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Sexual behaviour of the green capsid bug

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
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Stellingen behorende bij het proefschrift:

Sexual behaviour of the green capsid bug

1. Ruim 40 jaar onderzoek naar seksferomonen bij motten heeft een gedegen kennis opgeleverd van seksferomonen bij deze groep insecten. Helaas is deze kennis maar beperkt bruikbaar om de seksuele communicatie van andere insecten te begrijpen.
   Dit proefschrift.

2. Hoewel de EAG-techniek geleid heeft tot een groot aantal seksferomoon-identificaties, kunnen EAG-resultaten ook verwarring scheppen omdat sommige chemische signalen, die essentieel zijn in een communicatie-systeem van een insect, minimale EAG-activiteit kunnen geven.
   J.N.C. van der Pers & C. Löfstedt, 1986. Signal-response relationship in sex pheromone communication. In: T.L. Payne, M.C. Birch and C.E.J. Kennedy (eds), Mechanisms in insect olfaction. Oxford University Press.

3. Het benoemen van signaalstoffen (zoals seksferomonen en alarmferomonen) suggereert soms onterecht kennis over de werking van die stoffen.
   Dit proefschrift.

4. Hoewel cuticulaire koolwaterstoffen van insecten minder vluchtig zijn dan de meeste geïdentificeerde seksferomonen, spelen deze stoffen waarschijnlijk een belangrijkere rol in de seksuele aantrekking op afstand dan tot nu toe is aangenomen.
   W. Francke, ISCE-meeting Marseille, November 1999.

5. Het identificeren van stoffen die mogelijk belangrijk zijn in biologische systemen is zinloos als de biologische betekenis van de geïdentificeerde stoffen niet bepaald wordt.
   Dit proefschrift.

6. De uitdrukking 'sperma-competitie' benadrukt, zoals traditioneel gebruikelijk in de biologie, de actieve mannelijke rol in man-vrouw interacties, terwijl dit een misleidend beeld kan zijn omdat daarmee de invloed van vrouwtjes op de concurrentie tussen mannetjes, en de uitkomst daarvan, onderschat wordt.
   W.G. Eberhard, 1998. Female roles in sperm competition. In: T.R. Birkhead & A.P Møller (eds), Sperm competition and sexual selection. Academic Press.

7. De hersenen van de mens kunnen vergeleken worden met de veren van een pauw; het intellect is ontwikkeld om een seksuele partner te verleiden.
   M. Ridley, 1993. The Red Queen. Penguin books.

8. De enorme toename van communicatie-mogelijkheden heeft geleid tot een enorme toename van inhoudloos gebabbel.

9. Dat meisjes en vrouwen met kort haar bijna dagelijks met 'meneer' worden aangesproken geeft aan hoe slordig mensen waarnemen.

10. Discriminatie van vrouwen kan in aanvang vermeden worden door op formulieren de vraag naar geslacht te schrappen.

11. Om een einde te maken aan de ontelbare levensgevaarlijke situaties op en rond fietspaden zouden burgemeesters en wethouders minimaal eens per jaar verplicht moeten worden tot een fietstocht over alle fietspaden in hun gemeenten.

12. Massale inzamelingsacties voor mensen in nood in tweede en derde wereldlanden dragen bij aan superieure gevoelens van Nederlanders ten opzichte van buitenlanders, en hebben daarmee een negatief effect op de ontwikkeling van onze multiculturele samenleving.

13. Bonsai-boompjes zijn een uiting van agressie.
A. van Dis, VARA-tv B & W, 26-5-2000.

14. Dat er nu ook al PC-privé projecten zijn waarbij het principe geldt hoe meer iemand verdient, hoe minder iemand hoeft in te leveren (in dit geval voor een computer voor privé-gebruik thuis), geeft aan hoe kapitalistisch onze maatschappij is geworden.

15. Nog interessanter dan de vragen die een kind stelt, zijn de vragen die bijna niemand stelt.

Astrid T. Groot
Wageningen, 15 september 2000

Aan alle wantsen die het leven hebben moeten laten voor dit project

A different perspective:

What is your profession?

I'm a biologist.

So you're working on AIDS?

No, I'm working with insects.

Ah, there are deadly insects in your country!? ...

(acquaintance conversation with a schoolteacher in a township in South Africa, April 1999)

Contents

Abstract

The green capsid bug (*Lygocoris pabulinus* (L.), Heteroptera; Miridae) is an unpredictable pest in fruit orchards in North-Western Europe, since it migrates twice a year. An efficient monitoring system to predict bug damage in an orchard may reduce the use of insectices. Such a monitoring system may be developed by exploiting the sex pheromone, as *L. pabulinus* males are attracted by virgin females. However, attempts to identify the pheromone in the past decade have failed, which may be due to a lack of understanding of their sexual behaviour as a whole. Therefore, the sexual behaviour of the green capsid bug was studied in detail.

Males and females are sexually mature 4-5 days after the final moult. EAG-responses suggest that males are more sensitive to insect-produced pheromone-type compounds, whereas females are more sensitive to plant compounds. This correlates with their behaviour, as males are attracted to virgin and mated females at long range, with and without plants. The attraction is mediated by a sex pheromone, not an aggregation pheromone, since males are not attracted to males, and females are not attracted to either sex. Sex pheromone emission is inhibited by hexyl butanoate, the alarm pheromone of *L. pabulinus.* Females do not show a specific calling behaviour. At close range, males are attracted to female-specific, low volatile compounds, present on female legs. These compounds are also deposited on the substrate on which females walk. At long and close range, males vibrate with their abdomen when they perceive signals from females.

Matings last only 1-2 minutes, during which a compartmentalized spermatophore is formed in the spermatheca of the female. A mating plug is part of the spermatophore, inducing a refractory period in females for about 24 hours. After 24 hours, sperm is released from the spermatophore and found throughout the spermatheca and the lateral oviducts. When males have mated, they do not respond to females for at least two hours. Multiply-mated females oviposit as many eggs and live as long as once-mated females. Virgin females also oviposit eggs, but these eggs do not hatch. Under summer conditions, females oviposit preferably in potato plants.

This study shows that long-range mate location by means of a sex pheromone is only part of the sexual behaviour of the green capsid bug. For pest management, the alarm pheromone of *L. pabulinus* may be exploited to prevent bug damage in fruit orchards.

Chapter 1

Introduction

The sexual behaviour of insects is as variable as the insects themselves. This behaviour is not present in all insects, as many species reproduce asexually (at some stage of their life, or exclusively). The sexual behaviour of insects can be roughly divided in the following successive phases: (1) long-range attraction or upwind flight orientation, (2) close-range attraction, (3) courtship behaviour, (4) mating (*i.e.*, sperm transfer), (5) sometimes mate guarding to prevent subsequent matings of the female for at least some time, (6) fertilization of eggs, and in conclusion (7) oviposition of fertilized eggs. Not every phase is included in the sexual behaviour of all insect species. For example, in gregarious species mates are not attracted from a long range. When the adults are not in each other's vicinity, the sexes may locate each other by means of visual, chemical and/or acoustic communication (*e.g.* Lewis, 1984). Usually, one of the sexes emits a signal to which the other sex is attracted. Especially chemical signals are very effective to attract insects from a distance (Cardé and Bell, 1995; Cardé and Minks, 1997). When chemical signals attract one of the sexes in a species, these signals are called sex pheromones. In many insect species males emit a sex pheromone to attract females. In many other species females emit a pheromone to attract males. Or males attract females from a long range, after which females attract males at close range. Or vice versa. Sex pheromone production and release in the emitting sex, and its response in the perceiving sex, depends on age, mating status, physiological state, and several environmental factors (McNeil, 1991; Raina *et al.*, 1992, 1994; Landolt and Philips, 1997). For example, females may only release pheromone when they are on or near a host plant, which coordinates their reproductive behaviour with the availability of food for the offspring (Raina *et al.*, 1992). The exact location of a potential mate within a plant patch or even within a plant, may be assessed by other (visual, chemical or acoustic) signals (*e.g.* Ota and Čokl, 1991). After mate location, courtship behaviour can precede or be part of a mating. When a mating is successful, the behavioural sequences will result in sperm transfer.

The sexual behaviour of insects does not end with a mating. Females of many insect species mate more than once. When a female remates, sperm competition within the female reproductive organs will determine which sperm will fertilize the eggs. Most eggs are likely to be fertilized with sperm of the last mated

male (Thornhill and Alcock, 1983), although females can probably influence which sperm will fertilize the eggs (Eberhard, 1996). Males may guard a female after a mating (physically, mechanically, or chemically) to prevent females from remating for at least some period of time, to assure that their sperm is used to fertilize the eggs rather than the sperm of competing males (Thornhill and Alcock, 1983; Alcock, 1994). Many male insects also donate nutrients to females during sperm transfer. These nutrients may cause a (temporal) increase of egg production in females. When this is the case, such a donation can be regarded as an effort of males to fertilize as many eggs as possible before the female will remate with another male (Vahed, 1999). In conclusion, the sexual behaviour of males and females before, during, and after a mating determines to a large extent their reproductive success.

Sexual behaviour and pest management

Studying the sexual behaviour of insects is not only interesting in itself, knowledge on this subject is also useful for pest management in agricultural crops. For example, when long-range mate location can be disrupted, it may be possible to prevent matings, which can be an effective pest control method.

In many insects, long-range mate location is chemically mediated by means of a sex pheromone. Sex pheromones have been identified in numerous insect species (Cardé and Minks, 1997; Hardie and Minks, 1999). A number of lepidopteran pests can be successfully controlled by introducing large amounts of synthetic sex pheromone in the field, so that potential mates are unable to locate each other (Minks and Van Deventer, 1992; Sanders, 1997). However, effective pest control by means of sex pheromones is hampered in many cases, because only few matings may be sufficient to produce a large amount of offspring, so that economic damage thresholds will still be exceeded (*e.g.* Wyatt, 1997).

Pheromones can also be used in other ways. For example, they are powerful tools to monitor many different pest populations of crop and orchard pests (Wall, 1989), stored products (Burkholder, 1990), and forestry pests (Borden, 1990). For monitoring an insect pest species, synthetic sex pheromone traps are placed in the area where the presence of the species is suspected. Individuals are likely to be caught in such traps when they are in the surroundings, as sex pheromones are species specific and the responding sex is very sensitive to the pheromone. Application of insecticides will only be needed when (a certain number of) individuals are caught in these traps. The use of sex pheromones for monitoring pests has resulted in major reductions of insecticidal use (Wall, 1989).

Heteropteran pests

True bugs have been ranked fourth among the economically important insect orders (Arnett, 1993). They are found in all types of crops (vegetables, fibres, perennial

trees), and usually migrate to different crops during the year. In the past, bugs were often controlled by pesticides applied against other insects. However, the reduced use of pesticides and the use of more selective pesticides have resulted in a recurrence of bug pests, among other secondary pest (Minks *et al.*, 1998). Therefore, the need for better bug sampling methods has acquired new urgency (McBrien and Millar, 1999).

The presence of a long-range sex pheromone has been reported for a large number of heteropteran species. In the past decade, many studies have aimed to identify these sex pheromones (see McBrien and Millar, 1999). However, relatively few studies have been successful in the pheromone identification of bugs. For example, in at least 10 mirid bug species females have been found to attract males (Strong *et al.*, 1970; King, 1973; Smith, 1977; Boivin and Steward, 1982; Graham, 1987; Graham *et al.*, 1987; Chinta *et al.*, 1994; Smith *et al.*, 1991; Smith *et al.*, 1994; Millar *et al.*, 1997; Millar and Rice, 1998), but sex pheromone identification has been successful for only three species (Smith *et al.*, 1991; Millar *et al.*, 1997; Millar and Rice, 1998).

Sex pheromone identification in Heteroptera may be hampered by the abundant defensive secretions, present in the metathoracic scent gland, that are released upon disturbance (Aldrich, 1988, 1995). These defensive secretions may function as an alarm pheromone, causing dispersal of conspecifics. In this way, confrontations with predators are avoided (Blum, 1985; Staddon, 1986; Aldrich, 1988; Aldrich *et al.*, 1997). Release of defensive secretions (alarm pheromone) upon disturbance may overshadow possible sex pheromone compounds. An extra complication can be that alarm pheromone compounds may be (part of) the sex pheromone, when emitted in much smaller quantities (McBrien and Millar, 1999).

Sexual behaviour of Heteroptera

Sex pheromone identification in heteropterans may not only be hampered by their abundant defensive secretions, but also by a lack of understanding of their sexual behaviour as a whole. For example, in addition to long-range sex pheromones, close-range signals may be involved in the sexual communication as well. Such signals can be important for trap catches; when part of the attractive signal is missing, males are not likely to enter a trap. Or when females mate more than once, a refractory period can succeed a mating, during which sex pheromone is not released. Mate guarding may also affect sexual communication. And virgin females may be more attractive than mated females. Hence, it is important to understand the additional behaviours to long-range attraction. Summarizing information available on terrestrial bugs, their sexual behaviour can be outlined as follows.

In many heteropterans, the final moult is succeeded by a sexual maturation period of some days, during which individuals are sexually inactive (Loher and Gordon, 1968; Mitchell and Mau,1969; Strong *et al.*, 1970; King, 1973; Mau and Mitchell, 1978; Chatterjee, 1984; Graham *et al.*, 1987; Wang and Millar, 1997; see also Table 1). When heteropterans are sexually mature, one of the sexes usually

attracts the other from a long range. Some heteropteran adults are gregarious, these species probably lack a long-range sex pheromone (Loher and Gordon, 1968; Carroll and Loye, 1990; Carroll, 1991). In most heteropteran families males attract females (Harris and Todd, 1980; McLain *et al.*, 1990; Aldrich, 1995; Wang and Millar, 1997), although in mirid bugs females attract males from a long range (reviewed by McBrien and Millar, 1999). In two mirid species, a specific calling position in females has been recognized when males are attracted (*i.e.* probably during pheromone emission): a raising of the abdomen (King, 1973; Smith, 1977).

After long-range mate location has occurred, acoustic signals have been found to play an important role in close-range mate location and courtship behaviour of *Nezara viridula* (Pentatomidae) (Todd, 1989; Ota and Čokl, 1991; Ryan and Walter, 1992). Acoustic communication has also been found in some other heteropterans (Leston and Pringle, 1963; Gogala, 1969; Gogala *et al.*, 1974). Courtship behaviour includes males and females antennating each other (Mitchell and Mau, 1969; Strong *et al.*, 1970; Levinson and Bar-Ilan, 1971; Tostowaryk, 1971; Nilakhe, 1976; Chatterjee, 1984; Wang and Millar, 1997). In *Podisus modestus* (Pentatomidae) a strong odour is released during courtship (Tostowaryk, 1971). Some male bugs show a vibration behaviour of their abdomen as part of their courtship behaviour (Strong *et al.*, 1970; Fish and Alcock, 1973; Wang and Millar, 1997).

The mating event itself may last a few minutes (Mitchell and Mau, 1969; Strong *et al.*, 1970; Mau and Mitchell, 1978; Liquido and Nishida, 1985; Carroll 1991), although in most heteropterans prolonged matings of several hours to as long as 10 days have been observed (Mitchell and Mau, 1969; Loher and Gordon, 1968; Tostowaryk, 1971; McLain, 1985, 1989; Harris and Todd, 1980; Carroll, 1988, 1991; Carroll and Loye, 1990; Wang and Millar, 1997). Such prolonged matings are probably a form of mate guarding (McLain, 1985, 1989; Sillén-Tullberg, 1981; Carrol 1988, 1991; Carroll and Loye, 1990; Alcock 1994). All heteropterans in which prolonged matings have been observed are polyandrous. Hence, sperm competition in females is likely to occur, which reduces the chance of the first mated male that his sperm will be used to fertilize the eggs (Thornhill and Alcock, 1983). The idea that prolonged matings are a form of mate guarding originates from the fact that, in some species, copulations lasting for a few hours or even 5 minutes resulted in as many fertile eggs as copulations lasting for several days (Sillén-Tullberg, 1981; McLain, 1989; Carroll, 1991).

The act of copulation has hardly been studied in Heteroptera. Bonhag and Wick (1953) studied the copulation of *Oncopeltus fasciatus* (Pentatomidae) in detail by focussing on the erection of the aedeagus. They found that during copulation an elongate, coiled bladder (which when at rest is folded in the phallobase) is filled with "transparent erection fluid", thereby greatly enlarging in a balloon-like manner. Such a structure was also found in the blood-sucking bug *Rhodnius prolixus* (Reduviidae) (Davey, 1960), where the everted sac was called the spermatophore sac, as it contained a spermatophore when in copula for 10 minutes.

Although copulation has hardly been studied, sperm displacement has been determined in *Oncopeltus fasciatus* (Economopoulos and Gordon, 1972), *Lygaeus equestris* (Sillén-Tullberg, 1981) (both Lygaeidae), *Dysdercus koenigii* (Pyrrhocoridae) (Harwalkar and Rahalkar, 1973), *Nezara viridula* (Pentatomidae) (McLain, 1985), and *Jadera haematoloma* (Rhopalidae) (Carroll, 1991). Sperm displacement in these species was determined by allowing females to mate with different types of males, in such a way that information on which eggs were fertilized by which males could be retrieved. Most sperm was displaced in *L. equestris*, where the last mated male fertilized about 90 % of the eggs (Sillén-Tullberg, 1981). Least sperm was displaced in *N. viridula*, where the first mated male fertilized as many eggs as the second male (McLain, 1985).

With sperm, nutrients, which can be converted into eggs, are probably transferred from males to females in some heteropterans, as fecundity and/or egg hatch have been positively correlated to the number of copulations (Mau and Mitchell, 1978; Kasule, 1986; McLain *et al.*, 1990; Wang and Millar, 1997). In *Nezara viridula* such nutrients seem to be transferred in the form of giant, nonfertilizing sperm (Schrader, 1960; McLain *et al.*, 1990). However, in other heteropterans multiple matings have not been found to correlate with overall fecundity, and/or fertility (Nilakhe, 1976; Harwalkar and Rahalkar, 1973; Strong *et al.*, 1970). Female longevity has not been found to be affected by the number of matings in these species either.

When females do not mate (*i.e.* kept virgin), they oviposit eggs anyway (Strong *et al.*, 1970; Economopoulos and Gordon, 1972; Nilakhe, 1976; Carroll, 1991; Wang and Millar, 1997), often as many as once-mated females. These eggs do not hatch in most species, although parthenogenesis has been found in the anthocorid *Calliodis maculipennis* and the mirid *Campyloneura virgula* (Carayon, 1989).

Table 1. Sexual behaviour of Heteropteran species, general overview

Possible steps in sexual behaviour:	-	**Found in Heteroptera:**
1. Long-range attraction	-	Sex pheromone (males attract females or females attract males)
2. Close-range attraction	-	Acoustic signals - chemical signals?
3. Courtship behaviour	-	Male vibration with abdomen, sexes antennating each other
4. Mating (sperm transfer)	-	Spermatophore (described only in *Rhodnius prolixus*), nutrients?
5. Mate guarding	-	By means of prolonged matings
6. Fertilization of eggs	-	(Partial) sperm displacement or sperm mixing
7. Oviposition	-	Unfertilized eggs are also oviposited, but usually do not hatch

The green capsid bug

The subject of this PhD thesis is the green capsid bug (*Lygocoris pabulinus* (L.), Miridae), a pest in fruit orchards in North-Western Europe that is difficult to control. (Blommers, 1994; Ravn and Rasmussen, 1996). In autumn, females oviposit their overwintering eggs in the stem of the trees, after which the adults die. In spring, when fruit trees start to bloom, nymphs emerge from the eggs (see Figure 1). These nymphs feed on shoot tips, ovaries and young fruitlets, which cause rusetted malformations in the fruits (Blommers, 1994). As emerging nymphs damage the fruit, the yield of fruit growers is directly affected. Consequently, economic thresholds will be exceeded soon after the nymphs have emerged. Additionally, this threshold is exceeded at very low population densities (Van den Ende *et al.*, 1996). Fruit growers cannot predict whether their fruit will suffer from bug damage the following spring. In order to reduce the risk of damage, fruit growers apply insecticides against this pest before and after bloom each year. An efficient monitoring system can help to reduce the use of insecticides against this pest species.

A reduction of insecticide use is possible, because bug damage will probably not occur in the same orchard each year. The green capsid bug migrates twice a year, and insecticides should only be applied in orchards to which *L. pabulinus* has migrated. The migration of *L. pabulinus* is as follows (see Figure 1). After the third larval stage, *L. pabulinus* migrates from trees to herbaceous plants. This migration is obligatory, as *L. pabulinus* needs herbaceous plants to complete its life cycle (Blommers *et al.*, 1997). When an herbaceous underlayer is present in the orchard, the green capsid bug may remain in the orchard throughout the summer (Hill, 1952). In the absence of herbs, migration will occur to nearby herbaceous fields, for example potato fields. A summer generation will develop on herbaceous plants. When daylength shortens, adults migrate back to fruit trees to oviposit their overwintering eggs.

Aim of the project

Our aim was to develop an efficient monitoring system to predict the presence of *L. pabulinus* in fruit orchards, by identifying the sex pheromone. *Lygocoris pabulinus* males are attracted by virgin females in the field (Blommers *et al.*, 1988). Since the late 1980's, identification of the sex pheromone of the green capsid bug has been attempted, but without success. In a related species with a similar life cycle, *Campylomma verbasci* (Miridae), sex pheromone identification has been successful (Smith et al., 1991). Therefore, identification of the sex pheromone of *L. pabulinus* seemed feasible as well. This project consisted of two components: (1) a biological study of the sexual behaviour of the green capsid bug, and (2) a chemical study to identify the sex pheromone of this species. In this thesis the biological part is described.

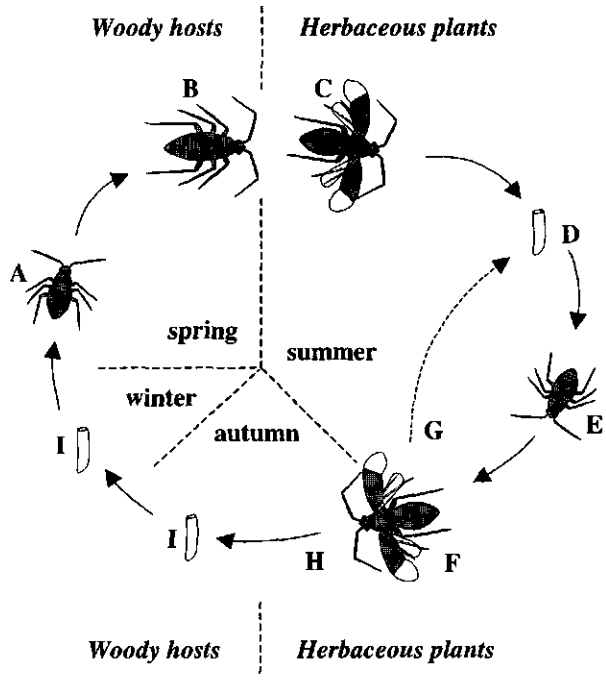

FIGURE 1. Life cycle of the green capsid bug. A: nymphs emerge in spring on woody hosts, B: after their thirdlarval stage (of five larval stages) they migrate to herbaceous plants, C-D: in summer females oviposit eggs in herbaceous plants, E: a summer generation develops on herbaceous plants, F: adults either oviposit eggs (D, I) in herbaceous plants (G) or in woody hosts (H) depending on the weather (in hot summers a second generation develops on herbaceous plants), H: when days get shorter in autumn, overwintering eggs are oviposited in woody hosts, after which the adults die (A, E: first instars, B: fourth instar, showing visible wing pads, C, F: adults).

Outline of the thesis

As identification of the sex pheromone was the aim of the project, our study focussed mainly on behavioural aspects that are related to sexual attraction. In the first part, general aspects of this behaviour and the perception of possible attractants is described for males and females (Chapter 2 and 3). The second part aims to quantify exactly to what sources males are attracted (Chapter 4 and 5). In the third part (Chapter 6, 7, and 8), the sexual behaviour is studied in more detail from the female perspective, to determine which factors may affect sex pheromone release and how their sexual behaviour is related to oviposition behaviour.

Chapter 2 describes general aspects of the mating behaviour in *L. pabulinus*, *i.e.* at what age females can be expected to emit sex pheromone, and at what age males will respond to this pheromone. We also determined whether attraction of males coincided with a calling behaviour of females and, thus, when

females supposedly emit sex pheromone. At close range, we studied the courtship behaviour in detail, and found a characteristic arousal behaviour of males, a vibration of the abdomen. This behaviour was useful as a bioassay to determine the attractivity of females and extracts.

The sex pheromone may be identified by determining to which compounds males (the attracted sex) are more sensitive than females (the emitting sex), because males are likely to be much more sensitive to sex pheromone compounds than females. Chemical signals are (mainly) perceived by sensilla on the antennae of insects. Which compounds are perceived can be assessed by means of electroantennogram-recordings (EAG's). In an EAG-setup, an antenna is placed between two electrodes, after which olfactory stimuli are applied to the antenna. When the antenna contains receptors that perceive the tested chemical, the resting potential of the antenna decreases. The larger the signal, the more sensitive is the antenna, and hence the more important the chemical is likely to be in the biology of the insect. In **Chapter 3** we tested a series of esters by means of EAG's. A number of these compounds have been found frequently in heteropterans in general, some in *L. pabulinus* in particular. In the tested series, all sex pheromone compounds, that have been identified so far in mirids, were included. A number of plant volatiles was tested as well, as mirid bugs (*i.e.* plant bugs) may be also attracted to plant odours.

In the field-assays that showed attraction of males towards females, females had been confined in a trap with a small potato sprout as a food source. Therefore, attraction of males may have been caused not only by females, but also by a combination of females and plant odours. In **Chapter 4** we determined the effects of plant volatiles on the sexual attraction in *L. pabulinus*.

In **Chapter 5** we used the vibration behaviour of males to determine the source of attractant in females. Sex pheromones are usually produced in specific glands. So far, these glands have not been identified in mirid bugs. When sex pheromone glands can be distinguished, elucidation of the sex pheromone would be possible by chemical analysis of their content. Heteropterans possess numerous glands (Staddon, 1979). Therefore, females were dissected into separate body parts, *i.e.* heads, wings, legs, and thorax plus abdomen. These parts were offered to males, to determine which parts elicited vibration behaviour.

Sexual communication in heteropterans may be disturbed by alarm pheromones (Blum, 1985; Staddon, 1986; Aldrich, 1988; Aldrich *et al.*, 1997). In **Chapter 6** we analysed the possible alarm pheromone of *L. pabulinus*, and the mechanism of how this may disturb sexual communication, *i.e.* are males repelled or do females stop to emit sex pheromone?

Sex pheromone production or release may not only stop when female insects are disturbed, but also after matings (Jurenka *et al.*, 1991; Raina *et al.*, 1994; Kingan *et al.*, 1995; Foster and Ayers, 1996). This may be temporary, for a short or longer period of time after females have mated, or sex pheromone production may be shut off permanently. Many female insects store sperm after a mating, and one mating may be sufficient to lay a full complement of eggs. As sperm transfer,

storage, and displacement may affect the sexual behaviour, we studied the fertilization in *L. pabulinus* females in detail (**Chapter 7**).

Migration of *L. pabulinus* females from herbaceous plants to fruit trees, to oviposit overwintering eggs, is not likely to occur over longer distances than a few km (Southwood, 1960, 1962). Therefore, knowledge on the possible location of the summer generation of the green capsid bug may predict bug infestation in different fruit orchards. In **Chapter 8** oviposition preference of *L. pabulinus* females under summer conditions was determined.

In **Chapter 9** all studied aspects of the sexual behaviour of the green capsid bug are summarized and discussed. The vibration behaviour of males suggests that acoustical signals are part of the sexual communication as well, and preliminary experiments on this subject are described. In conclusion, a possible control method to prevent bug damage in orchards is discussed.

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PART I:

Sex-related behaviour and perception of males and females

Chapter 2

COPULATION BEHAVIOUR OF *LYGOCORIS PABULINUS* UNDER LABORATORY CONDITIONS*

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ABSTRACT - As a prerequisite to the elucidation of the sex pheromone of the green capsid bug *Lygocoris pabulinus* (L.), we studied the bug's reproductive development and behaviour. When kept under long-day conditions (L18:D6) at 25°C, both males and females start to mate 4 days after the final moult. Second matings occur in some females, even on consecutive days. The premating period is 10 minutes on average and copulation lasts one to two minutes. At long range, when males are attracted to traps baited with live females, we did not observe a specific calling position of the females. At short range, a characteristic courtship behaviour of the females was not observed either. Males showed a characteristic vibration of the abdomen, which was repeated several times before copulation. This behaviour can be used as a bioassay to test potential sex pheromone compounds.

KEY WORDS: Heteroptera, Miridae, sex pheromone, long-range calling behaviour, close-range courtship behaviour, vibrational signal, ethogram

Introduction

The green capsid bug, *Lygocoris pabulinus* (L.) (Heteroptera: Miridae) is a serious pest in apple and pear orchards in Northwestern Europe (Blommers, 1994; Ravn and Rasmussen, 1996). *L. pabulinus* has two generations each year. In autumn adults lay overwintering eggs in woody plants, *i.e.* the fruit trees, and nymphs emerge the following spring when the fruit trees start to bloom. The summer generation feeds on herbaceous plants (Petherbridge and Thorpe, 1928; Kullenberg, 1946; Southwood and Leston, 1959). Damage occurs when nymphs emerging in spring feed on shoot tips, ovaries and young fruitlets, thereby causing russetted malformations of the fruits (Blommers, 1994). Control threshold is exceeded after observing only one single nymph per Crop Protection Unit, which is defined as a part of the orchard in which crop protection measures are comparable, *i.e.* plantings of similar age and flowering time (Van den Ende *et al.*, 1996). Since an efficient monitoring system is not available, fruit growers apply insecticides against this pest before and after bloom, in order to reduce the risk of damage (Blommers, 1994). Development of a monitoring

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system may be feasible by exploiting the sex pheromone of *L. pabulinus*, as Blommers *et al.* (1988) found that virgin females in traps attract males in the field. The use of sex pheromone traps is widely and successfully applied for the monitoring of lepidopterous pests (*e.g.* Minks and Van Deventer, 1992) and since a few years also for monitoring the mirid pest *Campylomma verbasci* (McBrien *et al.*, 1996).

Although the presence of sex pheromones has been demonstrated in several mirids (Strong *et al.*, 1970; King, 1973; Boivin and Steward, 1982; Graham, 1988; Smith *et al.*, 1994), the chemistry of these sex pheromones has only been elucidated in three species, *i.e. Campylomma verbasci* (Smith *et al.*, 1991), *Phytocoris relativus* (Millar *et al.*, 1997) and *P. californicus* (Millar and Rice, 1998). In general, one difficulty in identifying heteropteran sex pheromones is the presence of abundant defensive secretions in the scent glands (Aldrich, 1995; Staddon, 1986). Moreover, no well-defined gland which might serve as a source of sex pheromone has been found in this group of insects (Staddon, 1986). However, because it is expected that the production and use of sex pheromones coincides with reproductive receptivity of both sexes, we studied the reproductive development and the mating behaviour of *L. pabulinus* as a prelude to identifying its sex pheromone.

Mating behaviour can be divided into two distinct phases, long-range mate location and close-range courtship (Borges *et al.*, 1987). Long-range mate location is the upwind orientation and approach of one sex towards the other, resulting in the close proximity of the two sexes, while close-range courtship is the interaction of both sexes once they are in close proximity, which results in copulation (Borges *et al.*, 1987). At long range we concentrated on the behaviour of the females to determine if a calling behaviour associated with sex pheromone emission is visible. At close range we studied the courtship behaviour of both sexes.

Material and Methods

Rearing

Lygocoris pabulinus was reared on potted potato plants, cultivar Bintje, in wooden cages in greenhouses, which were maintained at 22 ± 2°C, 65 ± 5 % r.h. under a light regime of L18:D6 (Blommers, 1997). Rearing cages were checked daily for adults, which were then isolated. In this way, virgin males and females of known age were continuously available for our experiments. After isolating the sexes, bugs were transferred to a controlled environment room (22 °C, 65 % r.h., L18:D6), which was exclusively illuminated with artificial light, using high frequency fluorescent tubes (Philips type TLD 50W/84HF) connected to a Philips LPS 100 dimmer.

Preliminary mating experiments indicated that females without eggs copulated significantly less often than females with eggs. For this reason we measured egg production under different conditions in the climate room: the temperature was adjusted to either 20 °C or 25 °C, and the bugs were fed on either potato plants only or on potato plants plus pollen grains. Relative humidity and day length were not changed. After 7 days, the females were dissected and the number of

fully developed eggs in each individual was counted. Fully developed eggs of *L. pabulinus* can be recognized easily in females by the presence of a yellow rim, which is lacking in undeveloped eggs (Wightman, 1972). The optimal rearing condition was considered to be the one at which most females produced eggs, and at which the largest number of eggs per female was present.

Sexual maturation and copulation frequency

The age at which *L. pabulinus* start to copulate after the final moult was determined by mating experiments, using adult males and females of known age, reared at 25 °C, 65 % r.h. and L18:D6. To determine the sexual maturation period of each sex separately, first the age of the males was varied between 0.5 and 8.5 days with increments of one day, while the females used were 7.5 days old. In preliminary experiments 7.5-day-old females were reproductively active. Subsequently, the age of the females was varied between 0.5 and 10.5 days, using reproductively mature males of 5-7 days old, determined in the former experiments. Two to three hours before these test, each female was contained with a potato leaf in a plastic dish (diameter 10 cm, height 7 cm) sealed with a gauze lid and bottom. The experiment started by removing the potato leaf and placing one male into each dish. After one hour males were removed and females were dissected. If the spermatheca was swollen, it was filled a spermatophore (pers. obs.), hence copulation had occurred. All experiments were carried out between 12.00 and 15.30 hours (Greenwich mean time + 1 h). All combinations were repeated 30 times.

To measure sexual activity during the day, similar experiments were conducted but at different times of the day. The time period in which all previous mating experiments were carried out was considered as one time interval. Three additional time intervals were chosen, in which 30 5-8-day-old males and 30 females of 7.5-day-old were used: from 7.00 to 8.00 hours, which is at the onset of the light period; from 9.30 to 10.30 hours; and from 21.45 to 22.45 hours. No observations were made during the scotophase.

To determine if females copulated more than once, a different set of mating experiments was conducted in a greenhouse (22 ± 2 °C, 65 ± 5 % r.h., L18:D6). Three groups of females were observed for four sequential days, the first group of females being 4.5 days old at the start, the second group 5.5 days and the third group 6.5 days old. One to two hours in advance, each female was placed in a marked plastic dish with gauze lid and bottom containing a potato leaf. At the start of the experiments the potato leaf was removed and a 5-7-day-old male was placed into the dish. The pairs were observed for one hour, between 12.00 and 15.00 hours after which the males were removed and fresh potato leaves were added. If a mating lasting from one to two minutes was observed during the one hour observation period, this event was scored as a copulation. These copulation experiments were repeated over the following three days, using the same females at the same time of the day, but with fresh 5-7 day old males. All females were kept in the plastic dishes with potato leaves during the four-day period.

Long-range calling behaviour

To determine whether females show a specific calling behaviour at long-range, *i.e.* when males are attracted into traps, we conducted the following experiment in a large wind tunnel, described by Visser and Griepink (1996). Three rows of two potato plants were placed ~ 45 cm apart at the downwind side in the windtunnel. Approximately 60 reproductively mature males were released every three days on these plants. Two small delta traps (height 6.5 cm, width 6 cm, length 8.5 cm) were placed side by side near the upwind screen, 50 cm from the closest row of potato plants. The traps were divided in two equal parts, and the upwind half contained gauze at both sides. In one trap we caged two females between the gauze, with a supply of water and pollen grains. The females were replaced by new ones every day. Two females were needed to catch males at all, as it turned out in preliminary trials that no male bugs responded to traps with one female. The control trap contained water and pollen grains supply only. The downwind sides of the traps, where males could fly in, were glued with Tangle Trap®. Two video cameras were placed next to the trap containing the females, each camera viewed one half. The camera directed to the females was a CCD-color video camera (Sony model SSC-C370P), and equipped with a zoom lens (18-108 mm). The camera directed to the downwind side of the trap was a CCD-black and white camera, Sony model SSC-M370CE, equipped with a small zoom lens (6-12 mm). Both cameras were connected to one monitor (Panasonic, model TC-1470Y), in split screen mode by means of a video-effector (JVC, type TK-C50E). The monitor was connected to a video recorder (Panasonic SVHS, model AG6730E), to be able to tape and replay all observations.

When one of the two females remained motionless, the camera was zoomed onto her body, so that she was shown in detail on the monitor. When she started walking, the camera image was reduced to keep track of her. During the recordings we continuously observed the behaviour of the unfilmed female. The time around which one or more males would fly into the trap was used as an indication for the moment around which one or both females would emit sex pheromone. The behaviour of the females before and during the flight activity of the males was analysed by rewinding the video-tape and examining the recorded images.

Close-range courtship behaviour

To study courtship behaviour in detail, we video-taped reproductively mature pairs (6-9 days old) with a CCD-color video camera (Sony, model SSC-C370P), connected via a monitor (Panasonic, model TC-1470Y) to a video-recorder (Panasonic SVHS, model AG 6730E). The pairs were placed under a small hemi-spherical glass cylinder (diameter at base 5.9 cm, highest point 3.1 cm). The camera position allowed us to detect the direction in which the bugs were walking. The cylinder was placed on filter paper. After mating the male and female were replaced by fresh ones. If mating did not occur within half an hour, the pair was replaced as well. After each pair the cylinder was cleaned with hot water and acetone, and the filter paper renewed. After recording 15 matings, male and female precopulation behaviour were studied

separately by using the video-tapes. Since *L. pabulinus* started to copulate on average 10 min after introduction in this setup, the precopulation period used was 10 min before each mating. All distinguishable elements of male and female precopulation behaviour within these 10 min were sequenced, using the 'Observer' software (Noldus Information Technology 1995, Wageningen, The Netherlands). As a control, the behaviour of 15 reproductively immature pairs (2-4 days old) was recorded for 15 min, and the behavioural events of the last 10 min were sequenced as above. Ethograms were constructed from the two groups observed, each containing 15 pairs.

During the recording of precopulation behaviour, we observed one distinct behavioural element in the males, *i.e.* vibration of the abdomen. The sound produced from this vibration was analysed by means of an electromagnetic transducer (Strübing and Rollenhagen, 1988; De Winter and Rollenhagen, 1990). This setup records the vibratory signals transmitted through the substrate. Two males and one female of 6-8 days old were placed on a small potato leaf in a plastic petri dish (diameter 5.3 cm). The bottom of the petri dish had been excised and replaced by gauze. The petri dish was placed in a clamp. A pin was inserted through the midrib of the potato leaf, so that the tip of the pin protruded through the petri dish at the underside. A Neo Delta magnet 35 (type NE 33) was glued to the tip of the pin. The magnet was placed in front of the transducer. The signal was monitored on an oscilloscope and recorded on tape (Sony DAT recorder PCM0-7010, tape: Sony Dat PDP-124). Placing 2 males instead of 1 into the petri dish with a female induced the males to vibrate more readily. The 2 males did not vibrate simultaneously so that recordings could be made of vibration signals of one male at a time. During the recordings, the behaviour of the three bugs was closely observed to make sure that sounds recorded originated from a vibrating male. Recordings were made of 6 different groups of three bugs. Of all recorded calls, 8 were chosen to be analysed. The mean (± s.d.) duration of these calls and the mean (± s.d.) number of clicks per second were calculated.

Results and Discussion

Egg production

At 20°C and on potato plants only, 10 of the 21 dissected females contained mature eggs, with on average 6.6 ± 4.4 (s.d.) eggs per female. When pollen grains were added, 15 of the 25 dissected females contained mature eggs and the mean number of mature eggs per female almost doubled to 12.9 ± 8.1. This suggests the need for extra nutrients for egg development. When additionally the temperature was increased to 25°C, the number of females with mature eggs increased to 27 of the 35 dissected females, although the number of eggs per female decreased to 9.6 ± 7.8. Egg production in *L. pabulinus* is known to be temperature-related (Mols, 1990). Since females without eggs copulate significantly less often than those with eggs (unpubl. observations), bugs used for further experiments were reared at 25°C with pollen grains.

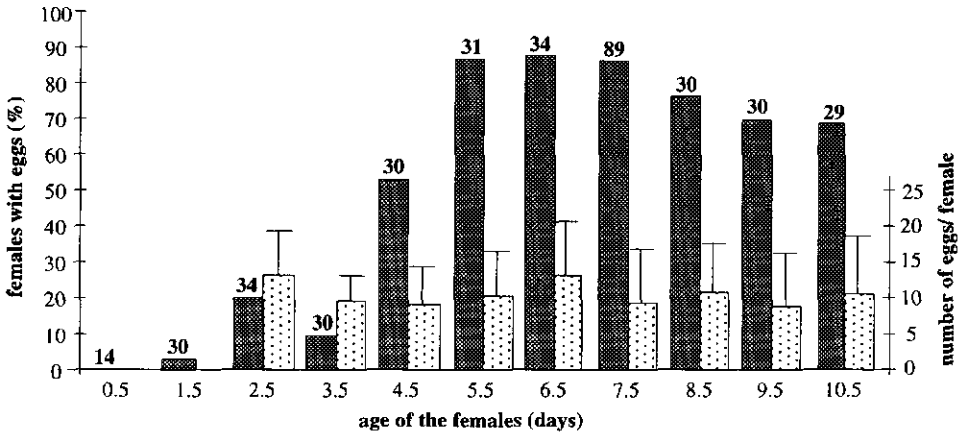

FIGURE 1. Egg production in virgin female *L. pabulinus*, at 25 °C with pollen. Dark bars: percentage of females with eggs, dotted bars: mean (± s.d.) number of eggs per female with eggs. On top of the bars the number of females dissected is indicated.

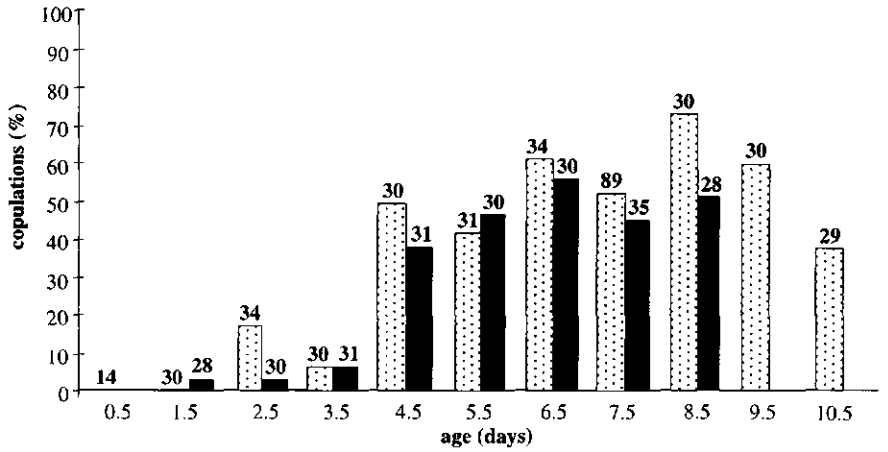

FIGURE 2. Sexual maturation and copulation frequency of *L. pabulinus*. Dotted bars: percentage of females copulating, black bars: percentage of males copulating. On top of the bars the number of bugs observed is indicated.

Under these conditions, fully developed eggs were present as early as 2.5 days after the final moult in some females, but after 4.5 days such eggs were observed in more than 50% of the females (Figure 1). When fully developed eggs were present, 9 to 14 eggs per female were observed, with a range of 1 to 20 (Figure 1).

Sexual maturation and copulation frequency

At 25°C, some females started to copulate as early as 2.5 days after the final moult, but more than 30 % of both males and females started copulating when 4.5 days old (Figure 2). The number of copulating males ranged from 38 to 57 % between the age of 5.5 and 8.5 days, while the proportion of females copulating ranged from 38 to 73 % between the age of 4.5 and 10.5 days. Copulations occurred throughout the photophase: from the experiment carried out between 7.00 and 8.00 hours 14 out of 29 dissected females contained a spermatophore, between 9.30 and 10.30 hours 13 out of 30 females had copulated, between 12.00 and 15.30 hours 41 of 61 dissected females contained a spermatophore, and between 21.45 and 22.45 hours 20 out of 31 females had copulated.

Sexual maturation and egg production are related to temperature. Under our rearing conditions at 25 °C copulation started about 4.5 days after the final moult, while at 20°C *L. pabulinus* started copulating after 7-8 days (Blommers, 1997). Sexual maturation in *L. pabulinus* is similar to other mirids; *Lygus elisus* and *L. desertinus* copulate when 2-4 days old at 26 ± 2°C (Graham *et al.*, 1987), *L. hesperus* starts copulating when 6-8 days old at 23-27°C (Strong *et al.*, 1970), *Distantiella theobroma* 4-5 days after the final moult (King, 1973) and *Nesidiocoris caesar* starts copulating after 2-5 days (Chatterjee, 1983) [no temperatures were given by King (1973) and Chatterjee (1983)]. The presence of 9-14 eggs in female *L. pabulinus* after egg production has started, is larger than in *L. hesperus*, where adult females only possess 2-4 eggs after 5-7 days (Strong *et al.*, 1970). *Distantiella theobroma* females also possess many mature eggs: on average 6 eggs after 4 days and 26 eggs after 7 days (King, 1973).

Figure 3 shows that 12 to 21 % of the females observed for one hour on four sequential days copulated more than once, which is three females in each group. Six of the second copulations occurred on consecutive days, and three occurred with one day in between. One of these females copulated three times, once on day 1, once on day 2 and once on day 4. During the four days of these experiments, one female died in each group. Second copulations have not been observed in mirids before, at least not on consecutive days (Blommers, 1997; Chatterjee, 1983; King, 1973; Kullenberg, 1946; Strong *et al.*, 1970). This suggests that in *L. pabulinus* a single copulation is not sufficient to ensure oviposition of a complete set of fertilized eggs.

Long-range calling behaviour of the female

During 16 days of testing in the wind tunnel, 30 times we observed males flying into the trap containing two females. Sometimes two to three males flew into the trap successively. Not all incoming males could be captured, as the Tangle Trap® was only applied at the bottom of the trap; we have seen several males flying into the trap without touching the bottom and then flying away. In the control trap no males were seen nor caught.

Of the 30 occasions that males flew into the trap, only 10 times we were able to zoom in, film and record the behaviour of the females. In 8 out of these 10 cases, at least one of the two females sat motionless for 5 to 30 minutes, the thorax

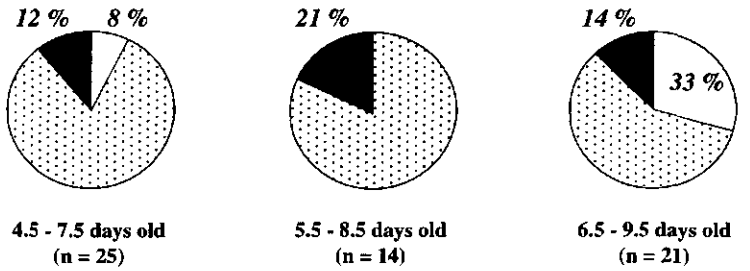

FIGURE 3. Frequency of copulations in groups of *L. pabulinus* females at different ages. White area: no copulations, dotted area: copulated once, dark area: copulated twice.

and abdomen slightly lower than the head, so that the body axis was in an angle of ± 20° with the substrate on which she sat. The legs were somewhat spread, and the proboscis was situated horizontally under the thorax. No movement of the abdomen could be distinguished. On one occasion that a male flew into the trap, both females were eating from the pollen grains, and on another occasion one female was grooming while the other was walking around.

In the mirid *D. theobroma* females emitting sex pheromone assume a characteristic "calling position" (King, 1973), in which the abdomen is raised from the substrate to the full extent of the hind legs, so that the body axis is horizontal or the thorax slightly higher than the head. A similar position has been recognized in *Helopeltis clavifer* (Smith, 1977). According to our observations in the present setup, females of *L. pabulinus* do not assume such a position when males are attracted to the traps from a distance of at least 50 cm. The position of the females during the flight activity of males is similar to their feeding position, the only difference is that the proboscis is situated horizontally under the thorax in the first case. If we could have distinguished a characteristic calling position that would indicate emission of sex pheromone, headspace collection of the sex pheromone from *L. pabulinus* females might have been possible without the interference of alarm or defensive volatiles.

Close-range courtship behaviour

During observations of 49 pairs of *L. pabulinus*, only 15 pairs copulated. Three random behavioural elements were arbitrarily distinguished: (1) resting, (2) grooming, which implied rubbing the antennae, thorax or abdomen with one (pair of) leg(s), and (3) walking. As possible precopulatory behavioural elements three additional elements were distinguished: (4) male antennating female, (5) female antennating male, and (6) male vibrating. Counting the number of pairs showing one of the behavioural elements at least once, the random elements occurred equally often in the mated pairs as in the control pairs, whereas the precopulatory elements occurred in more pairs of the former group than of the latter group (Table 1).

TABLE 1. Number of pairs (of a total of 15 pairs per group), showing the behavioural element at least once during the observation period. Mated pairs: reproductively mature pairs (6-9 days old); control pairs: reproductively immature pairs (2-4 days old)

Behavioural elements	Mated pairs	Control pairs
Random behaviour:		
1. resting	15	15
2. grooming	9	10
3. walking	15	15
Precopulation behaviour:		
4. male antennating female	13	9
5. female antennating male	11	8
6. male vibrating	14	2

Males and females antennated each other several times during close-range courtship, although this occurred in the control group as well. Antennation during courtship has also been described in the mirid *N. caesar* (Chatterjee, 1983) and in the pentatomid *Nezara viridula* (Borges *et al.*, 1987). In the latter species, males produce the sex pheromone instead of the females. This coincides with a reverse sequence of antennation: first the female antennates the male, then the male antennates the female, although in *L. pabulinus* these kinds of behaviour are not strictly alternating.

The vibration behaviour of the male differed between the two groups observed: 14 out of 15 males vibrated on average 8 (± 4.7 s.d.) times before mating, while in the control group only 2 out of 15 males vibrated (one male twice and one male 10 times). Of the 14 males that vibrated before mating, 5 vibrated before physical contact with a female and 9 after contact. Six of the 9 males that vibrated after contact, antennated the female before vibration, two of them were antennated by the female beforehand, and two of the 9 males antennated and were antennated by a female before they started vibrating. The vibration activity of the male before mating could be triggered by the physical contact with a female. Although 5 of the 14 males vibrated before antennation, touching could have occurred during introduction into the small glass cylinder, or before the 10 min precopulatory period that was analysed. To determine the need of contact to provoke vibration behaviour in the male, we observed males flying towards females in a glass cylinder in the wind tunnel (see Groot *et al.*, 1996). The females were located behind a gauze lid and could not be touched by the males. After landing on the lid the males showed the same vibration behaviour. As a result, we conclude that contacting a female is not essential to provoke male vibration behaviour. The vibration behaviour was never observed in the females.

Recordings of the signals of *L. pabulinus*, produced by male vibrations on the substrate, showed a pulsed pattern (Figure 4) with a tone-frequency of ± 200 Hz. The 8 analysed signals showed a mean duration of 2.4 (± 0.96 s.d.) seconds and a mean number of 12.4 (± 4.8 s.d.) clicks with 5.2 ± 0.7 clicks per second. We strongly suspect that vibration of the male capsid bugs is a sexual signal, as this occurs almost

FIGURE 4. Oscillogram of a vibration bout of one *L. pabulinus* male.

always and several times before copulation. Male vibration behaviour during courtship in mirids is also noted in *L. hesperus* (Strong *et al.*, 1970). In the Heteroptera acoustic signals during courtship have only been studied extensively in *N. viridula* (Todd, 1989; Ota and Cokl, 1991; Ryan and Walter, 1992). In this species both males and females emit specific vibrational signals to locate potential mates on plants (Ota and Cokl, 1991) and to initiate mating (Ryan and Walter, 1992). It is unlikely that *L. pabulinus* uses the vibrational signals for long-range mate location, because only males emit these signals.

From the 15 completed mating sequences an ethogram was constructed (Figure 5). Since females did not show characteristic precopulatory behaviour, only the ethogram of male precopulatory behaviour is given. In this diagram "female antennating male" is synonymous to "male being antennated by the female" from the original dataset, which was recorded during the analysis of male behaviour. In the ethogram the three precopulatory behavioural elements (*i.e.* male antennating female, female antennating male, male vibrating) were chosen as basis. From these elements the average frequencies were determined and expressed as percentages. Frequencies < 10 % are not included in the diagram. Since in < 10 % of the cases the grooming behaviour was preceded or followed by one of the three basic elements, this element is not included at all.

In general, courtship behaviour starts after walking or resting. These random behaviours are followed by the males vibrating or by the male or female antennating the other sex. After vibration, 66 % of the males resume walking, after which they may antennate the female once more. Female antennating male results mostly indirectly in the vibration behaviour of the male. After an average of 10 min, juxtaposition of the genitalia occurs, the male body axis being in an angle of 45° to 90° with the female body axis. The male curls his abdomen under that of the female, enabling the genitalia to make contact at the base of the ovipositor. Then the male inserts its ejaculatory organ in the genital opening of the female, attaches himself with the 2 parameres, after which they stay motionless in a position of about 45° for 1-2 minutes (see also Petherbridge and Thorpe, 1928; Kullenberg, 1946).

The mating event itself lasts for only 1-2 minutes in *L. pabulinus*, which is relatively short compared to other mirids. *Cyrtorhinus lividipennis* remains in copula for up to 15 min (Liquido and Nishida, 1985) and in *N. caesar* copulation can last as long as 3 h (Chatterjee, 1983).

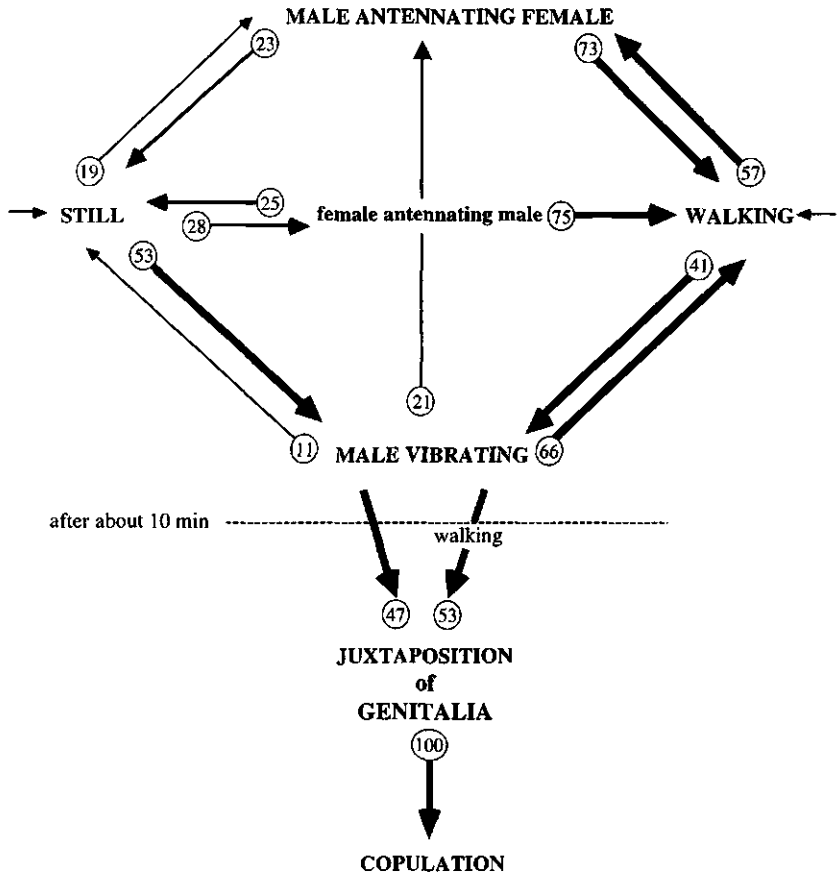

FIGURE 5. Ethogram of the precopulation behaviour of *L. pabulinus* males, based on the observations on 15 matings. 'Female antennating male' is synonymous to 'male being antennated by female' from the original dataset. The numbers next to the arrows are relative percentages, calculated from the originating behavioural element to determine how often this element is followed by one of the other behavioural elements. Percentages < 10 % are not mentioned in the diagram.

Conclusions

Provided that sexual receptiveness is associated with the production of sex pheromone, as Strong *et al.* (1970) suggest, sex pheromone production in *L. pabulinus* females starts at least 4 days after the final moult under our rearing conditions. This assumption also implies that sex pheromone production in *L.*

pabulinus occurs in mated females as well, as second matings have been observed. The long- and close-range behaviour of the females did not demonstrate a calling position or a specific moment of sex pheromone emission. However, the vibration behaviour of the male during courtship appears to be a characteristic courtship behaviour. This behaviour can be used as a bioassay to test potential sex pheromone components known to be present in other mirids (Groot *et al.*, 1998).

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PART I:

Sex-related behaviour and perception of males and females

Chapter 3

SEX-RELATED PERCEPTION OF INSECT AND PLANT VOLATILES IN *LYGOCORIS PABULINUS* (L.)*

ASTRID T. GROOT, RADBOUT TIMMER, GERRIT GORT, GERRIT P. LELYVELD, FALKO P. DRIJFHOUT, TERIS A. VAN BEEK AND J.H. VISSER

ABSTRACT - We recorded electroantennograms of male and female *Lygocoris pabulinus* antennae to 63 insect and plant volatiles. EAGs were between 100 and 500 μV. Overall, male EAGs were about twice the size of female EAGs. In both sexes, largest EAGs were recorded to *(E)*-2-hexenyl butanoate and *(E)*-2-hexen-1-ol. Response profiles were similar in both sexes. However, male antennae were more sensitive to a number of esters, especially the butanoates and pentanoates. Female antennae were more sensitive to 9 of the 19 plant volatiles, *i.e.*, to hexan-1-ol, heptan-1-ol, 1-octen-3-ol, 2-heptanone, (R)-carvone, linalool, geraniol, nerol, and methyl salicylate. Sexual differences in responses suggest that males are more sensitive to insect-produced pheromone-type compounds, whereas females are more sensitive to plant compounds for their orientation towards oviposition sites.

KEY WORDS: Heteroptera, Miridae, green capsid bug, sex pheromone, electroantennogram, odours, plant volatiles, esters, *(E)*-2-hexenyl butanoate, *(E)*-2-hexen-1-ol

Introduction

Female-produced sex pheromones are present in at least 10 mirid bug species (Strong *et al.*, 1970; King, 1973; Smith, 1977; Boivin and Steward, 1982; Graham, 1987; Graham *et al.*, 1987; Smith *et al.*, 1991; Chinta *et al.*, 1994; Smith *et al.*, 1994; Millar *et al.*, 1997; Millar and Rice, 1998). However, identification of the components and their active ratio has been elucidated in three species only (Smith *et al.*, 1991; Millar *et al.*, 1997; Millar and Rice, 1998). The pheromones of these species have been identified by analyzing chemical differences between male and female extracts. A sex pheromone in the green capsid bug *Lygocoris pabulinus* (L.) (Heteroptera: Miridae) has been established in the field, where males were attracted to traps baited with virgin live females caged on a potato shoot (Blommers *et al.*, 1988). The pheromone of this species is needed for further development of integrated pest management in fruit orchards in Northwestern Europe, where *L. pabulinus* is a serious pest (Blommers, 1994; Ravn and Rasmussen, 1996). Pheromone traps are

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widely and successfully used for monitoring lepidopterous pests (e.g., Minks and Van Deventer, 1992), and since 1990 also for monitoring the mirid pest *Campylomma verbasci* (McBrien *et al.*, 1994). In our quest to identify the sex pheromone of *L. pabulinus*, consistent chemical differences between the sexes have not yet been found (unpubl. data). In order to screen for potential attractants, perception of a number of volatiles was studied by means of electroantennogram (EAG) recordings.

The EAG technique is regularly used as a bioassay to test olfactory perception in insects (e.g., Dickens *et al.*, 1993a; 1993b; Visser and Piron, 1995; Visser *et al.*, 1996). In mirids, the technique has been applied to *Lygus lineolaris* (Chinta *et al.*, 1994; Dickens *et al.*, 1995), revealing olfactory receptors on the antennae that are responsive to insect and plant volatiles.

Two groups of volatiles were chosen for testing. The first consisted of esters which have been identified in other bug species (Knight *et al.*, 1984; Graham, 1988; Smith *et al.*, 1991; Chinta *et al.*, 1994; Aldrich, 1995; Dickens *et al.*, 1995; Millar *et al.*,1997; Millar and Rice, 1998). The second consisted of general plant volatiles, of which some may play a synergistic role in the sexual attraction of *L. pabulinus* (Groot *et al.*, 1996). EAGs of both males and females were recorded because volatiles eliciting larger EAGs in males could indicate a role in sexual attraction.

Material and methods

Insects

L. pabulinus was reared under summer conditions (22 ± 2 °C, 65 ± 5 % r.h., 18:6 hr L:D) on potted potato plants (Blommers *et al.*, 1997). Males and females were separated 0-2 days after the final moult to adult. *L. pabulinus* becomes sexually active about 4 days after the final moult (Groot *et al.*, 1998a), and so for EAG recordings, sexually mature, virgin adults of 4-10 days old were used. Prior to EAG recordings, bugs were collected from a rearing cage containing potato plants and sexed conspecifics.

Chemicals

Olfactory stimuli used are listed in Table 1. A series of esters was tested, comprising acetates, propionates, butanoates, pentanoates, and hexanoates. A series of plant-related volatiles were also tested, comprising one arbitrarily chosen nitrile (4-methoxy phenylacetonitrile), methyl salicylate, six monoterpenes, three ketones, five alcohols, and one aldehyde. *(E)*-2-Hexenyl acetate and *(Z)*-3-hexenyl acetate were tested in both series, since these compounds are insect-produced esters as well as general green leaf volatiles (see references in Table 1). Chemicals were obtained from commercial sources, or synthesized (Table 1). Newly synthesized esters (except *(E)*-2-butenyl and *(E)*-2-octenyl esters) were prepared by refluxing a 2-fold excess of the appropriate carboxylic acid with the corresponding alcohol for 5 hr, followed by

base extraction and partition into diethyl ether with removal of the solvent *in vacuo*. The *(E)*-2-butenyl and *(E)*-2-octenyl esters were prepared by refluxing 1 equivalent (eq) of the acid chloride with 1 eq of the corresponding alcohol for 1 hr, following Vogel (1989). *(E)*-2-Butenyl alcohol contained ~5% of the *Z*-isomer, *(E)*-2-octenyl alcohol was prepared by reducing *(E)*-2-octenal with $NaBH_4$. Purity was determined by GC-MS. The *(E)*-2-butenyl esters contained ~5% of the *Z*-isomer, which was in correspondence with the 5% *Z*-isomer in the starting material. For the EAG recordings, all chemicals were dissolved in paraffin oil at 1 % v/v, following Visser and Piron (1995).

Antennal preparation

An individual bug was anaesthesized with CO_2 for a few sec. The head was clipped off, and the distal tip of the terminal segment of one antenna cut off. The ground electrode was inserted into the open side of the head and the recording electrode was sleeved over the tip of the antenna. The electrodes consisted of two glass capillaries filled with 0.1 M KCl-solution. *L. pabulinus* antennae prepared this way showed a life span of 15 to 30 min.

EAG protocol

Ag-AgCl wires in the glass electrodes connected the antennal preparation to the amplification and recording devices consisting of an input probe and DC amplifier (Grass HIP 16A and P16D, rise time set at 30 msec), an oscilloscope (Philips PM3302), and a transient-recorder (Krenz TRC 4010, 12 bits ADC) connected to a personal computer (Estate 80386 and 80387). Stimulation cartridges were prepared by applying 25 µl of each paraffin oil solution onto a piece of filter paper which was subsequently placed in a Pasteur pipette. The antenna, placed perpendicularly 1-2 cm in front of a glass tube, was stimulated for 2 sec by pushing air (1 ml/sec) through the pipette into the tube with a continuous air flow of 40 cm/sec (30 ml/sec). The interstimulus time interval was 30 sec. In order to compare responses within an individual and among individuals, all responses were normalized by using a standard of 1 % *(E)*-2-hexenal in paraffin oil. The stimulation of each chemical was, thus, preceded and followed by the standard.

All chemicals were tested with 11-14 different individuals of each sex. Chemicals were tested in series of 10-18 compounds per antenna, each time offered in a different order. As a control, an EAG to 25 µl of paraffin oil was recorded during each series. EAGs of all chemicals were expressed as percentage responses relative to the responses of the adjacent standards. If the magnitude of the EAG of the adjacent standard was below 100 µV, the EAG was omitted. Means and 95% confidence intervals were calculated for each compound. Differences in volatility among compounds were not corrected for.

TABLE 1. List of volatiles used for EAG recordings of *L. pabulinus* males and females

VOLATILE	Source	Purity	referred to as insect-produced odour[ix]	referred to as plant-produced odour[ix]
Esters				
butyl acetate	Acros[ii]	> 99	5	
(E)-2-butenyl acetate	*a*	93 %		
pentyl acetate	Roth[iii]	> 99 %		
hexyl acetate[i]	Fluka[iv]	99 %	1, 2a, 3a, 4, 5, 6	22
(E)-2-hexenyl acetate	ICN/K&K[v]	99 %	2, 3, 4, 6, 7	23, 24, 25
(Z)-3-hexenyl acetate	Roth	99 %	2, 7	19, 20, 21, 22, 24, 25, 26
heptyl acetate	Roth	> 99 %		
octyl acetate	Roth	> 99 %	2, 3, 5	
(E)-2-octenyl acetate	*a*	62 %[vii]	2, 3a, 4, 5, 7, 8	
butyl propionate	Acros	> 99 %		
(E)-2-butenyl propionate	*a*	93 %		
pentyl propionate	*a*	97 %		
hexyl propionate	Roth	> 99 %		19
(E)-2-hexenyl propionate	ICN/K&K	98 %		25
(Z)-3-hexenyl propionate	ICN/K&K	> 99 %		25
heptyl propionate	*a*	96 %		
octyl propionate	*a*	96 %		
(E)-2-octenyl propionate	*a*	79 %[viii]		
butyl butanoate	Roth	> 99 %	1a, 4, 5, 6	
(E)-2-butenyl butanoate	*a*	93 %	1a	
pentyl butanoate	Roth	> 99 %		
hexyl butanoate[i]	Roth	> 99 %	1, 2, 3, 4, 5, 6, 9	23
(E)-2-hexenyl butanoate[i]	*a*	85 %[viii]	6, 10	
(Z)-3-hexenyl butanoate	*a*	92 %		19, 21, 20
heptyl butanoate	*a*	97 %		
octyl butanoate	Roth	> 99 %		
(E)-2-octenyl butanoate	*a*	92 %	2a, 3	
butyl pentanoate	*a*	95 %		
(E)-2-butenyl pentanoate	*a*	95 %		
pentyl pentanoate	*a*	96 %		
hexyl pentanoate	*a*	95 %		
(E)-2-hexenyl pentanoate	*a*	86 %[viii]		
(Z)-3-hexenyl pentanoate	*a*	97 %		
heptyl pentanoate	*a*	93 %		
octyl pentanoate	*a*	93 %		
(E)-2-octenyl pentanoate	*a*	88 %[viii]		
butyl hexanoate	Roth	> 99 %	4	23
(E)-2-butenyl hexanoate	*a*	95 %		
pentyl hexanoate	*a*	97 %		

hexyl hexanoate	*a*	97 %	5	
(E)-2-hexenyl hexanoate	*a*	92 %	5, 11	
(Z)-3-hexenyl hexanoate	*a*	84 %[viii]		
heptyl hexanoate	*a*	97 %		
octyl hexanoate	*a*	97 %		
(E)-2-octenyl hexanoate	*a*	92 %		
Aldehydes				
hexanal	Fluka	98 %	4, 7	19, 25, 26
(E)-2-hexenal (standard)	Roth	96 %	4, 6, 7, 11, 12, 13, 14, 15, 16	6, 19, 22, 24, 25, 26, 27
Alcohols				
hexan-1-ol[i]	Fluka	99 %	1, 3, 4, 7	10, 19, 21, 22, 25, 26, 27
(E)-2-hexen-1-ol	Roth	97 %	7	19, 22, 24, 25, 26, 27
(Z)-3-hexen-1-ol	Roth	97 %		6, 19, 20, 22, 25, 26, 27
heptan-1-ol	Fluka	99 %		6, 22, 25
1-octen-3-ol	Fluka	98 %		(1), 21, 25
Ketones				
2-heptanone	Aldrich[vi]	98 %	8, 12	25, 26
3-heptanone	Aldrich	98 %		25
3-octanone	Aldrich	99 %		25
Monoterpenes				
(1S)-ß-pinene	Fluka	99 %	7, 17	19, 25, 26
myrcene	Roth	91 %	17	20, 22, 25
(R)-carvone	Aldrich	98 %		25, 26
linalool	Fluka	97 %	7, 16, 18	6, 21, 22, 20, 25, 26
geraniol	Fluka	99 %		6, 22, 25, 26
nerol	Aldrich	97 %		6, 25, 26
Aromatics				
methyl salicylate	Fluka	99 %		20, 21, 24
4-methoxylphenylacetonitrile	Fluka	97 %		28

a. synthesized *de* novo; [i] compound found in both sexes of *L. pabulinus* (F.P. Drijfhout, unpubl. res.); [ii]Geel, Belgium; [iii]Karlsruhe, Germany; [iv]Buchs, Switzerland; [v]Costa Mesa, CA, USA; [vi]Zwijndrecht, The Netherlands; [vii] ~ 32 % of the rest product is 1-octenyl acetate, the remaining 5 % consists of the alcohol and the acid from which *(E)*-2-octenyl acetate was synthesized; [viii] the remaining % consist of the starting compounds, *i.e.*, the alcohol and the acid, from which the listed compounds were synthesized. [ix] numbers in the list refer to the following references: 1. Smith *et al.*, 1991 (**1a.** sex pheromone compound *Campylomma verbasci*; (1). from mold growing on mullein); **2.** Millar *et al.*, 1997 (**2a.** sex pheromone compound *Phytocoris relativus*); **3.** Millar and Rice, 1998 (**3a.** sex pheromone compound *Phytocoris californicus*); **4.** Aldrich and Yonke, 1975; **5.** Knight *et al.*, 1984; **6.** Chinta *et al.*, 1994; **7.** Aldrich, 1995; **8.** Blum, 1985; **9.** Graham, 1988; **10.** Dickens *et al.*, 1995; **11.** Leal and Kadosawa, 1992; **12.** Blum, 1996; **13.** Ishiwatari, 1974; **14.** Ishiwatari, 1976; **15.** Borges and Aldrich, 1992; **16.** Aldrich, 1988; **17.** Staddon, 1990; **18.** Aldrich *et al.*, 1993; **19.** Bernays and Chapman, 1994; **20.** Bolter *et al.*, 1997; **21.** Dicke *et al.*, 1990; **22.** Dickens *et al.*, 1993b; **23.** Fein *et al.*, 1982; **24.** Takabayashi *et al.*, 1994; **25.** Visser and Piron, 1995; **26.** Visser *et al.*, 1996; **27.** Visser *et al.*, 1979; **28.** Cole, 1976

Statistical analysis

The series of 45 esters was analysed separately from the series of plant compounds. *(E)*-2-Hexenyl acetate and *(Z)*-3-hexenyl acetate were tested and analysed in both series. Both series were analysed by using a mixed linear model, fitted with the procedure MIXED of the computer programme SAS version 6.12 (1997). Data were square-root transformed after addition of a constant in order to normalize and stabilize the variance. After fitting the mixed linear model with fixed main effects and interaction for gender and chemicals and a random effect for the antenna, the following comparisons were made:

A1. response to the control paraffin oil versus response to each of the tested esters in female antennae,

A2. response to the control paraffin oil versus response to each of the tested esters in male antennae,

A3. female versus male antennal response to each of the tested esters,

B1. response to the control paraffin oil versus response to each of the tested plant compounds in female antennae,

B2. response to the control paraffin oil versus response to each of the tested plant compounds in male antennae,

B3. female versus male antennal response to each of the tested plant compounds.

The significance level of each series was corrected for multiple comparisons through the Bonferroni method, and, therefore, set at: for A1 and A2 $P = 0.05 / 45 = 0.0011$; for A3 $P = 0.05 / 46 = 0.0011$; for B1 and B2 $P = 0.05 / 20 = 0.0025$, and for B3 $P = 0.05 / 22 = 0.0023$.

Results

The absolute peak EAG response of *L. pabulinus* to the standard *(E)*-2-hexenal was larger in males than in females; the male response was -308 ± 142 µV (mean ± sd; N=9), the female response was -149 ± 51 µV (mean ± sd; N=9).

Esters

A1. Responses in female antennae. Female antennae were most responsive to *(E)*-2-hexenyl butanoate (166 ± 13 % (mean ± c.i.) relative to the standard). Slightly lower responses were elicited by hexyl propionate, *(E)*-2-hexenyl propionate, hexyl butanoate, *(Z)*-3-hexenyl butanoate, *(E)*-2-octenyl butanoate, hexyl pentanoate, *(E)*-2-hexenyl pentanoate, and *(E)*-2-octenyl pentanoate. Lowest responses were recorded for the octyl esters and hexanoates, as in the male antennae. Octyl pentanoate and octyl hexanoate did not elicit a significant EAG response in female antennae.

A2. Responses in male antennae. The EAG profile of the males showed a distinct sensitivity for the array of esters. Largest EAGs were elicited by *(E)*-2-hexenyl propionate (195 ±15 % mean ± c.i.), and *(E)*-2-hexenyl butanoate (204 ± 15

TABLE 2. Anova-table of the series of esters, of which the response profiles are shown in Figure 1.
Tests of fixed effects

Source	NDF	DDF	Type III F	Pr > F	Covariance parameter	Estimate
gender	1	122	27.28	0.0001	Antenna (gender)	0.5603
chemical	45	1002	146.98	0.0001	Residual	0.9225
gender*chemical	45	1002	8.47	0.0001		

% mean ± c.i.). *(E)*-2-Butenyl butanoate, hexyl butanoate, *(Z)*-3-hexenyl butanoate, heptyl butanoate, *(E)*-2-octenyl butanoate, hexyl pentanoate, and *(E)*-2-hexenyl pentanoate elicited EAGs around 150 % relative to the standard in male *L. pabulinus* antennae. The smallest EAGs were recorded in response to octyl esters and hexanoates. Octyl hexanoate did not elicit a significant EAG in male antennae.

A3. Female versus male antennal responses to tested esters. Regarding the 5 groups of esters tested, none of the acetates showed significant differences in EAGs between female and male antennae. Of the propionates, *(E)*-2-hexenyl propionate and heptyl propionate elicited larger responses in males. Most butanoates also elicited larger relative responses in males, except hexyl butanoate, *(E)*-2-hexenyl butanoate, and *(Z)*-3-hexenyl butanoate, for which there was no difference between sexes. Male antennae responded more to most of the pentanoates as well, although both sexes responded similarly to *(Z)*-3-hexenyl pentanoate, octyl pentanoate, and *(E)*-2-octenyl pentanoate. The hexanoates did not elicit larger EAGs in male antennae. *(Z)*-3-Hexenyl hexanoate even elicited a larger relative EAG response in female antennae.

Plant volatiles

B1. Responses in female antennae. Largest EAGs in female antennae were elicited by *(E)*-2-hexen-1-ol (150 ± 18 % mean ± c.i.), and 1-octen-3-ol (126 ± 16 % mean ± c.i.). Hexan-1-ol, *(Z)*-3-hexen-1-ol, heptan-1-ol, *(E)*-2-hexenyl acetate, *(Z)*-3-hexenyl acetate, linalool, and methyl salicylate elicited about the same response as the standard *(E)*-2-hexenal. 3-Heptanone, (1S)-β-pinene, and myrcene did not elicit a significant EAG response in females.

B2. Responses in male antennae. Largest EAGs were elicited by *(E)*-2-hexen-1-ol (190 ± 21 % mean ± c.i.). The other alcohols, as well as hexanal, the acetates, methyl salicylate, and linalool elicited circa 50-100 % response relative to the standard *(E)*-2-hexenal. Nerol, (R)-carvone, and 4-methoxyphenyl acetonitrile elicited smaller EAGs, and the responses of male antennae to (1S)-ß-pinene and myrcene were not different from the control.

TABLE 3. Anova-table of the series of plant compounds, of which the response profiles are shown in Figure 2.
Tests of fixed effects

Source	NDF	DDF	Type III F	Pr > F	Covariance parameter	Estimate
gender	1	26	19.96	0.0001	Antenna (gender)	0.3761
chemical	20	438	81.76	0.0001	Residual	1.4271
gender*chemical	20	438	6.67	0.0001		

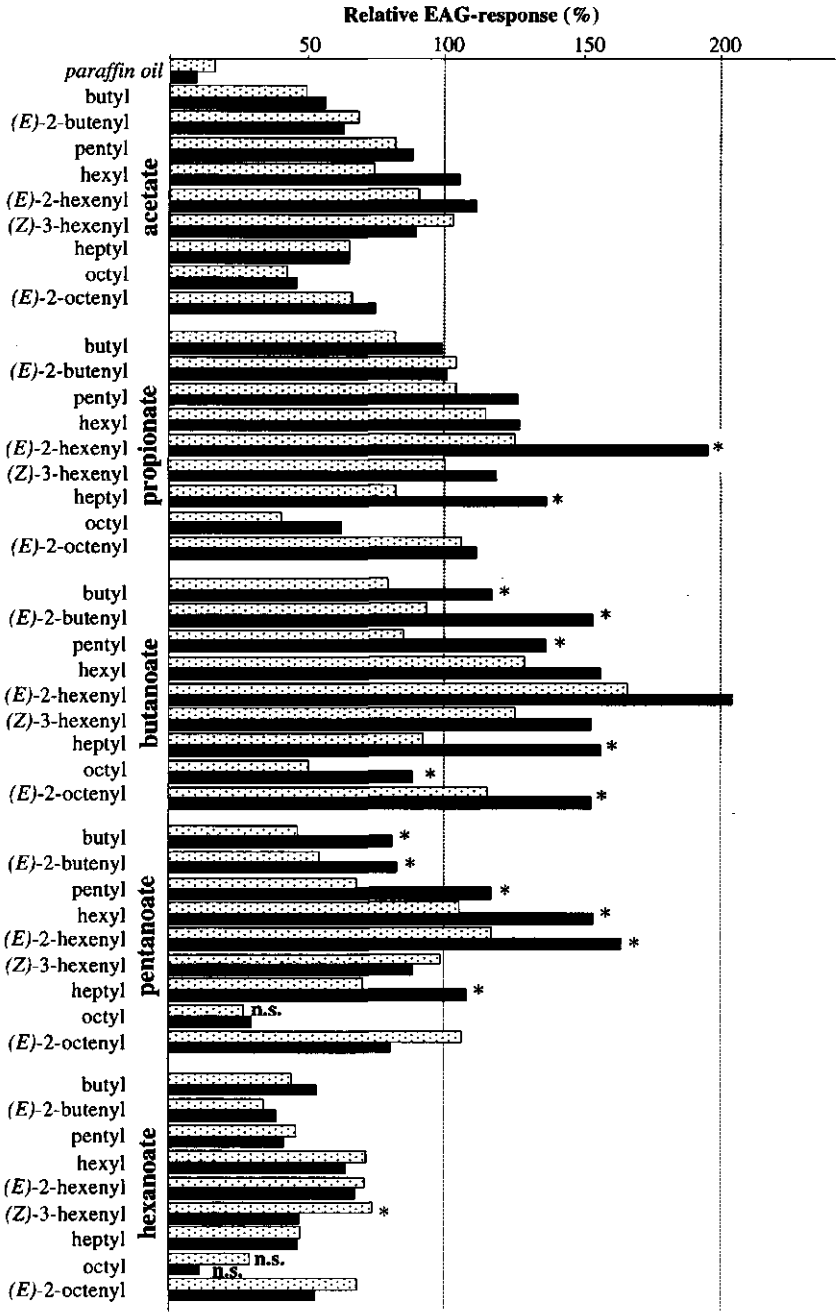

FIGURE 1. EAG responses of male and female L. pabulinus to the series of esters. The response bars are based on the backtransformed least-square means. Light bars: EAG response of female antennae; black bars: EAG response of male antennae. n.s.: EAG response not significantly different from the response to the control paraffin oil; * EAG response significantly different between the sexes (after correcting the significance level for multiple comparison through the Bonferroni method). See text for further explanation.

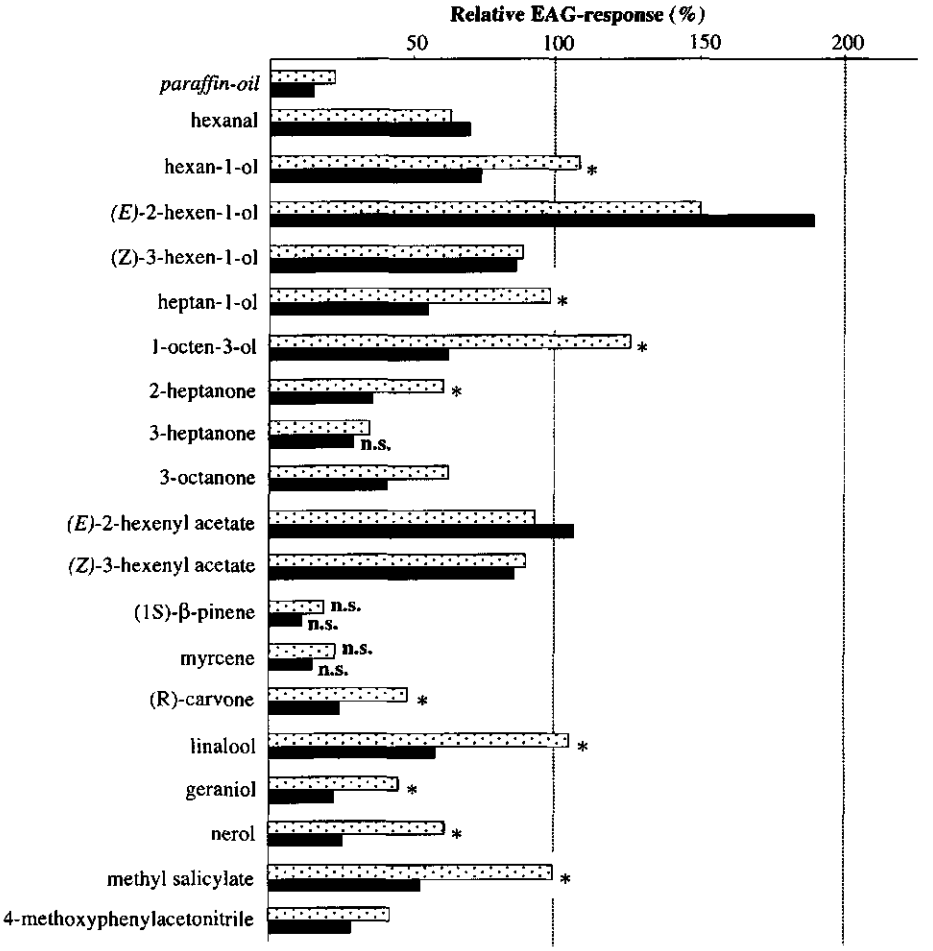

FIGURE 2. EAG responses of male and female L. pabulinus to the series of plant volatiles. The response bars are based on the backtransformed least-square means. Light bars: EAG-reponse of female antennae; black bars: EAG response of male antennae. n.s.: EAG response not significantly different from the response to the control paraffin oil; * EAG response significantly different between the sexes (after correcting the significance level for multiple comparisons through the Bonferroni method). See text for further explanation.

B3. Female versus male antennal response to tested plant volatiles. Female antennae showed larger relative EAGs to hexan-1-ol, heptan-1-ol, 1-octen-3-ol, 2-heptanone, (R)-carvone, linalool, geraniol, nerol, and methyl salicylate than male antennae.

Discussion

Responses to esters

Some of the esters tested were < 95 % pure, hence the recorded EAGs could in part be responses to the corresponding alcohols or, in case of *(E)*-2-octenyl acetate, to 1-octenyl acetate, since this comprised ~ 30 % of the solution. Despite these inpurities, the 5 groups of esters showed similar relative response patterns in both *L. pabulinus* male and female antennae. Larger EAGs to the different ester groups could be due to differences in volatilities, *i.e.*, acetates are more volatile (>) than propionates, propionates > butanoates > pentanoates > hexanoates. Since such correlations were not found for any of the ester analogs, the measured EAGs cannot be explained solely by differences in volatilities.

Esters consist of an alcohol and an acid part. With regard to the alcohol part of the esters, both sexes were highly sensitive when *(E)*-2-hexenol was present in the esters, *i.e.*, the *(E)*-2-hexenyl esters, and to a lesser extent to the hexyl esters, *(Z)*-3-hexenyl esters, and *(E)*-2-octenyl esters. The octyl esters elicited lowest EAGs. With regard to the acid part of the esters, both sexes responded least when hexanoic acids were part of the esters, *i.e.*, the hexanoates, followed by acetates, with largest EAGs to butanoates. Both sexes were most sensitive to *(E)*-2-hexenyl butanoate, which is one of the main compounds found in *L. lineolaris* (Gueldner and Parrot, 1978; Dickens *et al.*, 1995), and found in both sexes of *L. pabulinus* (F.P. Drijfhout, unpubl. res.). These results suggest that butanoates may play a role in the biology of *L. pabulinus*. Whether this role is intra- or interspecific, or attractive or repellent, cannot be concluded from the present data. EAGs only indicate that the insect perceives the volatiles and do not reveal their function in mediating behaviour.

Of the esters that elicited larger relative EAGs in male than in female antennae, three are known to play a role in the sexual communication of mirids. Butyl butanoate and *(E)*-2-butenyl butanoate are sex pheromone components in *Campylomma verbasci* (Smith *et al.*, 1991), and *(E)*-2-octenyl butanoate is a sex pheromone component of *Phytocoris relativus* (Millar *et al.*, 1997). In *L. pabulinus*, a possible sexual role of esters was assessed by offering 100 ng of compounds to males in petri dishes, after which the number of vibrating males was quantified (Groot *et al.*, 1998b). Butyl butanoate elicited male vibration response in 35 %, *(E)*-2-butenyl butanoate did not elicit vibration behaviour in any, *(E)*-2-hexenyl butanoate elicited a response in 22 %, and *(E)*-2-octenyl butanoate elicited a vibration behaviour in 87 % (Groot *et al.*, 1998b). Hexyl butanoate was also tested in this assay, since this is the major compound in the metathoracic scent gland of *L. pabulinus*, constituting up to 95 % of the total oil (F.P. Drijfhout, unpubl. res.). When 100 ng of hexyl butanoate was offered, 40 % of the males started to vibrate (Groot *et al.*, 1998b). Since this vibration behaviour is a specific sexual response of males (Groot *et al.*, 1998a), the compounds that elicited it may play a role in sexual communication in *L. pabulinus*.

Responses to plant volatiles

In comparing the relative EAGs, males were less sensitive than females to most of the tested plant volatiles, with the exception of *(E)*-2-hexen-1-ol, to which both sexes showed strong responses. The high response to *(E)-2*-hexen-1-ol may be due to similarity in structure between this fragment and *(E)*-2-hexenyl butyrate, which elicits high EAGs in both sexes as well. On the other hand, *(E)*-2-hexen-1-ol may play a role in general host plant orientation of *L. pabulinus*, as this is a common green leaf volatile (Visser *et al.*, 1979). The nine plant volatiles that showed larger EAGs in female than in male antennae are common plant volatiles as well (Visser, 1986). 1-Octen-3-ol, linalool, and methyl salicylate are also found in herbivore infested leaves of several apple cultivars (Takabayashi *et al.*, 1994). Female *L. pabulinus* may use these compounds for host orientation in autumn when they fly back from herbaceous summer hosts to apple orchards to lay winter eggs.

Some common plant volatiles are produced by Heteropterans and may play a role in sexual communication (Aldrich, 1988; 1995). In Miridae, these compounds have not been found, but since *L. pabulinus* males are mostly attracted to females on potato leaves (Groot *et al.*, 1996), some plant compounds may be involved indirectly in the attraction between the sexes.

In conclusion, male antennae are relatively more sensitive to a number of insect-produced esters, while female antennae are more sensitive to a number of plant volatiles. The same trend has been seen previously in *L. lineolaris* males and females (Chinta *et al.*, 1994). This sexual difference in response may be due to the fact that in mirids males are attracted to females, while females may use plant compounds for their orientation towards oviposition sites. Since hexyl butanoate, *(E)*-2-hexenyl butanoate, and *(E)*-2-octenyl butanoate elicited a vibration behaviour in some males, these or closely related compounds may be involved in sexual communication, at least at short range. Which of the volatiles that elicited large EAGs play a role in the sexual attraction at long range, remains to be studied.

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PART II:

The male perspective

Chapter 4

INFLUENCE OF HOST PLANTS ON SEXUAL COMMUNICATION IN *LYGOCORIS PABULINUS**

ASTRID T. GROOT AND J. HANS VISSER

ABSTRACT - Host plant volatiles may be involved in the sexual communication of insects in several ways. In the pheromone-producing sex, these volatiles may affect pheromone production or release, in the receptive sex plant volatiles may have a synergistic effect on the attraction to sex pheromone. We conducted three types of experiments to determine if and how plant volatiles are involved in the sexual communication of *Lygocoris pabulinus* (L.) (Heteroptera: Miridae), the females of which attract males. In a one-choice cylinder bioassay, females on potato leaves were as attractive as females on goosefoot leaves, but significantly more attractive than females without plant material. The latter result suggests an interaction between females and potato leaves. However, in two-choice flying and walking bioassays, using delta traps in a wind tunnel and a vertical Y-track olfactometer, males were attracted to females irrespective of the presence of potato leaves. This difference in result is probably due to the fact that in the latter assays females were confined with pollen as an alternative food source, while females in the one-choice assay had access to water only, so that they may have suffered from malnutrition. Males in the one-choice assay were also attracted to potato leaves from which females had been removed, indicating that attractive components from females are deposited and adsorbed to the substrate. Plants are probably only very indirectly involved in sexual communication, their surface merely functioning as a substrate from which pheromone is released. Males may subsequently be attracted to such plants or substrates. Clean plant material was not attractive to *L. pabulinus* males, hence plant volatiles alone do not seem to be used by these males as possible mate location cues.

KEY WORDS: Heteroptera, Miridae, sex pheromone production, pheromone release, male receptivity, host plant volatiles, adsorption.

**Submitted to Chemoecology*

Introduction

Host plant volatiles may be involved in the production, the release, or the perception of sex pheromones by insects (reviewed by McNeil and Delisle, 1989a; Landolt and Philips, 1997). Direct use of plant compounds as precursors for the biosynthesis of sex pheromone compounds has been found mainly in male insects (Wood, 1982; Eisner and Meinwald, 1987; Vanderwel and Oehlschlager, 1987; Baker, 1989; Landolt *et al.*, 1992a). When females produce sex pheromone, plant volatiles have been found to initiate pheromone production in a more indirect way: females start producing pheromone when in the odor of a host plant (McNeil, 1991; Raina *et al.*, 1989, 1992, 1997). Not only pheromone production, but also pheromone release may be affected by plant odors (Hendrikse and Vos-Bünnemeyer, 1987; McNeil and Delisle, 1989a; Landolt *et al.*, 1994). The ability of females to stop pheromone production or release until a suitable host is located, coordinates their reproductive behavior with the availability of food for the offspring (Raina *et al.*, 1992). At the receiver side, the attracted sex may be more attracted to a combination of pheromone and plant volatiles than to pheromone alone (Landolt *et al.*, 1992b; Dickens *et al.*, 1990, 1993; Light *et al.*, 1993; Hardie *et al.*, 1994).

Distinction between the role of host plants in sex pheromone production and pheromone release can be made when the sex pheromone has been identified, and when a pheromone gland has been recognized. The content of this gland can then be analysed under specified conditions. Pheromone release can be observed in insects that exhibit a specific calling behavior (*e.g.* King, 1973; Cardé and Taschenberg, 1984). A synergistic effect of pheromone with plant compounds in the receiving insect can be determined by differences in trap catches between pheromone traps with or without the addition of plant compounds (Dickens *et al.*, 1990, 1993; Light *et al.*, 1993; Hardie *et al.*, 1994; Lilley and Hardie 1996).

The insect of our present interest is the green capsid bug, *Lygocoris pabulinus* (L.) (Heteroptera, Miridae), a pest in Northern European fruit orchards. In this species, as in mirids in general, males are attracted by females, which has been confirmed in the field and in the laboratory (Blommers *et al.*, 1988; Groot *et al.*, 1996). Chemical identification of the sex pheromone has not been successful so far. Plant volatiles may be involved in the sexual attraction, as virgin females that attracted males were confined with plant material as a feeding source. In this study, we determined if and how host plants may be involved in the sexual communication of *L. pabulinus*. When plant compounds are directly involved in sex pheromone production, a difference in attraction is expected when virgin females are confined with different host plants or with no host plant at all. A possible distinction of indirect involvement of plant volatiles between pheromone production and pheromone release will be hampered in this species, since a sex pheromone gland has not been recognized in Miridae so far, and a specific calling behavior in *L. pabulinus* females has not been observed (Groot *et al.*, 1998). Whether host plants are involved at either the producer or the receiver side, *i.e. L. pabulinus* females or males respectively, is determined by placing females either upwind or downwind from the

host plant. Attraction may be different when flying or walking towards an odor source (Hardie *et al.*, 1992), therefore both flight and walking bioassays were conducted.

Material and Methods

Insects

Lygocoris pabulinus was reared on potted potato plants, cultivar Bintje, in wooden cages in a greenhouse at 22 ± 2°C, 65 ± 5 % R.H., L18:D6, following the procedure of Blommers *et al.* (1997). Every 2-3 days newly emerged adults were collected from the rearing cages, after which the sexes were placed in separate rearing cages. In this way, virgin males and females (see Groot *et al.*, 1998) of known age were continuously available for the experiments.

One-choice bioassay

To determine whether males are attracted to females with or without plant material, one-choice bioassays were conducted using glass cylinders (length 50 cm, diam. 11 cm, see Groot et al., 1996). The cylinders were placed horizontally on the floor in a wind tunnel of 3.0 (l) x 1.3 (w) x 0.8 (h) m with adjustable wind speed, light, temperature and R.H. (see Griepink (1997) for further details on the wind tunnel). At the downwind side of each cylinder we placed a plastic dish with gauze bottom and screen cone, containing 8-15 males of 5-8 days old. Against the upwind side a plastic dish with gauze lid and bottom was placed, in which an odor source was offered. In this way, each cylinder was a closed system, so that males from the downwind dish could only walk or fly towards the upwind dish that was offered in the specific cylinder. Two-three h before a test, the downwind dishes were filled with males, the upwind dishes with odor sources, and all dishes were placed in the wind tunnel. Eight different odor sources were tested: (1) empty dish, (2) five females on a fresh potato leaf, (3) five females with a small glass tube containing water, closed with oasis, (4) a potato leaf that had been in a dish with five females for 2-3 h prior to the experiment (females removed during the experiment), (5) a clean potato leaf, (6) five females on a fresh goosefoot leaf (*Chenopodium quinoa* L.), (7) a goosefoot leaf that had been in a dish with five females for 2-3 h prior to the experiment (females removed during the experiment), and (8) a clean goosefoot leaf. The wind speed through the cylinders was set at 0.20 m/s. After one hour the number of males sitting or walking on the upwind lid were counted as positive responders. All odor sources were tested 5-20 times. Differences in responses between the sources were compared with a Generalized Linear Model (GLM) for binomial data, using the the logit-link function in computer programme Genstat 5 (release 4.1, PC/Windows NT, 1997). In the model, the variance was assumed to be proportional to binomial variance. Overall effect of treatments was determined by performing an F-test for the ratio of the mean deviance for treatment and the mean deviance of the rest. If the overall test was

significant ($P < 0.05$), pairwise comparisons between treatment means on the logit scale were conducted, using the t-test.

Two-choice flight bioassay

To determine if females attract males when on a plant or in the odor of a plant, PVC-cages with two compartments were constructed. The compartments, each 7.5 cm long and 7 cm diam., were divided by gauze and closed at both sides with gauze lids (Figure 1). The cages were hung in delta traps (30 (l) x 20 (w) x 20 (h) cm) with replacable bottoms treated with Tangle Trap®, and placed in the wind tunnel, hanging from metal clamps 10-15 cm above the wind tunnel floor. Five different combinations were tested: (A) three females with wet oasis and pollen, and a small potato sprout in the downwind compartment, leaving the upwind compartment empty, (B) a small potato sprout in the upwind compartment, and three females with wet oasis and pollen in the downwind compartment, (C) three females with wet oasis and pollen in the upwind compartment, and a small potato sprout in the downwind compartment, (D) three females with wet oasis and pollen in the downwind compartment, leaving the upwind compartment empty, and (E) three *males* with wet oasis and pollen, and a small potato sprout in the downwind compartment, leaving the upwind compartment empty. The pollen was a mixture of different plant species, bought at a commercial bee-station (Bijenhuis, Wageningen, the Netherlands). To offer males a choice, a control trap was placed about 90 cm next to each test cage (so that both cages were about 20 cm from the sides of the wind tunnel), consisting of a similar cage with the two compartments containing the same as the test cage, only without bugs. Both traps were placed in the upwind part of the wind tunnel, about 1 m downwind from which 8-10 potato plants were placed. After arranging the cages and the plants in the wind tunnel, 35-40 virgin males of 5-8 days old were introduced at the downwind side of the wind tunnel. After 3-4 days the males caught in the Tangle Trap® in both traps were counted, and all males were recaptured. Percentage of the males trapped was calculated from the number of males recaptured.

Two-choice walking bioassay

To determine the influence of host plants on sexual attraction when males would walk towards an odor source, vertical Y-track olfactometer assays were conducted as described by Visser and Piron (1998). To suppress flight intention of males, the Y-track was placed in a black box under a halogen lamp (4-12 V DC, 10 VA), which was placed in a black socket sealed with a red filter, so that the light intensity at the base of the Y-track was 6.3-6.5 lux only. Two 250 ml glass bottles (h. 14 cm, diam. 6.5 cm) were placed outside the black box, one on each side, under a desk-lamp with a 25 W light bulb. One bottle contained the odor source to be tested, the other was the control bottle. Three odor sources were tested: (I) five *L. pabulinus* females with two small potato sprouts in wet oasis and pollen, (II) five females with two strips of green paper, wet oasis and pollen, (III) five *males* with two small potato sprouts in wet oasis and pollen. In all tests the control bottles contained wet oasis and pollen. The bottles were filled and placed in the setup 1 h before each test. All experiments

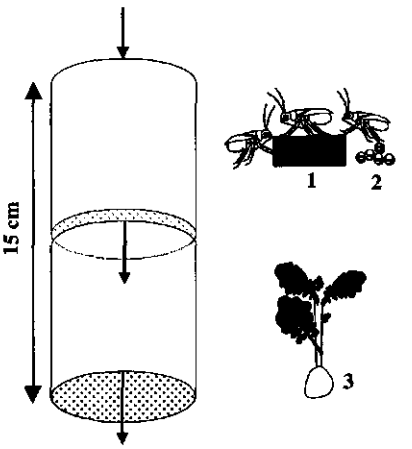

FIGURE 1. Cage with two compartments, each 7.5 cm long, diam. 7 cm. Bugs were always confined with wet oasis (1) and pollen (2). A small potato sprout (3; roots wrapped in wet cotton and aluminium foil to avoid dessication) was: (A) added to three females, (B) placed upwind from females, (C) placed downwind from females, (D) not added, (E) added to three *males*. See text for further explanation.

were conducted in three replicates. One replicate consisted of 20 males walking towards one of the sources. After every 5 males the bottles, glass tubes and connecting tubes were exchanged to correct for unforeseen asymmetry in the setup.

Results

One-choice bioassay

In total 1 out of 171 males responded to the empty control dish. Significantly more males responded to females on potato leaves than to clean potato or goosefoot leaves, goosefoot leaves from which females were removed, or to females with water only (Figure 2). Females on goosefoot leaves were similarly attractive as females on potato leaves, but also as attractive as all other odor sources tested, except for clean goosefoot leaves. Potato leaves from which females had been removed, *i.e.* leaves on which females had walked for 2-3 h prior to the experiment, elicited a similar response as females on potato leaves and females on goosefoot leaves. Females without plant material elicited a response in only 0.18 ± 0.05 (mean ± s.e.) of the males tested.

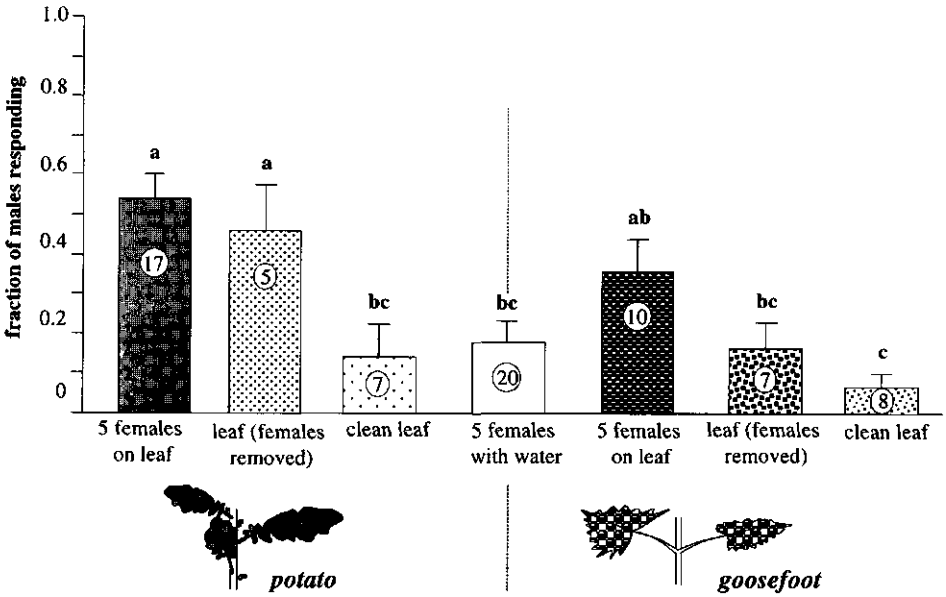

FIGURE 2. Mean fraction (+ s.e.) of males responding in cylinder bioassay. Numbers in bars: number of replicates. Each replicate consisted of 8-15 males. Letters above the bars indicate significant differences at the 5% level on the logit scale, using GLM. See text for further explanation.

Two-choice flight bioassay

When females were confined with a potato sprout (combination A), the first time 9 males were trapped in the Tangle Trap® versus one male in the control trap, and the second time 11 males were trapped versus one male in the control trap (Figure 3). Females compartmentalized downwind (B) or upwind (C) from a potato sprout, caught similar amounts of males, and significantly more than the control traps. Traps without plant material and only females in the downwind compartment (D), caught slightly lower amounts of males, but in total 23 males versus 2 males in the control traps were caught. In the traps with *males* and potato sprouts, in total only three males were caught versus two males in the control traps.

Two-choice walking bioassay

In total 43 males walked towards females on potato leaves, versus 17 males walking towards the control (Figure 4, I)). 40 Males walked towards females without plant material, while again 17 males walked towards the control (II). When the test bottle contained males with potato leaves (III), 32 males walked towards the test bottle versus 27 males walking towards the control.

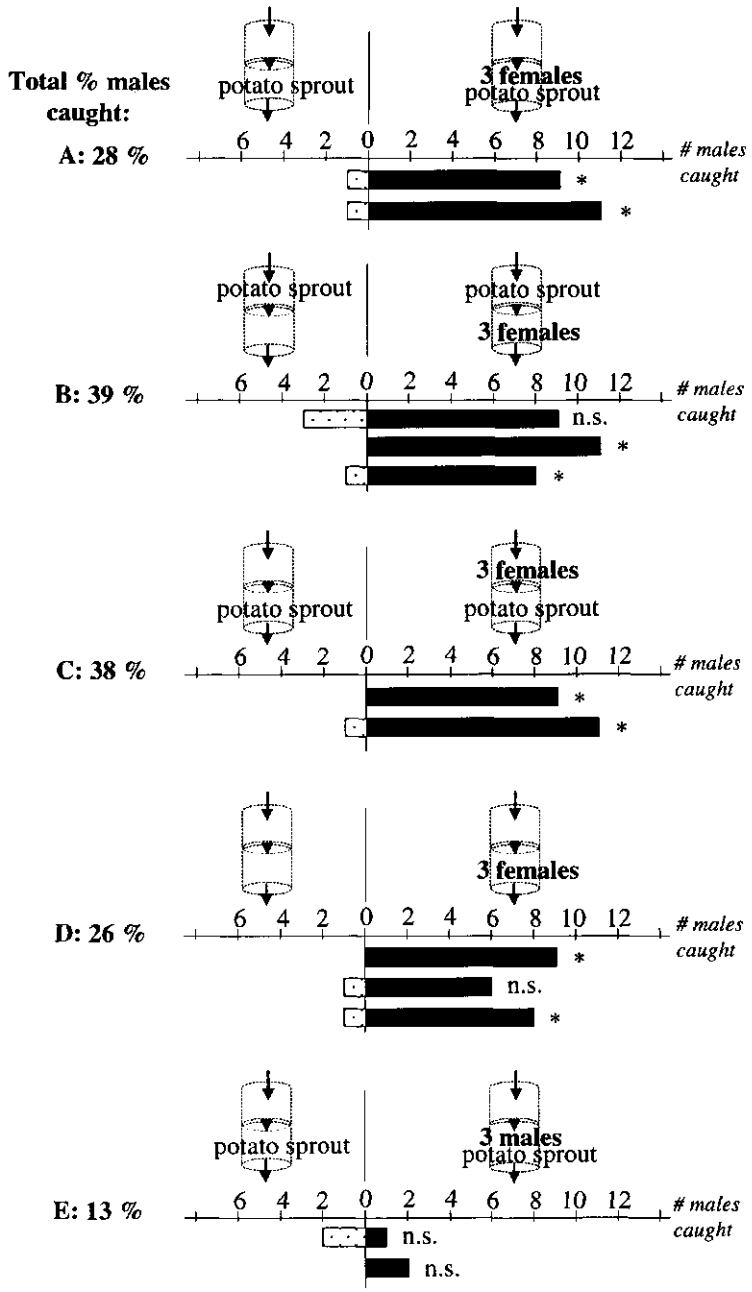

FIGURE 3. Numbers of males caught in traps. Per replicate 35-40 males had been released. Bugs were always confined with pollen grains and wet oasis. Significant differences were determined per test, using the two-sided binomial test. *$P < 0.05$, n.s.: $P > 0.05$.

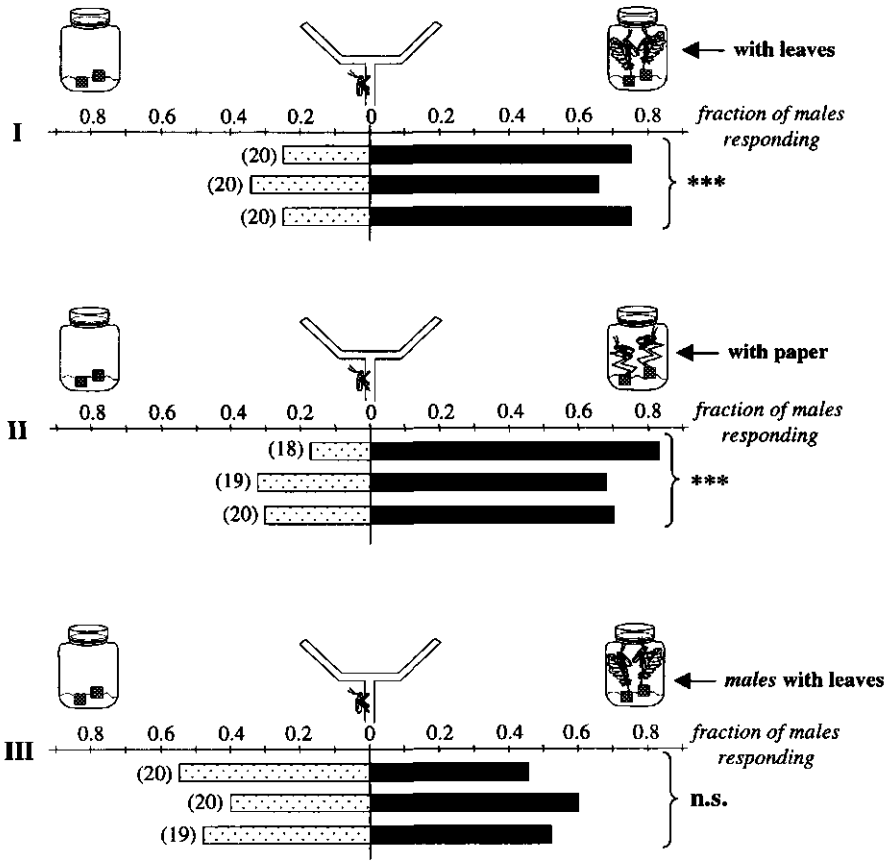

FIGURE 4. Y-track olfactometer tests with (I) 5 females on two potato leaves, (II) 5 females on green paper, (III) 5 *males* on two potato leaves. All bottles contained wet oasis and pollen grains. Significant differences between test source and control were determined using the two-sided binomial test after summation of male responses in the three experiments per test source. *** $P < 0.001$, n.s.: $P > 0.05$.

Discussion

Since attraction towards females on potato leaves was not significantly different from attraction towards females on goosefoot leaves, specific plant compounds do not seem to be involved in sex pheromone production in *L. pabulinus* females. However, the fact that females on potato leaves were significantly more attractive than females without plant material, does suggest an interaction between females and potato leaves. On the other hand, the results of both two-choice assays do not confirm such

interaction, because females without plant material attracted similar amounts of males as females with potato sprout, containing potato leaves. The significant difference in the one-choice assay is probably due to malnutrition of the females without plant material, as they were confined with water only. In the two-choice assays pollen was added as a food source. We frequently observed *L. pabulinus* actively feeding on the pollen. Pollen have large nutritive value (McNeil and Delisle, 1989b), which is expressed in *L. pabulinus* by a higher production of eggs in females reared with pollen than in females reared without it (Groot *et al.*, 1998). Malnutrition is likely to stop pheromone production or release, as energy is needed for somatic maintenance.

Summarizing all results, we conclude that plant compounds are not directly involved in pheromone production in *L. pabulinus* females. Female bugs do not need to be in the odor of plants to attract males either: females upwind from a potato sprout attracted similar amounts of males as females downwind from a potato sprout. As a consequence, *L. pabulinus* females do not seem to coordinate their reproductive behavior with the availability of food for offspring. For *L. pabulinus* males, all females with pollen were attractive in the flight and walking bioassays, irrespective of the presence of plant material. Hence, the perception of sex pheromone in *L. pabulinus* males does not seem to be influenced by plant volatiles either.

Our findings may not apply to field bugs, as laboratory-reared insects may respond differently to host odors in relation to sex pheromone than field insects, which has been demonstrated for *Heliothis virescens* (Raina *et al.*, 1997). However, *H. virescens* was reared continuously on an artificial diet. When plant material is lacking completely for a number of generations, it is likely that life-history aspects, such as reproduction, are not associated with plant volatiles anymore. *Lygocoris pabulinus* has been reared continuously on potato plants, which reduces the chance that possible associations with plant volatiles have disappeared.

There are more examples that plant volatiles do not affect sex pheromone production, release or perception (Cardé and Taschenberg, 1984; Guldemond *et al.*, 1993; Den Otter *et al.*, 1996). Cardé and Taschenberg (1984) suggest that such kairomonal interactions are less likely to occur in polyphagous species. *L. pabulinus* is polyphagous and host-alternating, laying overwintering eggs in woody plants, such as fruit trees, while the summer generation feeds on herbaceous plants (Petherbridge and Thorpe, 1928; Kullenberg, 1946; Southwood and Leston, 1959). Also, plant volatiles probably do not influence sexual communication when eggs are not deposited near the site where females call. Such spatial discontinuity between calling activity and oviposition has been described for some insects (Showers *et al.*, 1976; Noldus *et al.*, 1991), and should be studied more closely in *L. pabulinus*.

One result of the one-choice assay needs further attention, *i.e.* the fact that *L. pabulinus* males were similarly attracted towards females on potato leaves as towards potato leaves, from which females had been removed. This is probably due to deposition of attractive components from females on the substrate on which they walked (Groot *et al.*, 2000). Most likely these components are female-specific cuticular hydrocarbons (Drijfhout *et al.*, 2000). In a similar assay used for *Mamestra*

brassicae, males were attracted to leaves on which female sex pheromone was adsorbed as well (Noldus *et al.*, 1991). In our assay wind was blown over the potato leaves for 1 h, which may enhance evaporation of low-volatile compounds. Adsorption of pheromone to a substrate increases the surface area from which pheromone evaporates, thereby increasing both the rate of volatilization and the possible communication distance (Colwell *et al.*, 1978).

In conclusion, plants are probably only very indirectly involved in sexual communication, their surface merely functioning as a substrate from which pheromone is released. Males may subsequently be attracted to such plants or substrates. Since clean plant material is not attractive to *L. pabulinus* males, plant volatiles alone do not seem to be used by these males as possible mate location cues.

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PART II:

The male perspective

Chapter 5

CLOSE-RANGE SEX PHEROMONE OF *LYGOCORIS PABULINUS* (L.) - SOURCE OF ATTRACTANT*

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ABSTRACT - Sexual communication in insects may include close-range courtship pheromones. Males of the green capsid bug, *Lygocoris pabulinus*, exhibit a specific courtship behaviour, a vibration of the abdomen. We used this behaviour to determine the presence and source of close-range sex pheromone from females. Live and dead females elicited similar responses. When females were dissected into separate body parts, heads, wings and legs elicited equally strong responses, while thorax plus abdomen elicited a much weaker response. When separate body parts were extracted with dichloromethane, the leg extracts elicited significantly stronger response than any other extract. This suggests that female *L. pabulinus* legs are either the source of a close-range sex pheromone (or at least the target deposition site after synthesis in thorax or abdomen), or pheromone is accumulated on the legs due to grooming behaviour. Substrates on which females had walked elicited similar responses as female legs, indicating that the pheromone is deposited on the substrate. This enlarges the functional range of low-volatility compounds, that are thought to function only when sexes are in close vicinity or in contact.

KEY WORDS: Heteroptera, Miridae, legs, contact sex pheromone, trail sex pheromone, cuticular hydrocarbons, male vibration, dichloromethane extracts

Introduction

Sex pheromones are commonly used by insects to locate mates at long range and to stimulate mating at close range (Carlson *et al.*, 1971; Cardé *et al.*, 1975; Muhammed *et al.*, 1975). Long-range sex pheromones have first been described and chemically identified in moths (Butenandt *et al.*, 1959), and are now widely used for monitoring of lepidopterous pests (e.g. Minks and Van Deventer, 1992; Cardé and Minks, 1997). In recent years identification of non-lepidopteran sex pheromones has received growing attention (Hardie and Minks, 1999). In mirid bugs, where virgin females attract males, long-range sex pheromones have been identified for three species up to

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now (Smith *et al.*, 1991; Millar *et al.*, 1997; Millar and Rice, 1998; McBrien and Millar, 1999).

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

In order to identify close-range sex pheromones, a specific arousal or courtship behaviour of one of the sexes should be distinguished. Males of the green capsid bug (*Lygocoris pabulinus* (L.), Heteroptera: Miridae) exhibit a characteristic courtship behaviour, a repeated vibration of the abdomen (Groot *et al.*, 1998). This behaviour is sex-specific, males only vibrate in the presence of females and only when they are sexually mature (Groot *et al.*, 1998). In the present study the vibration behaviour of male *L. pabulinus* is used to determine the source of attraction in females at close-range. The source of long-range pheromones in mirids has been suggested to be the metathoracic scent gland (Aldrich, 1988), or at least the thoracic region (Millar *et al.*, 1997), although Graham (1988) identified the ovipositor region as source of attraction. Since the chemical nature of long-range pheromones may differ completely from close-range pheromones, their sources probably differ as well.

Materials and Methods

Insects

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

Bioassays

One to two hours before each test, virgin males of 6-9 days old were collected from the separate rearing cages and isolated in small glass tubes. Glass Petri dishes of 5 cm diameter were cleaned with acetone, and the bottoms were covered with white filter paper discs of the same diameter. The stimuli to be tested were placed in the

Petri dishes, after which one male per dish was introduced. Stimuli consisted of one bug equivalent per Petri dish, and originated from bugs that were virgin and 6-9 days old. All males were observed for 15 min. If a male in a dish started to vibrate within this period, that dish was set aside and counted as a positive response. The number of Petri dishes with positive responses were calculated as a fraction of the total number of Petri dishes in which the stimulus had been applied. Different stimuli were tested at the same time, and stimuli were tested on several different days. All experiments were carried out at 19-23 °C between 10.00 and 14.00 hours C.E.T.

Stimuli tested

First, we tested a series of live females, dead females, live males and dead males. Bugs were killed by anaesthetizing them with CO_2, after which the heads were clipped off. In a following series freshly anaesthetized females were dissected into heads, wings, legs, and thorax plus abdomen (see Figure 1). Thorax and abdomen were not subdivided, since clipping would mean cutting through several organs and glands that run from thorax to abdomen, which may then release a variety of chemicals. Thirdly, extracts were made of the different body parts of females, *i.e.* heads, wings, legs, and thorax plus abdomen. After anaesthetizing fresh females with CO_2, the body parts were dissected and placed in 1 or 4 ml vials. After dissecting all available females, 15-50 µl dichloromethane per female was added, the amount of which was set as one female equivalent of the regarding extract. The extracts were stored in a freezer (-20 ± 2° C) until used in bioassays. All extracts were used 1-14 days after the initial dissections.

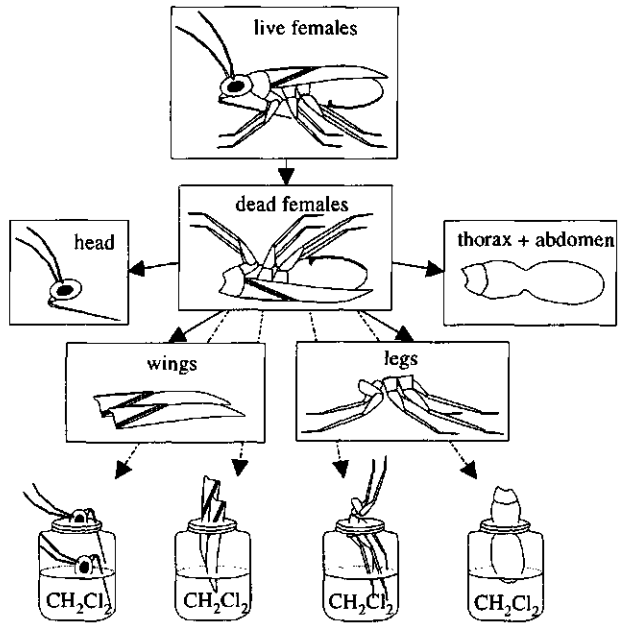

FIGURE 1. The diffferent parts of *L. pabulinus* females offered to males.

Statistical analysis

If males responded to a source, differences in responses towards the different sources were statistically analysed by fitting a logit regression model with overdispersion to the daily observed counts of responses of a test (McCullagh and Nelder, 1989), using the computer programme Genstat 5 (release 4.1, PC/Windows NT, 1997). In the model, source was taken as explanatory variable and the variance was assumed to be proportional to binomial variance. First a chi-square test for the residual deviance was conducted to determine overdispersion. Overall effect of treatments was determined by performing an F-test for the ratio of the mean deviance for treatment and the mean deviance of the rest. If the overal test was significant ($p < 0.05$), pairwise comparisons between treatment means on the logit scale were conducted, using the t-test.

Results

Live and dead females elicited similar responses, the fractions of males vibrating being 0.88 ± 0.08 and 0.74 ± 0.09 (mean ± s.e.), respectively (Figure 2A). Live and dead males elicited vibration responses in few males, reconfirming sex specificity of male vibration behaviour (Groot *et al.*, 1998). When the bodies of females were dissected, the head, wings and legs were equally attractive and as attractive as dead females, while the thorax plus abdomen of females were significantly less attractive (Figure 2B). After extraction of the separate body parts of females in dichloromethane, leg extracts elicited significantly more vibrational response than all other extracts (Figure 2C). Extracts from thorax plus abdomen did not elicit a response from males, which may be due to defensive compounds in the metathoracic gland. Therefore, extracts were also made of females' thorax plus abdomen, from which the metathoracic gland was removed by gently cutting the cuticle with two sharp tweezers, trying to destroy as little tissue as possible. Few males did respond to this extract (Figure 2C). When differences in responses between freshly dissected body parts and their corresponding extracts were statistically compared, male response to the leg extracts was not significantly different from response to freshly dissected legs, while wing and head extracts elicited significantly lower response ($P < 0.05$) than freshly dissected wings and heads.

Response to freshly dissected wings and heads may be due to grooming, which spreads attractive compounds over the body surface. For confirmation of presence of attractive pheromone on the whole body surface, small pieces of filter paper were rubbed over female bodies (after anaesthesia and clipping off heads). When these pieces of paper were offered in clean Petri dishes, almost half of the tested males (0.41 ± 0.07, mean ± s.e.) started vibrating (n=46).

The above-described results suggest that female legs may be the source of close-range sex pheromone. To determine whether the source of attraction could be defined more precisely, female legs were subdivided into (a) forelegs, middle legs and hindlegs, or into (b) coxae plus femorae, and tibiae plus tarsi. Per Petri dish one

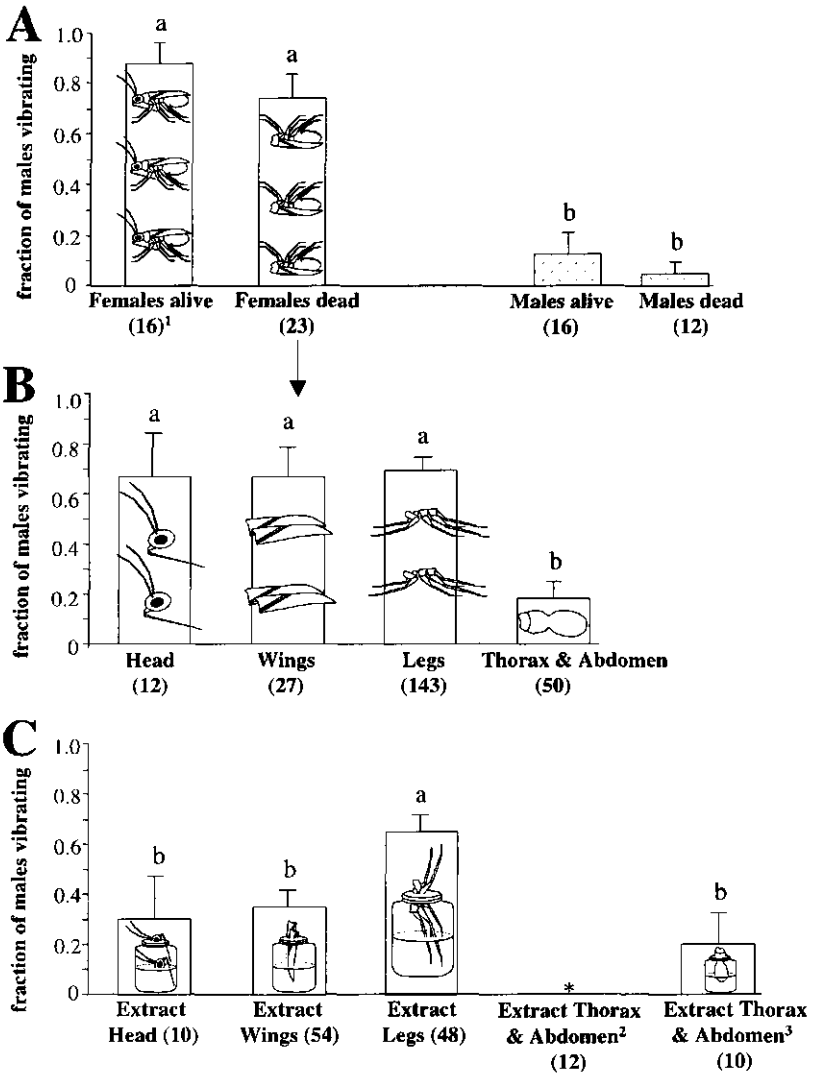

FIGURE 2. Male *L. pabulinus* responses (mean ± s.e.) to different stimuli. A: whole insects, B: body parts of females, C: extracts of female body parts. 1Total number of males tested, 2Metathoracic gland left in thorax, 3Metathoracic gland removed from thorax. Significant differences were determined between sources within one group (A, B, C). Different letters above the bars indicate significant differences in each group at the 5 % level. See text for statistical methods used. *Not included in the statistical analyses, as no males had responded.

pair of forelegs, middle legs or hindlegs of three females were placed, so that 6 legs per dish were offered. In series b the 6 coxae plus femorae of one female were placed in one Petri dish, and the 6 tibiae plus tarsi in another. Table 1 shows that all parts of the legs were equally attractive (no significant differences were found between any

TABLE 1. Male vibration response to different parts of female legs

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

[1]Different letters indicate signifant differences between pairs ($p < 0.05$), [2]different letters indicate significant differences between groups ($p < 0.05$). See text for statistical methods used.

pair). However, when the overall response to fore, middle and hind legs was compared to the overall response to coxae plus femorae and tibiae plus tarsi, response to entire legs was significantly stronger than to parts.

When legs contain attractive compounds, these compounds may be deposited on the substrate on which female *L. pabulinus* walk. To determine possible deposition of attractive compounds, we tested three different substrates: a piece of potato leaf (cultivar Bintje), a piece of green bean leaf (*Phaseolus vulgaris*, cultivar Miracle) and the glass of an empty Petri dish. One *L. pabulinus* female was allowed to walk in each dish for 75-140 min. As a control we tested pieces of potato leaf or empty dishes on which males had walked for 60-120 min, as well as pieces of potato leaves on which no bug had walked. Table 2 shows that males did respond to substrates on which females had walked, while no males showed vibration behaviour in any of the control dishes. During the 75-140 min that females walked around in the dishes, a characteristic pheromone-laying behaviour was not observed.

TABLE 2. Male vibration response to substrate on which females had walked

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

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

Discussion

Legs have been recognized as the site of sex pheromone release in the aphid *Megoura viciae* (Marsh, 1972), the mosquito *Culiseta inornata* (Lang, 1977), the tsetse fly *Glossina morsitans morsitans* (Carlson *et al.*, 1978), the housefly *Musca domestica* (Schlein *et al.*, 1980), and the parasitoid braconid *Ascogaster reticulatus* (Kainoh and Oishi, 1993). Depending on their volatility, these pheromones are active at some distance, as in *M. viciae* (Pickett *et al.*, 1992), or they elicit response at close range or upon contact, as in the other species mentioned. In some species, specific glands in the legs have been identified as the site of sex pheromone excretion (Marsh, 1972; Schlein *et al.*, 1980). In *L. pabulinus*, response to fore, middle and hind legs was similarly strong. The lower response to parts of the legs compared to entire legs may be due to the lower amount of leg biomass per Petri dish in the latter group. From these experiments no specific site of possible glands in legs became apparent.

Contact sex pheromones may not be synthesized by specific glands in the legs. For example, *M. domestica* synthesizes its pheromone in the abdomen (Dillwith *et al.*, 1981). Contact pheromones that have been chemically identified are cuticular hydrocarbons (e.g. Muhammed *et al.*, 1975; Carlson *et al.*, 1978; Bolton *et al.*, 1980; Dillwith *et al.*, 1981; Blomquist *et al.*, 1993; Gu *et al.*, 1995; Fukaya *et al.*, 1996; Doi *et al.*, 1997). Cuticular hydrocarbons are probably synthesized by oenocytes, large cells that are rich in smooth endoplasmatic reticulum and mitochondria, which appear to be restricted to epidermal tissue in thorax and abdomen (Gu *et al.*, 1995; Schal *et al.*, 1998). After synthesis, attractive hydrocarbons may be deposited at specific target sites, as in the German cockroach *Blattella germanica*, where the wings accumulate large amounts of pheromone (Gu *et al.*, 1995). In *L. pabulinus* females, low-volatile hydrocarbons seem to be involved in the close-range attraction (Drijfhout *et al.*, submitted). The cuticle of legs may hence be the specific target deposition site of the attractive compounds.

The presence of close-range sex pheromone on legs may also be due to grooming. Grooming may either accumulate pheromone from other body parts on the legs (Howard and Blomquist, 1982), or it may spread the pheromone from leg glands over the whole body surface, as in polistine wasps (Beani and Calloni, 1991), whose territorial marking pheromones from leg glands function as sex attractants as well. *Lygocoris pabulinus* males and females groom frequently (Groot *et al.*, 1998) and the attractive compounds are not only present on female legs, but also on other body parts, witness the male response to female wings and heads, and to pieces of filter paper rubbed over female bodies. In short, the site of sex pheromone production does not have to be the site of pheromone release, specific glands are not necessarily involved, and grooming may enhance chemical dispersion or accumulation at specific sites. When specific compounds will be identified as close-range sex pheromone for *L. pabulinus*, the site of synthesis and excretion of these compounds may be clarified.

Irrespective of how close-range sex pheromones are accumulated on insect legs, they may be deposited on the substrate. This may occur passively (Kainoh and Oishi, 1993), or actively, as hymenopteran insects do when scent marking their territory (Williams *et al.*, 1984; Beani and Calloni, 1991). Male *L. pabulinus* showed strong responses to substrates on which females had walked. The fraction of males responding to potato leaves was even similar to responses to female legs. As a characteristic pheromone-laying behaviour was not observed, we suspect that pheromone deposition on the substrate occurs passively, or that pheromone is adsorbed to the substrate.

Adsorption or deposition of attractive compounds on the substrate increases the probability of sex encounters, as it elicits intensive search by males in these areas (Colwell *et al.*, 1978; Fauvergue *et al.*, 1995). Adsorption of pheromone to a substrate also increases the surface area from which pheromone evaporates, thereby increasing both the rate of volatilization and the possible communication distance (Colwell *et al.*, 1978). Males may follow a gradient of intensity, created by the decay of the compounds over time, to orient their movements towards females (Fauvergue *et al.*, 1995). In this way, the functional range of low-volatile cuticular hydrocarbons would be greatly enlarged. In a consecutive paper identification of female-specific compounds of *L. pabulinus* will be described (Drijfhout *et al.*, submitted).

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PART III:

The female perspective

Chapter 6

DISTURBANCE OF SEXUAL COMMUNICATION IN *LYGOCORIS PABULINUS* BY HEXYL BUTANOATE*

ASTRID T. GROOT, FALKO P. DRIJFHOUT, ANNEKE HEIJBOER, TERIS A. VAN BEEK AND J. HANS VISSER

ABSTRACT - The metathoracic scent gland in *Lygocoris pabulinus* contains mostly hexyl butanoate. As secretions of this gland in Heteroptera may serve as alarm pheromone, we determined if hexyl butanoate is released by disturbed bugs, and how this compound disturbs sexual attraction of *L. pabulinus* males towards females. Undisturbed males and females, and disturbed males released less than 100 ng/h hexyl butanoate, while disturbed females released a highly variable amount, ranging from 25 ng/h to more than 1 µg/h. In the field, traps with virgin females and rubber septa containing 20 mg hexyl butanoate, caught a total of one male in a month. In control traps without hexyl butanoate, 36 males were caught in the same period. In Y-track olfactometer tests, males were *not* attracted to virgin females when a dispenser with 20 mg hexyl butanoate was placed in the bottle with females. Males were attracted to females when the dispenser was placed downwind from the females. These results suggest that males are not repelled by hexyl butanoate, but that this compound inhibits sex pheromone release in females. Application possibilities for pest management are discussed.

KEY WORDS: Heteroptera, Miridae, metathoracic scent gland, alarm pheromone, dispersal behaviour, sex pheromone inhibition, pest management

Introduction

True bugs are notorious for their defensive secretions when they are attacked or disturbed (Remold, 1963; Carayon, 1971; Staddon, 1986; Aldrich, 1988; Aldrich *et al.*, 1997). Intraspecifically, these secretions may function as alarm pheromone, causing dispersal and escape from confrontations with predators (Blum, 1985; Leal and Kadosawa, 1992; Leal *et al.*, 1994). As dispersal will reduce the chance of sexual encounters, alarm pheromones may disturb sexual communication.

In adult heteropterans, the metathoracic scent gland is generally described as the gland where defensive secretions are stored or synthesized (Remold, 1963; Carayon, 1971; Games and Staddon, 1973; Staddon, 1986; Aldrich, 1988). The

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content of this gland has been chemically analysed in many species (Levinson and Bar Ilan, 1971; Games and Staddon, 1973; Aldrich and Yonke, 1975; Oetting and Yonke, 1978; Staddon and Daroogheh, 1981; Aldrich *et al.*, 1984; Knight *et al.*, 1984; Lockwood and Story, 1987; Kou *et al.*, 1989; Aldrich *et al.*, 1996; 1997; Blatt *et al.*, 1998). Actual toxicity of compounds from this gland for predators was demonstrated only by Remold (1963) and Aryeeti and Kumar (1973). A few other studies have determined that these compounds cause intraspecific dispersal behaviour (Levinson and Bar Ilan, 1971; Lockwood and Story, 1987; Kou *et al.*, 1989; Blatt *et al.*, 1998). When chemical identification of compounds is not succeeded by bioassays showing their effect, possible functions remain subject to speculation.

In mirids, the content of the metathoracic scent gland is similar among species. For example, the major components in *Lygus lineolaris, L. elisus* and *L. hesperus* are hexyl butanoate and (*E*)-2-hexenyl butanoate (Gueldner and Parrott, 1987; Aldrich *et al.*, 1988), in *Pilophorus perplexus* they are butyl butanoate and hexyl butanoate (Knight *et al.*, 1984), and in *Blepharidopterus angulatus* the major components are hexyl hexanoate and (*E*)-2-hexenyl hexanoate (Knight *et al.*, 1984). In this family, females attract males by means of a sex pheromone (reviewed by McBrien and Millar, 1999). To our knowledge, so far no study has determined if or which of these compounds may disturb sexual communication in mirid bugs, and how such disturbance would affect sexual attraction of males towards females.

The metathoracic scent gland of *Lygocoris pabulinus* (L.) (Heteroptera: Miridae) contains up to 90 % hexyl butanoate (F.P. Drijfhout, unpubl. res.). In this study we determined: (A) whether this compound is released by males and females in undisturbed and disturbed conditions, (B) whether hexyl butanoate disturbs sexual attraction of males towards females, and (C) which sex is actually disturbed by hexyl butanoate, *i.e.* are the males repelled by this compound, or do females stop attracting males?

Material and Methods

Insects

Lygocoris pabulinus was reared on potted potato plants, cultivar Bintje, in wooden cages in greenhouses (t = 22 ± 2°C, r.h. = 65 ± 5 %, L18:D6), following Blommers *et al.* (1997). Every 2-3 days newly emerged adults were collected from the rearing cages, after which the sexes were isolated in separate rearing cages. In this way, virgin females and males of known age were continuously available (see Groot *et al.*, 1998).

A. Release rates of hexyl butanoate in undisturbed and disturbed bugs

To determine amounts of hexyl butanoate released by *L. pabulinus*, five virgin females or males of 5-8 days old were placed in a 250 ml glass bottle (h. 14 cm, diam. 6.5 cm) together with wet oasis and pollen. The inlet of these glass bottles

were connected to the air flow of the Y-track olfactometer setup, as described below (see also Figure 1). One h prior to sampling, the bottles were placed in the setup to avoid possible release of hexyl butanoate due to disturbance before the experiment. After one h the outlet of the bottle was connected to a glass tube filled with 200 mg Tenax®, on which the headspace was collected during 1 h. To collect headspace from undisturbed bugs, females and males were left untreated for one h. Headspace from disturbed bugs was sampled by vigourously shaking the bottles for one min, 3-4 times in one h. After collection, the headspace was thermally desorbed at 250 °C (5 min) and analysed by GC (see Drijfhout *et al.*, 2000). The release rate of hexyl butanoate was determined by comparing the peak area of hexyl butanoate with that of an external standard.

B. Disturbance of sexual communication

To determine whether hexyl butanoate disturbs sexual communication, a field test was conducted from 6 August to 2 September 1999, at the experimental orchard 'Schuilenburg', Kesteren, The Netherlands. Two kinds of traps with virgin females were tested in order to compare the number of males caught: (a) six traps with rubber septa containing hexyl butanoate, and (b) six traps with rubber septa without hexyl butanoate. All traps contained three virgin, 5-8-day old females, caged in a PVC-cage (ø 7 cm, length 7.5 cm, closed at both sides with gauze lids), with pollen and a small potato sprout, of which the roots were wrapped in wet cotton and aluminium foil to delay desiccation. Around each cage with females two rubber septa were placed, both in front of the gauze lids. These septa were filled with 100 µl dichloromethane, in which 4 mg 2,6-di-*t*-butyl-*p*-cresol and 2 mg 2-hydroxy-4-methoxybenzophenone were dissolved as anti-oxidants. Additionally, for traps (a) 20 mg hexyl butanoate was dissolved in each septum. The septa for traps (b) only contained the anti-oxidants. All cages were placed in delta traps (30 (l) x 20 (w) x 20 (h) cm), after which the 12 traps were arranged alternately in six 0.4 ha plots in apple trees at ± 1.50 m height, with at least 10 m or three rows of apple trees between adjacent traps. Numbers of males caught were counted every 3-4 days. Every week the cages were cleaned and refilled with pollen, new potato sprouts and fresh females of 5-8 days old, and all dispensers were renewed. The release rate of a rubber septum with 20 mg hexyl butanoate was measured by weighing an empty septum, then weighing the septum filled with the anti-oxidant and 20 mg hexyl butanoate. Subsequently, the dispenser was placed outside in a delta trap and weighed again after 24, 48 and 62 hours. This procedure was conducted twice.

C. Are males AND/OR females disturbed by hexyl butanoate?

To determine which sex is disturbed by hexyl butanoate, Y-track olfactometer assays were conducted as described by Visser and Piron (1998), with specific adjustments (see Figure 1). The Y-track was placed in a black box under a halogen lamp (4-12 V DC, 10 VA). To suppress flight intention of males, this lamp was placed in a black socket sealed with a red filter, so that the light intensity at the base of the Y-track was 6.3-6.5 lux only. One hour before each experiment five virgin *L. pabulinus*

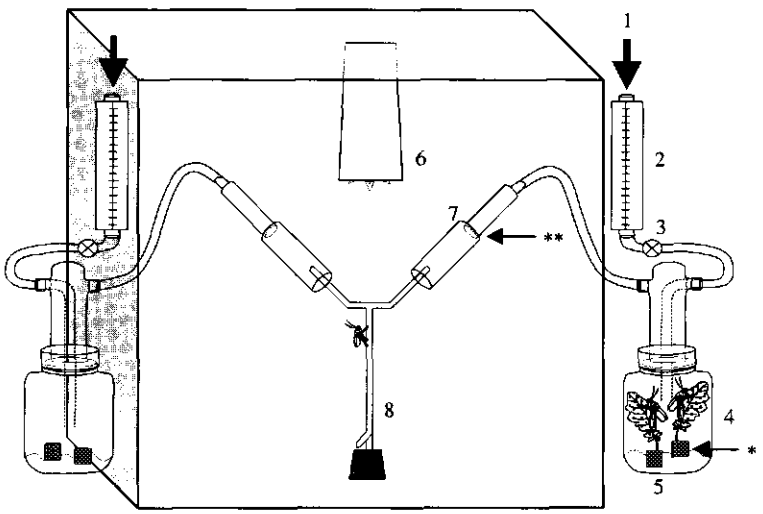

FIGURE 1. Schematic drawing of open Y-track olfactometer positioned in black box. The open front is covered by a black cloth during experiments. 1) incoming clean air, 2) air flow control, 3) open/close valve, 4) glass bottle under a desk lamp (25 W), 5) square of wet oasis on a bottom of wet cotton, 6) lamp in black cylinder with red filter, 7) glass tube with gauze (arrow), 8) brass rod (diameter: 4 mm, length to junction: 13 cm) with extension at base for placing a male. Arrows indicate position of rubber septum in experiment I and III (*) and experiment II (**).

females of 5-8 days old were placed in a 250 ml glass bottle (h. 14 cm, diam. 6.5 cm) together with two small potato sprouts in wet oasis, and pollen. The control glass bottles contained only wet oasis and pollen. Both bottles were placed outside the black box, one on each side, under a desk-lamp with a 25 W light bulb. Three situations were tested: (I) a similar rubber septum as used in the field test, containing 20 mg hexyl butanoate, was placed in the bottle together with the females, (II) the rubber septum with 20 mg hexyl butanoate was placed downwind from the females, in the glass tube slipping over the Y-track, behind gauze (indicated by the arrow in Figure 1), (III) a rubber septum was filled with only 0.2 mg hexyl butanoate, and placed in the bottle with the females. Hence, in situation (I) and (III) females were in the odour of hexyl butanoate, in situation (II) they were not. For males, situation (I) and (II) did not differ in the sense that they were offered 20 mg hexyl butanoate together with the odour of females in both situations. In situation (III) males were offered a lower dose of hexyl butanoate. All experiments were repeated 3-5 times. One test consisted of 20 males, tested individually, that were offered an odour source versus control. After testing 5 males the bottles, glass tubes and connecting tubes were exchanged among the olfactory arms to correct for possible positional effects. The release rate of hexyl butanoate from the septa in the Y-track olfactometer was determined by placing one dispenser with hexyl butanoate in a similar glass bottle as used in the tests. The inlet of this glass bottle was connected to the air flow of the

olfactometer setup, the outlet to a glass tube filled with Tenax®. Headspace of a septum with 20 mg hexyl butanoate was collected for 15 min, headspace of a septum with 0.2 mg hexyl butanoate was collected for 30 min. The release rates were determined by comparing the peak area of hexyl butanoate with that of an external standard (as described above).

Results

A. Release rates of hexyl butanoate in undisturbed and disturbed bugs

Undisturbed and disturbed males released similar amounts of hexyl butanoate, about 10 ng/h (Figure 2), which was the smallest amount that could be measured. Once, disturbed males released 45 ng/h. Undisturbed females released 10-92 ng/h hexyl butanoate (mean: 47 ng/h). Upon disturbance the released amount was highly variable: the lowest amount measured was 25 ng/h, the highest was 1081 ng/h.

B. Disturbance of sexual communication

In traps (a), with septa containing hexyl butanoate, in total one male was caught over one month. In traps (b), with septa containing only the anti-oxidants, 36 males were caught in the same period (Figure 3). The release rate of a septum with 20 mg hexyl butanoate in the field was 3.7-3.9 mg/day, which is 154-163 µg/h.

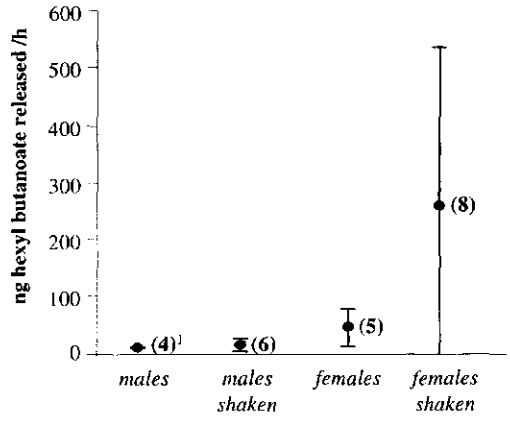

FIGURE 2. Amount (± 95 % c.i.) of hexyl butanoate, released from five males or females in one h, in glass bottles that have been shaken or not. [1]Number of replicates. See text for further explanation.

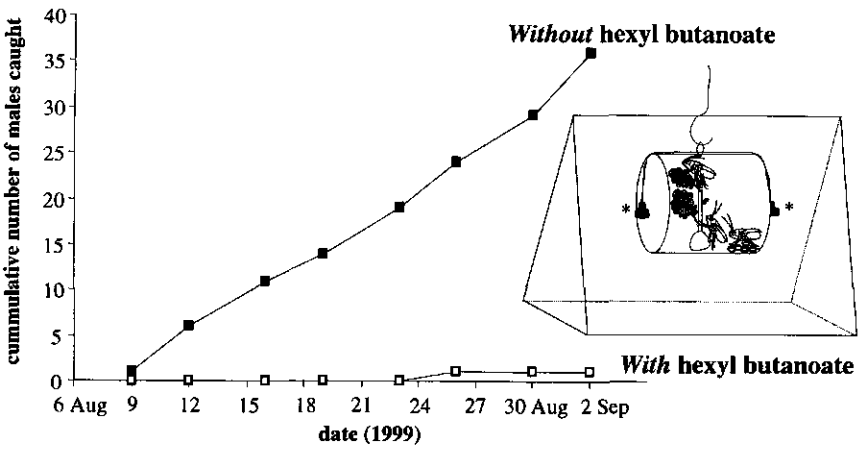

FIGURE 3. Trap catches of traps with 3 virgin females plus two rubber septa (*) loaded either with 20 mg hexyl butanoate each, or with anti-oxidants only. See text for further explanation.

C. Are males AND/OR females disturbed by hexyl butanoate?

In the Y-track olfactometer, when a dispenser with 20 mg hexyl butanoate was placed in the odour source bottle with females, males did not preferentially walk towards the females (Figure 4 I). Overall, significantly more males walked towards the control instead. When a similar rubber septum with hexyl butanoate was placed in the glass tube downwind from the females, significantly more males walked towards the females than towards the control (Figure 4 II), which resembles the reaction towards virgin females in this bioassay (see Groot and Visser, 2000). Since attraction was restored in the latter setup, experiments with 0.2 mg hexyl butanoate dispensers were only conducted with a dispenser placed in the bottle with females. Females with these dispensers were attractive to males as well (Figure 4 III). The release rate of a septum with 20 mg hexyl butanoate was about 92 µg/h, while the release rate of a septum with 0.2 mg hexyl butanoate was about 1 µg/h.

Discussion

Hexyl butanoate is present in similar amounts in both sexes of *L. pabulinus*, one adult may contain up to 100 µg (F.P. Drijfhout, unpubl. res.). Males do not seem to emit the scent upon disturbance, at least they did not when the glass bottles were vigourously shaken. Females did release additional hexyl butanoate upon disturbance, although a maximum of 1 µg released by 5 females in one hour is only a fraction of the amounts present in these females. Probably, upon sudden attack much larger amounts can be released. If it would be possible for females to release their total gland content at once, in the field maximally 300 µg would be released by the

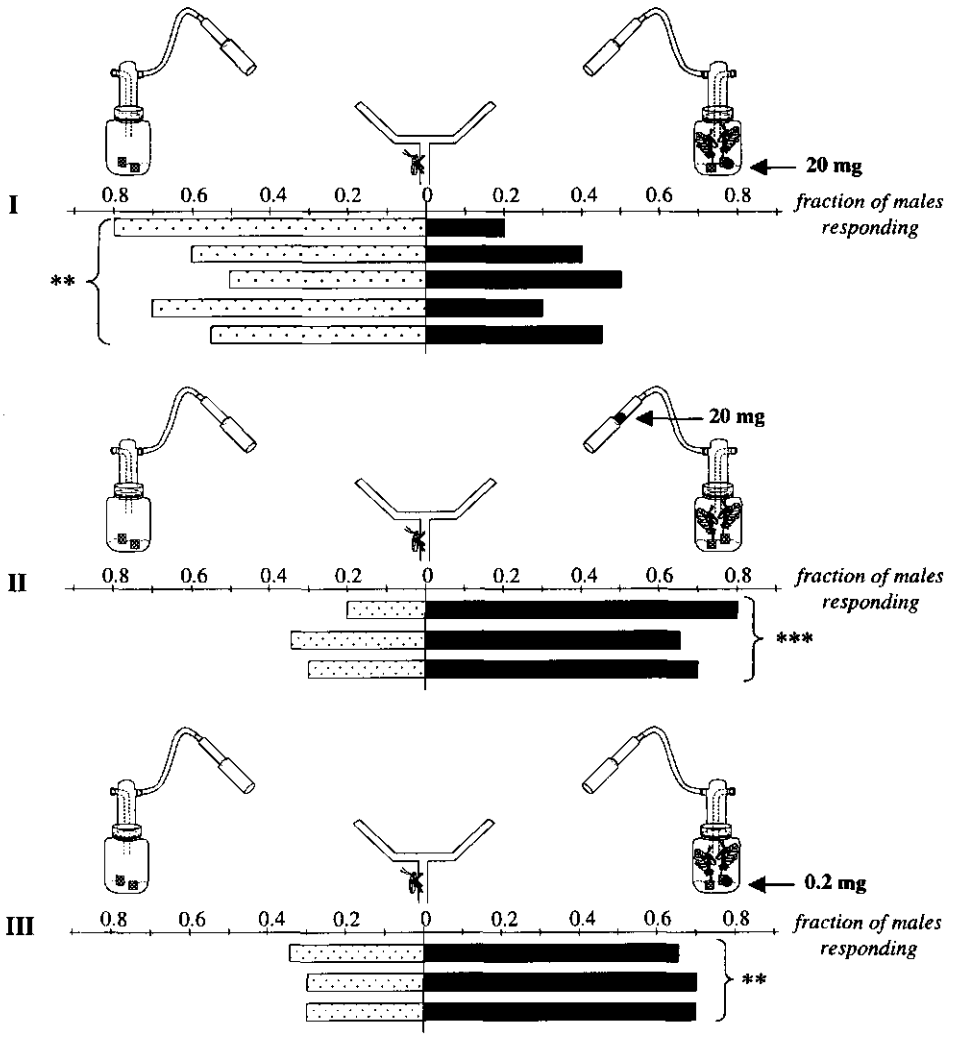

FIGURE 4. Y-track olfactometer tests. Odour sources tested: 5 females and a rubber septum, filled with 20 mg hexyl butanoate, (I) placed in the bottle with females, (II) placed downwind from the females, (III) filled with 0.2 mg hexyl butanoate, placed in the bottle with females (arrows indicate position of septa). Each bar represents one test with 20 males choosing. Preferences for odour sources were determined by using the two-sided binomial test for the mean results (after summing the replicates); ** $0.01 < P < 0.001$; *** $P < 0.001$.

three caged females, which is similar to the amount released by the two rubber septa in one h. However, males in the field are not constantly exposed to odour released from a trap. This only happens when they are in the vicinity of the trap. A male, flying in the surroundings of a trap with two dispensers, each with 20 mg hexyl

butanoate, would experience about 5-5.5 µg hexyl butanoate in one minute. In the Y-track olfactometer, in one minute each male would be exposed to about 1.5 µg hexyl butanoate from the 20 mg dispenser. Although males preferentially walked towards the control instead of towards females with this amount of hexyl butanoate (experiment CI), males were attracted to the odour source when this dispenser was placed downwind from the females (experiment C II). Hence, these amounts of hexyl butanoate did not elicit dispersal behavior in *L. pabulinus* males. For this sex the compound does not seem to function as an alarm pheromone at this concentration.

Unlike males, females did respond to the amounts of hexyl butanoate released from 20 mg dispensers, by not attracting males anymore when exposed to hexyl butanoate. This suggests that large amounts of hexyl butanoate inhibit sex pheromone release in *L. pabulinus* females. Females were not able to disperse away from the scent in the field cages or in the Y-track experiments. In the field traps, females were constantly exposed to the scent for a whole week. Under such circumstances, habituation or sensory adaptation to hexyl butanoate is likely to occur (Calam and Youdeowei, 1968; Oetting and Yonke, 1978; Blum, 1985; Lockwood and Story, 1987; El-Agami and Haynes, 1992; Blatt *et al.*, 1998). If habituation or sensory adaptation would have occurred, males would have been caught in the traps with hexyl butanoate some days after females were placed in the traps. As in one month only one male in total was attracted, such adaptation to the scent did not seem to occur in females.

Because of the persistent effect of hexyl butanoate in the field, this compound may be used to inhibit matings in fruit orchards, where *L. pabulinus* is a pest. Its pest status is generated by the nymphs, emerging in spring from overwintering eggs laid in fruit trees. These nymphs feed on plant ovaries and young fruitlets, which cause russetted malformations on the fruits (Blommers, 1994). *L. pabulinus* oviposits its overwintering eggs in autumn, after migration from herbaceous plants to woody hosts (Petherbridge and Thorpe, 1928; Kullenberg, 1946; Southwood and Leston, 1959). When adults of the summer generation could be prevented to mate so that winter eggs would not be deposited, no nymphs would emerge the following spring to cause damage. Population densities of *L. pabulinus* during remigration to woody hosts seem to be low. Bus *et al.* (1985) caught a maximum of 316 green capsid bugs in one 0.4 ha plot in two months time. Blommers *et al.* (1988) caught on average about 5 males per week per virgin female-baited trap, and in 1998 we caught an average of 4 males per week in similar traps (A.T. Groot, unpubl. res.). In such low densities it may be feasible to prevent matings. Before and during migration to woody hosts, large amounts of hexyl butanoate dispensers should be introduced in fruit orchards. Also, dispensers should be placed at potential summer sites, such as potato fields (Groot *et al.*, 2000), because mating may occur before migration. When additionally alternative oviposition sites for winter eggs are offered in the periphery of the orchard, for example by placing black currant plants (Wightman 1968), the density of damaging nymphs the following spring in orchards may be minimized.

Hexyl butanoate may not only be effective in disturbing sexual attraction in *L. pabulinus*, but also in other mirids. During the same period in which we caught 36 *L. pabulinus* males in virgin female traps without hexyl butanoate, we also caught 28 other male bugs (*Phytocoris longipennis* Flor, *P. dimidiatus* (Kirschbaum), and *P. varipes* Boheman, all identified by B. Aukema, Wageningen, The Netherlands), while the traps with hexyl butanoate were devoid of these bug species.

In conclusion, hexyl butanoate does not seem to function as an alarm pheromone for *L. pabulinus* males, but to inhibit sex pheromone release in females. As sex pheromone inhibition will reduce sexual encounters, and subsequently the amount of eggs oviposited, this compound has potential for pest management. To optimize such a management strategy, the time and place of matings in the field before ovipositing overwintering eggs should be studied in detail.

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PART III:

The female perspective

Chapter 7

POLYANDRY IN *LYGOCORIS PABULINUS* (L.) - EFFECTS ON SEXUAL COMMUNICATION AND FECUNDITY*

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ABSTRACT - *Lygocoris pabulinus* females are polyandrous under laboratory conditions (Groot *et al.*, 1998). In order to determine the effects of multiple matings, we first studied the morphology of the female reproductive tract and the process of sperm transfer and storage in detail, as female genitalia in mirids have only been studied partially so far. The bursa copulatrix consists of 4 plates, which are dorsally enclosed by a circular-shaped plate of the median oviduct, posteriorly and ventrally by a membrane. A spermatheca is connected anteriorly to the bursa. At copulation a spermatophore is formed in the spermatheca. The spermatophore is compartmentalized, consisting of a sperm-containing portion, a large sperm-free portion and a mating plug. After 24 h the spermatophore is partially disintegrated. The mating plug is still intact but reduced in size. Sperm is found throughout the spermatheca and in the median and lateral oviducts, where most likely fertilization takes place. The amount of male-derived substances transferred to females during first matings was ca. 5.2 % relative to male body weight. Mated males were not attracted to virgin females for 2 h after mating, and only 23 % of the tested males mated again within 24 h. Together these results suggest a high paternal investment, that may affect reproductive output. To determine effects of multiple matings on fecundity and longevity, we conducted two oviposition experiments. However, neither factor seemed to be influenced by multiple matings. We also determined sexual attraction of mated females at long range and at close range. At long range females were unattractive for only 1-2 h after mating, while they remained attractive at close range. Hence, females may not cease to attract males, but males may be unresponsive to females after mating.

KEY WORDS: Heteroptera, Miridae, female reproductive tract, spermatophore, mating plug, fecundity, longevity, sexual attraction, sexual communication

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Introduction

Many insect species attract mating partners by means of a sex pheromone. In species where females attract males, sex pheromone production is not continuous, but related to female receptivity. After mating, pheromone production can be shut off permanently in monoandrous species (Jurenka *et al.*, 1993). Polyandrous females generally show a refractory period after each mating, in which pheromone production may be shut off temporarily (Raina *et al.*, 1994; Kingan *et al.*, 1995; Foster and Ayers, 1996). In this refractory period females are not receptive (Graig, 1967; Tzanakakis *et al.*, 1968; Leopold, 1976; Gillot and Friedel, 1977) and most oviposited eggs in this period will have been fertilized by the lastly mated male (Thornhill and Alcock, 1983). Males can influence the refractory period of females by transferring substances with their ejaculate, that induce the unreceptive state of females (Miller *et al.*, 1994). Some of such substances have been identified (Ramalingam and Graig, 1977; Gillot, 1988; Kingan *et al.*, 1995). For example, pheromonostatic peptides (PSP's) from *Helicoverpa zea* males were found to evoke the depletion of sex pheromone (Kingan *et al.*, 1995).

In mirid bugs, females emit a sex pheromone that attracts males (*e.g.* Blommers et al., 1988; Smith *et al.*, 1991; Millar *et al.*, 1997; Millar and Rice, 1998; McBrien and Millar, 1999). So far, sex pheromone emission has not been related to female mating status in this group of insects, although polyandry is found in some mirids (Smith, 1977; Chatterjee, 1983; Strong *et al.*, 1970), as well as in pentatomid bugs (Mitchell and Mau, 1969; McLain, 1985; Kawada and Kitamura, 1983; Wang and Millar, 1997). In *Lygocoris pabulinus* (L.), (Heteroptera: Miridae) polyandry has been observed in the laboratory, also on consecutive days (Groot *et al.*, 1998). Although one mating may be sufficient for life-time fecundity, there are several possible advantages for females to be polyandrous, *i.e.* production of genetically diverse progeny, sperm competition in the female which may ensure that eggs are fertilized by the most competitive sperm, and increased female fecundity due to nutrients present in the seminal fluid (*e.g.* Thornhill and Alcock, 1983; Ridley, 1988). These nutrients may be used directly for egg production, or indirectly for somatic maintenance of the female, so that females may switch their behavior from foraging and feeding to oviposition (Boggs and Gilbert, 1979; Simmons, 1988; Boggs, 1990; Ward and Landolt, 1995; Oberhauser, 1997; Rooney and Lewis, 1999). Seminal fluid may also contain chemical stimulants that act directly on the brain of the female to stimulate oviposition (Huignard, 1983; Chen, 1984; Eberhard, 1997; Wolfner, 1997; Wilson *et al.*, 1999; see Vahed, 1999 for a detailed review).

To get an understanding of implications of multiple matings for *L. pabulinus* females, this study addresses the following questions:

1) How is sperm transferred, stored and displaced in *L. pabulinus* females? To be able to determine this, we first studied the morphology of the female reproductive tract in detail.

2) What is the effect of matings on sexual communication and receptivity in females and males?

3) What is the effect of multiple matings on fecundity and longevity?

Materials and Methods

Insects

Lygocoris pabulinus was reared on potted potato plants, cultivar Bintje, in wooden cages in greenhouses (t = 22 ± 2°C, r.h. = 65 ± 5 %, L18:D6), following Blommers *et al.*, (1997). Every 2-3 days newly emerged adults were collected from the rearing cages, after which the sexes were isolated in separate rearing cages. In this way, virgin males and females of known age were continuously available (see Groot *et al.*, 1998). When mated bugs were needed, sexually mature males and females of 5-8 days old were individually collected from the rearing cages in small glass tubes, after which one pair was introduced in glass Petri dishes (diameter 5 cm) and observed until mating.

Histological analysis of female reproductive organs and sperm transfer

Histological procedures were adapted from Smid (1998). Female *L. pabulinus* were allowed to mate as described above. The duration of a copulation was determined. Females in copula, or at a given time after mating (Table 1), were rapidly decapitated and immediately perfusion-fixed, by means of an injection of approximately one ml of fixative into the abdomen. Females that were fixed 24 and 48 h after mating, were kept in a greenhouse during this period, in plastic dishes with gauze lids containing a potato sprout and pollen. The pollen was a mixture of different plant species, bought at a commercial bee-station (Bijenhuis, Wageningen, the Netherlands). For analysis of polyandrous females, 15 additional females were fixed, that had been caged together with 8 virgin 5-8 day-old males for three days in a small rearing cage with potato plants and pollen grains.

TABLE 1. Number of females fixed at specific times after mating.

n females	x time mated	fixed x time after mating	spermatophore present/absent	spermatophore compartmentalized/disintegrated
3	< 60 sec	during mating	present in all	compartmentalized in all
4	1-2 min	after 2-5 min	present in all	compartmentalized in all
2	1-2 min	after 10 min	present in all	compartmentalized in all
2	< 1 min	after 20 min	present in all	compartmentalized in all
4	30 sec - 2 min	after 40-70 min	present in all	compartmentalized in all
1	10 sec	after 60 min	absent	
4	1-2 min	after 24 hours	present in all	disintegrated in all
2	1-2 min	after 48 hours	present in all	disintegrated in all
2	1-2 min	directly after second mating	2 present in both	one disintegrated, one compartmentalized

As fixation fluid, we used Bouin Hollande Sublimé (BHS) (Vieillemaringe *et al.*, 1984). After the perfusion procedure, abdomens were separated from the thorax and stored for one h in BHS. The female reproductive tissues, stabilized by the former procedure, were then gently dissected from the abdomen and post-fixed in BHS overnight, washed in several changes of 70 % ethanol overnight, dehydrated and embedded in Paraplast-Plus (Sigma). Serial sections of 10 µm were mounted on poly-L-lysine coated slides. Sections were stained with hematoxylin-eosin according to standard procedures, dehydrated, and mounted in DePeX (Fluka).

Effects of matings on sexual communication and receptivity

Long range attraction. To determine attractivity of mated females from a distance, Y-track olfactometer assays were conducted as described by Visser and Piron (1998). To suppress flight intention of *L. pabulinus* males, the Y-track was placed in a black box under a halogen lamp (4-12 V DC, 10 VA), which was placed in a black socket sealed with a red filter, so that the light intensity at the base of the Y-track was reduced to 6.3-6.5 lux. Females were first allowed to mate. Subsequently, they were immediately placed in a 250 ml glass bottle (h. 14 cm, diam. 6.5 cm) together with two sprouting potato stems in wet oasis plus pollen. In total 5 females were introduced in one bottle. The control glass bottles contained only wet oasis and pollen grains. Both bottles were placed outside the black box, one on each side, under a desk-lamp with a 25 W light bulb. Five experiments were conducted 1-2 hours after females had mated, and four experiments 3-5 hours after mating. Each experiment consisted of virgin 20 males (5-8 days old after the final moult), choosing between females or control. After every 5 males the bottles, glass tubes and connecting tubes were exchanged to correct for unforeseen asymmetry.

Close-range attraction. Specific courtship behaviour of male *L. pabulinus*, *i.e.* vibration of the abdomen (Groot *et al.*, 1998) was used to determine attraction of mated females at close range. Females were allowed to mate as described above. Directly after mating, they were transferred to clean Petri dishes, one per dish. One virgin male, collected from the rearing 1-2 hours in advance, was then introduced into a dish with a mated female. When a male started to vibrate within 15 min after introduction, this was counted as a positive response. To determine if males would still show this specific courtship behavior after they had mated, recently mated males (0-90 min ago) were placed in Petri dishes with virgin, 5-8 day-old females, and observed for 30 min.

Receptivity after mating. Mating experiments were conducted as above, after which mated females were placed in a rearing cage with a similar amount of virgin males and potato plants, forming group A. The mated males were caged with virgin females, forming group B. As a control, 40 virgin males were caged with 40 virgin females. 24 Hours later, all bugs were isolated, and the females dissected. In group A the number of females containing two spermatophores were counted as remated females, in group B the number of spermatophores in females was counted as the number of remated males.

Effects of multiple matings on fecundity and longevity

Two experiments were conducted to determine the effect of multiple matings on fertility and fecundity of female *L. pabulinus*. In experiment I, oviposition rate of individual females was determined from the start of their sexual maturity (*i.e.* 5-7 days after the final moult, see Groot *et al.*, 1998) to death. Virgin, 5-7-day-old females were collected from the rearing and placed in glass cylinders (h 22 cm, diam 5 cm), one female per cylinder. These cylinders were placed over individual potato stems, that were planted out in trays filled with soil. Some pollen grains were added on the soil near every potato stem. Every two days the cylinders with females were transferred to new trays with potato stems. The number of eggs laid in the used potato stems were counted using a stereo microscope.

46 Females were divided into three groups. In group A, females were allowed to mate at the start of the experiment by introducing one 6-8-day-old male per cylinder for 24 h. After one week this procedure was repeated to allow for a second mating. In group B, two males were constantly present in each cylinder to allow females to mate throughout the experiment. Every time the cylinders were transferred, both males were renewed. In group C, females were kept virgin.

In experiment II, oviposition rates of three groups of 40 females were determined from their sexual maturity until less than 5 females were alive in one group. 120 Virgin 5-10-day-old females were collected from the rearing and divided over three empty rearing cages with potato plants. To create suboptimal feeding conditions, no pollen was added, so that possible nutritive advantages for multiple matings would be more obvious. In cage A and B 40 males (5-8 days old) were added and removed after 24 hours. The females in these cages were checked for the presence of a spermatophore, which is visible through the cuticle and thus an indication for their mating status. This procedure was repeated every week for cage B, so that females in that cage could mate once a week. The number of males introduced was similar to the number of females alive. In cage C no males were added throughout the experiment. Every 3-4 days the number of surviving females was determined, and the plants were exchanged for new ones.

Fertility of eggs was estimated in experiment II for the eggs laid in plants at day 29 and day 42, by storing these plants in separate rearing cages. Hatchlings were counted after 14 days.

Results

Morphology of female genitalia

Figure 1 shows schematic drawings of the female reproductive tract from different views. Letters and numbers in the subsequent description correspond with the letters and numbers in the figures. Exteriorly the ovipositor is clearly visible on the ventral side of the abdomen (Fig. 1A, insertion). The ovipositor consists of two pairs of gonapophyses, the posterior (1) and the anterior (2) gonapophyses (Fig. 1A).

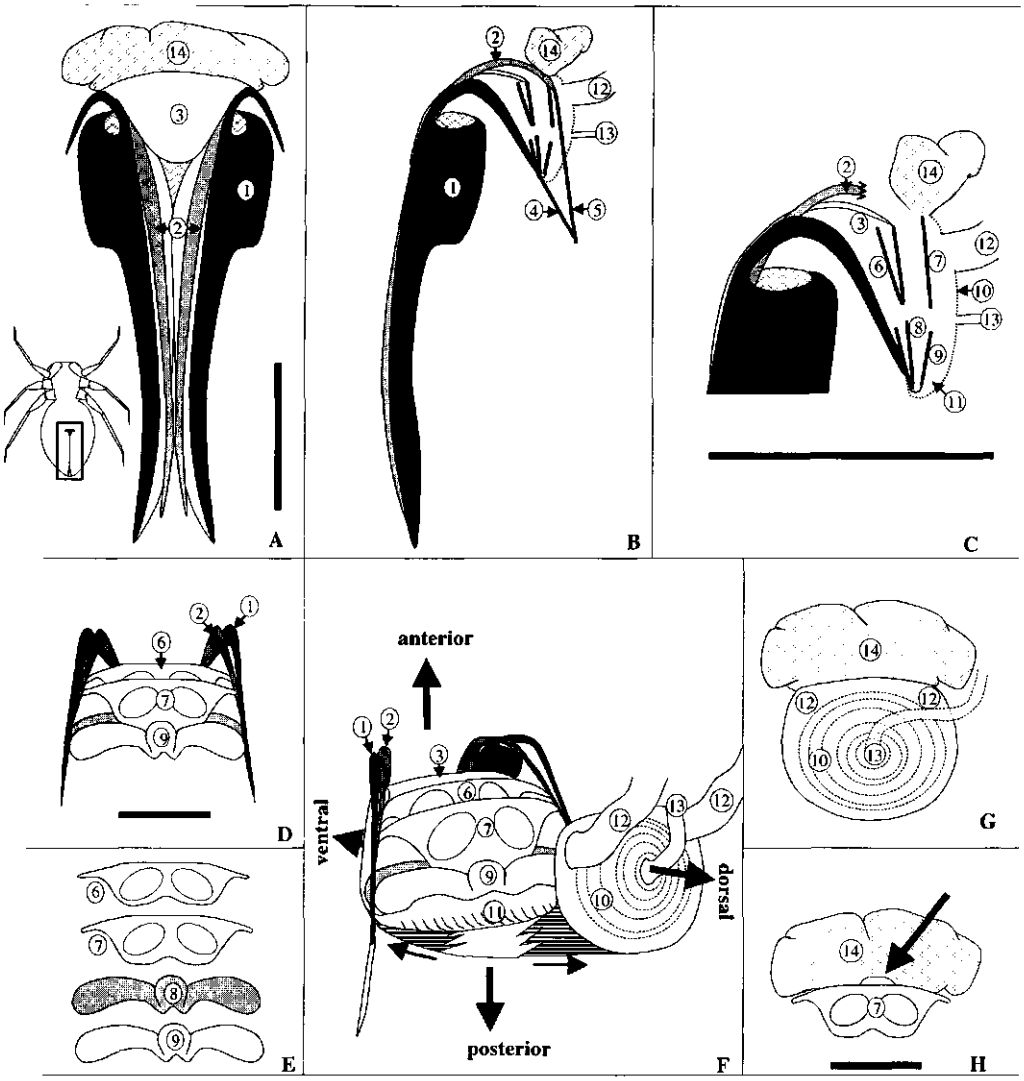

FIGURE 1. Schematic drawings of *L. pabulinus* ovipositor. Scale bars: ± 0.5 mm. **A**: ventral view of ovipositor dissected from body, showing posterior (1) and anterior (2) gonapophyses with subgenital plate (3) (insert: position of ovipositor on female body); **B**: lateral view with 'Verbindungsstucke' (4 and 5) of gonapophyses; **C**: enlargement of interior plates connecting to gonapophyses; **D, E**: position and shapes of inner plates of gonapophyses ((6) and (7): ventral and dorsal plate of anterior gonapophyses, (8) and (9): ventral and dorsal plate of posterior gonapophyses); **F**: schematic view of the bursa copulatrix ((11): median oviduct space, (12): lateral oviducts, (13) spermathecal gland; spermatheca omitted for clarity); **G**: connection of spermatheca to plate of median oviduct (10) (lateral oviducts omitted for clarity); **H**: connection of spermatheca to the dorsal plate (7) of anterior gonapophyses (arrow indicating medial opening).

Ventrally, both blades of the posterior gonapophyses bear a membrane. Dorsally, the posterior gonapophyses are also connected by a membrane, thus forming a gutter. The genital opening of *L. pabulinus* is covered by the subgenital plate (3), which is connected to the lateral sides of the posterior gonapophyses. The genital opening is opened by rotation of the gonapophyses (see below).

Both pairs of gonapophyses curve into the females' interior, the posterior gonapophyses posteriorly and the anterior gonapophyses anteriorly (Fig. 1B-C). Inside the female, in between both gonapophyses, two sclerotized plates are laterally connected, plate 6 and 7 to the anterior gonapophyses (only partly shown in the drawing), plate 8 and 9 to the posterior gonapophyses. The subgenital plate, also curving into the females' interior, is medially connected to plate 6. Both ventral plates, *i.e.* plate 6 and 8, consist of two layers (Fig. 1B-C). Plate 6 and 7 contain two ringed sclerites (Fig. 1D-F), and are only laterally connected to each other via the anterior gonapophyses. Plates 8 and 9 of the posterior gonapophyses are laterally connected as well as, but in addition they are posteriorly connected by means of a membrane (Fig. 1C). Together, plates 6 to 9 enclose the space for the mating plug (see below), and, thus, form the bursa copulatrix of *L. pabulinus* females.

Dorsally from the bursa copulatrix, a circular plate (10) constitutes the dorsal part of the median oviduct, which is enclosed by a membrane connected to the posterior rim to this plate and to the ventral side of plate 8 (Fig. 1F). The lateral oviducts (12) debouch into the median oviduct through plate 10. The accessory gland (13) connects to plate 10 central-dorsally (Fig. 1F, G). This gland is long, tubular and coils around the spermatheca.

The spermatheca (14) is ventrally connected to the anterior rim of plate 7, and dorsally to plate 10 (Fig. 1G, H). This connection is entirely over the rim of plate 10, but only partially on plate 7, thereby leaving a medial opening (Fig. 1H).

Sperm transfer, storage and displacement

In copula, the male aedeagus enters the genital opening posterior from the subgenital plate, thereby causing both gonapophyses to rotate almost 90 degrees from the females' abdomen (Fig. 2, insertion). The aedeagus enters the bursa copulatrix, and the endophallus enters the spermatheca via the medial opening on plate 7, after which the contents of the spermatophore are deposited in the spermatheca. In all females but one (n=24), that were fixed in copula up to one hour after mating, a complete spermatophore was found, even in the ones that had been in copula for only 30 sec (Table 1). The female not containing a spermatophore had mated for only 10 sec.

The spermatophore is compartmentalized into three compartments (Fig. 2, Fig. 3a). A dark-colored substance (15) fills the entire bursa copulatrix and protrudes ventrally into the spermatheca (Fig. 2A, D, Fig. 3b). Since this substance blocks entrance to the bursa copulatrix and spermatheca, we call this part of the spermatophore the mating plug. In the spermatheca, antero-medially of the mating plug, is a sperm-containing compartment (16), and to both lateral sides a large sperm-free mass is attached (17). To get an idea of the amount of male-derived

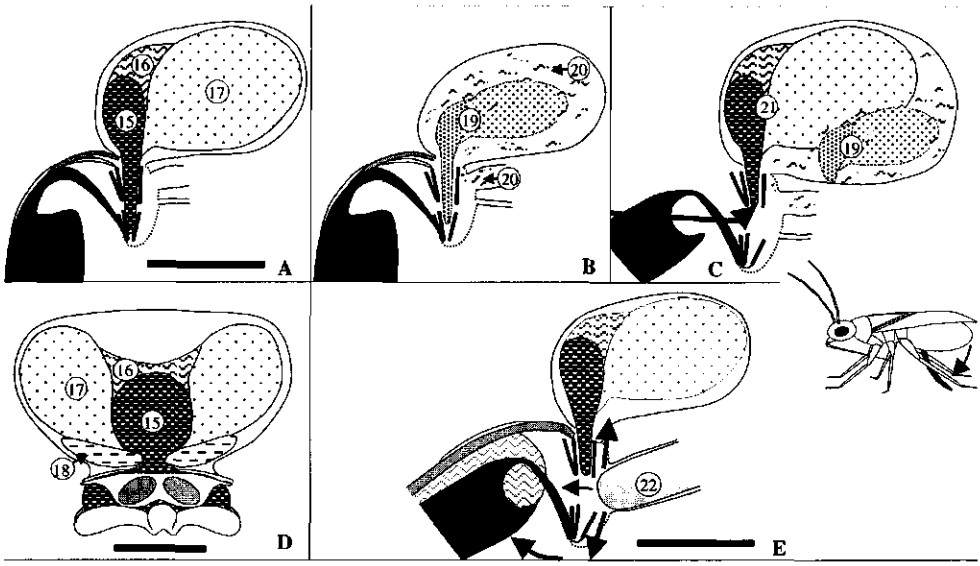

FIGURE 2. A-D: Schematic drawings of spermatophore in female. Scale bars: ± 0.5 mm. **A**: Lateral view of spermatophore ((15): mating plug protruding into bursa copulatrix, (16) sperm-containing compartment, (17) sperm-free mass); **B**: reduced spermatophore (19) after 24 h, with sperm (20) loose in spermatheca and in lateral oviducts; **C**: Second spermatophore (21) forces reduced spermatophore inside spermatheca (arrow indicates position of aedeagus when spermatophore is formed); **D**: ventral view of spermatophore in spermatheca with mating plug between the plates of gonapophyses ((18) amorphous substance). **E**: Schematic reproduction of spermatophore movement during oviposition ((22): egg in lateral oviduct). The mating plug does not break, but is lifted in anterior direction. A small portion of the mating plug remains between the plates of the posterior gonapophyses. Inserted drawing shows position of ovipositor during mating and oviposition).

substances transferred to females, spermatophores were weighed relative to the weight of males and females. After matings, spermatophores were dissected from mated females. Directly after dissection, each spermatophore was weighed on a Cahn C33-microbalance, as well as mated males and virgin females. The amount of male derived substances transferred to females during first matings was ca 5.2 % relative to male body weight and ca 2.6 % relative to female body weight (Table 2).

TABLE 2. Spermatophore weight in relation to bug weight

	spermatophores	**mated males**	**virgin females**
n:	14	15	17
mean weight (g) ± s.d.:	0.23 ± 0.04	4.06 ± 0.35	7.07 ± 0.81
min - max weight (g):	0.14 - 0.32	3.38 - 4.67	4.98 - 8.70
% weight (min - max):		***5.2 % (3 - 9.4)***	***2.6 % (1.6 - 6.4)***

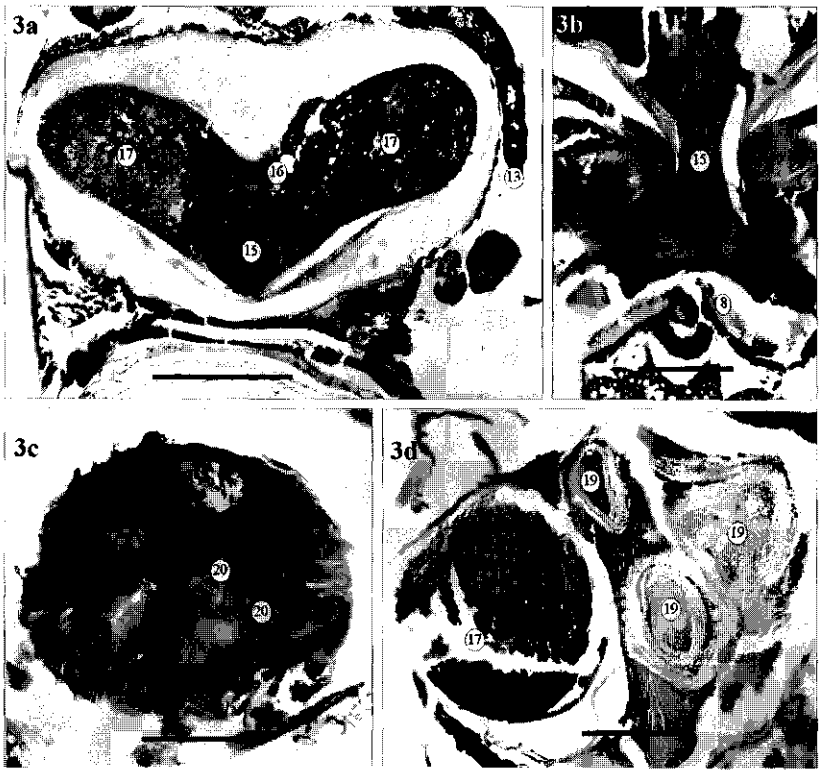

FIGURE 3. a-d: Sections of female reproductive organs, fixed at various times after matings. **3a**: Longitudinal section fo compartmentalized spermatophore, showing part of the mating plug (15), the sperm-containing compartment (16), and the sperm-free mass (17) (one h after mating; (13): spermathecal gland). Scale bar: 250 µm. **3b**: Longitudinal section of the mating plug (15) protruding into bursa copulatrix, also showing part of the ventral plate of the posterior gonapophyses (8) (one h after mating). Scale bar: 100 µm. **3c**: Cross section of the lateral oviduct with loose sperm (20) (24 h after mating). Scale bar: 75 µm. **3d**: Cross section of the spermatheca, showing three disintegrated, reduced spermatophores (19) and one large, compartmentalized spermatophore (cut through the sperm-free mass (17)) (one h after the last of four matings). Scale bar: 150 µm.

All females fixed 24 h after copulation, contained less voluminous spermatophores that were partially disintegrated (Table 1, Fig. 2B), *i.e.* the mating plug was still intact, but the separation between the sperm-containing compartment and sperm-free mass in the spermatheca had disappeared. Both the sperm-free mass and sperm were found throughout the spermatheca. In all these females sperm was also found in the median and lateral oviducts (Fig. 3c). Sperm was not found in or along the mating plug, indicating that sperm passes from spermatheca to oviduct through the medial opening of the spermatheca dorsally of plate 7 (Fig. 1H). In females fixed after 48 h the mating plug was still present, but reduced. In the two

females fixed directly after a second mating, the spermatheca contained dorsally one small disintegrated spermatophore with a disrupted mating plug, and ventrally one large compartmentalized spermatophore with mating plug protruding into the bursa copulatrix (Fig 2C). Of the females that had been caged for three days with males, four did not contain a spermatophore (hence they had not mated), four contained one compartmentalized spermatophore (*i.e.* recently mated females), one contained a disintegrated spermatophore (mated more than 1 h ago), and five contained 1-3 small spermatophores without a mating plug and one large, compartmentalized spermatophore (mated 2-4 times in three days; Fig. 3d).

The presence of a mating plug in the bursa copulatrix up to three days after mating raised the question how eggs pass through to the ovipositor. To determine this, we dissected recently mated females using a stereo microscope, and gently pushed a pair of small tweezers through the median oviduct towards the ovipositor. Through this movement the mating plug was lifted in anterior direction between plate 6 and 7 of the anterior gonapophyses, thereby allowing a passage of eggs towards the ovipositor (Fig. 2E).

Effects of matings on sexual communication and receptivity

At long range, *i.e.* in the Y-track olfactometer, females mated 1-2 h before the tests, were not significantly more attractive to males than the control when the five experiments were pooled (Fig. 4). Three to five hours after mating, significantly more males walked towards the females than towards the control, which is a similar response as towards virgin females (Groot *et al.*, 2000).

At close range, 16 of the 17 males vibrated within 15 min when placed in Petri dishes with females that had mated 0-30 min before. In comparison, 14 of the 16 males vibrated when exposed to virgin 5-8 day-old females. None of the males that had mated 0 - 90 min before, started to vibrate within 30 min when exposed to virgin females (n=19).

24 H after the first mating, 6 % of the females from group A (mated females x virgin males) contained two spermatophores (n=35). Hence, 24 h after a mating females may be attractive, but not receptive. In group B (virgin females x mated males) 23 % of the females contained a spermatophore, which is a measure of the number of males that remated (n=26). In the control group (virgin females x virgin males), all females contained a spermatophore (n=40).

Effects of multiple matings on fecundity and longevity

The mean oviposition rate of individual females (experiment I) significantly differed between the three groups (Fig. 5). Total number of eggs laid was highest in group A, where males were introduced twice for 24 h, less in group B, and lowest in group C, the virgin females. That females of group B oviposited less eggs than females of group A, suggests sexual harassment of males, as these females were constantly confined with two males. The mean oviposition rate of the three groups of females (experiment II) did not significantly differ between the three groups, although the mean oviposition rate in virgin females (group C) was 1-3 eggs per day, while mated

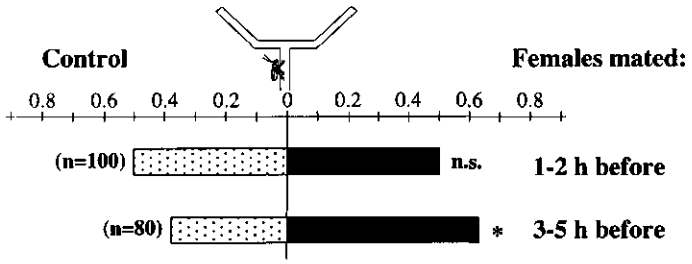

FIGURE 4. Attraction of recently mated females in Y-track olfactometer. Each bar represents the mean fraction of males choosing, after summing all tests. Bar 1-2 h after females had mated: five tests pooled; bar 3-5 h after females had mated: four tests pooled. Data were analysed by using the two-sided binomial test; n.s.: $P > 0.05$, * $P < 0.05$.

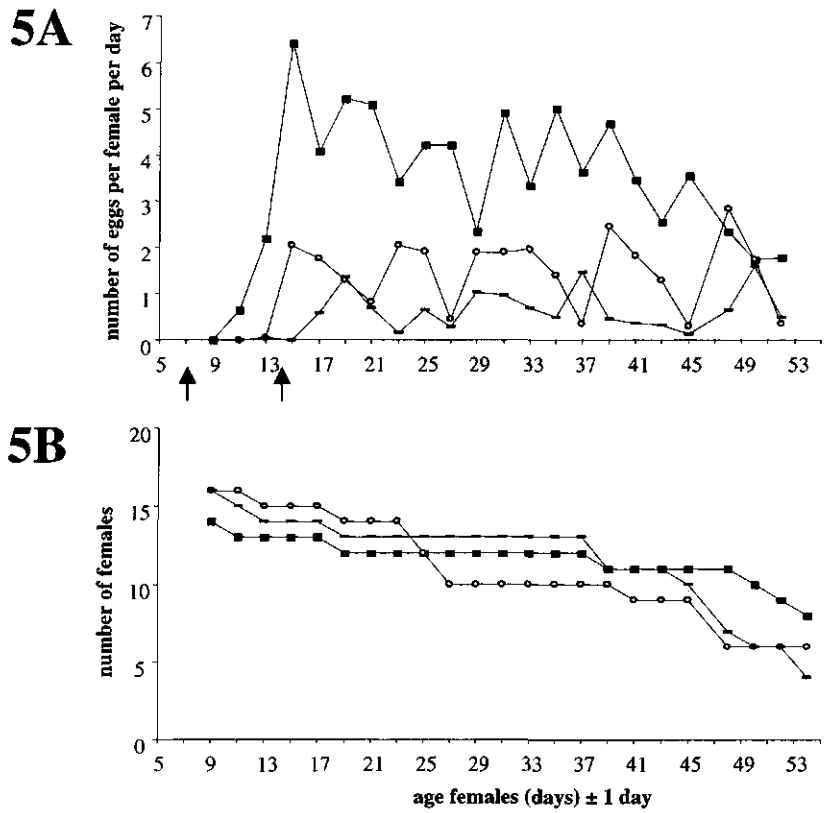

FIGURE 5. Effects of multiple matings on fecundity and longevity, experiment I. 5a: Mean oviposition rate of individually caged females, 5b: number of females surviving. Black squares: group A, females with males for 2 x 24 h (day 7 and day 14, arrows). White circles: group B, females constantly with 2 males. Stripes: group C, virgin females. Overall fecundity difference between the groups was determined with ANOVA, after square-root transforming all data to fit a normal distribution. As $P < 0.05$, fecundity differences between the three groups were compared with Fisher's least significant difference procedure. All groups significantly differed from each other ($P < 0.05$).

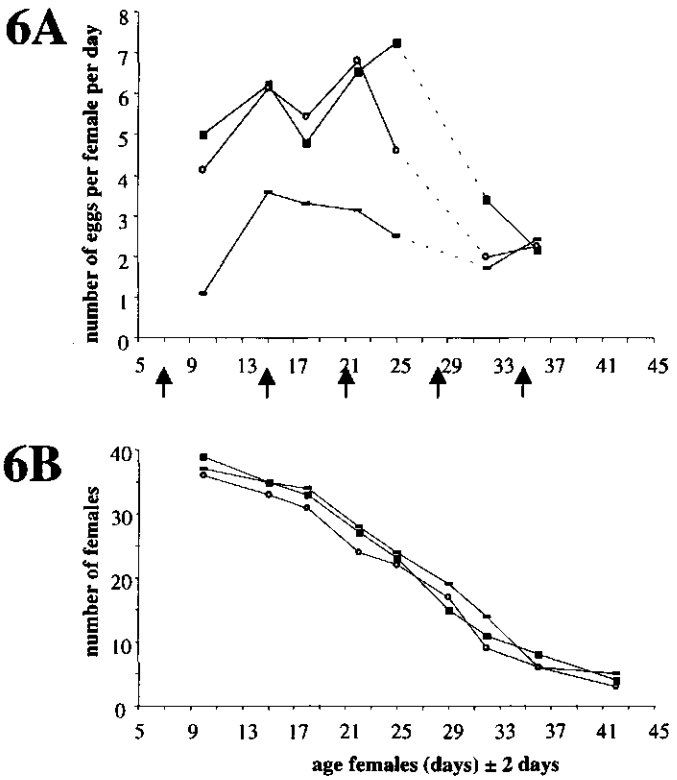

FIGURE 6. Effects of multiple matings on fecundity and longevity, experiment II. 6a: Mean oviposition rate of individually caged females, 6b: number of females surviving. Black squares: group A, females with males 1 x 24 h (first arrow). White circles: group B, females with males once every week for 24 h (arrows). Stripes: group C, virgin females. Overall fecundity difference between the groups was determined with ANOVA; $P > 0.05$.

females of group A and B laid 4-7 eggs per day (Fig. 6). Differences in fertility between group A and B in experiment II were not apparent either, the number of nymphs emerging from the plants after 14 days was ca 80-100 in both groups for the oviposited eggs laid at day 29, and ca 50-60 in both groups for the eggs laid at day 42. No eggs hatched from group C. In both experiment I and II the number of surviving females was similar in the three groups (Fig. 5 and 6), which means that we did not observe differences in longevity.

Discussion

Morphology of female genitalia

Mirid female genitalia have been studied to some extent (Petherbridge and Thorpe, 1928; Kullenberg, 1946; Slater, 1950; Davis, 1955; Strong *et al.*, 1970). However, the entire female reproductive organs have not been studied in such detail as presented here. Slater (1950) analysed specific structures of female genitalia (*i.e.* the sclerotized rings on plate 6 and 7) for taxonomic purposes, but only after treatment with 5 % hot potassium hydroxide solution, so that the overall structure of reproductive organs was destroyed. The presence of a spermatophore in the spermatheca after mating has not been recognized before in Miridae, which is probably due to general or partial analyses. Some other structures distinguished here have not been described before either, *i.e.* both plates (8 and 9) of the posterior gonapophyses and the circular plate (10) of the median oviduct. To allow for comparison of this study with existing descriptions on female insect genitalia in general and mirid genitalia in particular, we summarized existing terms used in mirid studies (Table 3) and chose for terms referring to the position in the female and their function where possible.

Sperm transfer, storage and displacement

Spermatophore formation. As only complete spermatophores were found in fixed females, even when they had been in copula for as short as 30 sec, we have not been able to study spermatophore formation. We never found spermatophores in dissected virgin, sexually mature males, and strongly suspect that formation takes place in the female, as in most insects (Gillot, 1988). Bonhag and Wick (1953) studied erection of the aedeagus in the pentatomid bug *Oncopeltus fasciatus* in detail, and found that the endophallus is surrounded by a so-called vesica. This is described as an elongate, coiled bladder, folded in the phallobase when at rest and filling with "transparent erection fluid" during copulation, thereby greatly enlarging in a balloon-

TABLE 3. Terminology of female reproductive structures, found in literature on mirids

terms used here:		terms used by other authors:
gonapophyses:	-	valvulae[1]
anterior gonapophyses[2, 3] (2)*:	-	first valvulae[1]; gonapophyses VIII[2]
posterior gonapophyses (1):	-	second valvulae[1]; gonapophyses IX[2]
interior connection between gonaophyses (4 and 5)	-	rami[1]; Verbindungsstuck[4]
ventral plate of anterior gonapophyses (6):	-	ventral labiate plate[1]; vorderer Wand der Bursa Copulatrix[4]
dorsal plate of anterior gonapophyses (7):	-	dorsal labiate plate[1]; hinterer Wand der Bursa Copulatrix[4], dorsal wall of the bursa copulatrix[5]
sclerotized rings (in plate 6 and 7)[5]:	-	ringed glands[1]; Chitingschlinge[4]
spermatheca (14):	-	seminal depository[1,6]; receptaculum seminis[3]

*numbers between brackets refer to numbers used in Figures. [1]Davis 1955; [2]Dupuis & Carvalho, 1956; [3]Tuxen 1956, [4]Kullenberg 1946; [5]Slater 1950; [6]Strong et al, 1970

like manner. In *L. pabulinus* pairs that were fixed in copula, a similar balloon-like structure seemed to erect from the aedeagus (data not shown), extending into the spermatheca and surrounding the spermatophore. Such a structure was also found in the blood-sucking bug *Rhodnius prolixus* (Davey, 1960), where the everted sac was called the spermatophore sac, as it contained a spermatophore when in copula for 10 min. Spermatophores can be formed in such a short a period as 30 sec, as has been demonstrated in ticks (Feldman-Muhsam and Borut, 1983).

We refer to the part of the spermatophore, that blocks the entrance to the bursa copulatrix and spermatheca, as the mating plug. Mating plugs blocking the female reproductive tract have been found in a number of insects (Thornhill and Alcock, 1983; Lachmann, 1998). In *Lygus hesperus* a mating plug was also described, when Strong *et al.*, (1970) noted a substance "which fills the genital pouch after mating, similar to that found only in the medial pair of male accessory glands", although this was not identified as a mating plug or as part of a spermatophore. In *Rhodnius* bugs, mating plugs have been described as well (Davey, 1960). Mating plugs may serve a nutritional function once dissolved, but act as a mating plug until then, since females cannot accept additional spermatophores before dissolution (Thornhill and Alcock, 1983). As such, mating plugs induce a mechanical refractory period (Vahed, 1999). In the pentatomid bug *Thyanta pallidovirens*, a refractory period was induced by a "sclerotized rod blocking the spermathecal opening" as well (Wang and Millar, 1997). The refractory period in *L. pabulinus* females induced this way lasts up to 24 h, after which the mating plug seems to have lost its effectiveness, since 6 % of the females remated successfully within 24 h. This loss of effectiveness is not due to a disruption, as we did not find any disrupted mating plugs in females that had mated only once, but is probably due to a reduction of the mating plug. Fertilization of eggs most likely takes place in the lateral oviducts, as sperm is released from the spermatophore into the median and lateral oviducts within 24 h.

Sperm displacement. L. pabulinus females fixed directly after a second mating contained a partly disintegrated spermatophore with a disrupted mating plug, and a large and intact spermatophore. As sperm was still found in the oviducts of these females, sperm displacement by the second male does not seem to occur. More likely, after disintegration of the new spermatophore, sperm mixing takes place in the oviducts. Sperm mixing was also found in the Southern green stink bug, *Nezara viridula* (McLain, 1985).

Effects of matings on sexual communication and receptivity

Lygocoris pabulinus females seem to resume sex pheromone release a few hours after mating, as males were attracted to females 3-5 h after the latter had mated. At close range, no effect was found, males vibrated as readily when exposed to mated females as when exposed to virgin females. However, we did not investigate a possible preference of males for virgin or mated females. As males have an increased chance of fertilizing eggs in virgin females, mated females may be less attractive than virgin females in the field, which has been shown in the tettigoniid *Requena verticalis* (Lynam *et al.*, 1992). Also, mated apple moth females produce smaller

amounts of pheromone, suggesting that virgin females may elicit responses from males at greater distances than do mated females (Foster and Ayers, 1996).

Sexual communication in *L. pabulinus* is also affected by the mating status of males. For two h after males had mating, they did not respond to virgin females. Also, only 23 % of the males mated for a second time within 24 h. In addition, males transferred ca 5 % of their body weight to females during a mating, which is in the range of spermatophore masses found in Lepidoptera, where spermatophore masses range from 1 to 15 % of male body mass (Vahed, 1999). After transferring such an amount, males probably need a refractory period to be able to produce a new spermatophore. Collectively, these results suggest a high paternal investment. As a consequence, an increased fecundity and/or longevity in polyandrous females may be expected.

Effects of multiple matings on fecundity and longevity

In spite of the relatively large amount of male-derived substances transferred into females during mating, no positive effect was found on total fecundity or longevity in females that had mated multiply. However, in experiment I such an effect may have been nullified by sexual harassment, as each female was constantly confined with two virgin males, that were refreshed every two days. On the other hand, in many insects multiple matings do not result in an overall increased fecundity and/or longevity (Kanoh *et al.*, 1983; Simmons, 1988; Vahed, 1999). An increase in fecundity may occur only directly after mating (Boggs and Gilbert, 1979; Huignard, 1983; Simmons and Parker, 1989; Oberhauser, 1997; Eberhard ,1998). This would not result in a life-time fecundity increase, but in a temporary increase in oviposition directly after each mating. When such a temporary shift is caused by male-derived nutrients, and the nutrients are incorporated into eggs that are fertilized by the same male, these nutrients can be considered as a form of paternal investment of males (Trivers, 1972; Simmons, 1988; Vahed, 1999).

In some insects incorporation of male-derived nutrients into eggs has been found within 24 h (Huignard 1983; Simmons and Parker, 1989). When fecundity is temporarily increased by oviposition stimulants, or by using male-derived nutrients for somatic purposes so that females can switch from feeding to oviposition, male investment is regarded as mating effort (Simmons and Parker, 1989; Vahed, 1999). Specifically, Simmons and Parker (1989) defined mating effort as the effort of males to increase the proportion of eggs he fertilizes from a given female, or by increasing mating opportunities. To distinguish between these two possibilities, the effort to increase the proportion of eggs fertilized may be more appropriately called fertilization effort.

Paternal confidence is highest in virgin females where sperm competition is lacking. In *L. pabulinus* this paternal confidence lasts until sperm of a second male arrives in the oviducts, *i.e.* after disintegration of the first male mating plug and the release of sperm from a second male spermatophore into the oviducts. Fertilization effort may hence be enlarged when larger spermatophores are transferred to virgin females. Spermatophore size was shown to affect female refractory period in many

Lepidoptera, larger spermatophores causing a longer refractory period, sometimes even resulting in females never remating (*e.g.* Boggs, 1981; Oberhauser, 1989; Torres-Villa *et al.*, 1997). As a consequence, males may transfer larger spermatophores in virgin females than in mated females.

Spermatophore size may not only depend on the mating status of females, but also on the mating status of males. For example, the mating status of male moths affected spermatophore size and the length of refractory period in females (Royer and McNeil, 1993; Foster and Ayers, 1995). Also, a trend towards lower fertility and fecundity of successive mating partners of a given male has been determined (Royer and McNeil, 1993; Ofuya, 1995). It would be interesting to determine if such mechanisms are not restricted to Lepidoptera, but occurring in other insect orders such as Heteroptera as well.

In conclusion, although polyandry in *L. pabulinus* does not seem to affect fecundity or longevity at first sight, multiple matings of males and females may affect the reproductive output in a less obvious way. This study does show an effect of multiple matings on sexual communication. At long range females were unattractive for a few hours after mating, although at close range they remained attractive. However, mated males were not responsive at close range to females for at least two hours after they had mated. Hence, sexual attraction of *L. pabulinus* males towards females may not depend on whether or not females release sex pheromone, but more on whether or not males are responsive to females.

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PART III:

The female perspective

Chapter 8

OVIPOSITION PREFERENCE OF *LYGOCORIS PABULINUS* IN RELATION TO PLANTS AND CONSPECIFICS*

ASTRID T. GROOT, ANNEKE HEIJBOER, J. HANS VISSER AND MARCEL DICKE

ABSTRACT - To predict possible locations of *Lygocoris pabulinus* (L.) in the field during the summer, we determined their oviposition preference under summer conditions. With *L. pabulinus* reared on potato, oviposition preference was determined for potato, tomato or green bean. As preference may depend on larval or early adult experience, oviposition preference was also determined of bugs, reared on green bean for 3 generations, and of bugs captured from the field 12 h prior to the experiment. All females showed a strong preference for potato plants, on which fecundity was higher. Hence, although *L. pabulinus* is a generalist in its feeding habits, the summer generation seems to be an oviposition specialist. Aggregation of ovipositing females does not seem to occur; similar amounts of eggs were oviposited in plants with clip cages containing conspecifics than in plants without conspecifics. More eggs were oviposited in damaged plants than in undamaged plants. Plant volatiles released upon damage may aid *L. pabulinus* females in finding suitable oviposition sites.

KEY WORDS: Heteroptera, Miridae, *Solanum tuberosum*, *S. lycopersicum*, *Phaseolus vulgaris*, fecundity, feeding generalist, oviposition specialist

Introduction

The spatial distribution of insect species is determined to a large extent by the oviposition behaviour of females, when their larvae have limited dispersal capabilities (Huignard *et al.*, 1986; Hanks, 1999). Females select host plants as oviposition sites and may exhibit an oviposition preference for specific plants. Such a preference can be influenced by several factors. For example, oviposition preference can be induced by previous experience of females as larvae. This has been demonstrated in some species (Jermy *et al.*, 1968), but not in others (Propoky *et al.*, 1982; Holopainen, 1989; Ntonifor *et al.*, 1996). Oviposition preference may also be influenced by early adult experience (Traynier, 1984; Jaenike, 1990; Vet and Papaj, 1992; Turlings *et al.*, 1993), as experience on a previous host may affect fertility on a

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subsequent host (Jaenike, 1990; Cunningham *et al.*, 1998). Other factors influencing oviposition preference are competition for oviposition sites (Rothschild and Schoonhoven, 1977; Wood, 1982; Finch and Jones, 1987; Jaenike, 1990; Thompson and Pellmyr, 1991; Dicke, 2000), the presence or absence of natural enemies (Root, 1973; Andow, 1991; Propoky and Lewis, 1993; Ohsaki and Sato, 1994; Dicke, 2000), and distribution of potential host plants (Wiklund, 1982; Stanton, 1983; Andow, 1991; McLain, 1992; Päts *et al.*, 1997). Females are likely to discover or encounter large host plant patches sooner than small patches (Southwood, 1987). Favourable conditions in these patches for growth, survival, and reproduction may attract other searching females, and inhibit emigration or dispersal from the area (Stanton, 1983; Andow 1991). Oviposition preference for specific plants will decrease when the egg load in searching females increases (Singer, 1982; Jaenike, 1990; Mangel, 1993).

This study investigates oviposition preference of the green capsid bug, *Lygocoris pabulinus* (L.) (Heteroptera; Miridae), an unpredictable pest in fruit orchards in Northwestern Europe. Its presence is unpredictable, because *L. pabulinus* migrates twice a year. In autumn, females oviposit overwintering eggs in woody fruit trees, such as apple, pear, or raspberry (Hill, 1952; Blommers, 1994; Ravn and Rasmussen, 1996), after which the adults die. In spring, nymphs emerge from these eggs and feed on shoot tips and flower buds, thereby causing rusetted malformations in the fruits, which determines their pest status. When the nymphs become adult, they migrate to herbaceous plants, where a summer generation develops (Petherbridge and Thorpe, 1928; Kullenberg, 1946; Southwood and Leston, 1959). Adults of this summer generation migrate back to woody hosts to oviposit overwintering eggs when daylength shortens and temperature drops. As migration of ovipositing females in autumn is not likely to occur over distances larger than a few km (Southwood, 1960, 1962), knowledge on the possible location of the summer generation may predict chances of oviposition in different fruit orchards, and hence bug damage the following spring.

Summer hosts to which *L. pabulinus* migrates are potentially numerous; a long list of plants belonging to many different families have been reported to contain green capsid bugs (Bech, 1969; De Jong, 1972). However, feeding habits of adults may not reflect oviposition behaviour in bugs (Pannizi, 1997), *i.e.* females may be specialists in their choice for oviposition sites, and generalists in their feeding behaviour. When a herbaceous underlayer is present in fruit orchards, *L. pabulinus* seems to remain in these orchards throughout the summer (Hill, 1952). In the absence of a herbaceous underlayer, migration of the summer generation is obligatory, as *L. pabulinus* needs herbaceous plants to complete its life cycle (Blommers et al, 1997). Fruit growers in the Netherlands suggest that potato fields are favorite summer shelters for the green capsid bug, as their orchards always seem to have bug damage when potato has been the neighbouring crop the previous year. Potato is usually grown in large fields. In such large crop patches, population development of the green capsid bug may be augmented by increased ovipositions, and searching females may be attracted by ovipositing females (Stanton, 1983;

Southwood, 1987). Eggs of *L. pabulinus* are usually laid individually, but have been found in groups as well (Petherbridge and Thorpe, 1928; Wightman, 1968), indicating that some aggregation may occur.

In this study we determined oviposition preference of *L. pabulinus* for three different host plants: potato, tomato, or green bean. Potato and tomato both belong to the Solanaceae, so that a possible preference for this plant family may be determined. Green bean was arbitrarily chosen to distinguish oviposition preference for a plant, belonging to a different family, in this case the Fabaceae. Specifically, this study addressed the following questions:

1. Do *L. pabulinus* females have an oviposition preference for one of the three plants species?
2. Is oviposition preference related to host plant experience?
3. Is oviposition preference affected by infestation with conspecifics?

Material and Methods

Insects

Lygocoris pabulinus was reared on potato plants (*Solanum tuberosum* L., cultivar Bintje), under summer conditions (t = 22± 2 ° C, RH = 65 ± 5 %, L:D = 18:6, following Blommers *et al.*, 1997). In the rearing, newly emerged adults were collected from the rearing cages every 2-3 days, after which the sexes were placed in separate rearing cages (see Groot *et al.*, 1998). For all oviposition experiments, virgin bugs 5-8 days after their final moult were used, which had been reared on potato for 12-25 generations, unless stated otherwise.

Plants

All plants used were grown in a separate greenhouse with similar climatic conditions, except for raspberry plants, which were seedlings from a raspberry orchard and used directly in the experiment (see below). Potato plants were grown from tubers from Cebeco and Agrico, Zeewolde, the Netherlands. Tomato plants (*Solanum lycopersicum* L., cultivar Moneymaker) were grown from seeds from Fa. Janssen, Dinxpelo, the Netherlands. Green bean plants (*Phaseolus vulgaris* L., cultivar Miracle) were grown from seeds of Pieterpikzonen bv. Heerenveen, the Netherlands.

1a. Oviposition in different host plants

Before testing oviposition preference for certain host plants, oviposition was observed in no-choice situations. A wind tunnel (300 (l) x 130 (w) x 80 (h) cm, laminar airflow ca 12 cm/sec, climatic conditions similar as above; see Griepink, 1997) was filled with either potato plants, tomato plants, or green bean plants. Three small trays with pollen were placed between the plants. 40 Females and a similar amount of males were introduced and recaptured 3-4 days later. As *L. pabulinus* oviposit its eggs *in* the stem of plants and only the operculum of each egg is visible

(see Wightman, 1972), the number of eggs in the plants were counted using a stereo microscope. These experiments were conducted twice with potato and green bean, and three times with tomato.

1b. Oviposition preference

Oviposition preference for potato, tomato or green bean was determined in the wind tunnel, by alternating two rows of one plant species with two rows of another plant species, and three small trays with pollen between the plants. In this way, three choice experiments were conducted: potato versus tomato, potato versus green bean, and tomato versus green bean. All experiments were conducted twice. In each experiment 40 females and a similar amount of males were introduced and recaptured after 3-4 days, after which the eggs in all plants were counted using a stereo microscope.

2. Oviposition preference in relation to host plant experience

To determine if oviposition preference is related to experience on a specific host plant, oviposition preference experiments were conducted with bugs of a different history than the ones reared on potato: (a) bugs reared on green bean, (b) bugs freshly captured from a raspberry field.

2a. Oviposition preference of bugs reared on green bean

A rearing on green bean was started by collecting 45 virgin *L. pabulinus* females and 15 virgin males from the potato rearing, 0-4 days after their final moult. They had been reared on potato for 10 generations. These bugs were placed into a rearing cage with green bean plants. The plants were renewed every 6-7 days. Every 2-3 days, the number of nymphs and adults emerging from these plants were counted, after which the nymphs were placed in a new rearing cage with green bean. The adults were used for oviposition to rear a new generation. After 3 generations, the following oviposition experiment was conducted.

15 Females and 8 males were introduced in a metal cage (50 (l) x 29 (w) x 40 (h) cm), because per time unit less than 20 females developed from the rearing, an amount too small to fill the wind tunnel. The cage contained one potato plant and one green bean plant, and a small tray with pollen. For comparison, a similar cage was filled with 15 females and 8 males from the potato rearing. Both cages were placed in a greenhouse with similar temperature, relative humidity and photoperiod as above. Four days later all bugs were recaptured, and the number of eggs in the the plants were counted using a stereo microscope.

2b. Oviposition preference of bugs collected from the field

Bugs were collected in midsummer from herbaceous plants in an IPM orchard of raspberry (*Rúbus idaéus* L., Rosaceae) in Oirschot, the Netherlands. From the abundant undergrowth in this orchard, 24 *L. pabulinus* females and 22 males could be captured, mainly from a bush of *Solanum nigrum* L. For 12 hours, they were placed in a rearing cage with potato plants, that was transferred to a climate room

with similar conditions as mentioned earlier. 12 H after collection from the field, all bugs were introduced in the wind tunnel, filled with four potato plants, alternating with four green bean plants and three raspberry plants. Three small trays with pollen were placed between the plants. After 5 days, all bugs were recaptured, and the number of eggs per plant was determined as above.

3. Oviposition preference for plants with or without conspecifics

To determine oviposition preference of *L. pabulinus* females for plants containing conspecifics, 20 clip cages (diam. 2.4 cm, height 1.0 cm) were attached to 2 potato plants, 10 per plant. Each clip cage contained a sexually mature, virgin female. As a control, two other potato plants were filled with 20 similar but empty clip cages, and two potato plants were left untreated. The six plants were placed in three rows in the wind tunnel, in such a way that each row contained two different treatments. In a second experiment, two plants were added to the setup, each with 10 clip cages containing one sexually mature, virgin male per cage. In both experiments three small trays with pollen were placed between the plants. 40 Females and a similar amount of males were introduced and recaptured after 3 days, after which the eggs in all plants were counted, using a stereo microscope. Eggs laid by the caged females were not included.

Results

1a. Oviposition on different host plants

In no-choice situations in the wind tunnel, *L. pabulinus* females reared on potato oviposited a similar amount of eggs when potato plants were offered as when green bean plants were offered (Table 1). Less eggs were oviposited in tomato plants. When more tomato plants were offered, the total amount of eggs oviposited per day was less: 60 eggs in 6 plants, 26 eggs in 10 plants, and only 16 eggs in total in 14 plants.

TABLE 1. Oviposition of *L. pabulinus* in plants in wind tunnel (3-4 days)

Plants offered	n plants	Total # eggs laid/day	
potato	6	128	a
potato	14	151	
tomato	6	60	
tomato	10	26	b
tomato	14	16	
green bean	10	87	a
green bean	10	108	

Different letters indicate significant differences between the number of eggs in each plant species (tested with ANOVA for overall significant difference (P = 0.0099), followed by Multiple Range test).

1b. Oviposition preference

When potato plants were alternated with green bean plants, *L. pabulinus* females reared on potato preferentially oviposited in the potato plants (Figure 1). Mixing potato plants with tomato plants once resulted in significantly more eggs in potato plants than in tomato plants, and once a similar amount of eggs laid in both plant species. Tomato plants that were alternated with green bean plants, resulted twice in an oviposition preference for green bean plants. Mean fecundity was lowest in the setups where tomato plants were included (ranging from 1.1 to 1.9 eggs per female per day), and highest when only potato plants had been offered (Table 2).

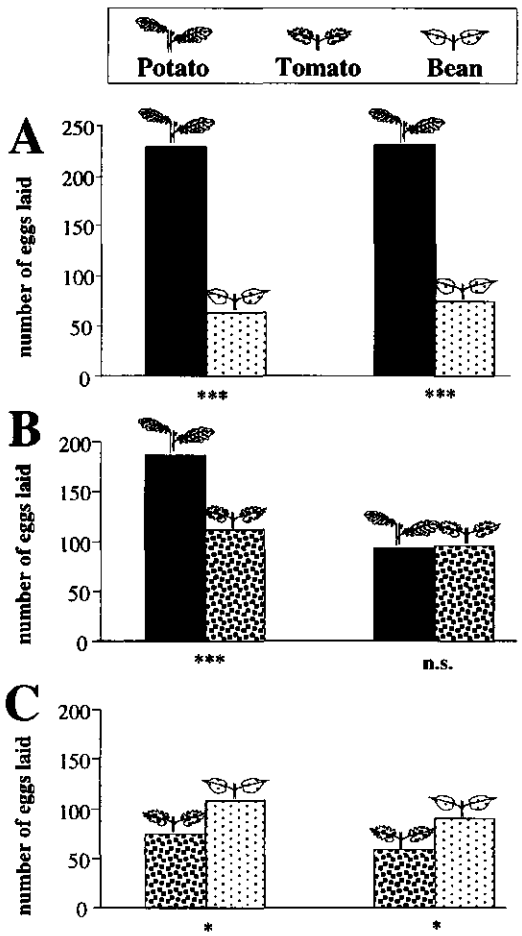

FIGURE 1. Oviposition preference of *L. pabulinus* females in three choice assays. A: potato plants alternated with green bean plants, B: potato plants alternated with tomato plants, C: tomato plants alternated with green bean plants. Black bars: number of eggs in potato, blocked bars: number of eggs in tomato, light bars: number of eggs in bean. *** $P < 0.001$; * $P < 0.05$; n.s.: not significant (Chi-square test for observed and expected frequencies, null hypothesis: number of eggs laid is equal in all plants).

TABLE 2. Fecundity of *L. pabulinus*. Summary from all experiments conducted.

plant	(w/m)[1]	experiment I	experiment II	experiment III	
potato	w	**3.2** (512:4:40)[2]	**3.8** (452:3:40)		**a**
tomato	w	**1.5** (181:3:40)	**0.9** (104:3:40)	**0.4** (64:4:40)	**b**
green bean	w	**2.3** (364:4:40)	**2.7** (324:3:40)		**ac**
potato vs green bean	w	**2.4** (289:3:40)	**2.5** (303:3:40)		**ac**
potato vs tomato	w	**1.2** (188:4:40)	**1.9** (298:4:40)		**bc**
tomato vs green bean	w	**1.1** (183:4:40)	**1.2** (149:3:40)		**b**
potato with clip cages	w	**3.5** (420:3:40)	**3.8** (461:3:40)		**a**
potato vs green bean	m	**1.1** (64:4:15)[3]	**0.3** (19:4:15)[4]		
potato vs bean/raspberry	w	**0.3** (41:5:24)[5]			

[1]W: wind tunnel, m: metal cage; [2]Eggs:days:females, [3]Rearing on potato; [4]Rearing on green bean; [5]Bugs captured from field. See text for further details. Differences in fecundity between the 7 experiments in the wind tunnel were determined by means of the Kruskal-Wallis test ($P = 0.049$), followed by a Box-and Whisker Plot. Fecundity of bugs captured from the field were not included, as the age and history of the females were unknown.

2a. Oviposition preference of females reared on green bean

When being reared on green bean, the number of adult bugs emerging decreased in time. After 7 generations the number of females available for oviposition to rear a new generation was so small, that the rearing was ended. Females that had been reared on green bean for three generations, oviposited 18 eggs in potato and only 1 egg in green bean (Figure 2b). For comparison, the females that had been reared on potato oviposited 61 eggs in potato and 3 eggs in green bean (Figure 2a).

2b. Oviposition preference of females captured from the field

Lygocoris pabulinus females captured from the raspberry orchard 12 hours earlier, oviposited 40 eggs in total in potato plants, 1 egg in green bean, and no eggs were oviposited in the raspberry plants (Figure 2c).

3. Oviposition preference for potato plants with or without conspecifics

Significantly more eggs were oviposited in plants with clip cages than in plants without clip cages, when both experiments are considered (Table 3). The clip cages themselves damaged the plant; in the course of the three days that the experiments lasted, the leaf sections within the cages had wilted. No differences were found between the number of eggs in plants with conspecifics and plants without conspecifics (*i.e.* plants with clip cages plus conspecifics and plants with empty clip cages).

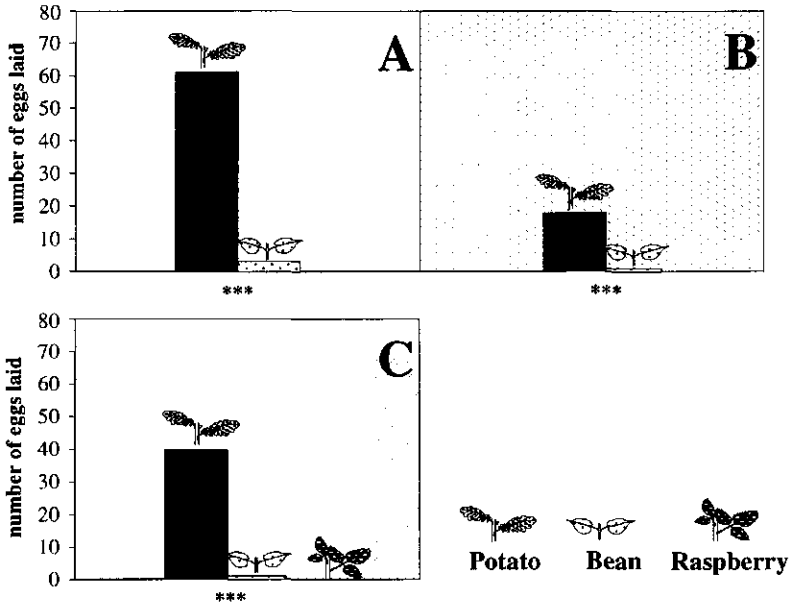

FIGURE 2. Oviposition preference of *L. pabulinus* females with different backgrounds. A: Bugs reared on potato, B: bugs reared on green bean, C: Bugs collected from the field 12 h prior to the experiment. Black bars: number of eggs potato, light bars: number of eggs in bean. *** $P < 0.001$ (Chi-square test for observed and expected frequencies, null hypothesis: number of eggs laid is equal in all plants).

Discussion

When *L. pabulinus* females had no choice between plant species, they oviposited in the plants that were offered. When females were offered a choice, females preferably oviposited in potato plants when present, irrespective of their history. Females captured from the field were collected from a solanaceous plant, and were placed in a rearing cage with potato plants for 12 hours prior to the experiment. Hence, early adult experience may have influenced their oviposition preference for potato. However, if oviposition preference would be induced by larval or early adult experience, females reared on green bean would have oviposited most eggs in green bean. Hence, oviposition preference of *L. pabulinus* for potato does not seem to be related to host plant experience, but seems to be innate. This was probably already recognized in the beginning of the 20th century, as *L. pabulinus* used to be called the potato capsid bug (Awati and Wolfe-Barry, 1914).

In one case, the strong oviposition preference for potato was not prevalent, *i.e.* when potato and tomato were offered simultaneously. This may suggest that *L. pabulinus* females do not distinguish between plants of the same family, or that they preferably oviposit not only in potato, but in solanaceous plants in general. This is also suggested by cpatures in the raspberry field, where *L. pabulinus* was found

TABLE 3. Oviposition preference for potato plants with or without conspecifics.

number of eggs in:	first experiment	second experiment
2 plants without clip cages	66	88
2 plants with empty clip cages	213	143
2 plants with females in clip cages	141	124
2 plants with males in clip cages	-	106
total number of oviposited eggs:	***420***	***461***

Whether similar amounts of eggs were oviposited in each group of plants per experiment, was determined with a Chi-square test for observed and expected frequencies (null hypothesis: number of eggs laid is equal in all plants). $P < 0.0001$ in the first experiment, $P = 0.002$ in the second experiment.

mainly on *Solanum nigrum*. However, in the choice experiments between tomato and green bean, *L. pabulinus* females preferred green bean over tomato. Also, fecundity was significantly lower when tomato plants were included in the experiments than when they were not (see Table 2). These results contradict a possible preference for Solanaceae. On the other hand, tomato plants specifically may not be suitable plants for oviposition. The fact that less eggs were laid as more tomato plants were available, may suggest that more time was spent on searching for better oviposition sites. Tomato plants have long, glandular hairs, especially on the stem of the plant, which may inhibit oviposition. More Solanaceae should be tested to determine oviposition preference for this plant family.

Specialization on one host plant species or family is predicted when there is a positive correlation between performance and preference for a host (Via, 1991; Fry, 1996; De Kogel, 1998). In our experiments, *L. pabulinus* females oviposited preferably in potato, then in green bean, and tomato was not preferred. This preference correlated with their fecundity on the different plants, as fecundity was highest on potato, and lowest when tomato plants were involved. Also, the rearing on green bean was not successful in due time, suggesting a lower fecundity on these plants as well, compared to fecundity on potato. Extrapolating these results to field situations, a stronger population growth of *L. pabulinus* can be expected in large, monocrop potato fields, which in turn increases infestation chances in nearby fruit orchards.

Population growth of *L. pabulinus* on green bean was reversed, as the amount of adults developing from this rearing diminished in each generation. In our initial attempts to start an additional rearing on a host plant other than potato, belonging to a different family, one other plant seemed suitable as well, *i.e.* sweet potato (*Ypomoea batata* L., Convolvulaceae). This plant seemed suitable, because nymphs emerged from eggs, that were oviposited by the initial 45 females obtained from the potato rearing. However, a total of only 6 females and 7 males developed from these nymphs (unpubl. data). Hence, population development of *L. pabulinus* has not been successful on several plant species other than potato, at least not under laboratory conditions. Specialization for oviposition sites may be needed for *L. pabulinus* females, as they need to obtain sufficient nutrients for egg development

(see Groot *et al.*, 1998), and provide offspring with sufficient nutrients until at least their third larval stage, after which the larvae can disperse (Petherbridge and Thorpe, 1928; Blommers *et al.*, 1997).

Aggregation of ovipositing *L. pabulinus* females does not seem to occur, as similar amounts of eggs were oviposited in plants with conspecifics as in plants without conspecifics. Fewest eggs were oviposited in undamaged plants, hence females may be attracted to plant volatiles released from damaged plants. Plants constitutively emit blends of volatiles that can attract herbivores (Visser 1986), but damage increases the emission of plant volatiles enormously (Dicke *et al.*, 1990; Turlings *et al.*, 1990; Vet and Dicke, 1992). Volatiles released from damaged plants attract a number of herbivores (reviewed by Dicke and Vet, 1999). Some plant volatiles are perceived very well by *L. pabulinus* females (Groot *et al.*, 1999). These volatiles may aid in finding suitable host plants (Chew and Renwick, 1995).

In conclusion, although *L. pabulinus* is considered a polyphagous bug, ovipositing females of the summer generation seem to prefer potato. Their preference is not influenced by host plant experience, but correlates with performance, which may have induced oviposition specialization. Ovipositing *L. pabulinus* females do not prefer plants with conspecifics, but prefer damaged plants over undamaged plants. This study supports the idea that chances of bug infestation in a fruit orchard are increased when potato fields are grown in the surrounding.

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Chapter 9

Discussion

The sexual behaviour of the green capsid bug comprises several aspects, a number of which are described in this thesis. Considering the successive steps from long range attraction to oviposition of fertilized eggs, the sexual behaviour of *L. pabulinus* can be summarized as follows (see also Table 1).

Both males and females start to be sexually active 4-5 days after their final moult (Chapter 2). When they are sexually mature, males are attracted to virgin females with *and* without plant material at long range (Chapter 4). Thus, plant volatiles are probably not involved in long-range attraction. Males are not attracted to males (Chapter 4) and females are not attracted to males or females (unpublished results). Hence, long-range mate location is mediated by a sex pheromone, not an aggregation pheromone, emitted by the female. Sex pheromone emission is inhibited when *L. pabulinus* females are exposed to larg amounts of hexyl butanoate (Chapter 6), a compound that is present in large quantities in the metathoracic scent gland of both sexes (F.P. Drijfhout, unpubl. data) and probably functions as a defensive allomone. Sex pheromone emission is also inhibited when females have mated, but only for a few hours (Chapter 7). Females do not assume a specific calling behaviour when they attract males (Chapter 2), contrary to the mirids *Distantiella theobroma* (King, 1973) and *Helopeltis clavifer* (Smith, 1977), that raise their abdomen when calling.

At close range, low volatile cuticular hydrocarbons are probably involved in the attraction of males to females (Chapter 5; Drijfhout *et al.*, 2000). These compounds are present on the legs of females, and are deposited on the substrate on which females walk. Similar compounds have been found to attract male bees pollinating orchids (Schiestl *et al.*, 1999), which suggests that cuticular hydrocarbons may be active at a longer range than is usually assumed. In addition, deposition of low volatile compounds on the substrate enlarges their functional range (Colwell *et al.*, 1978). Recently mated males are not responsive to virgin females at close range (Chapter 7). This indicates that sexual attraction depends on the mating status of both males and females.

In their courtship behaviour, a male and a female antennate each other frequently (Chapter 2). Possibly, sex-specific compounds are perceived through antennating. Such perception and behaviour also occurs in social insects for

nestmate-recognition (e.g. Howard, 1993; Howard and Akre, 1995; Smith and Breed, 1995). The courtship behaviour also includes a characteristic vibration behaviour in males. Such behaviour has been found in other male bugs as well (Strong *et al.*, 1970; Fish and Alcock, 1973; Wang and Millar, 1997) and will be discussed further in the next section.

Matings in *L. pabulinus* last 1-2 minutes only (Chapter 2), which is exceptionally short for heteropterans (see Introduction). However, in the mirid *Lygus hesperus* matings last similarly short (Strong *et al.*, 1970), and in other heteropterans sperm transfer is realized within 5 minutes (Carroll, 1991; Sillén-Tullberg, 1981). Prolonged matings of some hours to days are probably a form of mate guarding (see Introduction), a behaviour not observed in *L. pabulinus*.

Sperm transfer occurs by means of a compartmentalized spermatophore, formed when the aedaegus is in the spermatheca of the female (Chapter 7). In heteropterans, the presence of a spermatophore has only been described by Davey (1960). The fact that spermatophores have not been found in other heteropterans is likely to be due to the omission of detailed studies on the female reproductive tract. For example, when we dissected a mated female of another mirid, *Campylomma verbasci*, we found a similar spermatophore present in the spermatheca (pers. obs.). The spermataphore of *L. pabulinus* can be formed in a period as short as 30 seconds. Part of the spermatophore blocks the entrance to the bursa copulatrix and spermatheca, and can thus considered to be a mating plug. A block to the bursa of the female after mating has also been recognized in other heteropterans (Strong *et al.*, 1970; Davey, 1960; Wang and Millar, 1997). The mating plug induces a mechanical refractory period in *L. pabulinus* females, lasting for about 24 hours (Chapter 2 and 7). Hence, instead of prolonged matings mechanical mate guarding occurs in *L. pabulinus*. After 24 hours the spermatophore is partly disintegrated and sperm is found throughout the spermatheca, as well as in the oviducts, where fertilization of eggs is likely to occur. Mechanical mate guarding may thus be a means to inhibit remating before sperm is released from the spermatophore. When females remate, the reduced spermatophore of the first male is pushed dorsally into the spermatheca. However, as sperm is still present in the oviducts, most likely sperm mixing occurs, which has been found in *Nezara viridula* as well (McLain, 1985).

A large part of the spermatophore in *L. pabulinus* is a sperm-free mass (Chapter 7). These male-derived substances may be converted into eggs, to enhance female fecundity, fertility and/or longevity. However, females that had mated only once oviposited as many eggs and lived as long as females that had mated more than once (Chapter 7). Maybe fecundity is only temporarly increased directly after mating.

Under summer conditions, *L. pabulinus* females preferably oviposit their eggs in potato plants, compared to tomato or green bean plants (Chapter 8). Unfertilized eggs are also oviposited, but do not hatch (Chapter 7). Hence, parthenogenesis does not seem to occur in *L. pabulinus*.

Table 1. Sexual behaviour of the green capsid bug, *L. pabulinus*

Steps in sexual behaviour:	-	**Found in *L. pabulinus*:**
1. Long-range attraction	-	Females attract males when not disturbed
2. Close-range attraction	-	Female specific compounds, present on their legs; acoustic signals?
3. Courtship behaviour	-	Male vibration with abdomen, sexes antennating each other
4. Mating (sperm transfer)	-	Spermatophore, nutrients?
5. Mate guarding	-	By means of a mating plug, which is part of the spermatophore
6. Fertilization of eggs	-	Probably sperm mixing
7. Oviposition	-	In summer preferably in potato plants; unfertilized eggs are also oviposited, but do not hatch

Do acoustic signals play a role in sexual communication?

The male vibration behaviour, observed in *L. pabulinus* as well as in other heteropterans, may suggest that in addition to chemical communication acoustic communication is part of mate location. Such communication can be species-specific as well (for example Ewing, 1984; Claridge, 1985; De Vrijer, 1986; De Winter and Rollenhagen, 1990; Hunt *et al.*, 1992; De Winter, 1994). The sound produced by *L. pabulinus* male vibrations in closed Petri dishes could be heard even by the unaided ear. *Lygocoris pabulinus* males not only start vibrating at close range, but is also visible and regularly observed on plants at least 30 cm downwind from virgin-female-baited traps in the wind tunnel. This suggests that not only at close range, but also at a longer range males start vibrating after perceiving sex pheromone from females. These vibrational signals possibly help to locate females in a plant patch. After arriving in a host plant patch, males may perceive female-specific compounds, deposited on the plant surface. However, females may have moved to another plant long before male arrival, as such compounds will evaporate slowly from the substrate. By vibrating, males possibly verify whether females are still on the plant. If the vibration behaviour is part of an acoustic communication system, females should respond behaviourally. Such behavioural response can be phonotactic, *i.e.* females moving towards the source of the vibration (*e.g.* Ewing, 1984; Bailey, 1991), or acoustic (*i.e.* females producing a sound), as has been found in *Nezara viridula* (Ota and Čokl, 1991).

Sound production has been recorded for a number of terrestrial heteropterans (Leston and Pringle, 1963). Acoustic communication has been recognized in *Canthophorus dubius*, *Tritomegas bicolor* (both Cydnidae) (Gogala, 1969; Gogala *et al.*, 1974), and *Nezara viridula* (Pentatomidae) (Borges *et al.*, 1987; Ota and Čokl, 1991; Ryan and Walter, 1992). In *N. viridula*, males attract females by a long-range sex pheromone, but once the sexes have aggregated on the same plant, acoustic signals of both sexes mediate the final approach of males towards females (Ota and Čokl, 1991).

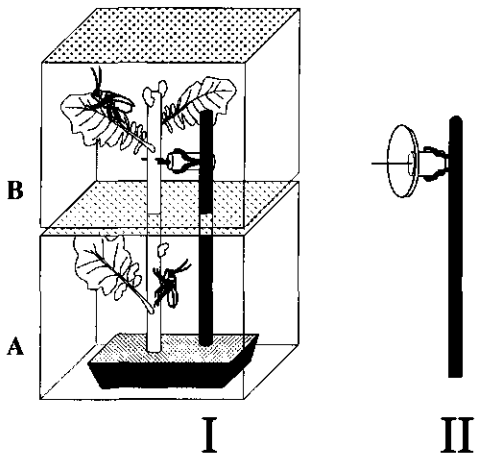

FIGURE 1. Schematic drawing of the acoustic experimental setup. Two plexi-glass boxes (A and B) were stacked with gauze in between and on top (dotted areas). A potato stem protruded into the upper box. To determine if *L. pabulinus* females responded acoustically to vibrating males, one male was introduced in the lower box, and one female in the upper box. A pin was inserted through the potato stem near the female, and sounds were recorded via an electromagnetic transducer (I). To determine if males would respond to the recorded sound, the transducer was replaced by a speaker (II). A pin attached to the speakers' coil was inserted in the potato stem. In this setup the lower box (A) was left empty, in the upper box (B) one male was introduced. See text for further explanation.

To determine whether *L. pabulinus* females produce an acoustic signal in response to the vibration signals of males, we conducted the following preliminary experiments. A potato plant, collected from a rearing cage with virgin females, was placed in a small plexi-glass box (A) without top, through which the upper half the plant protruded (see Figure 1). On top of this box a similar box (B) was placed with a gauze top and without bottom. Between both boxes gauze was tightened, so that bugs could not move from one box to the other via the potato stem or in another way. Through the potato stem in box (B) a pin was inserted. A Neo Delta magnet 35 (type NE 33) was attached to the pin. The magnet was placed in front of an electromagnetic transducer, so that acoustic signals transmitted through the substrate could be recorded (see De Vrijer, 1984; Strübing and Rollenhagen, 1988; De Winter and Rollenhagen, 1990; Chapter 2). In box (A) a virgin 5-8-day-old male was placed, and in box (B) a virgin 5-8-day old female was placed. As the plant was collected from a rearing cage with virgin females, it most likely contained the female-specific compounds, that induce vibration behaviour in the males (Chapter 5). The male started vibrating after introduction on the potato stem. The female was closely observed and all sounds were recorded. During 10 days of observations and recordings, three times a distinct, repetitive signal was recorded (Figure 2), which differed from the characteristic acoustic signals originating from males (see Chapter 2). However, during the recordings of these signals no obvious behaviour or particu-

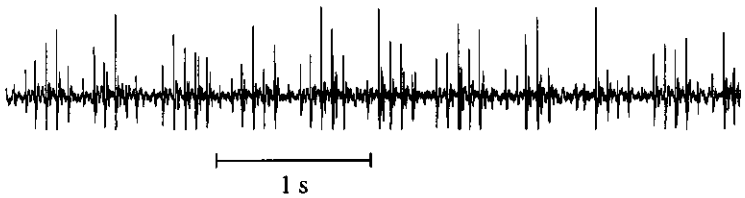

FIGURE 2. Oscillogram of a part of a sound recorded in the first setup, showing a repetitive pattern. This sound may originate from an *L. pabulinus* female, in response to vibrations of a male. A similar pattern was recorded three times, each time with a different female in the upper box. However, no obvious behaviour or particular movements from the females were observed during the recordings, and males did not start to vibrate in response to these signals.

lar movements from the female were observed. A phonotactic response of females towards vibrating males was not observed either.

Whether the recorded signals are an acoustic response of a female may be determined by playing back this signal to isolated males, placed on a clean potato stem. Upon perceiving the vibrations of the recorded signals, males may start vibrating in response. For such confirmation a similar setup as above was used, only now a clean potato plant was placed in the boxes, no female was introduced, and the transducer (I) was replaced by a small speaker (II) (Figure 1; see for more details on the setup De Vrijer, 1984). A pin attached to the speakers' coil was inserted in the potato stem. One male was introduced in box (B). In 3 days of trials, no male started vibrating in response to the recorded signal. We did not determine whether males responded phonotactically, *i.e.* moved towards the recorded signal. In conclusion, it remains unclear whether *L. pabulinus* females have produced the repetitive sound recorded and, if so, there is no evidence that the male responds to this. Thus, whether females respond acoustically to vibration signals from males and vice versa remains unclear. Further study is necessary to determine possible acoustic communication in *L. pabulinus*.

Feasibility of sex pheromone traps for monitoring

The aim of our study was to develop an efficient monitoring system to predict the presence of *L. pabulinus* in fruit orchards, by identifying its sex pheromone. However, even in virgin-female-baited traps, trap catches have always been low. For example, Blommers *et al.* (1988) caught on average about 5 males per week per virgin female-baited trap. In 1998 we caught an average of 4 males per trap per week in similar traps (107 males in total in one month; unpublished results). In 1999, when 12 virgin-female baited traps (half of which with 2 dispensers per trap containing 20 mg hexyl butanoate each) were distributed over six 0.4 ha plots (see Chapter 6), we caught a total of 36 males in one month in the six virgin-female-baited traps without hexyl butanoate, which is an average of 1-2 males per week per trap. Such low trap

catches raise questions about the probability that males are *not* caught in virgin-female-traps, and hence that the presence of a bug population in the orchard remains unnoticed.

If sex pheromone traps are to be used for monitoring this pest in fruit orchards, their reliability should be high, so that decisions of fruit growers to apply insecticides against this pest the following spring, can be based on the presence or absence of males in virgin-female traps. In general, when sex pheromone traps are used to monitor pest insects, the main difficulty is to correlate trap catches with population densities in order to determine whether or not control thresholds are expected to be exceeded (see for example Arn and Bues, 1989; Wyatt, 1997). For *L. pabulinus*, catching one male in a pheromone trap may already indicate that control thresholds will be exceeded the following spring, as few nymphs can cause considerable damage (Van den Ende *et al.*, 1996). Therefore, it is especially important that the monitoring method is reliable also at very low population densities. In both years that we conducted field experiments (1998 and 1999), population densities in the experimental orchard were estimated to be high, as adult bugs were regularly observed on the trees. The fact that under these circumstances we caught such a low number of males, generates serious doubts on the usefulness of sex pheromone traps to monitor the green capsid bug. In fact, human observations on bug presence in an orchard seem to be at least as effective as trap catches.

Potential of alarm pheromone for bug control

As hexyl butanoate was found to disturb the sexual communication in *L. pabulinus* (see Chapter 6), this compound may useful to control bug damage in fruit orchards. In general, the use of alarm pheromones as a possible insect control method has not been given much attention, because habituation or sensory adaptation has frequently been observed within 30 min after exposure in laboratory experiments (Calam and Youdeowei, 1968; Oetting and Yonke, 1978; Blum, 1985; Lockwood and Story, 1987; El-Agami and Haynes, 1992; Blatt *et al.*, 1998). However, these observations mainly concern nymphs, or adults feeding on a plant. Adult females searching for suitable oviposition sites are likely to avoid less suitable sites when they have a choice. Avoidance of oviposition sites treated with alarm pheromone has been found in female thrips, if some plants or parts of plants were left untreated (Teerling *et al.*, 1993). Also, alate aphids avoid landing on plants treated with alarm pheromone, and treatments in a greenhouse reduced aphid colonization (Wohlers, 1981; Dawson *et al.*, 1982).

In *L. pabulinus*, hexyl butanoate is not an alarm pheromone in the sense that it repels males, but it inhibits sex pheromone release in females (see Chapter 6). Habituation or sensory adaptation did not occur. Females were in the scent all week, but did not attract males during this period. We did not determine whether hexyl butanoate repels females. When females would be repelled, oviposition inhibition in fruit trees may be an effective control method for *L. pabulinus*. Exploiting the

observed inhibition of sex pheromone release to prevent matings, as discussed in Chapter 6, has as a major drawback that females can oviposit eggs more than 40 days after they have mated (Chapter 7). Hence, matings should be prevented long before migration to fruit orchards occurs. When aiming at oviposition inhibition in fruit trees, the migration behaviour of *L. pabulinus* is an important target that may be interfered with to control this pest. The migration period in autumn is a distinct choice period for *L. pabulinus* females, when they change from ovipositing on summer hosts to woody hosts, on which winter eggs are oviposited. In this period, fruit trees treated with hexyl butanoate are likely to be avoided when hexyl butanoate is repellent for females, especially when alternative oviposition sites for winter eggs would be offered in the periphery of the orchard, for example by placing black currant plants (Wightman, 1968). Removing these plants in winter time, after the ovipositing females have died, should minimize population development the following spring. This control method may have general application possibilities in insects where females are repelled by an alarm pheromone. When females oviposit at alternative sites instead of in the crop to be protected, the developing pest can be lured away from the crop. Such a strategy has been termed the push-pull tactic (see Miller and Cowles, 1990; Borden, 1997; Hardie *et al.*, 1999). In this tactic, negative semiochemical stimuli to push the pest insect away from the stand at risk, are coupled to attractive semiochemical-baited trees or traps that pull the insect away. Since many female insects use plant volatiles to locate suitable hosts for oviposition (e.g. Visser, 1986; Cardé and Bell, 1995; Dicke and Vet, 1999), these volatiles may be used as attractants in the push-pull strategy.

In conclusion, sex pheromone traps are probably not efficient or reliable tools for monitoring *L. pabulinus* in fruit orchards. Instead, bug damage may be controlled by exploiting its alarm pheromone. Aiming at a control method has the major advantage that insecticides could become dispensable for this pest, whereas monitoring can only promote a more selective use of insecticides, after presence of the pest has been established. And as long as insecticides are needed, further development of integrated pest management in European fruit orchards will be hampered (Minks *et al.*, 1998).

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Samenvatting

Seksueel gedrag van insecten in het algemeen

Het seksuele gedrag van insecten is zo gevarieerd als de insecten zelf. Niet alle insecten vertonen seksueel gedrag, aangezien veel soorten zich (gedeeltelijk of geheel) aseksueel voortplanten. Bij de seksuele voortplanting kan een reeks van gedragingen aan de bevruchting van eieren voorafgaan.

Veel mannetjes en vrouwtjes leven niet in elkaars nabijheid, zodat ze elkaar eerst moeten vinden. Gewoonlijk zendt of scheidt één van de seksen signalen uit waardoor de andere sekse wordt aangetrokken. Dit kunnen visuele, chemische of akoestische signalen zijn. Vooral chemische signalen zijn erg effectief om partners vanaf een afstand te lokken. Zulke signalen worden seksferomonen genoemd.

Seksferomonen worden niet continu uitgescheiden. Of seksferomonen uitgescheiden worden, hangt af van een aantal factoren, zoals leeftijd, of ze al dan niet gepaard hebben, en een aantal omgevingsfactoren. Sommige vrouwtjes scheiden bijvoorbeeld alleen feromoon uit als ze in de omgeving van een geschikte waardplant zijn, waarop ze direct daarna eieren kunnen leggen. Als de seksen eenmaal in elkaars nabijheid zijn, op dezelfde plant bijvoorbeeld, kunnen weer andere visuele, chemische of akoestische signalen ervoor zorgen dat ze elkaar ook daadwerkelijk ontmoeten. Nadat de partners elkaar gevonden hebben kan baltsgedrag aan de paring vooraf gaan. De paring is succesvol als er sperma-overdracht plaatsvindt.

Een succesvolle paring zegt echter nog niets over het reproductieve succes van een insect. Veel vrouwtjes kunnen meer dan eens paren. Wanneer een vrouwtje paart met een volgend mannetje kan sperma van het vorige mannetje verdrongen worden. Een mannetje dat gepaard heeft is daarom nog niet zeker of zijn sperma gebruikt zal worden voor de bevruchting van eieren. Meestal wordt het grootste deel van de eieren bevrucht door het mannetje waarmee het vrouwtje het laatst gepaard heeft. Sommige mannetjes proberen gedurende een bepaalde periode te verhinderen dat vrouwtjes opnieuw paren door een aantal uren of zelfs dagen in copula te blijven. Een andere gebruikte strategie is om, samen met sperma, nutriënten aan het vrouwtje te geven die gebruikt kunnen worden voor de aanmaak van (extra) eieren. Als er meer eieren gemaakt worden kunnen er ook meer eieren bevrucht worden door sperma van het mannetje die de nutriënten gegeven heeft. De aanmaak van extra eieren verhoogt bovendien het reproductieve succes van het vrouwtje. Kortom, het

reproductieve succes van een insect hangt voor een groot deel af van het seksuele gedrag voor, tijdens en na een paring.

Seksueel gedrag en plaagbestrijding

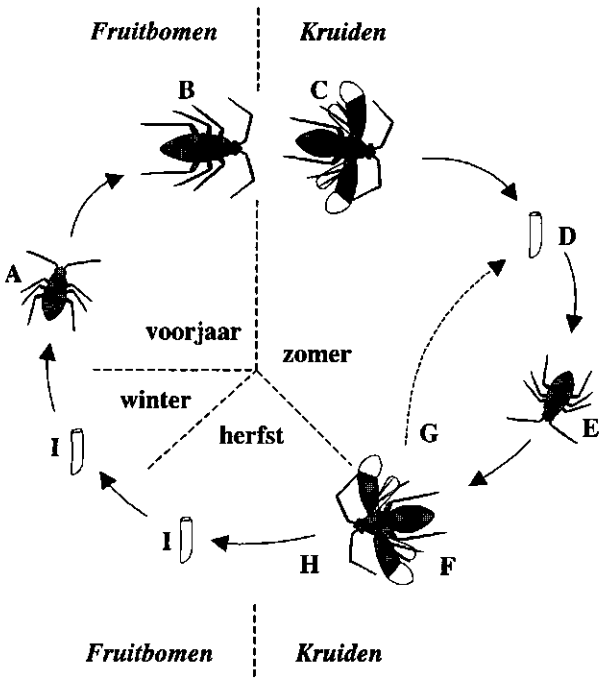
Bestudering van het seksuele gedrag van insecten is niet alleen interessant op zichzelf, kennis hierover is ook nuttig voor de bestrijding van insecten in landbouwgewassen. Zo kan bijvoorbeeld een aantal motten succesvol bestreden worden door grote hoeveelheden synthetisch (nagemaakt) seksferomoon in het veld te introduceren, waardoor potentiële partners elkaar niet meer kunnen vinden en er dus geen paringen meer optreden. Feromonen kunnen ook op andere manieren gebruikt worden, bijvoorbeeld om de aanwezigheid van een schadelijk insectensoort te signaleren. Vallen met synthetisch seksferomoon worden dan geplaatst in het gebied waar een plaagpopulatie verwacht wordt. Als zo'n populatie in de omgeving is, zullen hoogstwaarschijnlijk een aantal individuen gevangen worden, omdat seksferomonen soortspecifiek zijn en de aangetrokken sekse heel gevoelig is voor het betreffende seksferomoon. Toepassing van insecticiden is dan alleen nodig als (een bepaalde hoeveelheid) individuen gevangen wordt in deze vallen. Het gebruik van seksferomonen voor de signalering van schadelijke insecten heeft geleid tot een aanzienlijke reductie in het gebruik van insecticiden.

De groene appelwants

Het onderwerp van dit proefschrift is de groene appelwants (*Lygocoris pabulinus* (L.), familie Miridae). Deze wants veroorzaakt schade in fruitboomgaarden. In het najaar leggen vrouwtjes hun eieren in de stam van de bomen. De eieren overwinteren, de volwassen wantsen gaan dood. In het voorjaar, wanneer de fruitbomen gaan bloeien, komen de eieren uit. De larven prikken met hun monddelen de jonge vruchtbeginsels aan, wat vervormingen en beschadigingen aan de appels veroorzaakt.

Om verschillende redenen is de groene appelwants een lastig te bestrijden insect. Fruittelers kunnen niet vooraf bepalen of ze het komende voorjaar last zullen hebben van wantsenschade omdat de eieren ín de stam worden gelegd, en dus nauwelijks te vinden zijn. Bovendien kan tijdens de bloei, als de larven uitkomen, niet gespoten worden met bestrijdingsmiddelen, omdat dat schadelijk is voor de bijen, die altijd uitgezet worden om de bloemen in de fruitbomen te bestuiven.

Aangezien een klein aantal larven al aanzienlijke economische schade kan veroorzaken, spuiten fruittelers voor de zekerheid ieder jaar vlak voor en vlak na de bloei tegen de groene appelwants. Dat is waarschijnlijk vaak overbodig, omdat de wantsen twee keer per jaar migreren en dus ieder jaar in andere boomgaarden hun eieren kunnen leggen. De eerste migratie vindt plaats aan het eind van het voorjaar: de larven die in het voorjaar uit zijn gekomen migreren als ze volwassen worden naar



FIGUUR 1. Levenscyclus van de groene appelwants. A: larven komen in het voorjaar uit in fruitbomen, B: na het derde larvale stadium (van 5 stadia) migreren ze naar kruiden, C-D: in de zomer leggen vrouwtjes eieren in kruiden, E: een zomergeneratie ontwikkelt in de kruidlaag, F: volwassen vrouwtjes leggen eieren (D, I) in kruiden (G) of in fruitbomen (H), afhankelijk van het weer (tijdens hete zomers ontwikkelt een tweede generatie in de kruidlaag), H: als de dagen korter worden in de herfst, leggen vrouwtjes overwinterende eieren in fruitbomen, waarna de volwassenen doodgaan (A, E: eerste larvale stadium, B: vierde larvale stadium, waarbij de vleugelaanleg te zien is, C, F: volwassenen).

kruiden (zie Figuur 1). Dat kan een (on)kruidlaag zijn in of langs de randen van de boomgaarden, of kruidgewassen zoals aardappelvelden in de omgeving van boomgaarden. Nadat ze gemigreerd zijn naar een kruidgewas, worden hierin nieuwe eieren afgezet en ontwikkelt zich een zogenaamde zomergeneratie. De volwassen wantsen van deze generatie leggen in lange, warme zomers opnieuw eieren in kruiden. Als het kouder wordt, en de dagen korter, migreren de vrouwtjes weer naar fruitbomen om hun overwinterende eieren te leggen.

Fruittelers zouden al erg geholpen zijn als ze met zekerheid zouden kunnen bepalen of ze in het voorjaar wantsenschade kunnen verwachten. Met andere woorden, als er een betrouwbare signaleringsmethode zou zijn. Dan zou overbodig spuiten, en de daarmee gepaard gaande kosten en moeite, achterwege kunnen blijven. Zo'n betrouwbare signaleringsmethode zou gericht moeten zijn op het najaar, wanneer de overwinterende eieren gelegd worden, zodat bepaald kan worden in welke boomgaarden die eieren gelegd worden.

Doel van het project

Ons doel was om het seksferomoon van de groene appelwants te identificeren, om zo een betrouwbare signaleringsmethode te ontwikkelen. In het verleden is gebleken dat mannetjeswantsen worden aangetrokken door maagdelijke vrouwtjes, dus waarschijnlijk produceren deze vrouwtjes seksferomoon. Sinds eind jaren '80 is geprobeerd om dit seksferomoon te identificeren, maar helaas zonder succes. Dit is niet uitzonderlijk, want maar weinig studies hebben geleid tot succesvolle feromoon-identificaties bij wantsen. Waarschijnlijk komt dat doordat deze groep insecten grote hoeveelheden defensieve stoffen uitscheidt zodra ze verstoord wordt. Deze defensieve stoffen worden geproduceerd in een specifieke klier, de metathoracale klier, en uitgescheiden als er gevaar dreigt. Door de uitscheiding van defensieve stoffen kunnen mogelijke seksferomoon-componenten geheel overschaduwd worden. Maar dat is misschien niet de enige reden dat er nog maar zo weinig seksferomonen geïdentificeerd zijn bij wantsen. Er is ook gebrek aan kennis over het gehele seksuele gedrag van wantsen. En zoals hierboven al is opmerkt bestaat het seksuele gedrag uit veel meer dan het lokken van partners op afstand alleen. Daarom bestond dit project uit twee onderdelen, (1) een biologische studie naar het seksuele gedrag van de groene appelwants, en (2) een chemische studie om het seksferomoon te kunnen identificeren. In dit proefschrift wordt het biologische deel beschreven.

Opbouw van het proefschrift

Omdat identificatie van het seksferomoon het doel van het project was, is deze studie voornamelijk gericht op gedragingen die verband houden met de seksuele aantrekking. In het eerste deel (hoofdstuk 2 en 3) worden vooral fysiologische aspecten beschreven van zowel mannetjes als vrouwtjes. In het tweede deel wordt bepaald waardoor mannetjes precies worden aangetrokken (hoofdstuk 4 en 5). In het derde deel (hoofdstuk 6, 7 en 8) wordt het seksuele gedrag in meer detail bestudeerd vanuit het vrouwelijk perspectief, om te bepalen welke factoren invloed hebben op seksferomoon-uitscheiding en hoe het seksuele gedrag van vrouwtjes gerelateerd is aan hun eileggedrag.

Hoofdstuk 2 beschrijft op welke leeftijd vrouwtjes seksueel volwassen zijn, en op welke leeftijd mannetjes daarop zullen reageren. Ook is bepaald of vrouwtjes een specifiek lokgedrag vertonen. Daarnaast is het baltsgedrag in detail bestudeerd, waarbij mannetjes een specifiek gedrag blijken te vertonen, namelijk een vibratie met hun achterlijf. Dit gedrag was goed bruikbaar als biotoets om te bepalen wanneer en waarvan mannetjes seksueel geactiveerd worden.

De identiteit van het seksferomoon zou opgehelderd kunnen worden door te bepalen welke stoffen veel beter worden waargenomen door mannetjes dan door vrouwtjes. Immers, mannetjes zullen erg gevoelig zijn voor de stoffen waarmee ze vrouwtjes kunnen vinden, terwijl vrouwtjes zich daar niet op hoeven te richten.

Insecten nemen stoffen (voornamelijk) waar met hun antennen. Welke stoffen waargenomen worden, kan bepaald worden door middel van elektro-antennogram-afleidingen (EAG's). In een EAG-opstelling wordt een antenne van een insect tussen twee elektroden geplaatst, waarna een stof over de antenne wordt geblazen. Als op de antenne receptoren zitten waarmee deze stof kan worden waargenomen, verandert het rust-potentiaal van de antenne. De grootte van het signaal is een maat voor de gevoeligheid van de antenne voor de geteste stof. En hoe gevoeliger de antenne, hoe belangrijker waarschijnlijk de stof in de biologie van het insect. In **Hoofdstuk 3** hebben we een aantal kandidaatstoffen getest op mannetjes- en vrouwtjes-antennen met behulp van een EAG-opstelling. Deze kandidaatstoffen zijn esters, die in wantsen vaak voorkomen. Ook hebben we een aantal plantenstoffen getest, omdat vrouwtjes daar juist gevoeliger voor zouden kunnen zijn, aangezien zij planten moeten zoeken om hun eieren te kunnen leggen.

In de veldproeven, waarin is vastgesteld dat groene appelwants-mannetjes aangetrokken worden door vrouwtjes, waren vrouwtjes gekooid met een aardappelscheutje dat als voedsel diende. Deze kooitjes werden vervolgens opgehangen in zogenaamde deltavallen met lijmbodems, waar invliegende mannetjes in bleven kleven. De aantrekking zou daarom niet alleen veroorzaakt kunnen zijn door de vrouwtjes, maar ook door een combinatie van vrouwtjes en geuren van aardappelscheutjes. In **Hoofdstuk 4** hebben we bepaald wat de invloed van plantengeuren is op de attractie van mannetjes.

In **Hoofdstuk 5** hebben we het vibratiegedrag van mannetjes gebruikt om de bron van aantrekkelijke stoffen in vrouwtjes te vinden. Seksferomonen worden geproduceerd in specifieke klieren. Als bekend is in welke klieren deze feromonen gemaakt worden, kan het seksferomoon geïdentificeerd worden door de inhoud van deze klier chemisch te analyseren. Wantsen bezitten een groot aantal klieren, maar een specifieke seksferomoonklier is (nog) niet gevonden. Daarom hebben we groene appelwantsvrouwtjes gedissecteerd in kop, vleugels, poten, en lijf. Deze verschillende onderdelen werden aangeboden aan mannetjes om te zien of die vervolgens vibreerden, zoals ze doen bij levende vrouwtjes.

De seksuele communicatie in wantsen kan verstoord worden door defensieve stoffen die ook kunnen fungeren als alarmferomonen. Bij behandeling voor chemische analyses of experimenten kunnen wantsen al gauw verstoord raken. Dit kan tot gevolg hebben dat mannetjes worden afgeschrikt, in plaats van aangetrokken, of dat vrouwtjes stoppen met het lokken van mannetjes. In **Hoofdstuk 6** is het mogelijke alarmferomoon van de groene appelwants geanalyseerd, en bepaald hoe dit de seksuele communicatie verstoort.

Niet alleen alarmferomonen maar ook paringen kunnen seksferomoon-productie in vrouwtjes stopzetten. Dat kan gedurende een korte of lange periode na de paring aanhouden, of vrouwtjes kunnen in het geheel stoppen met het lokken van mannetjes nadat ze gepaard hebben. Insectenvrouwtjes kunnen namelijk meestal sperma opslaan en hebben vaak aan één paring genoeg voor de bevruchting van al hun eieren. Omdat sperma-overdracht, -opslag en -verdringing een grote invloed kan

hebben op het seksuele gedrag, hebben we in **Hoofdstuk 7** de bevruchting in groene appelwantsvrouwtjes in detail bestudeerd.

Migratie van groene appelwantsvrouwtjes van kruiden naar fruitbomen (om hun overwinterende eieren te leggen) vindt waarschijnlijk over niet meer dan een paar kilometer plaats. Daarom kan kennis over de mogelijke verblijfplaats van de zomergeneratie inzicht geven in de kans op infectie in verschillende boomgaarden. Vrouwtjes kunnen voorkeur hebben voor bepaalde planten om hun eieren in te leggen. In **Hoofdstuk 8** is deze voorkeur van groene appelwantsvrouwtjes onder zomerse condities bepaald.

Het seksuele gedrag van de groene appelwants

Als alle gevonden resultaten op een rijtje worden gezet, kan het seksuele gedrag van de groene appelwants, van het lokken op afstand tot eileg, als volgt worden samengevat (**Hoofdstuk 9**; zie ook Tabel 1).

Zowel mannetjes als vrouwtjes zijn 4 tot 5 dagen na de laatste vervelling seksueel volwassen. Dan worden mannetjes van een afstand aangetrokken door vrouwtjes, of er nu wel of geen plantenmateriaal aanwezig is. Dus plantenstoffen spelen waarschijnlijk geen rol bij deze aantrekking. Mannetjes worden niet aangetrokken door mannetjes en vrouwtjes worden niet aangetrokken door mannetjes of vrouwtjes, dus partners vinden elkaar door middel van een seksferomoon dat uitgescheiden wordt door vrouwtjes. Het seksferomoon wordt niet uitgescheiden als vrouwtjes blootstaan aan grote hoeveelheden hexylbutanoaat, het alarmferomoon van de groene appelwants. Vrouwtjes scheiden ook geen seksferomoon uit als ze gepaard hebben, maar dat geldt maar voor een paar uur. Ze vertonen geen speciaal lokgedrag als ze seksferomoon uitscheiden.

Nadat mannetjes van een afstand zijn gelokt, worden ze aangetrokken door specifieke stoffen die op de poten van vrouwtjes voorkomen. Deze stoffen worden ook afgegeven aan het substraat waarop vrouwtjes lopen. Uit chemische analyses bleek dat deze stoffen hoogstwaarschijnlijk voornamelijk cuticulaire koolwaterstoffen zijn. Dat zijn niet erg vluchtige stoffen. Door afgifte aan het substraat kan de communicatie-afstand van deze weinig vluchtige stoffen worden vergroot. Deze stoffen zouden belangrijk kunnen zijn om mannetjes daadwerkelijk in een val te lokken. Nadat mannetjes over langere afstand aangetrokken zijn, kunnen ze namelijk nog specifieke stimuli nodig hebben om ergens te landen. Dat zou kunnen betekenen dat mannetjes niet gevangen worden zonder deze stimuli.

Tijdens de balts raken mannetjes en vrouwtjes elkaar regelmatig aan met hun antennen. Misschien worden op deze manier de sekse-specifieke stoffen waargenomen. Ook vibreren groene appelwantsmannetjes tijdens de balts. De mogelijke functie van dit vibratiegedrag wordt verder besproken in de volgende sectie. De paring zelf duurt maar heel kort, een tot twee minuten. Dat is extreem kort in vergelijking tot de meeste andere wantsen, waarbij paringen van een aantal uren of zelfs een aantal dagen gevonden zijn. In de groene appelwants wordt in die paar

minuten sperma overgedragen door middel van een spermatofoor (spermapakketje), dat gevormd wordt in de spermatheca (sperma-opslagorgaan) van het vrouwtje. Tot dusver zijn spermatoforen maar in één wantsensoort gevonden, wat hoogstwaarschijnlijk komt doordat het vrouwelijke geslachtsapparaat nooit in detail bestudeerd is. Toen wij bijvoorbeeld gepaarde vrouwtjes van een andere wants (de toortswants, *Campylomma verbasci*), dissecteerden vonden we ook daarin een spermatofoor.

Een deel van de spermatofoor blokkeert de toegang tot het reproductieve orgaan van het vrouwtje, zodat ze niet onmiddellijk zou kunnen paren met een volgend mannetje. Deze blokkade kan omhoog bewogen worden om eieren door te laten, als die gelegd worden. Na 24 uur is sperma uit de spermatofoor vrijgekomen en verspreid in zowel de spermatheca als de oviducten. De eieren worden dus hoogstwaarschijnlijk in de oviducten bevrucht. Na 24 uur kunnen vrouwtjes ook paren met een volgend mannetje.

Mannetjes die net gepaard hebben reageren tenminste twee uur na paring niet op vrouwtjes, zelfs niet op maagdelijke vrouwtjes. Waarschijnlijk duurt het even voordat ze weer een spermatofoor kunnen maken. Deze kan namelijk wel zo'n 5 % van hun lichaamsgewicht bedragen. De spermatofoor van groene appelwantsen bestaat maar voor een klein deel uit sperma, de rest zouden nutriënten kunnen zijn, die de eiproductie van vrouwtjes (tijdelijk) verhogen, zodat zoveel mogelijk eieren door het pas-gepaarde mannetje bevrucht worden. Als dat zo is, zouden vrouwtjes die vaker paren waarschijnlijk meer eieren leggen dan vrouwtjes die maar één keer paren. Een verschil in eileg tussen deze vrouwtjes hebben we echter niet kunnen aantonen.

Onder zomerse condities leggen vrouwtjes bij voorkeur eieren in aardappelplanten. Dat kan betekenen dat boomgaarden in de omgeving van aardappelvelden een grotere kans op wantsenschade hebben. Onbevruchte eieren worden ook gelegd, maar deze komen niet uit. Ongeslachtelijke voortplanting lijkt dus niet voor te komen in de groene appelwants. Dat is bij twee andere wantsen namelijk wel het geval.

Tabel 1. Seksueel gedrag van de groene appelwants, *Lygocoris pabulinus*

Stappen in seksueel gedrag:	**Gevonden in de groene appelwants:**
1. Aantrekking op lange afstand	- Vrouwtjes lokken mannetjes als ze niet verstoord worden
2. Aantrekking op korte afstand	- Specifieke stoffen op de poten van vrouwtjes; akoestische signalen?
3. Baltsgedrag	- Vibratie van mannetje, seksen raken elkaar aan met antennen
4. Paring (sperma-overdracht)	- Spermatofoor, nutrienten?
5. Tot ongeveer 24 uur na de paring	- Blokkade in vrouwtjes verhindert een volgende paring
6. Bevruchting van eieren	- In de oviducten
7. Ovipositie van eieren (eileg)	- In zomer bij voorkeur in aardappelplanten; onbevruchte eieren ook gelegd, maar die komen niet uit

Spelen akoestische signalen een rol?

Het vibratiegedrag van groene appelwantsmannetjes kan een aanwijzing zijn dat naast chemische communicatie ook akoestische signalen een rol spelen bij de seksuele aantrekking. Het geluid van vibrerende mannetjes was zelfs voor het menselijk oor duidelijk te horen. Stengels van planten kunnen dit geluid doorgeven, waardoor het geluid een redelijk groot bereik kan hebben. Dit is onder andere bij cicaden in detail bestudeerd, waar iedere soort z'n eigen geluid blijkt te hebben, en mannetjes en vrouwtjes elkaar door middel van dit geluid kunnen vinden. Ook bij een paar wantsensoorten (onder andere *Nezara viridula*) is vastgesteld dat seksuele partners elkaar door middel van akoestische signalen vinden als ze eenmaal op dezelfde plant zitten. Bij de groene appelwants spelen de stoffen die vrouwtjes afgeven aan substraat waarschijnlijk een belangrijke rol bij de lokalisatie van vrouwtjes, maar omdat deze stoffen langzaam verdampen kunnen vrouwtjes inmiddels vertrokken zijn naar een andere plant. Door vibratie zouden de mannetjes kunnen verifiëren of vrouwtjes nog steeds op de plant zitten, tenminste als vrouwtjes op deze vibraties reageren. Om te bepalen of akoestische communicatie bij de groene appelwants mogelijk een rol speelt, hebben we een paar inleidende experimenten uitgevoerd (zie **Hoofdstuk 9**). Tot drie keer toe hebben we een vibrerend geluid opgenomen, dat duidelijk afweek van het geluid dat door mannetjes geproduceerd wordt, en dat afkomstig zou kunnen zijn van vrouwtjes in antwoord op vibrerende mannetjes. Maar tijdens deze opnames zagen we geen specifiek gedrag of beweging in vrouwtjes, en toen we dit geluid weer terugspeelden naar mannetjes vibreerden deze niet. Mogelijk reageren de mannetjes niet met vibraties maar lopen ze in de richting van de trilling, die door vrouwtjes geproduceerd wordt. Diepgaander onderzoek is nodig om vast te stellen of akoestische communicatie plaatsvindt bij de groene appelwants.

Tot slot

Het doel van deze studie was om het seksferomoon van de groene appelwants te identificeren, om zo een betrouwbare signaleringsmethode te ontwikkelen waarmee de aanwezigheid van de groene appelwants in boomgaarden vastgesteld kan worden. Maar in vallen met maagdelijke vrouwtjes zijn eigenlijk altijd heel weinig mannetjes gevangen. Zo is in een proefboomgaard (De Schuilenburg te Lienden) in 1988 een gemiddelde van vijf mannetjes per val per week gevangen en in 1998 vier mannetjes per val per week. Het is onwaarschijnlijk dat deze lage valvangsten veroorzaakt zijn doordat bepaalde essentiële elementen ontbraken, omdat in deze vallen levende vrouwtjes gebruikt zijn. Zulke lage valvangsten roepen de vraag op wat de kans is dat mannetjes *niet* in vallen gevangen worden, en dus dat de aanwezigheid van een wantsenpopulatie onopgemerkt zal blijven. Als seksferomoonvallen in de praktijk gebruikt worden door fruittelers moeten die vallen natuurlijk wel betrouwbaar zijn,

zodat aan de hand daarvan besloten kan worden of er wel of niet gespoten moet worden. Maar zelfs in jaren dat wantsen regelmatig waargenomen werden in de boomgaard (1998 en 1999), werden maar heel weinig mannetjes gevangen. Daarom betwijfelen wij of feromoonvallen wel een betrouwbare signaleringsmethode voor de groene appelwants zouden kunnen zijn. Het lijkt erop dat menselijke observaties minstens zo effectief zijn als valvangsten.

In plaats van te proberen om een betere signaleringsmethode te ontwikkelen, zou het groene appelwantsprobleem in boomgaarden misschien opgelost kunnen worden door gebruik te maken van het alarmferomoon (hexylbutanoaat). Over het algemeen wordt er niet veel aandacht besteed aan de mogelijkheid om alarmferomonen te gebruiken in de bestrijding van wantsen, omdat ze snel gewend lijken te raken aan een alarmgeur en er dan niet meer op reageren. Maar vrouwtjes van de groene appelwants raken niet gewend aan hexylbutanoaat; in vallen waar ze een hele week met de geur in aanraking waren hebben ze gedurende de hele periode geen mannetjes aangetrokken (zie hoofdstuk 6). In de periode dat vrouwtjes moeten bepalen waar ze de overwinterende eieren gaan leggen (in het najaar) zullen ze waarschijnlijk niet kiezen voor een omgeving met alarmferomoon. Als in die periode een boomgaard wordt omgeven door verdampers met het alarmferomoon, en er langs de randen alternatieve boompjes worden geplaatst waarin vrouwtjes hun eieren kunnen leggen, is de kans groot dat vrouwtjes de alternatieve boompjes kiezen. Deze boompjes, bijvoorbeeld zwarte bes, zouden in verplaatsbare potten geplant kunnen worden, zodat ze in de winter, als de populatie volwassen wantsen dood is, weggehaald en vernietigd kunnen worden.

Samengevat zouden komende studies zich beter kunnen richten op het gebruik van alarmferomoon om te proberen wantsenschade te voorkómen, in plaats van zich te richten op een betere signaleringsmethode. Voorkómen kan insecticidengebruik tegen wantsen geheel overbodig maken, terwijl na signalering nog steeds gespoten moet worden. En het gebruik van insecticiden belemmert de verdere ontwikkeling van geïntegreerde bestrijding van ziekten en plagen in fruitboomgaarden.

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Nawoord

Alleen mijn naam staat op de kaft van dit proefschrift. Maar natuurlijk is het niet alleen mijn verdienste dat dit geheel tot stand is gekomen. Allereerst zou het project er niet geweest zijn als Leo Blommers en Teris van Beek het projectvoorstel niet geschreven hadden. Leo en Teris, bedankt, ook voor jullie suggesties gedurende het project.

Gelukkig was assistentie bij de aanvraag ingediend en goedgekeurd, wat de eerste anderhalf jaar werd ingevuld door Antje Schuurman en de laatste tweeënhalf jaar door Anneke Heijboer. Antje en Anneke, jullie bijdrage is groter dan jullie zelf waarschijnlijk vermoeden. Jullie hebben me altijd het gevoel gegeven dat we samen aan oplossingen werkten, jullie dachten mee, kwamen met onverwachte ideeën, en waren altijd zo flexibel dat alle ideeën ook uitgevoerd konden worden.

In de periode dat Antje niet meer bij het project betrokken kon zijn en Anneke nog niet gevonden was, heeft Els Drijfhout vrijwillig met de kweek geholpen. Els, ook met jou verliep de samenwerking uitstekend, en door jouw inzet kon alles gewoon doorgaan.

Veldwerk kon plaatsvinden in proefboomgaard "De Schuilenburg", waar naast Leo Herman Helsen en Fredy Vaal altijd behulpzaam waren. Jullie kennis van en ervaring met de groene appelwants is vooral bij het opstarten van het project van groot belang geweest. Fredy, ook hartelijk dank voor de jaren dat je een schaduwkweek van de groene appelwants in stand hield (voor het geval dat ...) en het kweken van de toortswants (voor het andere geval dat ...).

Het project bestond uit een biologisch en een chemisch deel, en Falko Drijfhout was de chemische aio. Falko, wij weten nu dat interdisciplinaire samenwerking ingewikkelder is dan vaak wordt gedacht. Voordat we in de gaten hadden dat we allebei een eigen taal spraken, wat leidde tot subtiele interpretatie-verschillen, waren we x tijd verder. Maar jij hebt altijd veel geduld getoond om mij chemische mechanismen en jouw daarmee verbonden denkwijze uit te leggen, en ik heb veel van je geleerd.

De dagelijkse begeleider van mijn deel van het project was Hans Visser. Hans, jouw direktheid en eerlijkheid heb ik altijd erg gewaardeerd. Je suggesties zetten me steeds weer aan het denken en je was er ook altijd als ik je nodig had. Kortom, je was voor mij de ideale begeleider.

Mijn promotor Marcel Dicke is na ongeveer een jaar bij het project betrokken geraakt. Marcel, jij hebt hebt je vanaf het begin geïnteresseerd voor m'n ideeën en experimenten. Ook jij had altijd tijd als ik daarom vroeg en door jou ben ik ook meer betrokken geraakt bij de vakgroep. Hans en Marcel, jullie gedetailleerde én positieve commentaar op alle stukken waren steeds een impuls om er nog iets beters van proberen te maken.

Erna van der Wal, Radbout Timmer, Renate Geerts en Jeroen Willekens zijn als student bij verschillende onderdelen van het onderzoek betrokken geweest. De bijdragen van Erna en Radbout zijn zichtbaar in hoofdstuk 2 en 3 van dit proefschrift,

beiden hebben ongeveer alle data voor deze hoofdstukken verzameld. Renate en Jeroen, jullie bijdrage is minder zichtbaar omdat jullie er veel korter waren, maar ook jullie inzet en hulp hebben me geholpen.

Hans Smid, bij jouw was *ik* weer student! Door jou heb ik het interne insect leren kennen en inzicht gekregen in mechanismen die een rol kunnen spelen bij de paring. Dit is voor mij het boeiendste onderwerp van m'n onderzoek geworden, wat volgens mij wel terug te zien is in ons gezamelijk artikel (hoofdstuk 7). Bovendien hebben de boeken en artikelen die jij hebt aangedragen mijn inzicht in seks bij insecten en in seks in het algemeen vergroot.

Voor vragen over statistische verwerking van gegevens kon ik altijd terecht bij Gerrit Gort. Gerrit, dat jij me steeds *gratis* geholpen hebt zal ik gedenken als een van de laatste relicten van een inmiddels afgesloten tijdperk.

Gedurende het project zijn er momenten geweest dat we (even) wilden bepalen of groene appelwantsmannetjes en vrouwtjes ook akoestisch communiceren. Peter de Vrijer was altijd bereid om ons daarbij te helpen.

Natuurlijk zijn er ook mensen die een meer indirekte bijdrage hebben geleverd aan de totstandkoming van dit proefschrift. Ten eerste de leden van de STW-gebruikerscommissie, Aede De Groot, Louis Schoonhoven, Cees den Otter, Henny Balkhoven, Bas Heijne, Job Klijnstra, Janusz Moskal, Chris Mombers en Monique la Grand. De eerste vergadering ging ik in met het idee dat een zwaard van Damocles boven m'n hoofd hing, maar al gauw bleek zo'n gemengd gezelschap verfrissende ideeën en suggesties te genereren. Bovendien vond ik het erg nuttig om eens per halfjaar alle gegevens weer even op een rij te 'moeten' zetten.

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Ook de mensen van de vakgroep Entomologie zijn altijd open en behulpzaam geweest. Met sommigen is het contact meer geworden dan alleen af en toe een praatje op de gang of commentaar geven op elkaars manuscripten tijden de aio-lunches.

Mensen van de ondersteunende diensten (van zowel Entomologie als PRI) wil ik bij deze ook graag bedanken voor hun ondersteunende hulp, met name Jan den Dunnen, Jan Knuiman, Jan Mooi, Hassan Isaaq, Dorrie Jansen, Aly Mastenbroek en Heleen de Lange.

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Curriculum vitae

Astrid Tatjana Groot was born on 17 July 1965 in Anna Paulowna, The Netherlands. After her secondary education at the Rijkscholengemeenschap, Schagen (Havo), she lived with a host-family in Pikeville, KY, USA (via the AFS-exchange programme) for a year, and attended Senior Highschool. When she returned, she moved to Amsterdam and had several jobs. After a year of working she decided to go back to school, and during her preparatory scientific education (VWO) at the evening school Contardo Ferrini, her interest for Biology emerged. At the University of Amsterdam, her first research training was in Entomology. A completely different research training followed in Tropical Ecology, for which she stayed in the Andes of Colombia for 6 months. With an Erasmus-scholarship she had the opportunity of an additional research training for three months at the University of Turku, Finland. After her graduation (cum laude), she conducted preliminary research on epiphytes in the primary and secondary Amazonian and Andean forests of Colombia, with a Nuffic scholarship. Since funding for further research did not seem feasible, she applied for an entomological PhD position in Wageningen, the results of which are described in this thesis. This research was conducted from August 1995 to August 2000 at the Research Institute Plant Research International (PRI), Business Unit Biointeractions and Plant Health, in cooperation with the Laboratory of Entomology and the Laboratory of Organic Chemistry of Wageningen University.

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