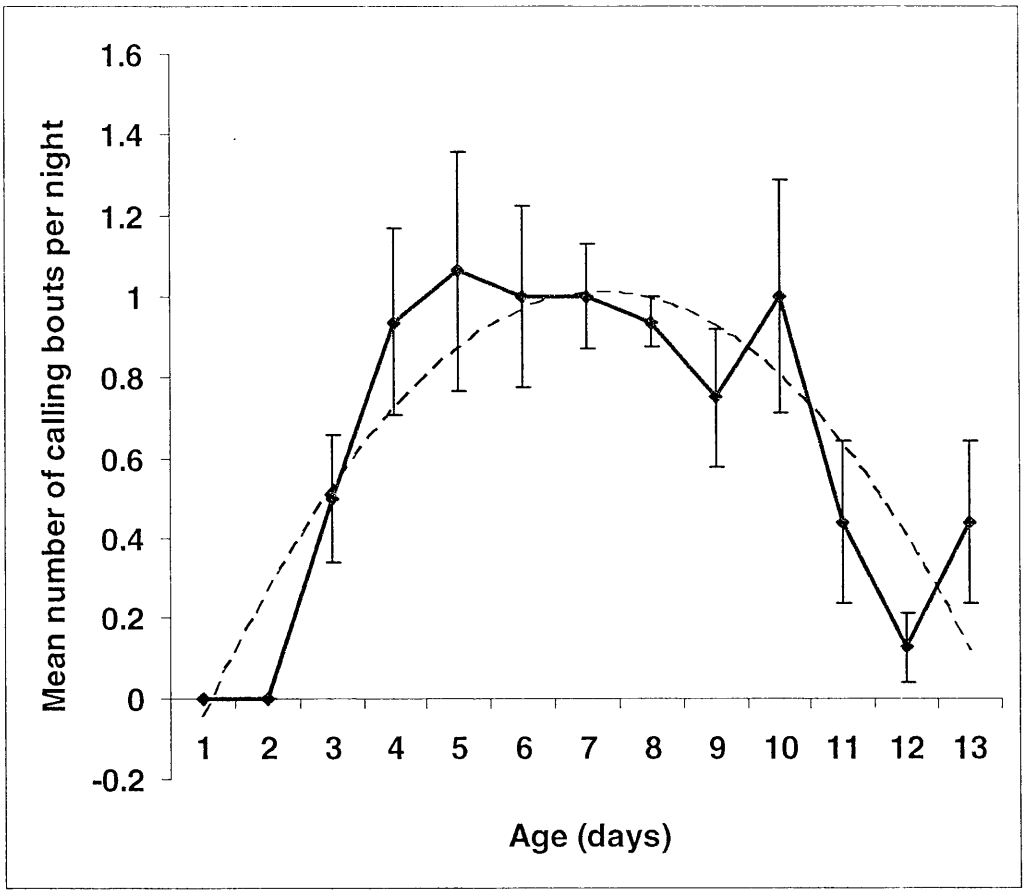


Figure 5.18. Mean number of calling bouts per night in *C. plebejana* females at different ages under 25°C and 16:8 L:D conditions (N=16). Bars are (± SEM).

Regression analysis for the data yielded the equation:

$$y = -0.027x^2 + 0.3922x - 0.413$$

Where y = number of calling bouts
x = age of moth

The regression was highly significant ($F_{1,142} = 16$, $P < 0.001$, $R^2 = 0.77$), indicating correlation between number of calling bouts with age. The R^2 value indicates that the model explains about 77% of the variation in number of bouts.

5.3.3 Wind tunnel bioassays

Experiment 1

Figure 5.19 shows various behavioural observations of *C. plebejana* males in the absence of a pheromone (blank or empty cage) and in the presence of the four individual compounds, 18Al, 18Ac, 18OH and 2:19Kt in the wind tunnel.

In all cases where males were presented with empty cages (blank, ie, no pheromone source), only 1% of them took off. Males that took off usually immediately came to rest on the walls or floor of the wind tunnel. A similar trend was observed when males were tested with the other three individual compounds 18Al, 18OH and 2:19Kt. Only the component 18Ac elicited upwind flight and approach behaviours. Of the males tested against 18Ac, 40% approached the pheromone source and 10% made contact with source. This result indicates that 18Ac appears to be an important component of the sex pheromone blend for *C. plebejana*.

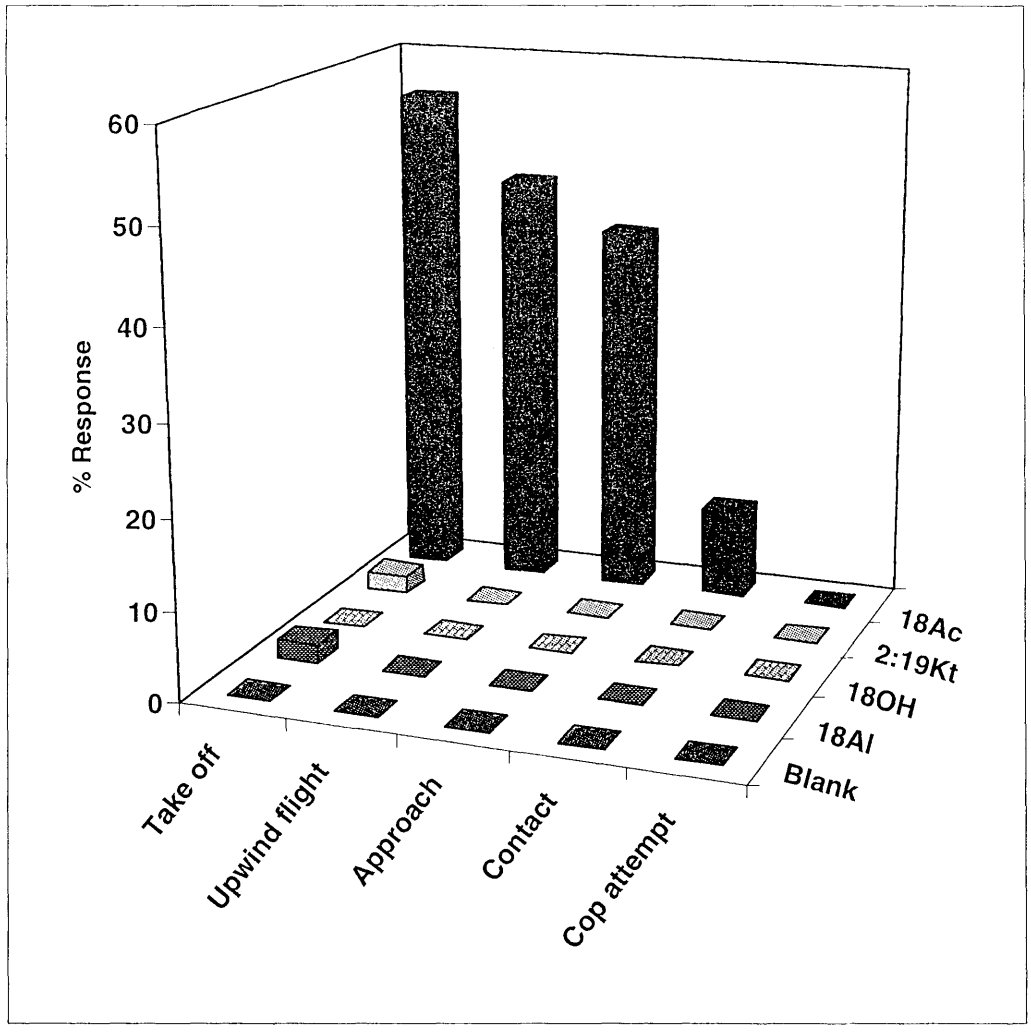


Figure 5.19. Experiment 1. Percentages of *C. plebejana* males exhibiting the different behaviours in the absence of a pheromone source (blank or empty cage) and in the presence of 18Al, 18OH, 2:19Kt and 18Ac. Males used were 4 days old. N= 50/treatment.

Experiment 2

Figure 5.20 shows the different behaviours observed in *C. plebejana* males in the presence of live females (FEM), the single component 18Ac (T11) and five multi-component synthetic pheromone blends (T12, T13, T21, T22 and T24).

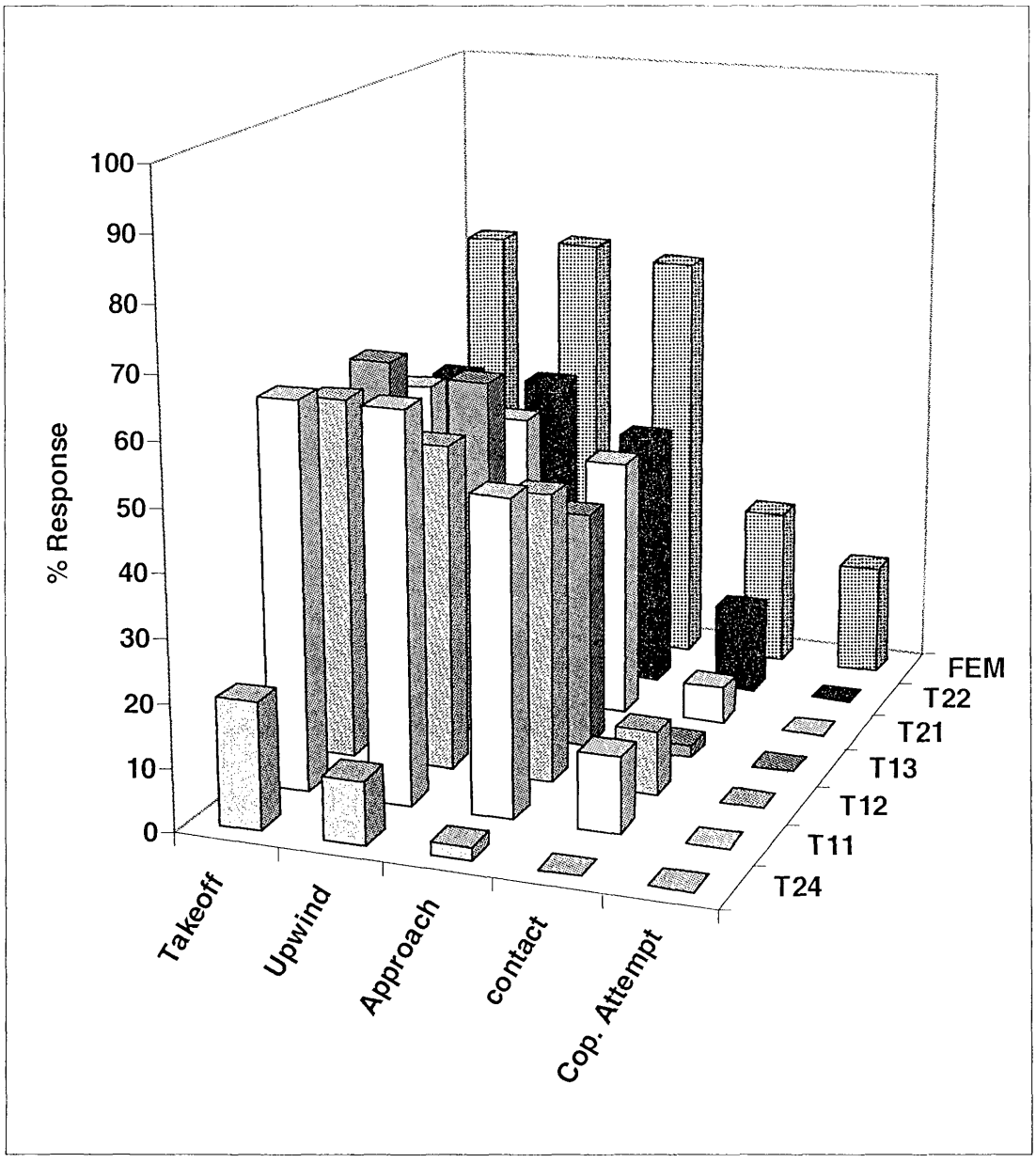


Figure 5.20. Experiment 2. Percentages of *C. plebejana* males exhibiting the different behaviours in the presence of females (FEM); 18Ac (T11); 2:2:1 ratio of 18Al, 2:19Kt and 18Ac (T12): 2:2:2:1 ratio of 18Al, 2:19Kt , 18OH and 18Ac (T13); 1:1 ratio of 18Al and 2:19Kt (T21); 2:1 ratio of 18Al and 18Ac (T22); and 2:2:1 ratio of 18OH, 2:19Kt and 18Ac (T24). Males used were 4 days old. N= 50/treatment.

In all cases where males were presented with pheromone source, over 50% of them took off except in blend T24 (2:1 ratio of 18OH and 18Ac) where only 20% of the males took off. Males usually took off and spent a little time upwind before coming to rest or approaching the source. Approach and contact behaviours were observed in most of the males tested with the synthetic pheromone blends. Approach was characterised when a male moth got near the pheromone source to about 5 cm. With contact behaviour, a male touched the pheromone source after approaching. However, no males attempted copulation with the pheromone blends, and only a few did so with conspecific females. Copulatory attempt was characterised by the extension of the claspers and curving them towards the pheromone source.

Data were analysed using GLM with a Chi-square test for significance of the blend for each of the observed behaviours, i.e. take off, upwind flight, approach, contact with source and copulatory attempt. The analysis of deviance table as an output of the GLM indicated that the effects of blend treatments were highly significant on take-off, upwind flight, approach, contact and attempt at copulation ($P < 0.001$ in all cases).

Comparison of the mean response of copulatory attempt using contrast in R indicated highly significant difference between females and the other six synthetic pheromone treatments ($P < 0.001$). A similar trend was obtained for the differences between the females and blend T24 for take-off, upwind flight, approach and contact. Between females and the other blends, however, differences ranged from significant ($P < 0.001$) in some cases to non significant ($P > 0.230$). These results are summarised in Table 5.4.

Behaviours	Comparison	P- Value
Take Off	Female vs T11	P = 0.423
	Female vs T12	P = 0.230
	Female vs T13	P = 0.317
	Female vs T21	P = 0.073
	Female vs T22	P = 0.047
	Female vs T24	P < 0.001
Upwind flight	Female vs T11	P < 0.042
	Female vs T12	P < 0.073
	Female vs T13	P = 0.231
	Female vs T21	P < 0.029
	Female vs T22	P < 0.047
	Female vs T24	P < 0.001
Approach	Female vs T11	P = 0.069
	Female vs T12	P = 0.027
	Female vs T13	P = 0.003
	Female vs T21	P = 0.009
	Female vs T22	P = 0.009
	Female vs T24	P < 0.001
Contact	Female vs T11	P = 0.020
	Female vs T12	P = 0.008
	Female vs T13	P < 0.001
	Female vs T21	P < 0.001
	Female vs T22	P = 0.046
	Female vs T24	P < 0.001
Copulatory attempt	Female vs T11	P < 0.001
	Female vs T12	P < 0.001
	Female vs T13	P < 0.001
	Female vs T21	P < 0.001
	Female vs T22	P < 0.001
	Female vs T24	P < 0.001

Table 5.4. Experiment 2. P-values showing the level of significance for comparison of various behaviours of *C. plebejana* males between the different treatments and conspecific female.

5.3.4 Field Bioassays

On the basis of the results from the wind tunnel bioassays, three treatments were tested in preliminary field trapping experiments. These were T11 (18Ac), T12 (2:2:1 blend of 18Al, 2:19Kt and 18Ac) and T13 (2:2:2:1 ratio of 18Al, 2:19Kt, 18OH and 18Ac). Table 5.5 shows trap catches for these blends. In this experiment, low catches of *C. plebejana* males were obtained in the traps. This was probably because the trial was conducted in a year with drought problems and low occurrence of non-crop hosts of *C. plebejana*. The experiment could not be replicated within sites because of small field sites with few available moths, due to the patchiness of the hosts. One of the traps had one female in addition to the eleven males. Although these results are only preliminary, the data suggest that male cotton tipworms can be attracted by pheromones into traps.

	Site 1		Site 2		Site 3		Site 4	
Blend	males	females	males	females	males	females	males	females
T11	10	0	1	0	11	1	1	0
T12	4	0	5	0	6	0	0	0
T13	7	0	2	0	46	0	5	0

Table 5.5. Trap catches for blends T11 (18Ac), T12 (2:2:1 blend of 18Al, 2:19Kt and 18Ac) and T13 (2:2:2:1 ratio of 18Al, 2:19Kt, 18OH and 18Ac) set up in marshmallow at sites 1, 2, and 4 in Boggabri and 3 in Gunnedah, NSW.

5.4 Summary and conclusion

The calling behaviour of *C. plebejana* was studied in the laboratory at 25°C and 16:8 light: dark conditions. As in many moth species, *C. plebejana* had wings slightly raised above the abdomen with full protrusion of the ovipositor when calling. The age at which *C. plebejana* called for the first time ranged between the third and seventh scotophases. The mean onset time of calling was about 5 hours into the scotophase, and did not significantly advance with age. Duration of calling ranged from 6 minutes on the 3rd scotophase to a maximum of 77 minutes on the 7th scotophase before dropping gradually to 4 minutes on the 12th scotophase. There was a high correlation between the number of calling bouts and age. Generally, the number of calling bouts increased with age. Calling behaviour and pheromone production of *C. plebejana* females was synchronous. Female gland extracts generally tended to contain about 10-12 ng/female as compared to 2 ng/female in the air collections. It appears that *C. plebejana* follows a calling and pheromone biosynthesis pattern that is common for many lepidopteran species such *as Sesamia nonagrioides* Lefebvre (Babilis & Mazomenos, 1992), *C. pomonella* (Backman *et al.*, 1997), *Heliothis subflexa* Guenée (Heath *et al.*, 1991), *Helicoverpa assulta* Guené (Kakimura & Tatsuki, 1993) and *H. zea* (Raina *et al.*, 1986b). In these species, pheromone production occurs during the period when females are calling.

Sex pheromonal compounds from the glands of *C. plebejana* have been identified as a mixture of octadecanal, 2-nonadecanone, octadecyl acetate and octadecanol, in a ratio of 2:2:1:2, respectively. In contrast with most lepidopteran sex pheromone systems which are multi-component, and in which the relative composition may be critical to be effective attractants, preliminary field trials with *C. plebejana* indicated the possibility of using only 18Ac as an effective trap attractant. A single component pheromone is always much easier to formulate than a multi-component blend. This, however, needs further field trials to compare

with the full blend. Further trials were not achieved in this study because of lack of available field sites with *C. plebejana* due to the drought years when this research was carried out.

Generally, prediction of tipworm abundance and infestation levels on cotton can be done based on the abundance and timing of senescence in the malvaceous weeds, *M. parviflora* and *A. cristate* (Hamilton, 1985). However, timing of infestation cannot be assessed by the abundance of the host plants. The sex pheromone developed for the tipworm has a potential use in managing this pest in cotton. Traps baited with the pheromone and used as monitoring tool could provide information on the timing of infestation. If further research with the pheromone provides data with good correlation between trap catches and level of infestation, then the pheromone could be used to assist in timing of other control measures like the use of insecticides. The use of pheromones in mating disruption is known to work well in insects that are less mobile with limited hosts and easily synthesised pheromone components (Suckling, 1993). The tipworm probably falls in this group and so there could be a potential use of the pheromone in this aspect as well (especially if it could be done with only 18Ac). More data need to be collected from field trials using the pheromones identified before any pheromone-based control method could be recommended. This study, however, has provided the first step in any pheromone application work, identification of the pheromone.

CHAPTER 6

SEX PHEROMONES OF THE GREEN MIRID, *CREONTIADES DILUTUS* (STÅL) (HEMIPTERA: MIRIDAE)

6.1 Introduction

In conventional (non-transgenic) cotton in the past, the green mirid, *Creontiades dilutus*, (GM) rarely reached economic levels because the populations were usually suppressed by insecticides sprayed to control the key pests, *Helicoverpa* spp. With an increase in the adoption of integrated pest management strategies in the cotton industry as well as the commercialisation of transgenic (*Bt*) cotton, broad spectrum insecticide use is being reduced. GM pest status is therefore increasing, and it is expected to become an important economic pest in cotton in Australia. Similarly in the US, data indicate resurgence of Heteroptera as pests with the implementation of *Bt*-cotton (Layton *et al.*, 1997; Layton, 2000).

Currently, some strategies in place for managing GM in cotton involve the following: (a) destruction of all alternate hosts including native weeds at least two weeks before planting of cotton; (b) establishment and management of lucerne as a trap crop at least two weeks prior to cotton planting; this is to attract mirids away from the cotton; (c) monitoring mirid damage and thresholds to determine if control is needed; mirids are then sprayed only when insect and damage counts are at the threshold levels; (d) avoiding the use of broad-spectrum insecticides

for control of green mirid which reduce the numbers of beneficials that feed on *Helicoverpa* and mites (Mensah & Khan, 1997).

The biology, behaviour, ecology including population dynamics, sources, host plants and movements of GM have been studied by Miles (1995) and Khan (1999). Female-baited Delta traps were used by Miles (1995) to demonstrate the attraction of males to females, presumably through pheromones, in the 1993/94 cotton season. The development of sex pheromone lures or other attractants for this species would complement current control methods. They might be useful for attract-and-kill, as monitoring tools of GM populations, or perhaps for mating disruption. Attract-and-kill might be suitable since GM have a relatively long adult pre-reproductive period (about 7 days; Khan, 1999), which would provide a long window in which males could be removed before mating, thereby reducing female oviposition. Also, since adult male mirids cause damage, reducing their numbers would be beneficial, independently of any reduction in oviposition.

Hemipteran pheromone systems, like those of other insects, are multi-component. A mixture of two female-specific components, butyl butyrate and (*E*)-2-butenyl butyrate in a ratio of 94:6 has been identified in the mullein bug, *C. verbasci*. In field studies, lures which released the pheromone at rates of 91 and 183 µl per day were found to be as attractive as five live virgin females (Smith *et al.*, 1991; McBrien *et al.*, 1994). The individual components on their own were found to be inactive. Attraction of males to live females, crushed females and volatiles of females feeding on mullein, *Verbascum thapsis* L., was also observed (Thistlewood *et al.*, 1989).

The pests of pistachio, *Phytocoris californicus* and *P. relativus*, on the other hand, use a 2:1 ratio of hexyl acetate, produced by both sexes, with the female-specific compounds (*E*)-2-octenyl acetate and (*E*)-2-octenyl butyrate, respectively (Millar & Rice, 1998; Millar *et al.*,

1997). While (*E*)-2-octenyl acetate did not inhibit *P. relativus* males, (*E*)-2-octenyl butyrate inhibited attraction of *P. californicus* males to traps (Millar & Rice, 1998).

This chapter discusses the isolation, identification and bioassays of the major sex attractant components of *C. dilutus*. A synthetic blend of these components was found to be attractive to adult *C. dilutus* males only. The chapter also describes some preliminary studies investigating whether these pheromone components might be useful for attract-and-kill or mating disruption.

6.2 Materials and methods

6.2.1 Insects

Nymphs of *C. dilutus* were collected from lucerne in Armidale, NSW, and were reared through to adults on fresh beans in the insectary at 25 ± 1°C and 13:11 light:dark (L:D) conditions (Chapter 3.1). About 5 – 10 mirids were held in 1litre plastic rearing containers with meshed tops. Fresh beans were supplied daily in the containers. Adults were sexed immediately and males and females were kept in separate containers.

6.2.2 Pheromone collection and analyses

Two methods of pheromone collection, air and whole body extracts, were adopted. For each sex, air collections were made from 3 to 4 unmated mirids aged 6 to 8 days. The method was similar to that described in Chapter 3.2 with the following modifications: a pod of green bean and a small branch of lucerne were supplied as food in the insect flask. Air was drawn into the flask at a rate of 60 ml/min and the volatiles trapped on a 100 mg filter of super Q. Collection was done for 15 hours. Trapped volatiles were eluted from the filter with 2-3 ml of hexane followed by ethyl acetate and concentrated as required under a gentle stream of nitrogen before analysis.

Whole body extractions were done following the procedure of Ho and Millar (2002). Test mirids were first immobilised in a freezer. The immobilised insects were then put on a small piece of filter paper held between aluminium foils. The foil was then placed on another filter paper and insects squashed by applying gentle pressure. The external filter paper was discarded and the foil with the inner filter paper was then transferred using forceps into the collection chamber of the air collection apparatus. Air was sucked through the system at 60 ml/min for 15 hours. Trapped volatiles were then eluted with hexane or ethyl acetate and concentrated as required before analysis.

GC-MS analyses were conducted on whole body and air extracts using the Hewlett Packard 6890 series gas chromatograph and HP 5973 mass selective detector (Hewlett-Packard, Palo Alto, USA) as well as the GC fitted with the FID. The conditions of run were as described in Chapter 3.3.

6.2.3 Field bioassays

A series of field experiments using Delta traps (Figure 3.4, Chapter 3.6.2) were conducted to test the attractiveness of single components as well as various ratios of a blend of the major component, hexyl hexanoate and the minor component, (*E*)-2-hexenyl hexanoate. After the initial optimisation, two other components (methyl salicylate and (*Z*)-3-hexenyl acetate) found in both the male and female extracts were added to the optimised blend to determine their effect on trap catches. The different blends used in the field trials (Experiments 1 – 6) were coded as shown in Table 6.1.

Field experiments using AgriSense® traps (Figure 3.5, Chapter 3.6.2) were conducted to compare trap designs and for attract-and-kill and mating disruption studies using green mirid pheromones (Experiments 7 – 10).

	Ratio			
Blend	Hexyl hexanoate	(*E*)-2-hexenyl hexanoate	Methyl salicylate	(*Z*)-3-hexenyl acetate
GM1	2	1	-	-
GM2	5	1	-	-
GM3	10	1	-	-
GM4	16	1	-	-
GM5	1	-	-	-
GM6	-	1	-	-
GM7	1	1	-	-
GM9	25	1	-	-
GM12	3	1	-	-
GM13	7	1	-	-
GM14	5	1	1	-
GM15	5	1	-	1
GM16	5	1	1	1
GM17	4	1	-	-
GM19	6	1	-	-
GMC	-	-	-	-

Table 6.1. Coding for blends of GM pheromone components used in field trials for blend optimisation. All blends contained butylated hydroxytoluene (BHT) as antioxidant, at a concentration of 10% of the total pheromone components. Blend GM2 was based on ratios obtained from air samples, other blends were arbitrarily selected to test the effects of departures from this ratio.

6.2.3.1 Experimental sites and designs

Field experiments were conducted at "Yanco" and "Kurralinden" near Cecil Plains, "Carbucky" near Goondiwindi and "Prospect" near Warra in Queensland, and at "Yarral" and ACRI near Narrabri, "Keranna Piallamore" near Dungowan and at "Jahlee" near Mullaley in New South Wales. All trapping experiments were designed as Latin Squares with treatment (pheromone blend), trap position and day as the factors, using Delta traps as described in

Chapter 3.5.2. The layout of trap designs varied between experiments, depending on the shape and size of the field. Where possible square layouts with equal inter-trap spacings were used, but sometimes the conditions of the field made this difficult. In all the experiments, traps were cleared and rotated daily.

Experiment 1 – Comparison of blend GM1 with single components GM5 and GM6

This experiment aimed to test a 2:1 blend of the major and the minor components, hexyl hexanoate and (*E*)-2-hexenyl hexanoate, respectively, against each individual component. A 4 x 4 Latin square design with 4 treatments (GM1, GM5, GM6, GMC or control), 4 rotation periods and 4 trap locations was set up in soybeans (flowering stage) at "Kurralinden", Cecil Plains, Qld. Delta traps were located 100 m within and between rows, and cleared every day before rotation. Each lure (Figure 6.1) was loaded with 2 mg of the blend and 0.2 mg BHT as anti-oxidant. The lure for the control trap (GMC) was loaded with the BHT only.

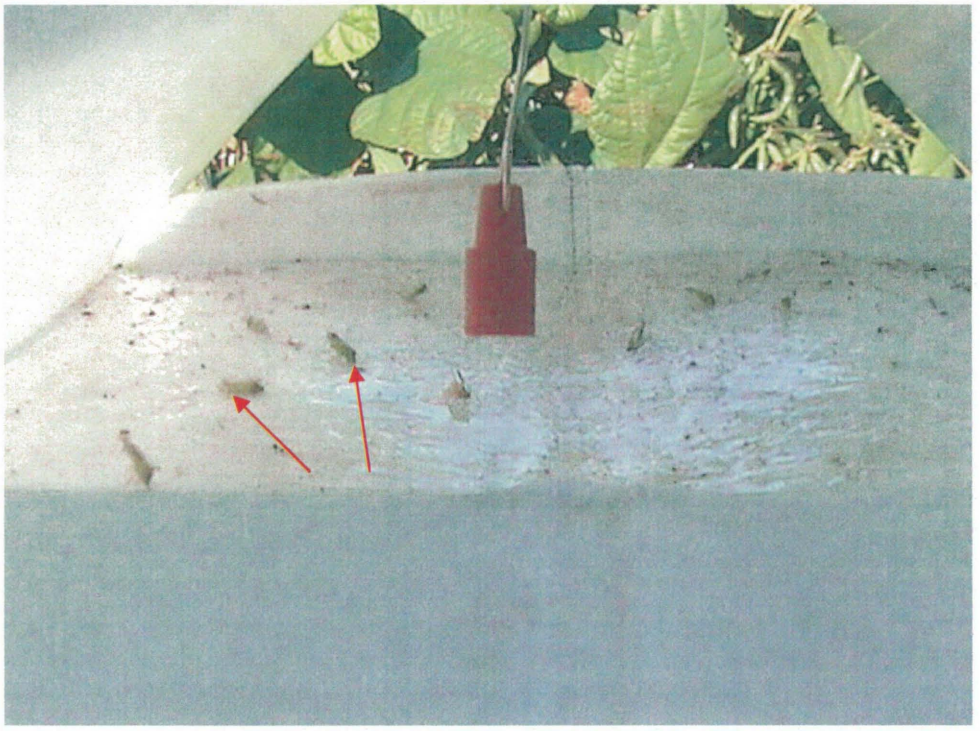


Figure 6.1. Sticky base of a Delta trap, showing rubber septa with 2 mg loading of green mirid pheromone and captured GM (arrows).

Experiment 2 – Blend optimisation (step 1)

In order to determine the optimal ratio of the blend needed to attract males, an initial experiment was carried out with a variation of the complete blend GM1. This experiment involved the use of blends GM1, GM2 and GM3. This was a 3 x 3 Latin Square design with 3 rotations, 3 blends and 3 trap locations set up in lucerne (slashing stage) at "Yarral", Narrabri, NSW. Traps were located 30 m apart within and between rows. The same blends were run concurrently in a rotational experiment in mature conventional cotton (Plot 16) at the Australian Cotton Research Institute (ACRI), Narrabri.

Experiment 2 - Blend optimisation (step 1b)

Run concurrently with the above experiment was the testing of blends GM2, GM7, GM9 and control (GMC). This experiment was also replicated twice in space in two different soybean (pod filling stage) farms located 1 kilometre apart at "Yarral", Narrabri, and hereafter referred to as site 1 and site 2.

Experiment 3 – Blend optimisation (step 2)

Three 3 x 3 Latin Square experiments were set up on two mung bean fields (pod filled stage) and one in lucerne (one week post-slashing), in "Jahlee", Mullaley, NSW. This experiment compared blends GM2, GM12, GM13. Traps were spaced at 25 m intervals within and between rows in the lucerne experiment and at 40 m in the mung bean experiments.

Experiment 4 - Blend optimisation (step 3)

Blends GM2, GM12, GM17 and GM19 were tested in a 4 x 4 Latin Square experiment in lucerne (pre-slashing stage) at "Keranna Piallamore" near Dungowan, NSW. Traps were spaced at intervals of 30 m within and between rows.

Experiment 5 - Loading effects on green mirid trap catches

This experiment aimed at testing the effect of septa loading on trap catches. A 5:1 ratio of hexyl hexanoate to (*E*)-2-hexenyl hexanoate was used in the three loadings tested. These were 2 mg (GM2), 20 mg (GM11) and 40 mg (GM10). The experimental design was a 3 x 3 Latin Square set up at two different sites in mung beans (pod filled stage) at "Jahlee", Mullaley, NSW. Traps were spaced at intervals of 50 m within and between rows.

Experiment 6 - Effects of methyl salicylate and (*Z*)-3- hexenyl acetate on blend GM2

This experiment aimed at testing the role of methyl salicylate and (*Z*)-3-hexenyl acetate identified in both male and female extracts, on the optimised blend. The hypothesis was that the addition of either methyl salicylate (GM14) or (*Z*)-3-hexenyl acetate (GM15) or both of these minor components (GM16) to a 5:1 ratio of hexyl hexanoate to (*E*)-2-hexenyl hexanoate (GM2) might increase the attractiveness of the pheromone blend. These four blends were used in a 4 x 4 Latin Square experiment conducted in lucerne (slashing stage) at "Keranna Piallamore" near Dungowan, NSW. Traps were spaced at intervals of 50 m within and between rows.

Numbers of green mirids caught in pheromone traps at various times

During Experiment 1, traps were checked to record the number of GM males caught at different times. For the first day, traps were inspected at 1200 h, 1800 h, 2400 h and 0600 h AEST. On the second day, traps were once again inspected at hourly intervals from 1800 h to 2400 h, and again at 0600 h AEST.

Experiment 7 - Comparison of AgriSense® and Delta trap designs using blend GM2

This experiment aimed to test the suitability of the AgriSense® funnel traps for trapping GM compared with the Delta traps. The experimental design is shown in Figure 6.2. There were

4 rotation periods, 2 trap locations and 2 trap types. The experiment was conducted on a cotton field (pre-squaring stage) at "Yanco" near Cecil Plains, Qld. Traps were located 50 m from each other within rows and 100 m between rows. Traps were located 100 m from the edge of the cotton field. Trap clearing was done daily before rotation.

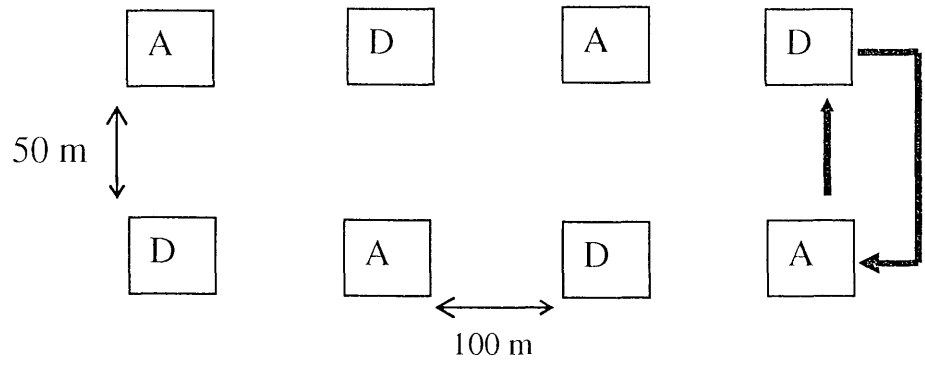


Figure 6.2. Experiment 7. Layout of pheromone traps at "Yanco", Cecil Plains, Qld. Traps were rotated daily in the direction of the black arrow. D – Delta trap; A – AgriSense® trap.

Experiment 8 – Effect of trap location on green mirid catches

This experiment aimed at determining the effect of trap location in relation to the crop site to catches of GM with AgriSense® traps. Four traps, located 100 m apart, were set up in the middle of a 700 m x 700 m cotton field (pre-squaring stage) at "Yanco", near Cecil Plains, Qld. Four other traps were located just outside the cotton, one at each corner of the field (about 12 m from the edge of the outer row of the cotton). Each trap had a GM pheromone lure with a 2 mg loading which was replaced every two days when the traps were serviced.

Experiment 9 – 'Attract-and-kill' experiment using suction sampling

This experiment was designed to investigate whether the mirid pheromone components identified through laboratory and field trapping experiments described above could be used in

a sprayable formulation for attracting male GM to specific rows of cotton, for possible application in an attract-and-kill system.

The trial was set up in a field of flowering faba beans at "Carbucky", near Goondiwindi, Qld. Magnet® base (Table 6.2) was used to formulate the treatments. Magnet® is a sprayable attract-and-kill formulation which contains plant volatiles and insecticides, and is being commercialised for use against *Helicoverpa* moths in Australian cotton (Gregg & Del Socorro, 2002). For the purposes of this experiment, the plant volatiles and insecticides were omitted. There were two treatments, Magnet® base alone and base containing the GM pheromone blend. The GM pheromone blend consisted of 1% hexyl hexanoate and 0.2% (*E*)-2-hexenyl hexanoate.

Ingredient	Purpose	(%)	Supplier
Canola oil (food grade)	Carrier	15.0	Various
Sucrose (food grade)	Feeding stimulant	20.0	Various
Sorbitan monostearate	Emulsifier	2.0	APS Cotter Food Services, Seven Hills, NSW, Australia
Xanthan gum	Thickener	0.1	Sigma-Aldrich, Castle Hill, NSW, Australia
Vitamin E	Antioxidant	0.1	Sigma-Aldrich, Castle Hill, NSW, Australia
Butylated hydroxytoluene	Antioxidant	0.1	Lancaster, Lanchashire, United Kingdom
Brilliant Blue (food colour 133)	Marker	0.1	Queen Fine Foods, Alderley, Qld, Australia
Water	Extender	62.5	Various

Table 6.2. Ingredients of Magnet® base used as carrier for pheromone components in Experiment 9.

Treatments were applied to 50 m strips of faba beans, arranged in a square pattern of four rows each containing one replicate of each treatment, with 50 m buffer strips between them.

Rows were separated by 50 m. Formulations were applied to the tops of plants in each replicate by hand (shaken from a plastic bottle) at 500 ml per 50 m.

The treatments were sampled using a large backpack suction sampler (D-Vac), based on a Solo Mist Blower Port 423. This machine has a sampling efficiency of 50-60% for GM in cotton (Stanley, 1997). Its efficiency in faba beans has not been determined, but the structure of the crop is comparable to cotton and similar efficiency might be expected. The nozzle was moved over the top of the plants at a slow walking speed and insects collected in a nylon bag, then transferred to plastic bags and frozen prior to counting. Treatments were sampled at 20, 31, 78 and 123 h post application. The 20 h sample was done in mid-morning, on the day following application, but all subsequent samples were done at night, around 2200-2300 h. This was because the trapping studies (Experiment 1) showed that most male mirids came to the pheromone in the early evening. On each sampling occasion, four control (untreated) 50 m sections were sampled from randomly chosen locations between the treated rows. The controls therefore represented sections from which insects had not been previously removed, whereas for the treated sections, most insects collected probably represent arrivals since the last sampling time.

Experiment 10 – Attract-and-kill and mating disruption trial for green mirids

This experiment aimed at testing formulations using mirid pheromones identified for attract-and-kill and for mating disruption. The trial was carried out in December 2004 in dryland cotton (pre-squaring stage) at "Prospect", near Warra, Qld. Two sites, about 400 metres apart, were chosen for this experiment. These sites were labelled as field S3 (conventional cotton) and field WL4 (Bollgard II® or transgenic cotton). Both fields had double skip rows, that is, two rows planted and two rows skipped, which is a common configuration for dryland cotton in Australia. Field WL4 was used as a distant control, additional to the control plot included

in the test field (S3). Trial layout and design was as shown in Figures 6.3 and 6.4. Treatment blocks were demarcated as 290 m x 300 m (8.7 ha) for field S3 as shown in Figure 6.4. Buffer zones of 300 m were left between treatments. For field WL4, no treatments were imposed but pheromone traps were laid out in a similar pattern to field S3, except that the dimensions of this field were slightly smaller (Figure 6.3). Populations were monitored on field WL4 as a control to detect possible area-wide changes in mirid numbers, unrelated to the treatments, and in case the effects of the treatments extended beyond the boundaries of the plots in field S3.

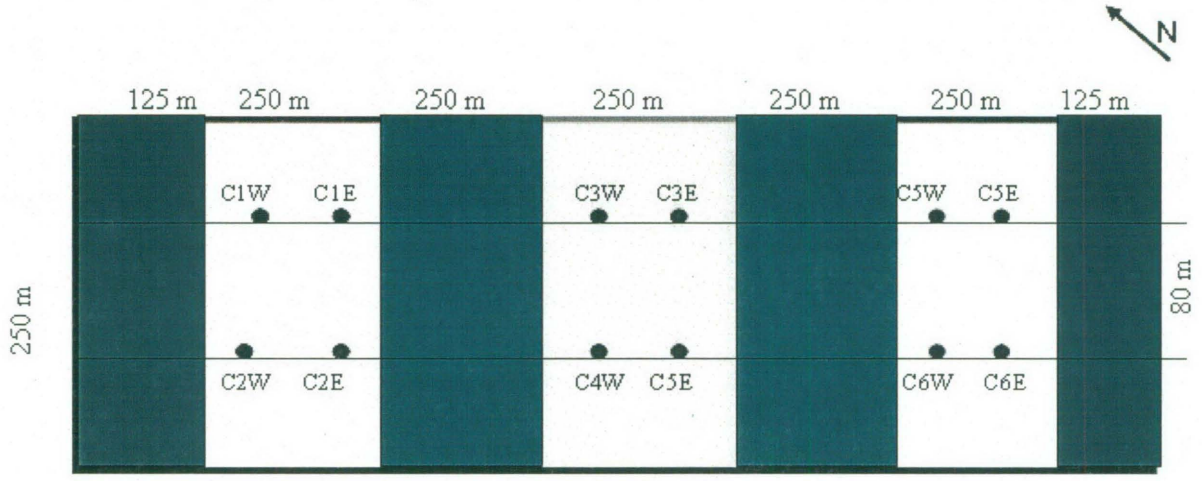


Figure 6.3. Layout of field WL4 (control) showing location of pheromone traps. • – pheromone traps

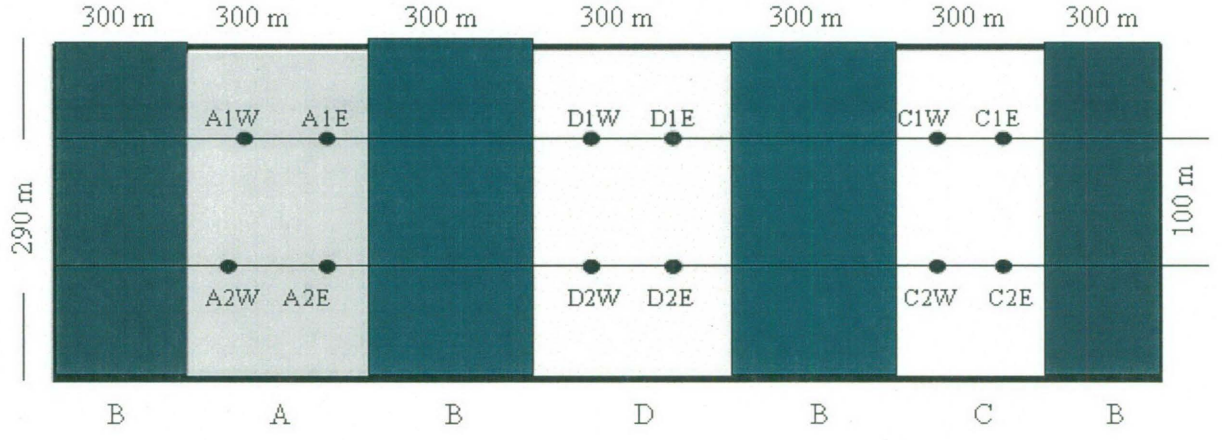


Figure 6.4. Layout of field S3 (treated) showing location of pheromone traps. • – pheromone traps, A – attract-and-kill, D – mating disruption, C - control , B – buffer zones.

Each treatment plot had 4 pheromone traps (AgriSense® design) containing mirid lures (5:1 hexyl hexanoate: (*E*)-2-hexenyl hexanoate at 2 mg loading) Traps were located one-third of the way in from each corner, in two rows (row 1 = northern, row 2 = southern), and coded for ease of identification as shown in Figures 6.3 and 6.4.

Attract-and-kill

For the attract-and-kill treatment, one row in every 32 (including skip rows) was treated with 500 ml/100 m of 1.2% mirid pheromone mix prepared in a base similar to Magnet® (Gregg & Del Socorro, 2002) as used in Experiment 9, but omitting the sugar and food dye. This mixture is shown in Table 6.3.

Ingredient	Purpose	(%)
Canola oil (food grade)	Carrier	15.0
Sorbitan monostearate	Emulsifier	2.0
Xanthan gum	Thickener	0.25
Vitamin E	Antioxidant	0.05
Butylated hydroxytoluene	Antioxidant	0.05
Water	Extender	81.0

Table 6.3. Ingredients of the carrier for pheromone components in the attract-and-kill trial.

A latex-based adjuvant and sticker (Bond®, Nufarm Aust. Limited, Laverton, Victoria, Australia) was then added to give a 1% concentration. Fipronil (Regent®, Bayer Australia Ltd, Pymble, NSW, Australia) was sprayed onto the treated rows as a cover spray, immediately after the pheromone, at a rate of 1.25 ml a.i./100 m. Fipronil is the most widely used insecticide against mirids, and the rate chosen was twice the normal rate to ensure mortality of the insect.

Mating disruption

For mating disruption, one row in every 16 (including skip rows) was treated with 500 ml/100 m of 2.4% mirid pheromone in the base described above. The pheromone quantity applied per hectare was therefore four times that in the attract-and-kill treatment (twice as concentrated, and applied to twice as many rows). However, there was no insecticide present.

Formulations were applied by spraying through a low pressure electric pump using a nozzle designed for liquid fertiliser application. A modified motor cycle was used. The appearances of the formulation freshly applied and after weathering, are shown in Figures 6.5 and 6.6.

Figure 6.5. Cotton plants on field S3 showing the freshly applied formulation of the pheromone in the mating disruption plot. Photo: Peter Gregg.

Figure 6.6. Appearance of formulation on leaves with weathering. Photo: Peter Gregg.

The treatments in field S3 were sampled using a large backpack suction sampler (D-Vac), as described for Experiment 9. Four samples were taken from 50 m strips of cotton that did not have any treatment application within the treatment blocks. Treatments were sampled at 0 day (pre-treatment), 1, 2, 3, 4 and 7 days post application.

6.3 Results and discussion

6.3.1 Pheromone analysis and identification

GC- MS analysis of air adsorbed and whole body extracts revealed several compounds from both virgin male and female insects (Figure 6.7). Compounds were identified by comparison of the mass spectral data with standard spectra for hexyl hexanoate, (*E*)-2- hexyl hexanoate, methyl salicylate and (*Z*)-3 hexenyl acetate, by co-injection with authentic standards. Apart from hydrocarbons ranging from dodecane to nonadecane, the rest of the compounds are shown in Table 6.4.

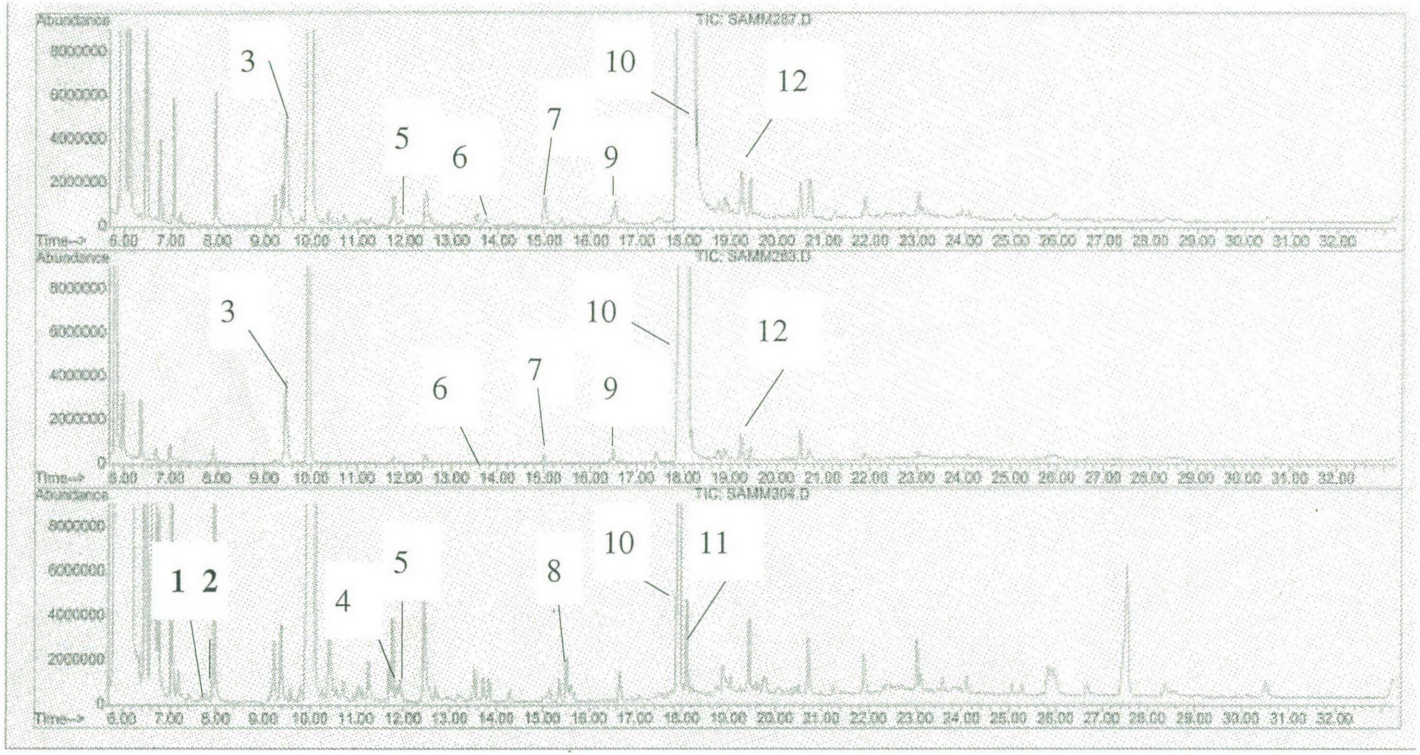


Figure 6.7. Gas chromatographs of male whole body extracts (A), female whole body extracts (B), and female air collections (C) of *Creontiades dilutus*.

compound 1 = 3-hexanone, 2 = 2-hexanone, 3 = hexanol, 4 = (*Z*)-3-hexenyl acetate, 5 = Octanal, 6 = nonanal, 7 = hexyl butyrate, 8 = methyl salicylate, 9 = pentyl hexanoate, 10 = Hexyl hexanoate, 11 = (*E*)-2-hexenyl hexanoate, 12 = heptyl hexanoate.

Compound		Whole body extract (n=3)		Air (n=3)	
	Retention time (min)	female	male	female	male
3-Hexanone	7.655	0.00 ± 0.0	0.00 ± 0.0	1.05 ± 0.5	5.89 ± 0.6
2-Hexanone	7.751	0.00 ± 0.0	0.00 ± 0.0	1.42 ± 0.7	4.97 ± 0.4
Hexanol	9.455	4.16 ± 2.4	3.16 ± 1.5	0.00 ± 0.0	0.00 ± 0.0
(*Z*)-3 Hexenyl acetate	11.869	0.00 ± 0.0	0.00 ± 0.0	4.05 ±2.2	2.40 ± 1.5
Octanal	11.932	0.32 ± 0.2	0.18 ± 0.1	6.30 ±3.8	24.46 ± 4.2
Nonanal	13.714	0.51 ± 0.3	0.24 ± 0.2	10.43 ± 4.2	40.85 ± 0.6
Hexyl butyrate	15.020	0.91 ± 0.5	0.79 ± 0.7	0.00 ± 0.0	0.00.± 0.0
Methyl salicylate	15.516	0.00 ± 0.0	0.00 ± 0.0	9.32 ± 2.8	4.90 ± 2.8
Pentyl hexanoate	16.524	3.44 ± 2.0	0.99 ± 0.8	0.00 ± 0.0	0.00 ± 0.0
Hexyl hexanoate	17.904	100	100	100	100
(*E*)-2- Hexenyl hexanoate	18.051	0.00 ± 0.0	0.00 ± 0.0	33.67 ± 0.9	0.00 ± 0.0
Heptyl hexanoate	19.254	2.37 ± 1.4	2.21 ± 1.3	0.00 ± 0.0	0.00 ± 0.0

Table 6.4. Calculated percent composition of air and whole body extracts of green mirid relative to hexyl hexanoate (mean ± s.e).

The mass spectra of the various components and some corresponding standard spectra are shown in Figures 6.8 to 6.14.

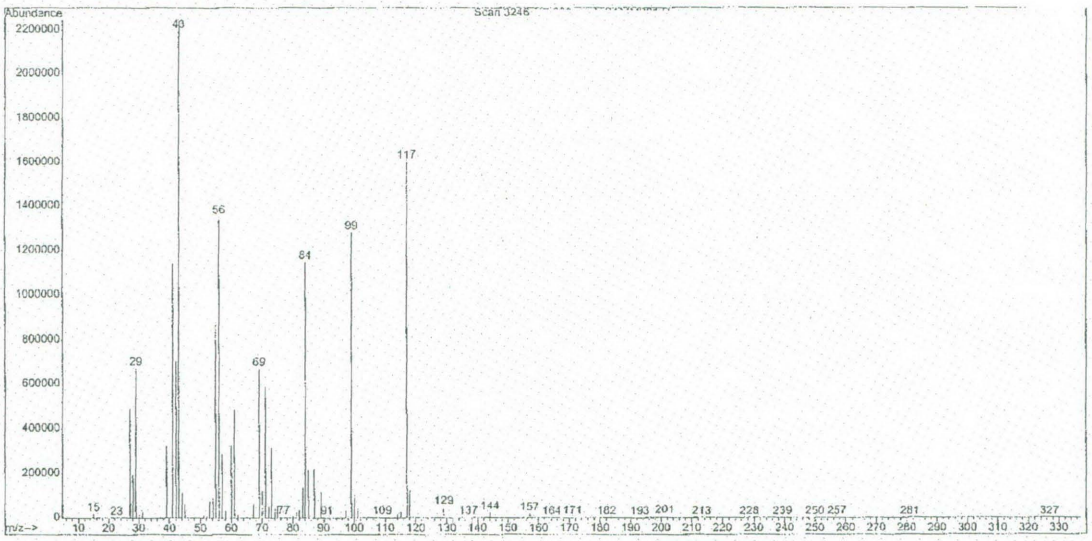


Figure 6.8. Mass spectra of compound 10.

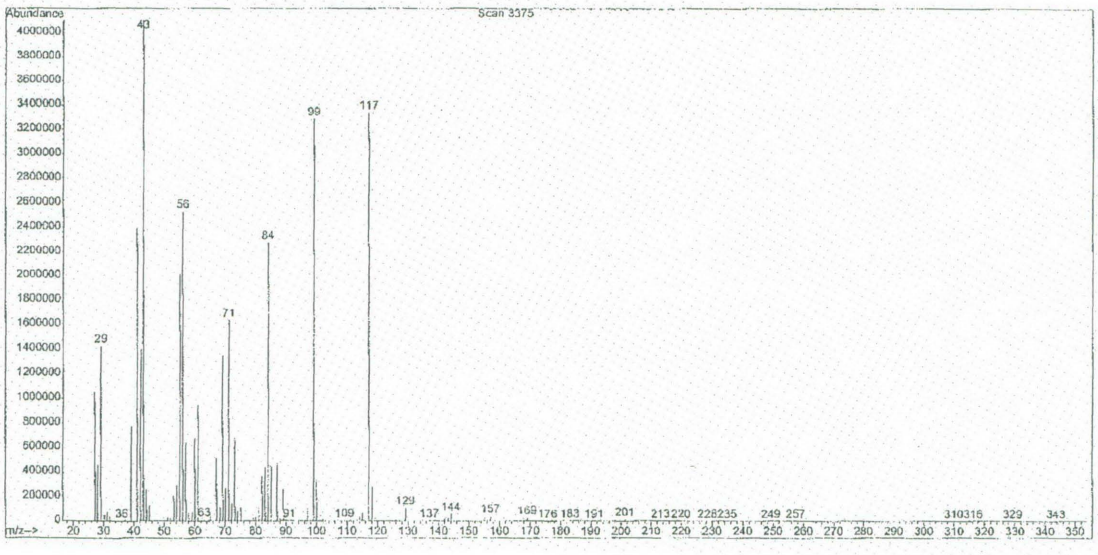


Figure 6.9. Mass spectra of standard hexyl hexanaote.

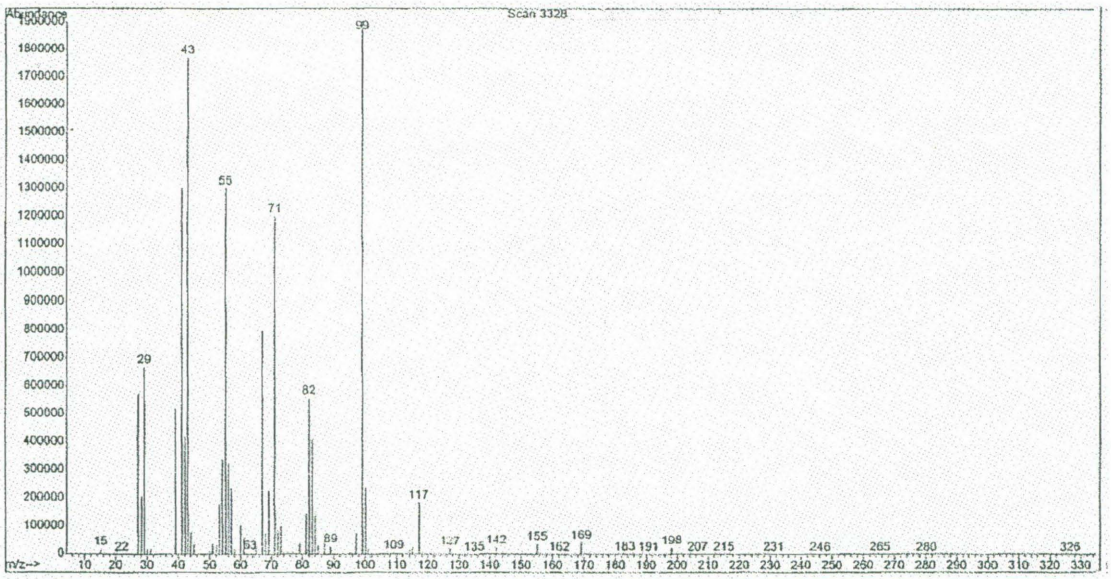


Figure 6.10. Mass spectra of compound 11.

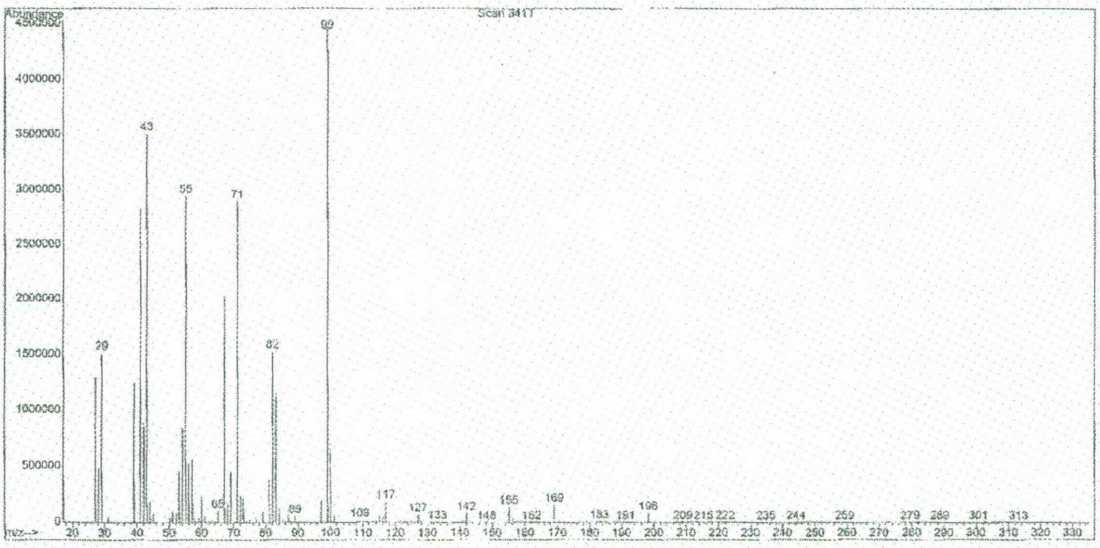


Figure 6.11. Mass spectra of standard (*E*)-2-hexenyl hexanoate.

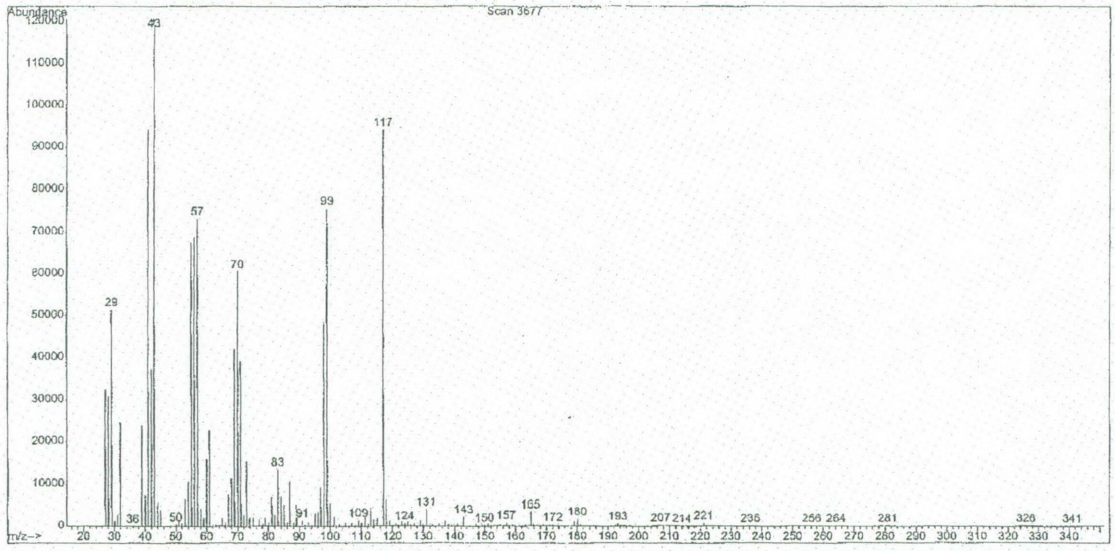


Figure 6.12. Mass spectra of compound 12.

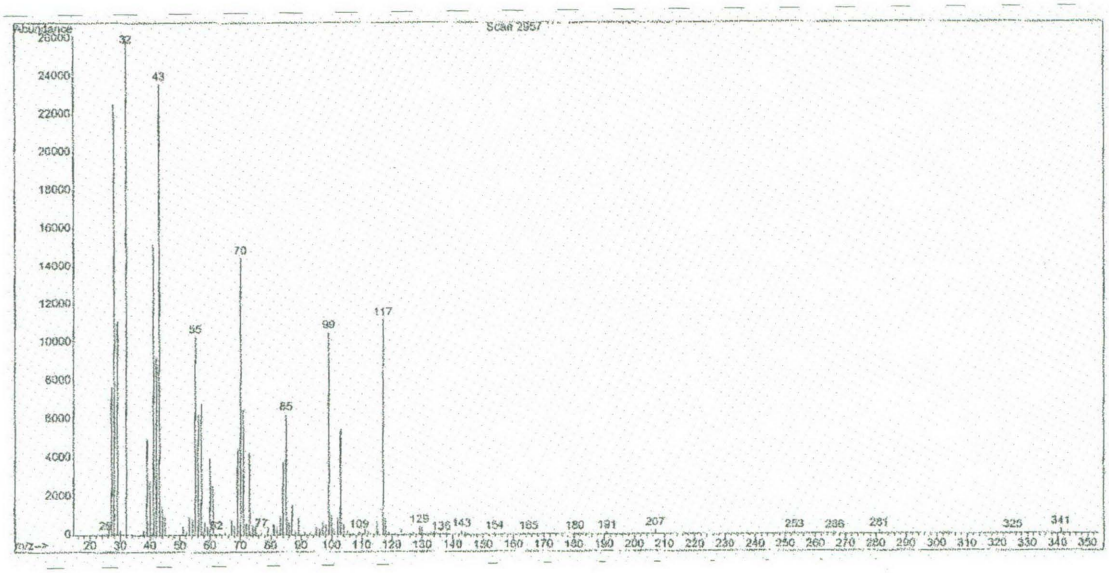


Figure 6.13. Mass spectra of compound 9.

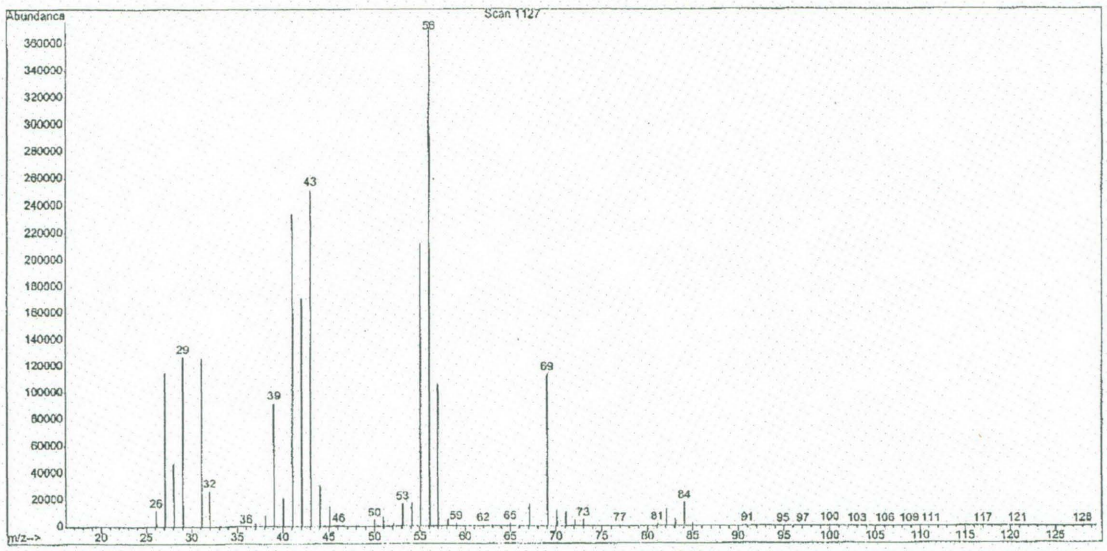


Figure 6.14. Mass spectra of compound 3.

Searching for differences between the chemical profiles of female and male extracts in insects is one of the ways of determining potential sex pheromones (i.e., sex-specific compounds). No sex-specific compound was found in the whole body extracts of GM. The whole body extracts from both males and females contained four additional compounds, hexan-1-ol, hexyl butyrate, pentyl hexanoate and heptyl hexanoate which were not detected in the air collections (Figure 6.7). These compounds could possibly be alarm pheromones. Hexyl butyrate has been identified in both air and methathoracic gland collections of other Miridae like *Lygus hesperus* (Ho & Millar, 2002), *L. lineolaris* (Gueldner & Parrott, 1978) and *Phytocoris californicus* (Millar & Rice, 1998). While Zhang and Aldrich (2003b) reported this compound as a male produced anti-sex pheromone of *P. relativus*, another study (Zhang & Aldrich, 2004) indicates it, in addition to (*E*)-2-hexenyl butyrate in a ratio occurring in *L. lineolaris* and other mirids, acts as a kairomone attracting large numbers of scavenging

Chloropid and Milichiid flies (Diptera). In *L. hesperus*, hexyl butyrate formed the major component of the metathoracic glands. Hexanol was present at 9.9% relative to the hexyl butyrate in both male and female air collections, and 1.2 and 1.9% in the female and male glands, respectively. However, in squashed samples values were 400 and 419% for female and male air collections, while in glands these values were 429 and 140%, respectively. In GM, hexanol was not present in the air collections but about 4% was detected in the squashed whole body extracts. Ho and Millar (2002) detected the presence of heptanol in extracts of *L. hesperus* spiked with heptyl butyrate which formerly did not contain either compound. They concluded that extensive hydrolysis of the esters may have been the reason for the high level of hexanol in the squashed samples. It is possible that the presence of hexanol in the squashed extracts of both the female and male GM was also the result of hydrolysis of the hexyl butyrate present. Pentyl hexanoate on the other hand, has only been cited as part of volatiles collected from apples which act as an attractant for the apple maggot fly, *Rhagoletis pomonella* (Walsh) (Zhang *et al.*, 1999).

In the present study, sex-specific differences existed in the air collected samples from GM. One of the major components in both whole body and air collections from both male and female was hexyl hexanoate. In addition, the female air collected samples contained a sex-specific compound, (*E*)–2-hexenyl hexanoate. Others included hydrocarbons like dodecane, tridecane, tetradecane and methyl salicylate (MeSA). Hydrocarbons have been suggested to function as "wetting and spreading agents" (Blum, 1978), promoting penetration of compounds like aldehydes and retarding evaporation of the more potent compounds. They have also been suggested to function as predator deterrents by possibly reducing olfactory perception. MeSA, on the other-hand, has been cited as an anti-aphrodisiac produced by male *Pieris napi* (Linnaeus) butterflies, synthesised and transferred at mating (Andersson *et al.*, 2000). As a herbivore-induced plant volatile, MeSA has been demonstrated as an attractant

for the green lacewing, *Chrysopa nigricornis* (Burmeister) (James, 2003) and the western flower thrips, *Frankliniella occidentalis* (Pergande) (Chermenskaya *et al.*, 2002). Its role in GM is not clear. However, MeSA and another volatile collected in air but not whole body extracts, (*Z*)-3 hexenyl acetate, are common plant volatiles, and they may have come from the pieces of lucerne and beans included with the GM, not from the insects themselves.

The female sex-specific compound (*E*)–2-hexenyl hexanoate identified in GM has also been reported in other bugs (Heteroptera:Alydidae). For example, males of the bean bug *Riptortus clavatus* (Thunberg) produce male specific compounds (*E*)-2-hexenyl–(*Z*)-3-hexenoate, (*E*)-2-hexenyl–(*E*)-2-hexenoate, myristyl isobutyrate and (*E*)–2-hexenyl hexanoate, the latter being an alarm pheromone (Leal & Kadosawa, 1992) and a blend of the others as attractant pheromones attracting adults and second instar nymphs (Leal *et al.*, 1995). In a similar species, *Riptortus serripes* (Fabricius) both males and females produced (*E*)-2-hexenyl hexanoate but its role as pheromone component is unknown (Aldrich *et al.*, 1993). In addition to octyl butyrate, the two compounds hexyl hexanoate and (*E*)-2-hexenyl hexanoate have been identified as pheromones of the rice leaf bug, *Trigonotylus caelestialium* (Kirkaldy) in Japan (Kakizaki & Sugie, 2001). A ratio of 10-100:1000:400-500 of octyl butyrate, hexyl hexanoate and (*E*)-2-hexenyl hexanoate was found to be attractive to males. (*E*)–2-hexenyl hexanoate and hexyl hexanoate have also been reported in secretions of *Blepharidopterus angulatus* (Fallen) (Miridae) (Knight *et al.*, 1984) and *Homoeocerus unipunctatus* (Thunberg) (Coreidae) (Kitamura *et al.*, 1984).

The source of pheromone secretions has been determined in other Miridae species. It is in the thorax for *P. relativus* (Millar *et al.*, 1997), abdomen tip for *L. hesperus* (Graham, 1988), head and thorax for *C. verbasci* (Thistlewood *et al.*, 1989) and is suggested to be in the metathoracic scent gland for *V. thapsis*, (McBrien & Millar, 1999) and *L. lineolaris* (Gueldner

& Parrott, 1978). The location of pheromone glands in the *C. dilutus* has not been determined in this study. Miles (1995), however, had previously identified hexyl hexanoate from the metathoracic glands of GM. It is therefore possible that the source of all pheromone compounds in this species might be the metathoracic glands.

6.3.2 Field bioassays

Experiment 1 - Comparison of blend GM1 with single components GM5 and GM6

Figure 6.16 below shows the mean (± s.e) catches per night of Delta traps baited with the two-component blend (GM1), the single components hexyl hexanoate (GM5) and (*E*)-2-hexenyl hexanoate (GM6), and the blank or control (GMC).

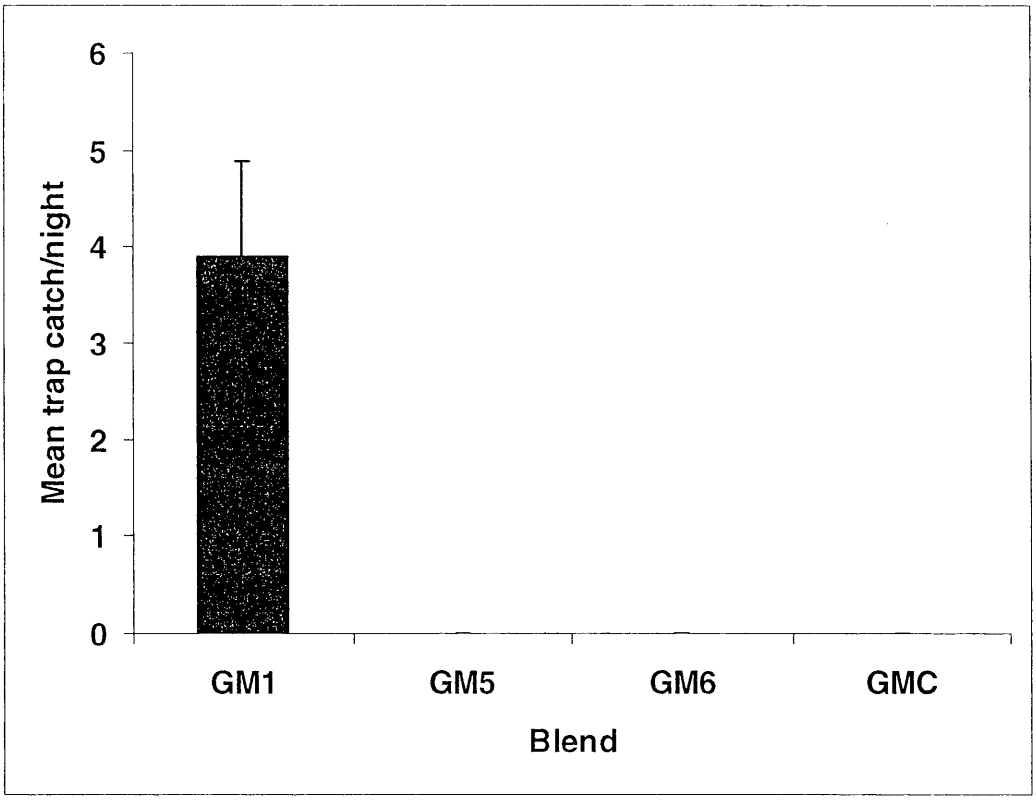


Figure 6.15. Experiment 1. Mean (± s.e) catches of Delta traps baited with the two-component blend (GM1), the single components (GM5 and GM6) and the blank or control, at "Kurralinden", Cecil Plains, Qld.

C. dilutus adults were only caught in traps baited with the 2:1 blend of hexyl hexanoate and (*E*)-2-hexenyl hexanoate (GM1) at an average of 3.9 GM per trap per night. All insects caught were sexed morphologically and found to be males. Traps baited with either hexyl hexanoate (GM5) or (*E*)-2-hexenyl hexanoate (GM6) only, and the blank or control traps, did not catch any GM. These results are similar to the situation in the rice leaf bug, *T. caelestialium*, where all the ten electroantennographic detector (EAD) active compounds on their own did not attract any males (Kakizaki & Sugie, 2001). These results also suggest that the two compounds, hexyl hexanoate and (*E*)-2-hexenyl hexanoate, are both required for the pheromone to work in GM. (*E*)-2-hexenyl hexanoate, being the minor component unique to the female, did not attract any males in the absence of the major component, hexyl hexanoate, which is produced by both sexes. This situation where the attractive blend is a mix of a female component(s) and one produced by both sexes has also been reported in two other mirids, *P. relativus* and *P. californicus* (Millar *et al.*, 1997; Millar & Rice, 1998). Another situation where both male and female extracts contain the same compounds but the pheromone is attractive to the males only has also been reported for the rice leaf bug, *T. caelestialium* (Kakizaki & Sugie, 2001) and *Phytocoris difficulis* (Zhang & Aldrich, 2003a).

Due to the inhibitory or defensive compounds produced by heteropteran bugs, previous researchers have shown that attempting a complete reconstruction of the pheromone blend is very difficult (McBrien & Millar, 1999). Experiments to optimise the GM pheromone blend were based on two compounds only, hexyl hexanoate and (*E*)-2-hexenyl hexanoate. The fact that only adult males of *C. dilutus* (and none of other species of mirids likely to be present in the field sites) were caught in the traps suggests that the sex pheromone is stage and species specific.

The analysis of variance indicated that effects of trap rotation and location ($P \geq 0.100$), and day factors ($P = 0.090$) were not statistically significant. The one highly significant factor influencing trap catch was the blend type ($P < 0.001$). Comparison of the means using contrast in the R program indicated highly significant differences between blend GM1 and GM5, GM6 and GMC ($P < 0.001$).

Experiment 2 - Blend optimisation (step 1)

In this experiment, various ratios of hexyl hexanoate to (*E*)-2-hexenyl hexanoate were compared in traps set up in a lucerne field. These were GM1 (2:1), GM2 (5:1) and GM3 (10:1). Mean male trap catches per night for blends GM1, GM2 and GM3 were 4.2, 7.2 and 2.7, respectively (Figure 6.16). Whereas there were no significant effects of trap rotation, location and day ($P \geq 0.200$), the analysis of variance yielded a significant effect of blend type ($P = 0.010$). Comparison of the means using contrast in the R program indicated significant differences between all three blends, with blend GM2 having the highest mean catch per trap.

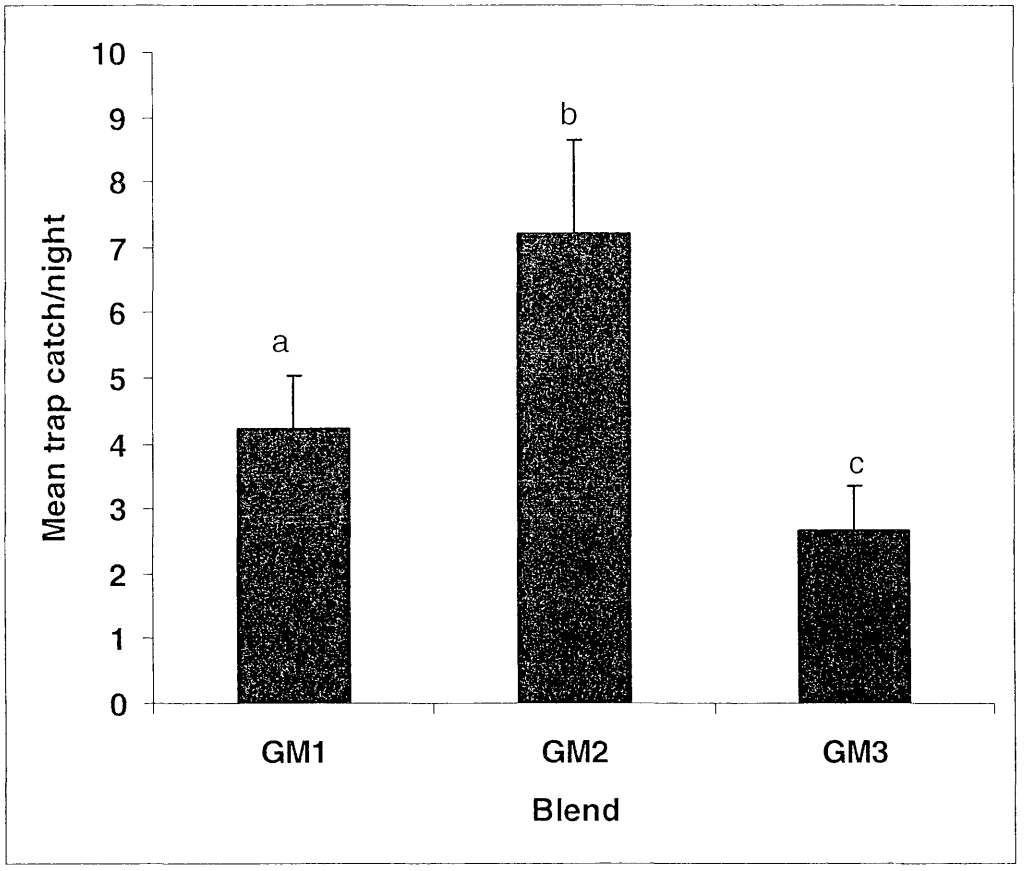


Figure 6.16. Experiment 2. Mean (± s.e) catches per trap per night of blends GM1 (2:1), GM2 (5:1) and GM3 (10:1) in lucerne, "Yarral", Narrabri, NSW. Columns with common letters are not significantly different ($P > 0.05$).

The same experiment using the same three blends as above were also carried out in maturing cotton. Mean trap catches per night of GM1, GM2 and GM3 were 0, 1.4 and 0.1, respectively (Figure 6.17). These means were much lower than those reported above (in lucerne) possibly because of low numbers of GM in this advanced cotton field. The effects of day, trap rotation and location were not significant ($P \geq 0.300$), while that of blend type was highly significant ($P < 0.001$).

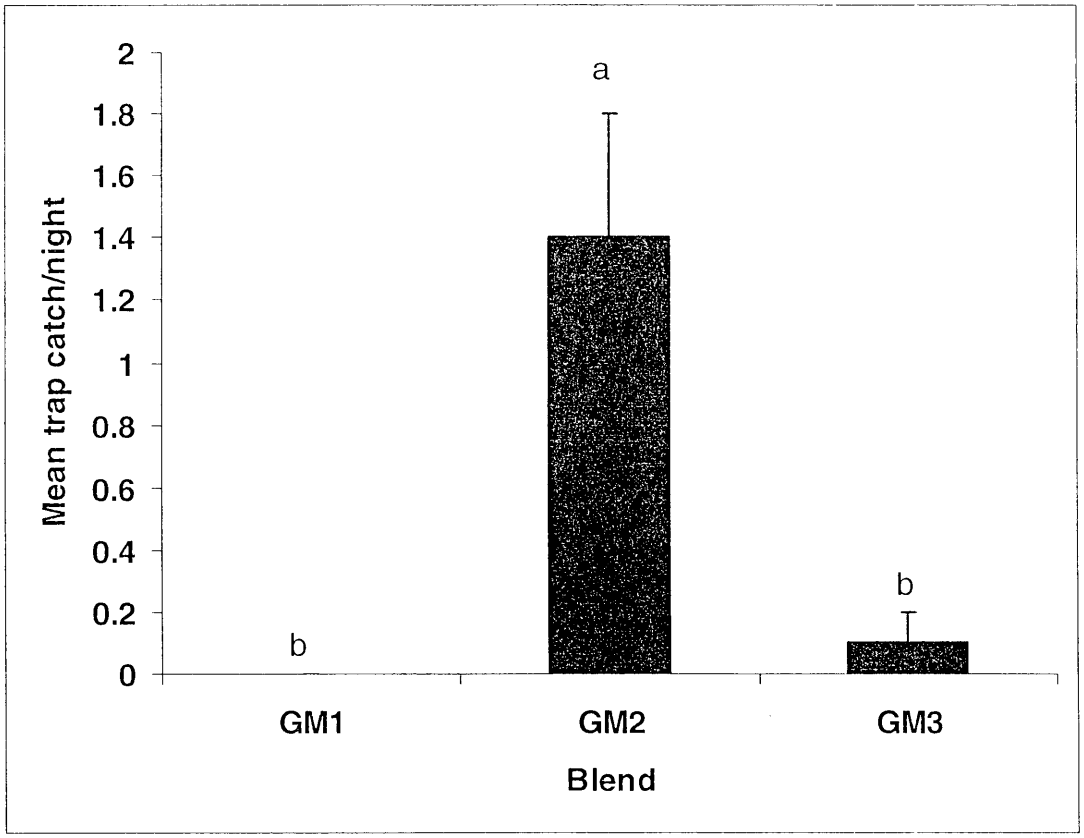


Figure 6.17. Experiment 2. Mean (± s.e) catches per trap per night of blends GM1 (2:1), GM2 (5:1) and GM3 (10:1) in cotton, ACRI, Narrabri, NSW. Columns with common letters are not significantly different ($P > 0.05$).

Experiment 2 - Blend optimisation (step 1b)

Results from both the lucerne and cotton traps in Experiment 1 above showed that GM2 consisting of a 5:1 ratio of hexyl hexanoate and (*E*)-2-hexenyl hexanoate, appeared to be attractive to GM males. In Experiment 2, GM2 was compared with two other blends with different ratios of hexyl hexanoate to (*E*)-2-hexenyl hexanoate, GM7 (1:1) and GM9 (25:1), and the blank or control (GMC), at two sites in soybeans.

Trap catches at the two different sites in soybeans were low, as shown in Figures 6.18 and 6.19. At site 1, blend type was the only significant factor influencing catches ($P < 0.001$). At

both sites, all the other factors were not significant ($P \geq 0.220$). The results suggest that a higher amount of the main component, hexyl hexanoate, reduced trap catches as seen in GM9. A 1:1 ratio of the two components also had lower catches compared to the 5:1 ratio in GM2. Since higher amounts of hexyl hexanoate are inhibitory, maybe this compound alone could function for mating disruption, although the limited previous attempts to use mirid pheromone components for mating disruption did not indicate the best results with the complete pheromone blends (McBrien *et al.*, 1997; Kakizaki, 2004).

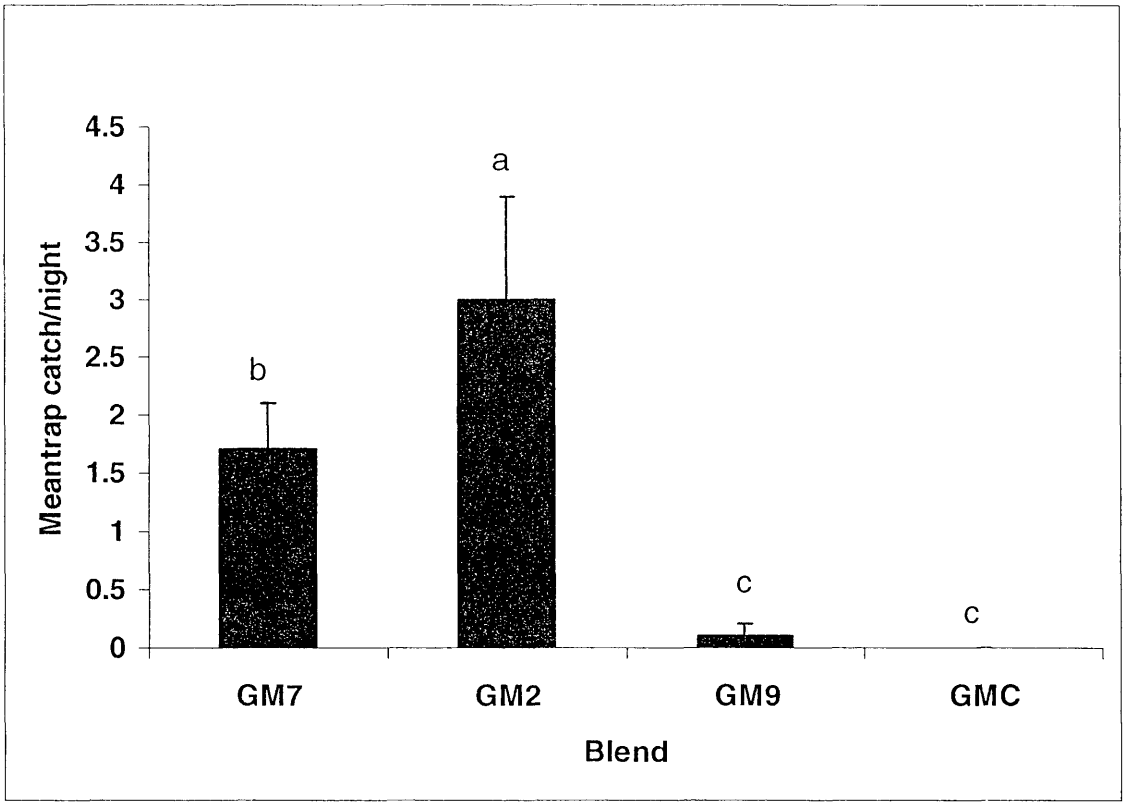


Figure 6.18. Experiment 2. Mean (± s.e) catches per trap per night of blends GM7 (1:1), GM2 (5:1), GM9 (25:1) and GMC set up in soybeans at site 1, "Yarral", Narrabri, NSW. Columns with common letters are not significantly different ($P > 0.05$).

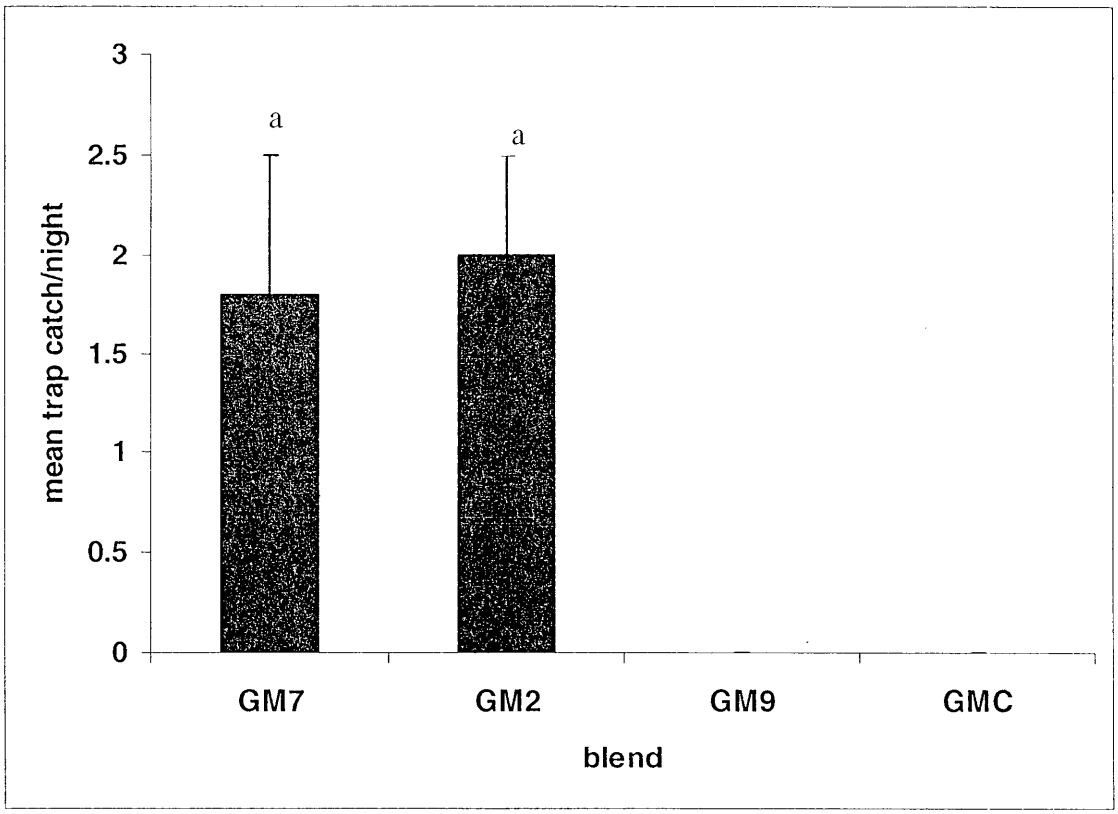


Figure 6.19. Experiment 2. Mean (± s.e) catches per trap per night of blends GM7 (1:1), GM2 (5:1), GM9 (25:1) and GMC set up in soybeans at site 2, "Yarral", Narrabri, NSW. Columns with common letters are not significantly different ($P > 0.05$).

Experiment 3 - Blend optimisation of blend (step 2)

In this experiment, GM2 (5:1) was compared with another two blends of different ratios of hexyl hexanoate to (*E*)-2-hexenyl hexanoate, GM12 (3:1) and GM13 (7:1), at two sites in mung beans and one in lucerne.

Figures 6.20 and 6.21 show mean trap catches for the various blends tested at two sites in the mung beans.

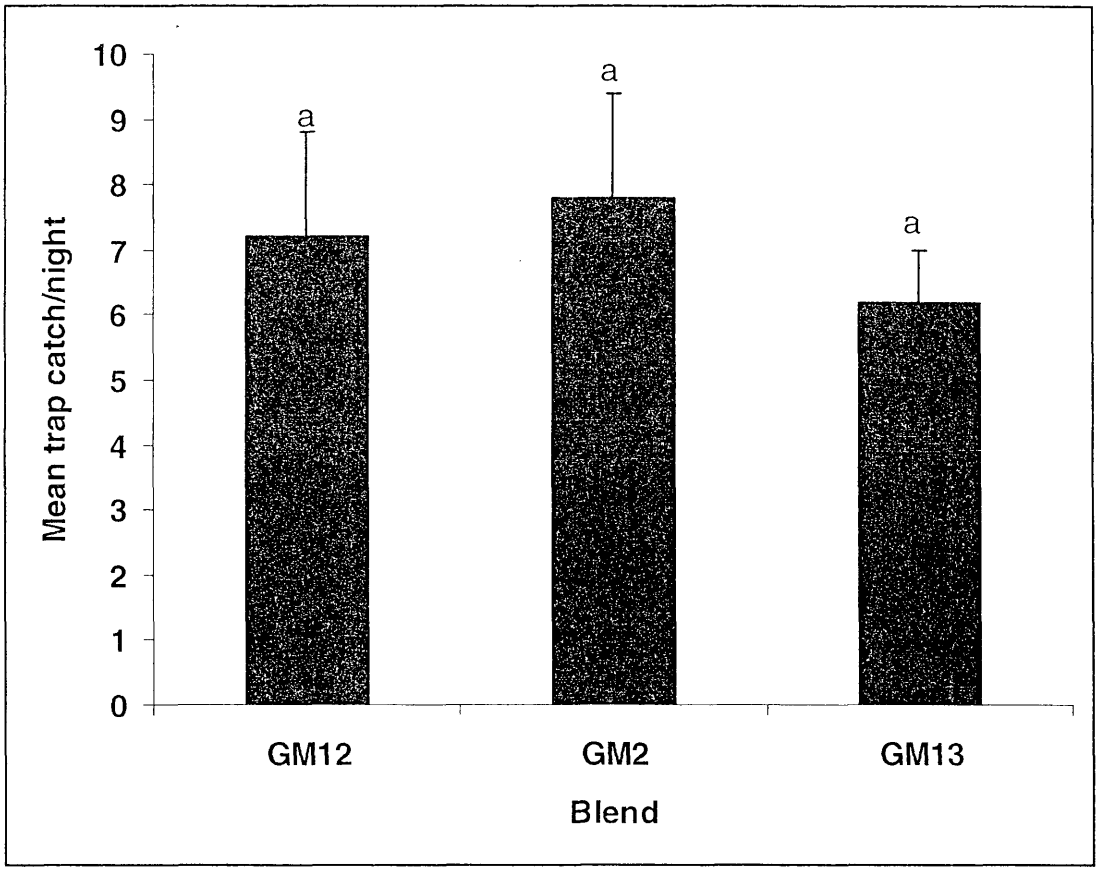


Figure 6.20. Experiment 3. Mean trap catches per trap per night for comparison of blends GM12 (3:1), GM2 (5:1) and GM13 (7:1) in mung beans at site 1, "Jahlee", Mullaley, NSW. Columns with common letters are not significantly different ($P > 0.05$).

In both sites, the analysis of variance showed no significant effects of blend, row, day or position ($P \geq 0.570$ and $P \geq 0.146$, respectively).

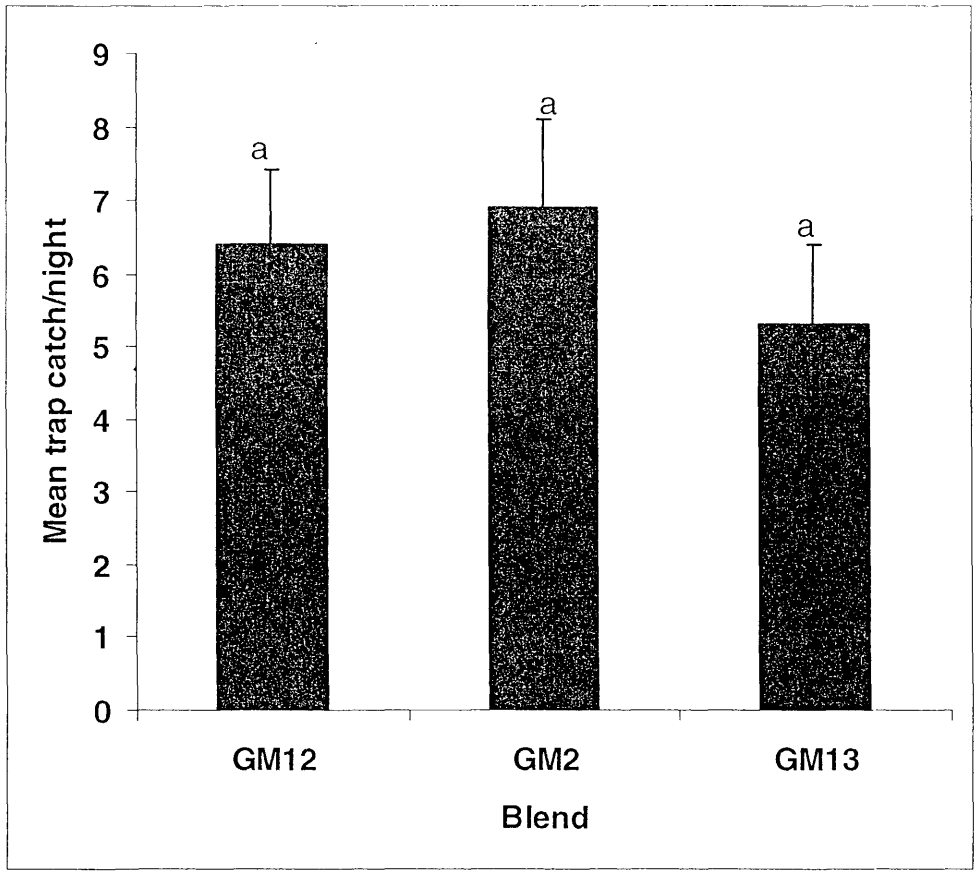


Figure 6.21. Experiment 3. Mean trap catches per trap per night for comparison of blends GM12 (3:1), GM2 (5:1) and GM13 (7:1) in mung beans at site 2, "Jahlee", Mullaley, NSW. Columns with common letters are not significantly different ($P > 0.05$).

Figure 6.22 shows trap catches for a similar experiment carried out in a harvested lucerne site. The analysis of variance did not indicate any significant effect of blend type, row and position ($P \geq 0.140$). The effect of day was, however, significant ($P = 0.016$), indicating that some nights may not have been suitable for trapping, perhaps because of low temperatures.

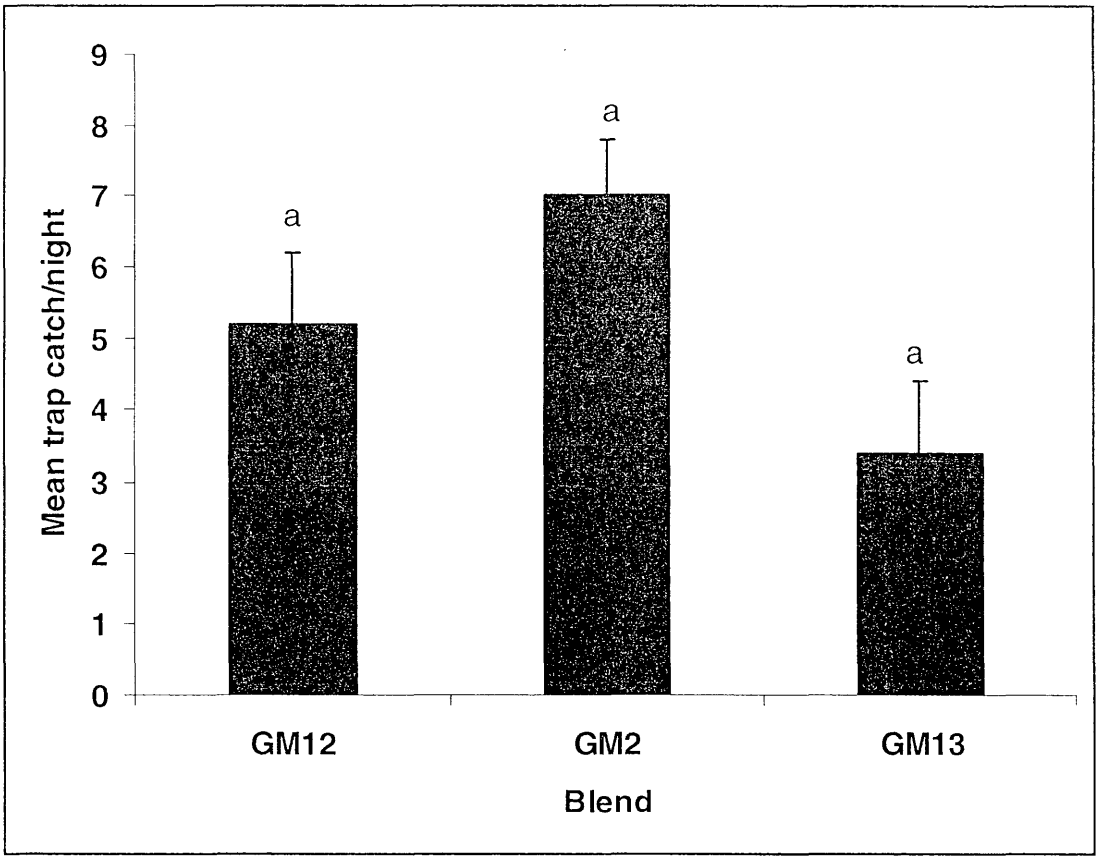


Figure 6.22 Experiment 3. Mean trap catches per trap per night for comparison of blends GM12 (3:1), GM2 (5:1) and GM13 (7:1) in lucerne, "Jahlee", Mullaley, NSW. Columns with common letters are not significantly different ($P > 0.05$).

The implication of the results from these three experiments is that blend ratios of between 7:1 and 3:1 of hexyl hexanoate to (*E*)-2 hexenyl hexanoate would be effective in attracting GM males. While no statistically significant differences could be found within this range, in all three experiments blend GM2 consistently had the highest mean catches. On this basis, blend GM2 was chosen for further comparison testing with other blends.

Experiment 4 - Blend optimisation (step 3)

Further testing of other ratios of the two-component blend was done in this experiment. GM2 which consisted of the 5:1 ratio of hexyl hexanoate to (*E*)-2-hexenyl hexanoate, was compared with GM12 (3:1), GM17 (4:1) and GM19 (6:1). Figure 6.23 shows mean trap catches for these blends. Analysis of variance indicated no significant differences ($P = 0.051$) between the 4 blends. The day however, had a highly significant effect ($P < 0.001$) on the trap catches, again perhaps because of low temperatures on some nights during this experiment. These results again suggest that GM males can respond to a range of ratios of the hexyl hexanoate to (*E*)-2-hexenyl hexanoate, between 7:1 and 3:1.

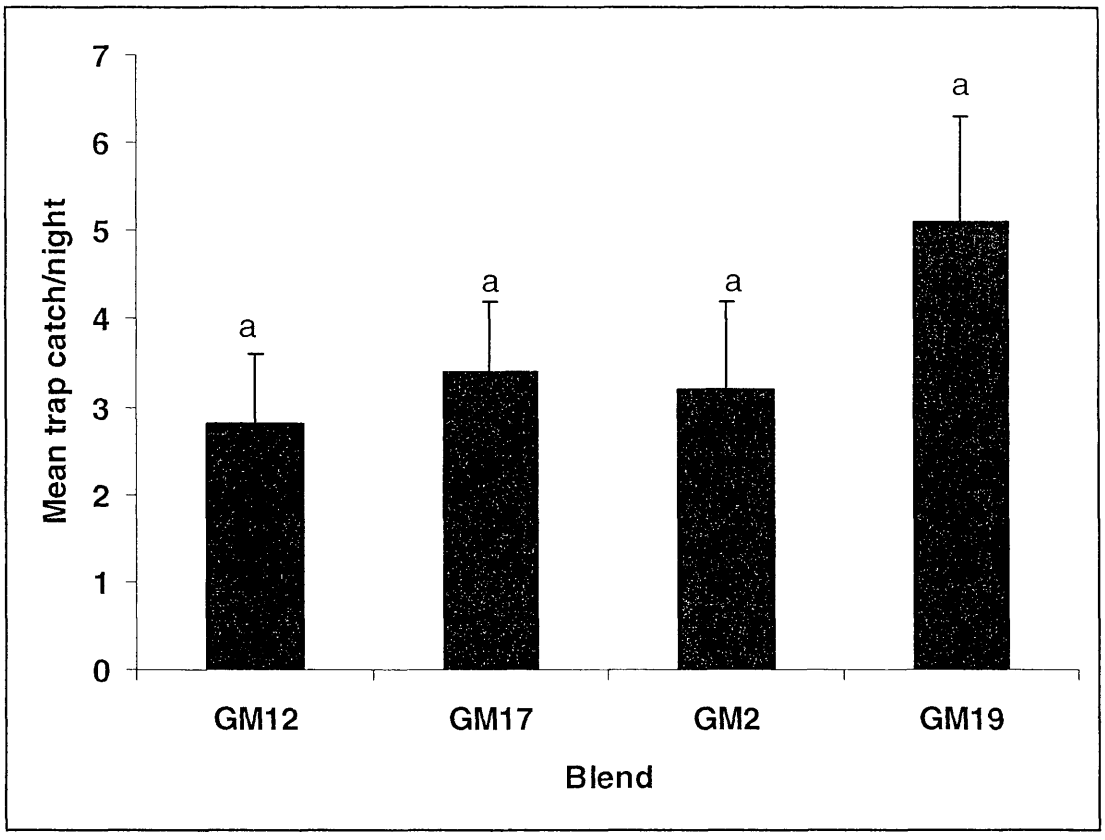


Figure 6.23. Experiment 4. Mean trap catches per trap per night for blends GM12 (3:1), GM17 (4:1), GM2 (5:1) and GM19 (6:1) in lucerne, "Kerrana Pillamore", Dungowan, NSW. Columns with common letters are not significantly different ($P > 0.05$).

Experiment 5 - Loading effects on green mirid trap catches

To determine the effects of pheromone loading on trap catches, three doses of the 5:1 ratio of hexyl hexanoate to (E)-2-hexenyl were compared. These were 2 mg (GM2), 20 mg (GM11) and 40 mg (GM10) per septum. Two of these experiments were done in mung beans, and the results are shown in Table 6.6. In both experiments, analyses of variance showed no significant differences between trap catches for the three doses ($P = 0.450$ for site 1, $P = 0.140$ for site 2). The effects of trap position, row and day were also not significant ($P \geq 0.210$).

Blend	Mean trap catches	
	Site 1	Site 2
GM2	1.2 ± 0.1^{a}	4.8 ± 1.8^{a}
GM10	1.2 ± 0.4^{a}	2.2 ± 0.8^{a}
GM11	3.0 ± 1.0^{a}	2.4 ± 0.7^{a}

Table 6.6. Experiment 5. Mean (± s.e) catches per trap per night of blends GM2, GM10, and GM11 in loading experiments in mung beans at sites 1 and 2, "Jahlee", Mullaley, NSW. Means in the same column followed by common letters are not significantly different.

These results suggest that pheromone loading between 2 and 40 mg might not be critical for GM. In another mirid species, the rice leaf bug, *T. caelestialium,* doses ranging from 4.29 µg to 14.3 µg loaded on capillary tubes of the three component blend (1000:400:30 ratio of hexyl hexanoate, (*E*)-2-hexenyl hexanoate and octyl butyrate) were most attractive to males, and doses greater than 42.9 µg significantly reduced the numbers of males captured (Kakizaki & Sugie, 2001). This inhibition of attraction by excessive amounts of the pheromone as reported in the rice leaf bug and *L. rugulipennis* was not seen in GM, though it is difficult to compare the release rates of this experiment with those of Kakizaki & Sugie (2001) and Innocenzi *et al.*, (2004), because different types of pheromone dispensers were used.

Experiment 6 - Effects of methyl salicylate and (Z)-3-hexenyl acetate on blend GM2

In this experiment, either methyl salicylate or (Z)-3-hexenyl acetate or both were added to GM2. The results are shown in Figure 6.24. There were no significant differences between the blends (P = 0.119), and the day factor was highly significant (P < 0.001).

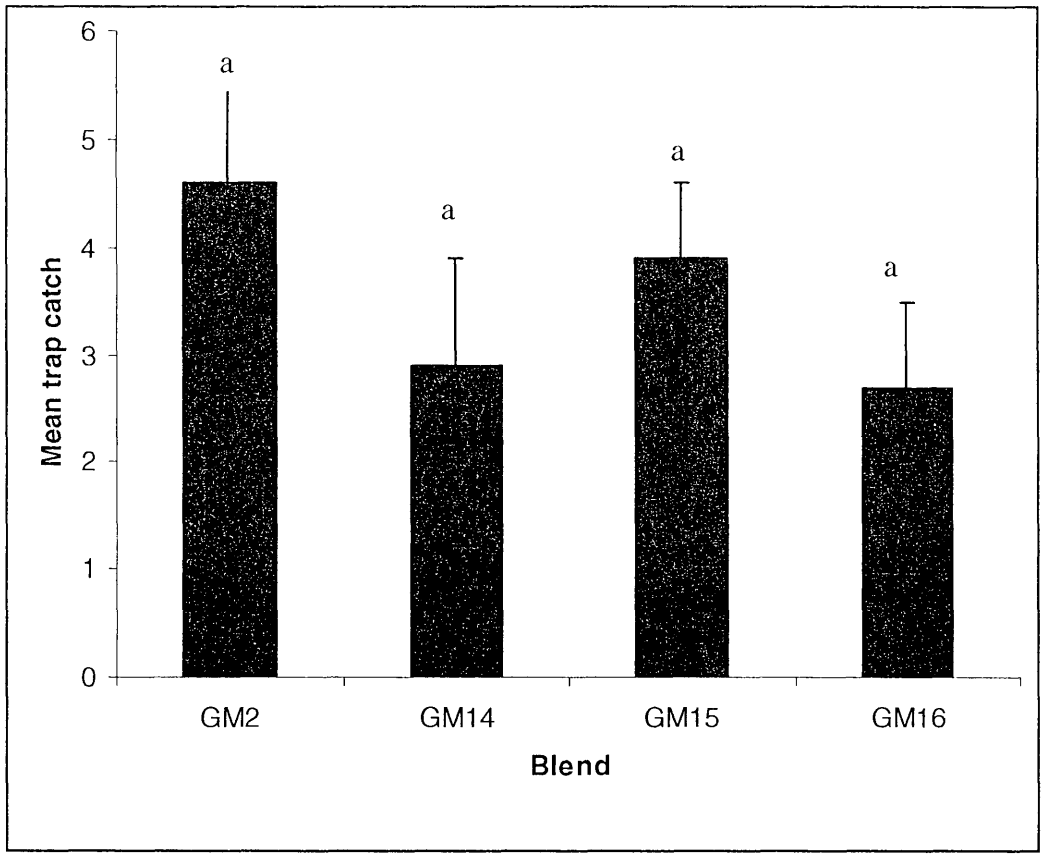


Figure 6.24. Experiment 6. Mean (± s.e) catches per trap per night of blends GM2, GM14, GM15 and GM16 set up in lucerne, "Keranna Piallamore", Dungowan, NSW. Columns with common letters are not significantly different (P > 0.05).

A blend containing methyl salicylate as a single addition (GM14) or in combination with (Z)-3-hexenyl acetate (GM16) reduced the trap catches. This reduction was, however, not statistically significant (P = 0.121). Likewise, the addition of (Z)-3-hexenyl acetate (GM15), did not significantly increase trap catches (P = 0. 159). These results thus suggest that the addition of either methyl salicylate or (Z)-3-hexenyl acetate or the combination of both to the

5:1 blend of hexyl hexanoate and (*E*)-2-hexenyl hexanoate (GM2) is not likely to improve the attractiveness of GM pheromone blend. As previously noted, it is possible that these compounds originated from the plant material held in the air extraction chamber along with the insects, and not from the insects themselves.

Experiment 7 – Comparison of AgriSense® and Delta trap designs using blend GM2

The AgriSense® trap was compared with the Delta trap using blend GM2 as the pheromone lure. The average numbers of mirids caught per night over the four-day period were 4.8 and 4.9 per trap, respectively, for the AgriSense® and Delta traps (Table 6.7). Statistical analysis showed highly significant effect of day ($P < 0.001$) but no significant effect of the trap type, row or position on mirid catches ($P \geq 0.49$). This result indicates that either trap type could be used for monitoring GM. While Delta traps are often recommended for small insects like GM, they are more difficult to service because of the sticky base, especially if there are larger insects which may be accidentally caught. AgriSense® traps are more easily serviced, and are familiar to consultants who monitor Australian cotton because they are used for *Helicoverpa* spp. Thus, the findings of this experiment are likely to facilitate the adoption of pheromone-based monitoring for GM.

	AgriSense® Trap	Delta Trap
Day 1	12.5 ± 6.2	7.5 ± 3.8
Day 2	5.3 ± 2.6	9.5 ± 4.8
Day 3	0.5 ± 0.3	1.3 ± 0.6
Day 4	1.0 ± 0.5	1.5 ± 0.8
Mean (± s.e)	4.8 ± 1.2	4.9 ± 1.2

Table 6.7. Experiment 7. Mean (± s.e) catches of GM per trap per rotation interval in AgriSense® and Delta traps in cotton, "Yanco", Cecil Plains, Qld.

Numbers of green mirids caught in pheromone traps at various times

Most insects have specific times of the day or night during which they respond to pheromones. Males of some mirid species have been known to be attracted to pheromones during the day, as in *D. theobroma* (Cotterell, 1926). Others are attracted in the first few hours after sunset or at night, as in *Helopeltis clavifer* Walker (Smith, 1977) and *C. verbasci* (Thistlewood *et al.*, 1989). So far, the lygus bug, *L. elisus* has been the only species found to have no distinct mating period, mating during both photophase and scotophase (Graham, 1987).

During Experiment 1, observations on the numbers of GM caught in the traps at various times were done to determine the periodicity of pheromone response of GM in the field. Results are shown in Figure 6.25 in relation to the ambient photoperiod.

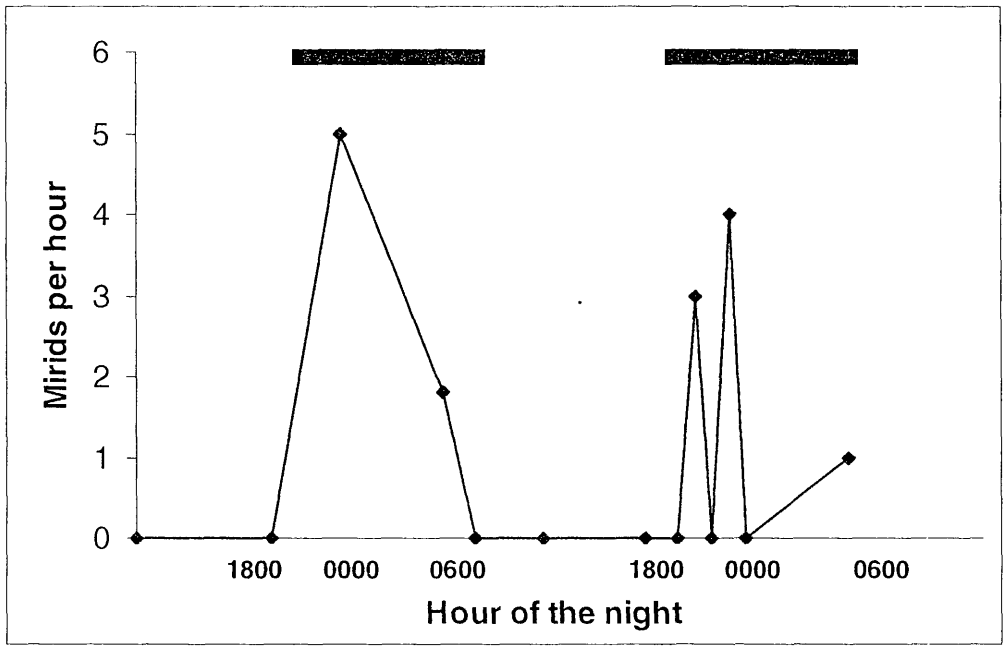


Figure 6.25. Timing of GM catches in pheromone traps during Experiment 1. Results are expressed as mirids per trap per hour for the preceding period (averaged per hour when the observation interval was greater than one hour). Only results from GM1 traps are included since other blends in this experiment caught nothing. Black bars at the top of the graph represents the night periods.

The data obtained in this experiment indicate that it is unlikely that green mirids are sexually active during the day. It is clear that GM males responded to pheromones during the night, and most strongly during the early part of the night. During the experiment, the nights were reasonably warm (sunset temperature about 20°C). It is possible that this behavioural pattern might be different if the temperature at sunset was too low for insect activity. No observations were made on an hourly basis from 12 midnight to 6 am, hence, the time when GM males ceased approaching the traps was not known. The results might also explain why Miles (1995) and Khan (1999) did not observe a single mating event during the day when they observed GM in the laboratory.

Experiment 8 – Effect of trap location on green mirid catches

Mean numbers of males caught in traps located within and outside the cotton field are shown in Table 6.8.

Location	Mean catch/trap/day (± s.e)
Within Cotton field	2.13 ± 0.75^{a}
Outside Cotton field	0.13 ± 0.04^{b}

Table 6.8. Experiment 8. Numbers of GM males caught in the AgriSense® traps at the two different locations, "Yanco", Cecil Plains, Qld. Means within the same column bearing different letters are significantly different ($P < 0.05$).

GM numbers in the traps were lower during this experiment than in previous ones. The analysis of variance showed a highly significant difference ($P < 0.05$) between catches in the traps at the two different locations. This result indicates that for monitoring GM it will be necessary to place traps within the field. This is in contrast to the usual practice of consultants

when monitoring for *Helicoverpa* spp., when traps are placed along roads bordering fields. This is partly to keep traps out of the way of machinery being moved in and out of the farm as well as to make it easier to service if the farm is wet after irrigation. It appears that this practice will not be adequate for monitoring GM, but a change to locating the traps in the fields should not prove too difficult.

Experiment 9 – 'Attract-and-kill' experiment using suction sampling

Mean numbers of male and female GM from each treatment on each sampling occasion are shown in Table 6.9. GM numbers were very low during this experiment. In the control sections they were always below 1 per 50 m. At the 20 h sample, there were no significant differences between treatments. This may have been because males which approached the pheromone during the night left again the next morning, before sampling time. For the 31 h sample, there was a tendency for more male mirids in the pheromone treatment, and the difference was almost significant ($F_{2,11} = 3.76$, $P = 0.065$). In the 78 h treatments there was a significant difference between the treatments in the case of males ($F_{2,11} = 5.72$, $P = 0.025$). For the females, however, there were no significant differences. For the males, the differences were mostly due to higher numbers in the pheromone treatment. This treatment was significantly different from all the others (Table 6.9). At the final sample time, 123 h, the trends were similar to earlier samples, but the differences were not statistically significant for the males ($F_{2,11} = 1.88$, $P = 0.208$).

When catches were summed over all sample intervals, there was a significant difference for males ($F_{2,11} = 4.98$, $P = 0.035$). Most of this was due to the pheromone treatment, which was significantly different from the others. Overall, this treatment yielded approximately 11 times the number of male GM compared to the control.

Treatment	Hours post spray	Males	Females	Total
Control	20	0.00[a]	0.00[a]	0.00[a]
Base	20	0.00[a]	0.50[a]	0.50[a]
Pheromones	20	0.75[a]	0.00[a]	0.75[a]
Control	31	0.25[a]	0.25[a]	0.50[a]
Base	31	1.00[a]	1.25[a]	2.25[a b]
Pheromones	31	3.50[b]	0.75[a]	4.25[b]
Control	78	0.25[a]	0.00[a]	0.25[a]
Base	78	0.75[a]	0.00[a]	0.75[a]
Pheromones	78	3.00[b]	0.75[a]	3.75[b]
Control	123	0.25[a]	0.50[a]	0.75[a]
Base	123	0.00[a]	0.00[a]	0.00[a]
Pheromones	123	2.00[a]	0.00[a]	2.00[a]
Control	Total	0.75[a]	0.75[a]	1.50[a]
Base	Total	1.75[a]	1.75[a]	3.50[a]
Pheromones	Total	8.50[b]	1.50[a]	10.00[b]

Table 6.9. Experiment 9. Numbers of GM males and females collected by suction sampling from 50m sections of treated rows in faba beans, "Carbucky", Goondiwindi, Qld. Means within the same column for the same sampling time which are followed by common letters are not significantly different ($P > 0.05$).

These results suggest that there may be potential for attract-and-kill of GM using pheromones. As well as increased numbers in the suction samples, green mirids were frequently observed sitting immobile on the foliage close to deposits of the sprayed pheromone formulation. They were not observed to contact this material, however. Such observations were made during both daylight hours and by night (using a torch), and no similar observations were made on untreated rows. These observations suggested that an appropriate strategy for attract-and-kill would be to treat the rows to which pheromone was applied with a cover spray of an insecticide effective against GM, such as fipronil. The data suggest that with an 11-fold

concentration factor over 120 h (and this is conservative because new control sections were sampled at each time), most of the males in the population could be removed by treating only occasional rows, which would allow survival of most of the beneficial insects.

Experiment 10 - 'Attract-and-kill' and mating disruption trial on mirids

Figures 6.26 and 6.27 show mean pheromone trap catches in field S3 (treated), both before and after the treatments, and in field WL4 (control), respectively. In field S3, mirid trap catches were not significantly different (P = 0.658) between the sites marked for the various treatments from day1 to day 12 (that is, during the pre-treatment phase).

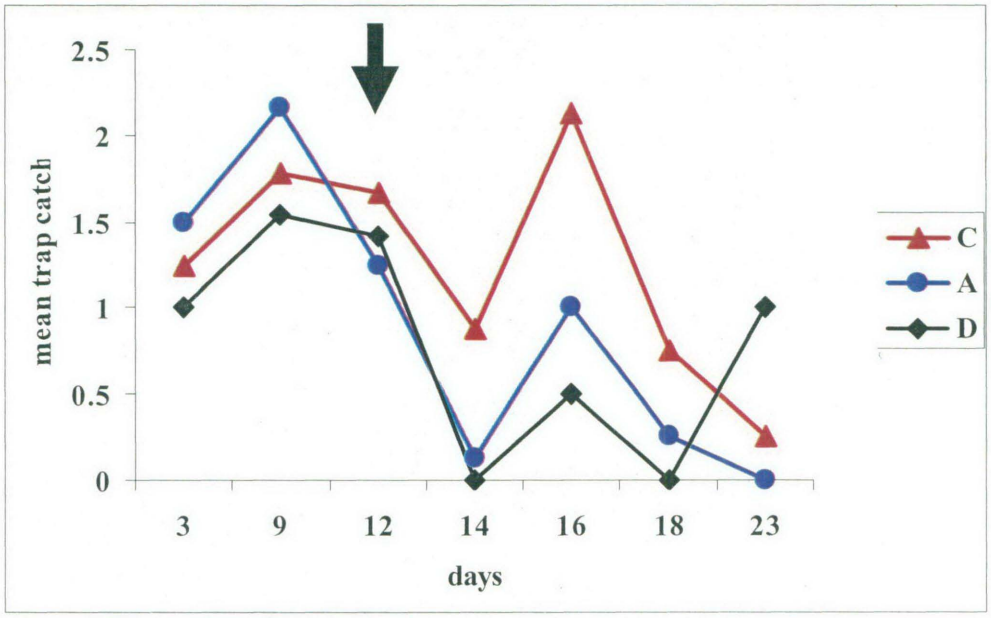


Figure 6.26. Experiment 10. Mean pheromone trap catches for the treated plots in field S3, "Prospect", Warra, Qld. C - control. A - attract-and-kill treatment D – mating disruption treatment. Black arrow indicates day of treatment application.

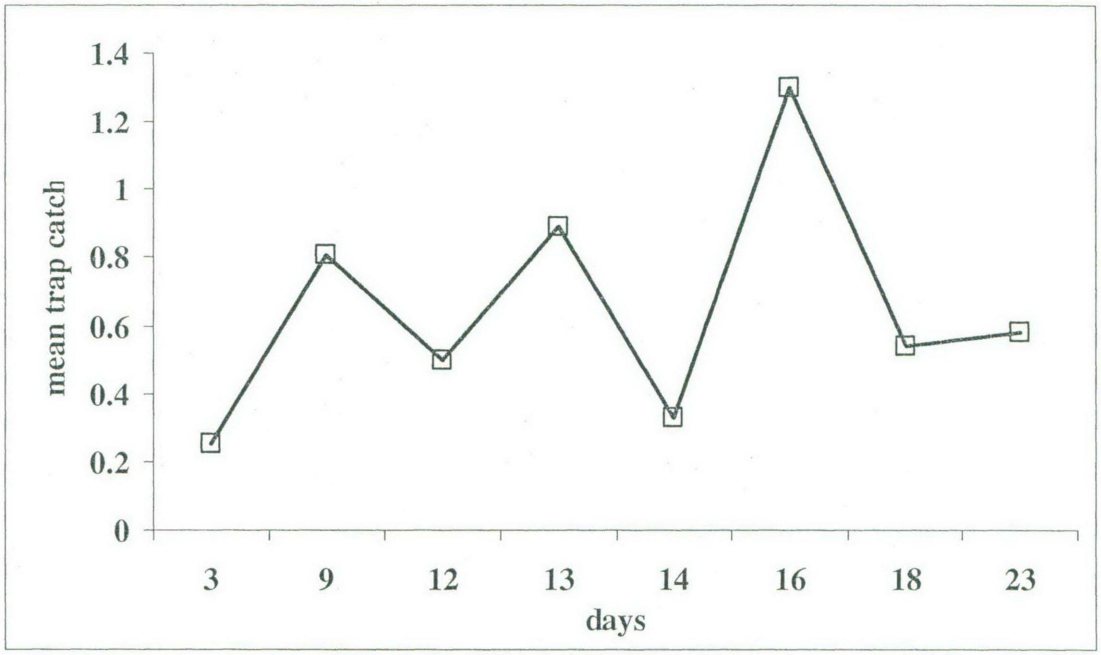


Figure 6.27. Experiment 10. Mean pheromone trap catches for the untreated field WL4, "Prospect", Warra, Qld.

Differences however, occurred between the control and the treatments after the application of the mating disruption and attract-and-kill formulations to field S3 on the 12th day. Catches of male GM in the control plot were significantly higher compared to the mating disruption and attract-and-kill plots for day 14 ($P = 0.003$, when no GM were found in any trap from either the attract-and-kill or the mating disruption treatment), day 16 ($P < 0.001$) and day 18 ($P = 0.004$). There was however, no significant difference between the treatments on the day 23 ($P = 0.06$). For field WL4, no significant differences existed between the summed trap catches from different parts of the field from days 1 to 23 ($P = 0.358$). Further analysis revealed no significant variation, either along or across the field, on any day. This further strengthens the inference that the differences between plots within field S3 on days 14, 16 and 18 was due to the treatments applied.

The result suggests a two-day total trap shut down for the mating disruption treatment and a further partial shut down for at least another 4 days. It should be noted that the lures in the pheromone traps were replaced on day 14, 2 days after the treatments were applied. With the fresh lures, it is possible that a surface effect which involved a temporarily higher release rate of the pheromone could have taken place, overcoming the mating disruption application temporarily, hence the appearance of a few mirids in the pheromone traps in the mating disruption plot on days 16-18.

Trap shutdown following the application of pheromones is generally regarded as an indication that mating would be disrupted, since if males are unable to locate a point source of pheromone, they will also be unable to locate a female (Jones, 1998). However, this result needs to be confirmed directly, for example by the use of sentinel females or by dissection of females caught in the treated area to determine their mated status. Final confirmation of mating disruption can be obtained by suppression of reproduction, demonstrated by a reduction in the number of eggs or immature insects. However, successful mating disruption does not always equate to reduced crop damage, since females which have mated outside the treated area may move in and oviposit (for example, Betts *et al.,* 1993). All these ecological questions need to be examined before it can be concluded that mating disruption is a viable option for mirid management. Also, the period over which trap shutdown occurs would need to be extended (perhaps by the use of controlled-release formulations) before the method is likely to become economically viable. Nevertheless, these results show promise for the use of mating disruption against GM, especially in view of the ready commercial availability of the two pheromone components, and their low price compared to Lepidopteran pheromone components. This experiment represents one of the few examples where trap shutdown has been demonstrated for a Hemipteran species, especially in a broad acre field crop.

Lower trap catches were also recorded from the attract-and-kill treatment, after the formulation was applied (Figure 6.26). This could have been because there were fewer males in the plot, because some had been killed by the insecticide applied to the treated rows. It could also have been because there was sufficient pheromone in the formulation to produce mating disruption, independently of the killing effect. An attempt was made to assess the impact of the insecticide by placing horticultural plastic along 5 m of treated row in three locations within the attract-and-kill plot and examining it at regular intervals for dead insects. No GM (males or females, or nymphs) were found. This may have been because numbers were so low, or because the fipronil did not kill mirids quickly enough to prevent them moving away from the treated area before dying. It is therefore not possible to be sure of the mechanism for trap reductions in this treatment. However, the reduction occurred over the same time frame as the mating disruption formulation, which suggests mating disruption as the most likely possibility.

Figures 6.28 and 6.29 show the mean numbers of GM males and females respectively caught in the D-Vac sampling from the three treatments- D (mating disruption), A (attract-and-kill) and C (control).

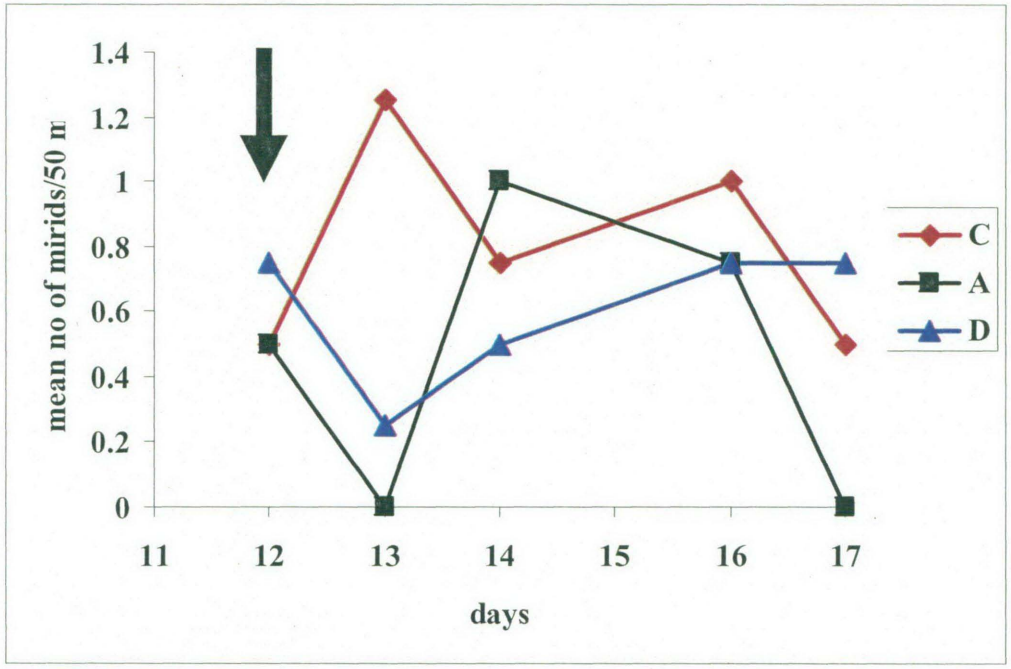


Figure 6.28. Experiment 10. Mean numbers of GM males per 50m caught in D-Vac sampling, "Prospect", Warra, Qld. C - control treatment A - attract-and-kill treatment D – mating disruption. . Black arrow indicates day treatment was applied.

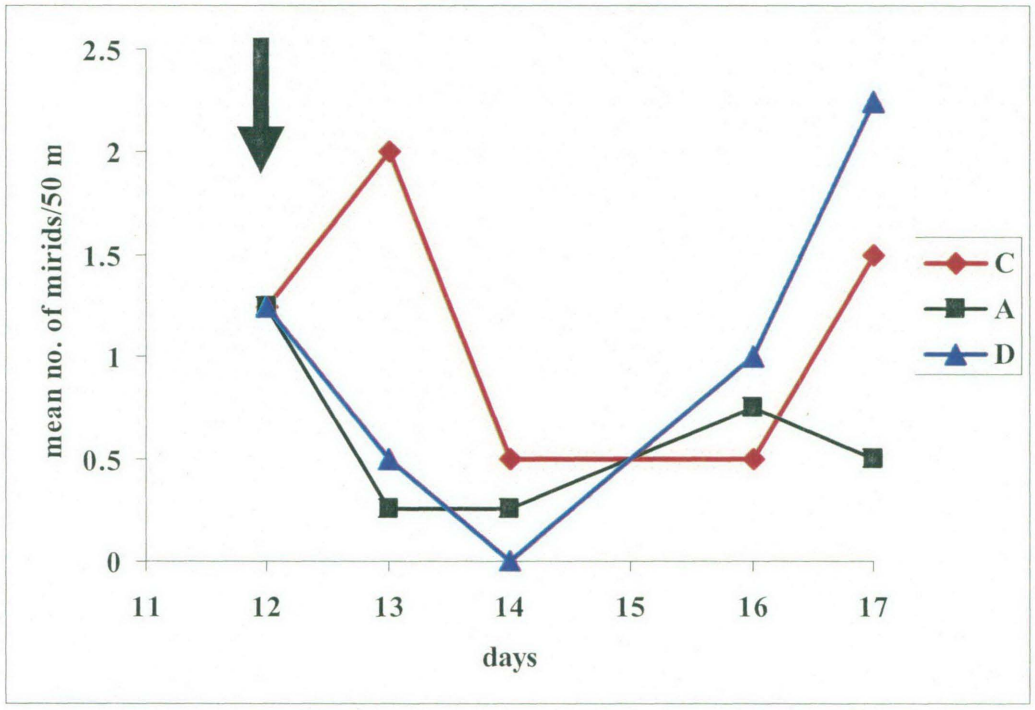


Figure 6.29. Experiment 10. Mean numbers of GM females per 50m caught in D-Vac sampling, "Prospect", Warra, Qld. C - control A - attract-and-kill treatment D – mating disruption treatment. Black arrow indicates day treatment was applied.

Analysis of the data showed no significant differences between the number of males sampled with the D-Vac among the various treatments from day 12 (pre-treatment) to day 17 (5 days after treatment). The P values were: day 12 (P = 0.98), day 13 (P = 0.20), day 14 (P = 0.80), day 16 (P = 1), day 17 (P = 0.28 It also suggests GM males were equally present in all the treatment plots. The differences observed in the trap catches in the attract-and-kill and mating disruption treatments could only then have come from the disruptive effects of the treatments applied. Similarly, no significant differences were observed in the number of females occuring per 50 metres of the treated fields on day 12 (P = 0.94), day 14 (P = 0.32), day 16 (P = 0.87), and day 17 (P = 0.39). However, there were significantly higher number of females in the control plot compared to the mating disruption plot (P = 0.012) on day 13 (1 day after treatment). It is possible that the females, unable to attract any males for mating because of the mating disruption treatment, moved into the buffer zones or control plots for a greater chance of finding mates.

6.4 Summary and general conclusions

The major sex attractant components produced by adult green mirid females have been isolated and identified. Gas chromatography-mass spectrometry analyses and field bioassays indicated that the pheromone is sex-specific and the synthetic pheromone blend was attractive to adult males only. The blend consistently caught green mirid males in traps set up in a range of crops (cotton, lucerne, soybeans and mung beans) at different locations in NSW and Queensland.

A total of 13 synthetic blends of the major component, hexyl hexanoate and the minor component, (*E*)-2-hexenyl hexanoate, in various ratios as well as the single components were

tested at various times in the field in an attempt to select the optimal blend. Results from the optimisation process indicated that the two compounds are needed together for the pheromone to work. (*E*)-2-hexenyl hexanoate alone (GM6), being the minor component unique to the female, did not attract any males in the absence of the major component, hexyl hexanoate (GM5) which is produced by both sexes. Various ratios of these two components between 3:1 and 7:1 (GM2, GM12, GM17 and GM19) statistically had the same effectiveness in attracting males to traps. The optimum ratio appeared to be 5:1 of the major component, hexyl hexanoate to the minor component, (*E*)-2-hexenyl hexanoate, but this ratio does not appear to be critical. The addition of either or both methyl salicylate and (Z)-3-hexenyl acetate which were found in both the male and female air collections to GM2, did not significantly enhance the trap catches. A dose response trial of doses ranging from 2 mg to 40 mg carried out with the optimal blend (GM2) did not show any significant effect of loading on trap catches. It is therefore likely that blends similar to GM2 have the potential for development as a commercial pheromone for the green mirids.

Green mirid males appeared to respond to pheromones during the night, especially in the early part of the night, at least when the temperatures are high enough to permit night flight. During the Mullaley trials (Experiments 3 - 5), which were conducted in March 2004 when night temperatures were relatively low, there were indications that trap catches were reduced. This suggests that responses to pheromones are temperature-sensitive, as they are in many other species. This effect has not been analysed in the current experiments because no accurate temperature recordings were taken.

The pheromone lure developed from this work has potential applications as a tool in managing green mirids in Australia. Sampling to enable control measures to be put in place is very difficult since GM are very mobile and easily disturbed, which makes visual scouting

inefficient. Also, populations are often low. The numbers found in D-Vac samples in this work, though low, are not atypical for Australian cotton. Tolerance for GM is very low, with the recommended early season thresholds being only 0.5 per metre in cool regions and 1 per metre (adult and nymphs in warm season areas in Australia) (Johnson & Farrell, 2003), and many consultants advising insecticide application when even fewer are present (P.C. Gregg, pers. comm. 2004). The blend developed in this thesis has been found to be very sensitive and further work is in progress to relate trap catches to the standard methods of sampling used by consultants in cotton (beat sheets, visual counts and D-Vacs). Good correlation between trap catches and field infestations under low and high density mirid populations would allow the use of this tool in monitoring GM in the field. It would be easier, less expensive and time saving, providing a good alternative sampling method for this pest.

Factors likely to affect adoption and practical application of pheromone lures developed include the efficiency of the trap type and how easily they can be serviced. Results from this research indicate that AgriSense® traps are equally efficient for trapping GM as Delta traps. Generally, Delta traps are suited for use with small insects, but more difficult to service compared to the AgriSense® traps. The use of AgriSense® traps is likely to be embraced by farmers and consultants who are very familiar with them, and find them easy to service. However, for monitoring purposes, consultants and farmers in Australia generally like to place pheromone traps outside the field of interest. Results from the trap location experiment suggest that this may not work for GM. Traps tend to catch more and possibly give a true reflection of what the insect numbers are when placed in the field than when placed on the outside.

The experiment on attract-and-kill with suction sampling (Experiment 9) was done under unfavourable conditions, early in the season, before GM numbers had built up. The actual

numbers of GM collected are probably an underestimate, since the suction sampler was only 50-60% efficient (Stanley, 1997). Nevertheless, the experiment showed a clear tendency of male (but not female) green mirids to accumulate in the rows treated with pheromone. If a contact foliar insecticide had been applied to these rows, it would have killed the males. We did not do this because dead mirids are very hard to find, especially at the densities present in this experiment. Instead we collected them live by suction sampling. Insecticides currently registered for control of GM in cotton, which have contact activity, include alpha-cypermethrin, beta-cyfluthrin, bifenthrin, chlorpyrifos-methyl, deltamethrin, dimethoate, endosulfan, fipronil, imidacloprid, lambda-cyhalothrin and omethoate (Johnson & Farrell, 2003). All these insecticides damage natural enemy populations, and the ability to control GM by treating only occasional rows with them and allowing natural enemies to survive in the other rows would be a considerable advance in cotton IPM. Killing male GM would reduce damage to cotton directly (since the males themselves feed on the crop), and indirectly by removing potential mates for the females, thus reducing the next generation. The magnitude of the indirect effect would depend on the extent of multiple mating, and the ability of mated female GM to move into the crop from outside sources. Both of these factors are not well understood for GM at present.

Mating disruption may also be possible with this pest. Though there have been few examples reported in the literature for Hemipterans, encouraging results have been reported for *C. verbasci* (McBrien *et al.*, 1996; 1997) and *T. caelestialium* (Kakizaki & Sugie, 2004). Initial data obtained from the field work done in Warra, Qld indicated a total trap shutdown for 2 days when the pheromone was used in a mating disruption experiment. The short trap shutdown period is thought to have arisen because of the formulation used. This needs further work to provide a slow and sustainable long term release formulation for use in mating disruption. In the attract-and-kill work, efforts made to locate and count dead mirids for

quantification did not work. Either the insecticide did not kill the mirids fast enough, resulting in their moving away from the treated row before dying, or the low numbers of mirids present made the sampling method ineffective. Taken together with the results from Experiment 9, however, the trap results suggest that attract-and-kill for male GM remains a promising option. As with mating disruption, however, further work needs to be done on a long lasting formulation to overcome potential problems with reinvasion of treated fields.

CHAPTER 7

SUMMARY OF KEY FINDINGS AND THEIR IMPLICATIONS FOR THE MANAGEMENT OF THE ROUGH BOLLWORM, COTTON TIPWORM AND GREEN MIRID IN AUSTRALIA

The primary objective of this thesis was to identify the sex pheromone components of three pests of cotton, namely, the rough bollworm (*Earias huegeliana*), the cotton tipworm (*Crocidosema plebejana*) and the green mirid (*Creontiades dilutus*). The development of pheromone lures for these species would be useful as monitoring tools, in mass trapping and mating disruption applications. This chapter summarises the key findings of the research and highlights the implications of these findings in the management of these pests. Future research needs arising out of this work are also presented.

7.1 Key findings

7.1.1 Sex attractants of the rough bollworm, *Earias huegeliana*

Previous studies on the sex pheromones of the *Earias* spp. have focussed on all the major pests but the one endemic to Australia, *E. huegeliana.* The study described in Chapter 4 examined through GC-MS the ovipositor washings and air collections for pheromones produced by *E. huegeliana* females. Compounds were identified as (*E,E*)-10,12-hexadecadienal ((*E,E*)10,12:16Al, (*E,E*)10,12-hexadecadienol ((*E,E*)10,12:16OH), (*Z*)-11-hexadecenal ((*Z*)-11:16Al), and (*Z*)-11-octadecenal ((Z)-11:18Al) in a ratio of 4:1:1:1 in the ovipositor

extractions. All of these compounds but (*E,E*)10,12-hexadecadienol, were also found in the air collections.

A total of 11 synthetic pheromone blends of the four compounds identified in various combinations were tested at Oakey, Cecil Plains and Mondure in Queensland. Attraction of *E. huegeliana* males to traps was generally effective with a two component blend of the major component, (*E,E*)-10,12:16Al and the minor component (*Z*)-11:16Al in a ratio of 4:1. These two compounds were essential for the pheromone blend to work.

The optimal blend of (*E,E*)-10,12:16Al and (*Z*)-11:16Al in a ratio of 4:1 was tested in the field using AgriSense® and Delta traps. Only the Delta traps caught *E. huegeliana* males, suggesting this trap as the more effective of the two for this species. In another experiment, this blend was compared with that of *E. vittella*, a closely related species found in parts of Asia and in Western Australia, which consists of a 5:1:1 ratio of (*E,E*)-10,12:16Al, (*Z*)-11:16Al and (*Z*)-11:18Al. Trap catches for these two blends were similar indicating that both *E. huegeliana* and *E. vittella* sex pheromones are not highly species-specific. The three components (*E,E*)-10,12:16Al, (*Z*)-11:16Al, and (*Z*)-11:18Al, found in *E. vittella* are also found in *E. huegeliana*. Hence, similarities between these species in morphology and pheromone components raise questions about their reproductive isolation.

E. huegeliana males responded to pheromones during the night, and most strongly during the latter part of the night, between 0400–0600 h AEST. Pheromone collection was done when females were calling in the laboratory during this time. It thus appeared that male response to the sex pheromone is synchronous with female calling and pheromone production.

Studies on lure stability in the field and storage in a freezer at -21°C showed that the formulated synthetic lures did not appear to lose much of the active ingredients when stored for about a month at -21°C. In the field, there appeared to be a linear loss of the active ingredients in the

lures over a 4-week period, suggesting a life span of 4 weeks for the 2 mg lure between a temperature range of 19.3 and 23.3°C.

7.1.2. Sex attractants of the cotton tipworm, *Crocidosema plebejana*

Chapter 5 describes a series of experiments aimed at isolating and determining the pheromone composition of the cotton tipworm, *C. plebejana*, and a series of wind tunnel studies to select an attractive pheromone blend. Compounds were identified as 18Al, 18OH, 2:19Kt and 18Ac in a ratio of 2:2:2:1 in the ovipositor extracts. All but 18OH and 2:19kt were found in the air collections.

The four individual components (18Al, 18OH, 2:19Kt and 18Ac) and a blank, were tested in the wind tunnel at a dose of 4 µg. Behaviours like takeoff, upwind flight, approach and contact were significantly different between 18Ac and the other components. All the other components were not significantly different from the blank. These results suggest that 18Ac should form an important part of an attractive pheromone blend.

Male responses to 18Ac and three synthetic pheromone blends consisting of two, three or all four components at different ratios in comparison with conspecific females were also studied in the wind tunnel. In all cases, all the observed behaviours of males in the presence of the females were significantly different from those in the presence of the synthetic pheromone blends. Preliminary field trials, however, have shown that 18Ac alone, the three-component blend of 18Al, 18Ac and 2:19Kt (2:1:2), and the four-component blend of 18Al, 18Ac, 2:19Kt and 18OH (2:1:2:2) consistently caught tipworms at the three sites despite the very low numbers of tipworms during the trials. Replicated experiments were not done due to scarcity of tipworm populations in the field. Nevertheless, preliminary findings support the possible general

suitability of all of these blends as attractants for cotton tipworm. Field trials should be conducted to further test the attractiveness of these blends when tipworms are more abundant.

Calling in *C. plebejana* females did not start until they were three days old. The age at first calling ranged between three and seven days after emergence. Calling activity was initiated during the 5th and 6th hours of the scotophase and this was synchronised with pheromone production. While mean onset time of calling did not advance with age, the number of calling bouts and mean duration of calling were significantly different between the ages.

7.1.3 Sex attractants of the green mirid, *Creontiades dilutus*

Miles (1995) first raised the possibility of the use of long-distance pheromones in mate attraction by green mirids. Initial attempts to characterise the pheromones from airborne volatiles and the metathoracic sac of green mirid females and males resulted in the identification of two principal components, hexyl hexanoate and hexyl acetate. No field trials were however, carried out to test the attractiveness of these compounds as sex pheromones for this species.

In this thesis, further attempts to identify the pheromone components of *C. dilutus* were conducted by whole body squashing and collection of airborne volatiles from 6-8 day-old females and males. Pheromone analyses were done by gas chromatography linked to a mass spectrometer. In collections from males and females using the whole body extracts, hexyl hexanoate was found to be the principal component. Other minor components identified in females included hexanol, octanal, nonanal, hexyl butyrate, pentyl hexanoate and heptyl hexanaote in a ratio of 9.5:0.3:0.5:0.9:3:2.4, respectively, to hexyl hexanoate. The presence of hexanol in the squashed samples but not in the air collection has been explained by extensive hydrolysis product of the hexyl butyrate. This has been inferred from a similar work done on heptyl butyrate by Ho and Millar (2002). In the air collections, hexyl hexanoate was again identified as the major component in both males and females. In females, the minor components

found were 2-hexanone, 3-hexanone, (*Z*)-3-hexenyl acetate, octanal, nonanal, methyl salicylate and (*E*)-2-hexenyl hexanoate in a ratio of 1.4:1.1: 4.1:6.3:10.4:9.3:33.7, respectively, to hexyl hexanoate. The only compound unique to the female air collection was (*E*)-2-hexenyl hexanoate.

Field trials were conducted using Delta traps on a variety of crops, including cotton, lucerne, soybeans and mung beans to test the attractiveness of the two individual components, (*E*)-2-hexenyl hexanoate and hexyl hexanoate, and 12 synthetic pheromone blends, with hexyl hexanoate as the major component. No mirids were caught in traps baited with either of the two individual components, indicating the need for both compounds to be present for any attraction to occur. Maximum attraction to lures in Delta traps was found to occur with a blend of hexyl hexanoate and (*E*)-2-hexenyl hexanoate in a ratio of 5:1. This ratio, however, did not appear to be critical, since blends in the range of 3:1 to 7:1 worked just as well. The addition of (*Z*)-3-hexenyl acetate and methyl salicylate to the optimised blend did not significantly increase the trap catches. The pheromone blend of hexyl hexanoate and (*E*)-2-hexenyl hexanoate in a 5:1 ratio was found to be attractive to adult green mirid males only, indicating it was stage and species specific.

Field observations have shown that green mirid males responded to pheromones during the night, and most strongly during the early part of the night, between 2000 and 0600 h AEST, during reasonably warm nights (eg, sunset temperature about 20°C). It is possible that this behavioural pattern might be different if the temperature at sunset was too low for insect activity. Male response to pheromone as indicated by catches in Delta traps was not affected by pheromone loading between 2 mg to 40 mg.

The type of pheromone trap (Delta or AgriSense® design) did not significantly affect green mirid catches. The implication of this finding is that the AgriSense® trap can be recommended

for use with trapping this species. They are easy to install and service compared to the Delta traps, and hence, would be more suitable and convenient to use by farmers.

Attempts at applying the pheromone in a formulation for mating disruption and attract-and-kill applications provided some mixed results. It was clear that mating disruption worked over a short period, but the effect of the application disappeared after a few days. This was attributed to the formulation used and further research needs to be done for a formulation that would provide a slow and long term release of the pheromone over a longer period of time. Similarly, further work on the feasibility of using the pheromone with an insecticide in an attract-and-kill system is needed.

7.2 Implications for pest management

The studies presented in this thesis represent the first successful and most comprehensive investigations into the sex pheromones of three endemic Australian insects which are occasional pests of cotton, the rough bollworm (*E. huegeliana*), the cotton tipworm (*C. plebejana*) and the green mirid (*C. dilutus*). The pheromone blends developed for *E. huegeliana, C. plebejana* and *C. dilutus* are likely to impact on decision processes in the management of these pests as the Australian cotton industry turns to softer options of controlling specific pests. Sex pheromones as monitoring, mating disruption or attract-and-kill tools could form a vital part of integrated pest management systems in cotton, aimed at reducing pesticide use and allowing beneficial insect numbers to build up in the farming ecosystem. Traps baited with the pheromone lures would be useful in monitoring field populations of these pests to complement the beat sheet method and visual sampling methods which are currently the principal methods of estimating their abundance in the field. This could help eliminate the reliance many consultants place on crop damage to determine treatments for mirids, and offer an alternative, proactive, approach. There is

also a great potential for use of their sex pheromones in mating disruption and attract-and-kill systems.

The three pests forming the subject of this thesis are all probably less mobile and less migratory than the key pests of cotton, *Helicoverpa* spp. This makes them more suitable targets for mating disruption and attract-and-kill applications as evident in the case of rough bollworm. In Pakistan and Egypt, where for example, mating disruption applications are used for the closely related *E. vittella* in cotton which has similar ecological characteristics to the rough bollworm. Similarly, mating disruption and monitoring programmes are in place overseas for the codling moth which has a similar ecology to the cotton tipworm. Little information is available on mating disruption work in mirids, but as has been demonstrated in this thesis, it looks promising, as has been reported overseas for *Campylomma verbasci* (McBrien *et al.*, 1996; 1997). The GM is a particularly suitable target for attract-and-kill because damage is not restricted to the immature stages like in *Helicoverpa.* The adults, including the males cause damage to cotton. Reducing male numbers would eventually lead to less plant injury and reduction in the number of the next generation of mirids. GM is very difficult to spot in crops coupled with potentially sudden early season immigration into cotton from other hosts, or from inland, easily disturbed but responsive to pheromones and hence, a pheromone monitoring tool would help for monitoring purposes, to complement the beat sheet method.

With Australian cotton growers tending to transgenic cotton and softer options of insect control that does not affect natural enemy populations, these environmentally friendly methods of insect control would be readily welcomed by growers and consultants. Basic machinery for insecticide applications already available could easily be adapted for sprayable formulations for mating disruption and attract-and-kill for the GM. Monitoring involving AgriSense® traps for *Helicoverpa* is already in use and should not be difficult to be applied to the GM. Trap

monitoring is likely to save time and complement the beat sheet method and would be readily welcome by Australian growers.

There are still many aspects that need to be examined in order to recommend the best trapping system to be used for all three insects studied. Other trap designs, of various sizes and shapes need to be experimented with. There is also a need for intensive trapping data with the three pheromone blends developed to be cross-checked with visual or the current insect checking methods by researchers and consultants to be able to set thresholds using sex pheromones for monitoring purposes in IPM. For monitoring purposes, it is essential that there is a correlation between trap catches and the numbers of the damaging stage of the pest in the field. A high priority should be given to studies aimed at determining whether such correlations exist, especially for GM. Further work needs to be done on the persistence of pheromones for all three pests, but especially for GM pheromones, which were relatively short-lived in sprayable formulations. The development of formulations which give a uniform rate of release over a prolonged period is crucial for the success of mating disruption or attract-and-kill methods. There are many questions which need research but the future looks promising.

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APPENDIX 1

STATISTICAL ANALYSES FOR CHAPTER 4

APPENDIX 1.1

Experiment 2a

(a) Analysis of variance on transformed data for experiment involving blends A, B, C and CTRL set up in cotton

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	3	5.71	1.90	41.74	9.1e-14
Day	3	0.75	0.25	5.51	0.0023
Row	3	0.10	0.03	0.73	0.5385
Posit	3	0.13	0.04	0.97	0.4123
Residuals	51	2.33	0.05		

(b) Results from comparison of transformed means of A, B, C and CTRL using contrast in R

	Estimate	Std. Error	t value	Pr(>|t|)
Blend A vs B -	0.167	0.0509 -	3.28	1.75e-01
Blend A vs C	0.246	0.0509	4.84	9.56e-06
Blend A vs CTRL	0.325	0.0509	6.38	2.84e-08
Blend B vs C	0.111	0.0480 -	2.32	2.36e-02
Blend B vs CTRL	0.190	0.0480 -	3.96	2.02e-04
Blend C vs CTRL	0.0392	0.0415	0.944	3.49e-01

APPENDIX 1.2

Experiment 2b

(a) Analysis of variance on transformed data for experiment involving blends A, E and F set up in cotton

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	2	9.74	4.87	286.10	2.3e-14
Day	2	0.03	0.01	0.87	0.44
Row	2	0.06	0.03	1.82	0.19
Posit	2	0.05	0.03	1.58	0.23
Residuals	18	0.31	0.02		

(b) Results from comparison of transformed means of A, E, and F using contrast in R

	Estimate	Std. Error	t value	Pr(>|t|)
Blend A vs E	0.425	0.0373	11.4	3.75e-11
Blend A vs F	0.425	0.0373	11.4	3.75e-11
Blend E vs F	0.000	0.0323	0.0	1.00e+00

APENDIX 1.3

Experiment 3

Analysis of variance on transformed data for experiment comparing AgriSense and Delta traps

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Trap	1	0.393	0.393	11.87	0.0015
Day	3	0.067	0.022	0.67	0.5749
Residuals	35	1.159	0.033		

Analysis of Deviance table for experiment comparing AgriSense and Delta traps

	Df	Deviance Resid.	Df Resid.	Dev	P(>|Chi|)
NULL		39	60.8		
Trap	1	25.0	38	35.8	5.9e-07
Day	3	6.2	35	29.7	0.1

APENDIX 1.4

Experiment 4

(a) Analysis of variance on transformed data for experiment involving comparison of sex pheromone of *E. vittella* and *E. huegeliana* (blends EH1, EH2, EH3, EH4 and EVA) conducted in cotton.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	4	0.332	0.083	3.15	0.0173
Day	4	0.431	0.108	4.08	0.0040
Row	4	0.325	0.081	3.08	0.0192
Posit	4	0.294	0.074	2.78	0.0302
Residuals	108	2.852	0.026		

(b) Results from comparison of transformed means of EH1, EH2, EH3, EH4 and EV using contrast in R

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.0961	0.0161	5.958	2.62e-08
Blend EH4 vs EV	0.0238	0.0323	0.739	4.61e-01
Blend EV vs EH3	0.0218	0.0323	0.676	5.00e-01
Blend EV vs EH2	-0.0504	0.0323	-1.564	1.20e-01
Blend EV vs EH1	-0.0672	0.0323	-2.084	3.93e-02
Blend EH4 vs EH3	0.0218	0.0323	0.676	5.00e-01
Blend EH4 vs EH2	- 0.0504	0.0323	-1.564	1.20e-01
Blend EH4 vs EH1	-0.0672	0.0323	-2.08	4 3.93e-02

Wind tunnel experiments

Experiment 6: Male responses to sex pheromones in the wind tunnel.

Analysis of deviance: Takeoff

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			89	32.728	
Treatment	2	4.454	87	28.274	0.108

Analysis of deviance: Upwind flight

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			89	87.229	
Treatment	2	22.310	87	64.920	1.431e-05

Contrast: Upwind flight

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			89	87.229	
B vrs K	1	0.109	88	87.121	0.741
B vrs M	1	17.687	88	69.542	2.603e-05
K vrs M	1	21.346	88	65.883	3.833e-06

Analysis of deviance: Approach

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			89	123.653	
Treatment	2	70.068	87	53.585	6.093e-16

Contrast: Approach

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			89	123.653	
B vrs K	1	0.270	88	123.383	0.603
B vrs M	1	45.015	88	78.638	1.956e-11
K vrs M	1	54.931	88	68.722	1.248e-13

Analysis of deviance: Contact

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			89	92.777	
Treatment	2	18.552	87	74.224	9.364e-05

Contrast: Contact

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			89	92.777	
B vrs K	1	0.905	88	91.871	0.341
B vrs M	1	6.653	88	86.123	0.010
K vrs M	1	13.078	88	79.698	0.0002988

Analysis of deviance: Attempt at copulation

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			89	70.681	
Treatment	2	11.051	87	59.630	0.004

Contrast: Attempt at copulation

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			89	70.681	
B vrs K	1	0.580	88	70.101	0.446
B vrs M	1	3.736	88	66.946	0.053
K vrs M	1	7.613	88	63.069	0.006

APPENDIX 2

STATISTICAL ANALYSES FOR CHAPTER 5

Onset time of calling

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Age	1	1.31	1.31	3.19	0.11
Residuals	9	3.70	0.41		

Coefficients for the regression

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	6.118	0.5258	11.64	0.000001
Age	-0.109	0.0611 -	1.78	0.107939

Mean onset of calling = 6.118-0.109age.

Mean calling duration

Residuals:

Min	1Q	Median	3Q	Max
-70.1	-63.1	-56.1	67.2	239.8

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	54.97	12.21	4.50	1.1e-05 ***
Age	1.16	1.54	0.76	0.45

Residual standard error: 83 on 206 degrees of freedom

Multiple R-Squared: 0.00276, Adjusted R-squared: -0.00208

F-statistic: 0.571 on 1 and 206 DF, p-value: 0.451

Mean number of bouts

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.6781	0.2416	6.94	1.3e-10
Age	-0.1031	0.0258	-4.00	0.00010

Residual standard error: 0.8 on 142 degrees of freedom

Multiple R-Squared: 0.101, Adjusted R-squared: 0.0948

F-statistic: 16 on 1 and 142 DF, p-value: 0.000103

Wind tunnel work with blends.

Analysis of deviance: Take off

	Df	Deviance Resid.	Df	Resid.Dev	P(>|Chi|)
NULL		3	49	483.82	
Blend	6	32.41	343	451.41	1.364e-05

Analysis of deviance: Upwind flight

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			349	485.20	
Blend	6	49.39	343	435.81	6.233e-09

Analysis of deviance: Approach

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			349	474.16	
Blend	6	60.90	343	413.26	2.958e-11

Analysis of deviance: Contact

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			349	227.558	
Blend	6	28.055	343	199.504	9.176e-05

Analysis of deviance: Attempt at copulation

	Df	Deviance Resid.	Df	Resid. Dev	P(>|Chi|)
NULL			349	90.818	
Blend	6	33.875	343	56.943	7.111e-06

APPENDIX 3

STATISTICAL ANALYSES FOR CHAPTER 6

APPENDIX 3.1

Experiment 1

(a) Analysis of variance on transformed data for experiment involving blends GM1, GM5, GM6 and GMC.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	3	3.81	1.27	42.90	5.6e-14
Day	3	0.20	0.07	2.25	0.093 .
Row	3	0.18	0.06	2.03	0.122
Posit	3	0.08	0.03	0.95	0.424
Residuals	51	1.51	0.03		

(b) Results from comparison of transformed means of GM1, GM5 and GM6 using contrast

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.141	0.0227	6.21	5.41e-08
Blend GM1 vs GM5	0.141	0.0393	3.59	6.74e-04
Blend GM1 vs GM6	0.141	0.0393	3.59	6.74e-04
Blend GM1 vs GMC	-0.422	0.0393	-10.76	1.24e-15

APPENDIX 3.2

Experiment 2a

(a) Analysis of variance on transformed data for experiment involving blends GM1, GM2 and GM3 set up in lucerne

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
BlendA	2	0.570	0.285	5.44	0.014
DayA	2	0.176	0.088	1.67	0.215
RowA	2	0.068	0.034	0.65	0.532
PositA	2	0.285	0.142	2.72	0.093
Residuals	18	0.944	0.052		

(b) Results from comparison of transformed means of GM1, GM2 and GM3 using contrast in R

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.676	0.0477	14.18	3.69e-13
Blend GM1 vs GM2	-0.178	0.0674	-2.64	1.43e-02
Blend GM1 vs GM3	0.	0.0674	2.64	1.43e-02
Blend GM2 vs GM3	0.178	0.0674	2.64	1.43e-02

APPENDIX 3.3

(a) Analysis of variance on transformed data for experiment involving blends GM1, GM2 and GM3 set up in cotton

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	2	0.621	0.310	12.66	0.00037
Day	2	0.007	0.003	0.14	0.87040
Row	2	0.004	0.002	0.08	0.92581
Posit	2	0.050	0.025	1.02	0.38198
Residuals	18	0.441	0.025		

(b) Comparison of transformed means of GM1, GM2 and GM3 using contrast in R

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.123	0.0278	4.44	1.73e-04
Blend GM1 vs GM2	-0.214	0.0393	-5.43	1.41e-05
Blend GM1 vs GM3	0.090	0.0393	2.29	3.12e-02
Blend GM2 vs GM3	0.090	0.0393	2.29	3.12e-02

APPENDIX 3.4

Experiment 2b

(a) Analysis of variance on transformed data for experiment involving blends GM2, GM7, GM9 and GMC set up in soy beans site 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	3	2.163	0.721	16.33	7.3e-07
Day	2	0.079	0.039	0.89	0.42
Row	3	0.096	0.032	0.72	0.54
Posit	3	0.203	0.068	1.53	0.22
Residuals	36	1.589	0.044		

(b) Results from comparison of transformed means of GM2, GM7, GM9 and GMC at site 1 using contrast in R

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.219	0.0305	7.19	6.09e-09
Blend GM2 vs GM7	0.140	0.0529	2.65	1.10e-02
Blend GM2 vs GM9	-0.194	0.0529	-3.67	6.43e-04
Blend GM2 vs GMC	-0.219	0.0529	-4.15	1.50e-04

APPENDIX 3.5

Experiment 2b

(a) Analysis of variance on transformed data for experiment involving blends GM2, GM7, GM9 and GMC set up in soy beans site 2

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	2	0.479	0.240	4.05	0.035
Day	2	0.565	0.283	4.78	0.022
Row	2	0.133	0.067	1.13	0.346
Posit	2	0.041	0.021	0.35	0.711
Residuals	18	1.065	0.059		

(b) Results from comparison of transformed means of GM2, GM7, GM9 and GMC at site 2 using contrast in R

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.718	0.0528	13.60	8.96e-13
Blend GM2 vs GM7	0.16	0.0746	2.17	4.03e-02
Blend GM2 vs GM13	-0.165	0.0746	-2.20	3.73e-02
Blend GM12 vs GM13	-0.165	0.0746	-2.20	3.73e-02

APPENDIX 3.6

Experiment 3

Analysis of variance on transformed data for experiment involving blends GM2, GM12 and GM13 set up in Mung beans site 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	2	0.052	0.026	0.35	0.71
Day	2	0.070	0.035	0.46	0.64
Row	2	0.087	0.044	0.58	0.57
Posit	2	0.004	0.002	0.03	0.97
Residuals	18	1.366	0.076		

APPENDIX 3.7

Experiment 3

(a) Analysis of variance on transformed data for experiment involving blends GM2, GM12 and GM13 set up in Mung beans site 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	2	0.065	0.033	0.84	0.450
Day	2	0.412	0.206	5.28	0.016
Row	2	0.167	0.084	2.14	0.146
Posit	2	0.094	0.047	1.21	0.322
Residuals	18	0.703	0.039		

(b) Analysis of variance on transformed data for experiment involving blends GM2, GM12 and GM13 set up in Mung beans site 2

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	2	0.224	0.112	1.21	0.32
Day	2	0.239	0.120	1.29	0.30
Row	2	0.363	0.181	1.96	0.17
Posit	2	0.189	0.094	1.02	0.38
Residuals	18	1.667	0.093		

APPENDIX 3.8

Experiment 4

Analysis of variance on transformed data for experiment involving blends GM2, GM12, GM17 and GM19 set up in lucerne

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	2	0.224	0.112	1.21	0.32
Day	2	0.239	0.120	1.29	0.30
Row	2	0.363	0.181	1.96	0.17
Posit	2	0.189	0.094	1.02	0.38
Residuals	18	1.667	0.093		

APPENDIX 3.9

Experiment 5

(a) Analysis of variance on transformed data for experiment involving blends GM2, GM11 and GM10 set up in Mung beans at site 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	2	0.226	0.113	2.22	0.14
Day	2	0.125	0.062	1.23	0.32
Row	2	0.202	0.101	1.98	0.17
Posit	2	0.086	0.043	0.85	0.44
Residuals	18	0.915	0.051		

(b) Analysis of variance on transformed data for experiment involving blends GM2, GM11and GM10 set up in Mung beans at site 2

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	2	0.191	0.095	0.84	0.45
Day	2	0.353	0.176	1.55	0.24
Row	2	0.385	0.193	1.70	0.21
Posit	2	0.083	0.041	0.36	0.70
Residuals	18	2.043	0.114		

APPENDIX 3.10

Experiment 6

Analysis of variance on transformed data for experiment involving blends GM2, GM14, GM15 and GM16 set up in lucerne

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blend	3	0.53	0.18	2.21	0.09795
Day	3	1.60	0.53	6.70	0.00067
Row	3	0.01	0.0022	0.03	0.99355
Posit	3	0.38	0.13	1.57	0.20696
Residuals	51	4.06	0.08		

Experiment 7

Analysis of variance on transformed data for Comparison of AgriSense and Delta trap designs

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Trap	1	0.19	0.19	0.48	0.49
Day	1	17.08	17.08	42.61	5.3e-07
Row	1	0.02	0.02	0.05	0.83
Posit	1	0.10	0.10	0.24	0.62
Residuals	27	10.83	0.40		

APPENDIX 3.11

Experiment 8

Analysis of variance on transformed data for experiment involving effect of trap location on green mirid catches

Response: log(Catch + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Location	1	3.2431	3.2431	20.2159	0.0006014
Day	1	0.8190	0.8190	5.1051	0.0416731
Residuals	13	2.0855	0.1604		

APPENDIX 3.12

Experiment 10

Analyses of variance on transformed data for experiment involving attract-and- kill and mating disruption on field S3 at Warra

Response: log(Day3 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.033	0.016	0.05	0.95
Residuals	9	2.893	0.321		

Response: log(Day9 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.175	0.088	2.01	0.19
Residuals	9	0.393	0.044		

Response: log(Day10 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.08	0.04	0.17	0.85
Residuals	9	2.16	0.24		

Response: log(Day12 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.072	0.036	0.44	0.66
Residuals	9	0.739	0.082		

Response: log(Day13 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	1.029	0.515	7.42	0.012
Residuals	9	0.624	0.069		

Response: log(Day14 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.561	0.280	3.5	0.075
Residuals	9	0.721	0.080		

Response: log(Day15 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	1.947	0.974	7.77	0.011
Residuals	9	1.128	0.125		

Response: log(Day16 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.631	0.315	1.81	0.22
Residuals	9	1.567	0.174		

Response: log(Day17 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.775	0.388	3.16	0.091
Residuals	9	1.105	0.123		

Response: log(Day18 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.320	0.160	3	0.1
Residuals	9	0.480	0.053		

Response: log(per9day + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.0896	0.0448	2.04	0.19
Residuals	9	0.1972	0.0219		

Response: log(per12day + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.0461	0.0230	0.66	0.54
Residuals	9	0.3153	0.0350		

Response: log(d15d16 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	1.147	0.573	16.1	0.0011
Residuals	9	0.320	0.036		

Response: log(d17d18 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.617	0.309	11.2	0.0036
Residuals	9	0.247	0.027		

Response: log(Day23 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.822	0.411	3.76	0.065
Residuals	9	0.985	0.109		

Response: log(d13d14 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.840	0.420	12.3	0.0027
Residuals	9	0.308	0.034		

Contrast Output

lm(log(Day13 + 1))

	Estimate	Std.Error	t value	Pr(>|t|)
(Intercept)	0.207	0.076	2.72	0.02346
Treatment control vs MD	-0.414	0.108	-3.85	0.00389
Treatment control vs AK	0.207	0.108	1.93	0.08621

lm(log(Day14 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.231	0.0817	2.83	0.0198
Treatment control vs MD	-0.289	0.1155	-2.50	0.0339
Treatment control vs AK	0.231	0.1155	2.00	0.0766

lm(log(Day15 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.987	0.102	9.66	4.77e-06
Treatment control vs MD	-0.428	0.145	-2.96	1.59e-02
Treatment control vs AK	0.539	0.145	3.73	4.68e-03

lm(log(Day17 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.323	0.101	3.19	0.0110
Treatment control vs MD	-0.299	0.143	-2.09	0.0664
Treatment control vs AK	0.323	0.143	2.26	0.0505

lm(log(d15d16 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.733	0.0545	13.46	2.88e-07
Treatment control vs MD	-0.397	0.0770	-5.15	6.00e-04
Treatment control vs AK	0.357	0.0770	4.63	1.23e-03

lm(log(d17d18 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.251	0.0478	5.24	0.000535
Treatment control vs MD	-0.299	0.0677	-4.41	0.001686
Treatment control vs AK	0.251	0.0677	3.71	0.004880

lm(log(Day23 + 1))

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.2648	0.0955	2.774	0.0216
Treatment control vs MD	0.0916	0.1350	0.678	0.5148
Treatment control vs AK	0.2648	0.1350	1.961	0.0815

lm(log(d13d14 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.235	0.0534	4.41	0.001690
Treatment control vs MD	-0.370	0.0755	-4.90	0.000851
Treatment control vs AK	0.235	0.0755	3.12	0.012317

Experiment 10

Analyses of variance on transformed data for experiment involving attract-and-kill and mating disruption on field WL4 at Warra

Response: log(Day3 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.24023	0.12011	1.2857	0.3227
Residuals	9	0.84079	0.09342		

Response: log(Day9 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.99897	0.49948	3.3186	0.08325
Residuals	9	1.35460	0.15051		

Response: log(Day10 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.15432	0.07716	0.4024	0.6802
Residuals	9	1.72559	0.19173		

Response: log(Day13 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.73850	0.36925	1.1381	0.3625
Residuals	9	2.92006	0.32445		

Response: log(Day14 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.08008	0.04004	0.3	0.748
Residuals	9	1.20113	0.13346		

Response: log(Day15 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.04526	0.02263	0.0935	0.9116
Residuals	9	2.17741	0.24193		

Response: log(Day16 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.15432	0.07716	0.474	0.6372
Residuals	9	1.46496	0.16277		

Response: log(Day17 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.88860	0.44430	8.2679	0.00916
Residuals	9	0.48364	0.05374		

Response: log(Day18 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.24023	0.12011	1.2857	0.3227
Residuals	9	0.84079	0.09342		

Response: log(per9day + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.24017	0.12009	3.1686	0.09083 .
Residuals	9	0.34109	0.03790		

Response: log(per13day + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.25949	0.12975	1.1542	0.3579
Residuals	9	1.01175	0.11242		

Response: log(d15d16 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.04349	0.02175	0.3165	0.7365
Residuals	9	0.61841	0.06871		

Response: log(d17d18 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.35039	0.17519	6.9622	0.01489
Residuals	9	0.22647	0.02516		

Experiment 10

Analyses of variance on transformed data for experiment involving D-vac sampling in attract-and-kill and mating disruption on field S3 at Warra

Response: log(m12 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.01	0.01	0.02	0.98
Residuals	9	3.25	0.36		

Response: log(f12 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.027	0.014	0.06	0.94
Residuals	9	2.045	0.227		

Response: log(m13 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.822	0.411	1.9	0.20
Residuals	9	1.945	0.216		

Response: log(f13 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	1.806	0.903	7.5	0.012
Residuals	9	1.084	0.120		

Response: log(m14 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.154	0.077	0.23	0.8
Residuals	9	2.997	0.333		

Response: log(f14 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.240	0.120	1.29	0.32
Residuals	9	0.841	0.093		

Response: log(f16 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.082	0.041	0.14	0.87
Residuals	9	2.572	0.286		

Response: log(m16 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.002	0.001	0.01	1
Residuals	9	1.928	0.214		

Response: log(m17 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.441	0.221	1.45	0.28
Residuals	9	1.365	0.152		

Response: log(m17 + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.441	0.221	1.45	0.28
Residuals	9	1.365	0.152		

Response: log(mbulk + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.16	0.08	0.2	0.82
Residuals	9	3.73	0.41		

Response: log(fbulk + 1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	0.552	0.276	1.49	0.28
Residuals	9	1.665	0.185		

Output for contrast where significance differences were observed

lm(log(m12 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.2986	0.174	1.7210	0.119
Treatment control vs MD	0.0240	0.245	0.0977	0.924
Treatment control vs AK	-0.0479	0.245	-0.1954	0.849

lm(log(f12 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.7269	0.138	5.283	0.000505
Treatment control vs MD	0.0338	0.195	0.174	0.866003
Treatment control vs AK	0.0338	0.195	0.174	0.866003

lm(log(m13 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.2648	0.134	1.973	0.0799
Treatment control vs MD	-0.3564	0.190	-1.878	0.0932
Treatment control vs AK	0.0916	0.190	0.482	0.6411

lm(log(f13 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.530	0.100	5.29	0.000503
Treatment control vs MD	-0.539	0.142	-3.81	0.004173
Treatment control vs AK	0.183	0.142	1.29	0.228530

lm(log(m14 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.4240	0.167	2.545	0.0314
Treatment control vs MD	-0.0240	0.236	-0.102	0.9212
Treatment control vs AK	0.1493	0.236	0.634	0.5420

lm(log(f14 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.173	0.0882	1.96	0.0811
Treatment control vs MD	-0.173	0.1248	-1.39	0.1983
Treatment control vs AK	0.173	0.1248	1.39	0.1983

lm(log(f16 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.448	0.154	2.903	0.0175
Treatment control vs MD	0.101	0.218	0.464	0.6534
Treatment control vs AK	-0.101	0.218	-0.464	0.6534

lm(log(m16 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.52968	0.134	3.965	0.00328
Treatment control vs MD	-0.01963	0.189	-0.104	0.91953
Treatment control vs AK	0.00982	0.189	0.052	0.95970

lm(log(m17 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.2648	0.112	2.356	0.0429
Treatment control vs MD	-0.0817	0.159	-0.514	0.6196
Treatment control vs AK	-0.1831	0.159	-1.152	0.2792

lm(log(f17 + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.6452	0.208	3.095	0.0128
Treatment control vs MD	-0.0479	0.295	-0.163	0.8744
Treatment control vs AK	-0.3226	0.295	-1.094	0.3023

lm(log(mbulk + 1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.2610	0.186	6.784	8.05e-05
Treatment control vs MD	-0.0828	0.263	-0.315	7.60e-01
Treatment control vs AK	-0.0828	0.263	-0.315	7.60e-01

lm(log(fbulk + 1))

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.6254	0.124	13.090	3.66e-07
Treatment control vs MD	-0.2383	0.176	-1.357	2.08e-01
Treatment control vs AK	-0.0433	0.176	-0.246	8.11e-01