



## Copulation behaviour of *Lygocoris pabulinus* under laboratory conditions

Astrid T. Groot<sup>1</sup>, Erna van der Wal<sup>2</sup>, Antje Schuurman<sup>1</sup>, J. Hans Visser<sup>1</sup>, Leo H.M. Blommers<sup>1</sup> & Teris A. van Beek<sup>3</sup>

<sup>1</sup>Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, The Netherlands;

<sup>2</sup>Wageningen Agricultural University, Laboratory of Entomology, P.O. Box 8031, 6700 EH Wageningen, The Netherlands;

<sup>3</sup>Wageningen Agricultural University, Laboratory of Organic Chemistry, P.O. Box 8026, 6700 EG Wageningen, The Netherlands

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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 copulate 4 days after the final moult. Second matings occur in some females, even on consecutive days. The pre-mating period is 10 min on average and copulation lasts 1 to 2 min. 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.

### 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 & 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 & Thorpe, 1928; Kullenberg, 1946; Southwood & Leston, 1959). Damage occurs when nymphs emerging in spring feed on shoot tips, ovaries and young fruitlets, thereby causing russeted 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 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 & 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 & 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 & 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). 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.

## Materials and methods

**Rearing.** *Lygocoris pabulinus* was reared on potted potato plants, cultivar Bintje, in wooden cages in greenhouses, which were maintained at  $22 \pm 2$  °C,  $65 \pm 5\%$  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.

**Egg production.** 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 with a spermatophore (pers. obs.), hence copulation had occurred. All experiments were carried out between 12.00 and 15.30 h (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 h, which is at the onset of the light period; from 9.30 to 10.30 h; and from 21.45 to 22.45 h. 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 ( $22 \pm 2$  °C,  $65 \pm 5\%$  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 h 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 & 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<sup>®</sup>. 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 & 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 was 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 & Rollenhagen, 1988; De Winter & 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 ( $\pm$  s.d.) duration of these calls and the mean ( $\pm$  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  $6.6 \pm 4.4$  (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  $12.9 \pm 8.1$ . 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  $9.6 \pm 7.8$ . 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.

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 h 14 out of 29 dissected females contained a spermatophore, between 9.30 and 10.30 h 13 out of 30 females had copulated, between 12.00 and 15.30 h 41 of 61 dissected females contained a spermatophore, and between 21.45 and 22.45 h 20 out of 31 females had copulated.

Sexual maturation is related to temperature as well. 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  $26 \pm 2$  °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 1 h 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 could be captured, as the Tangle Trap<sup>®</sup> was only applied at the bottom of the trap; we have seen several males

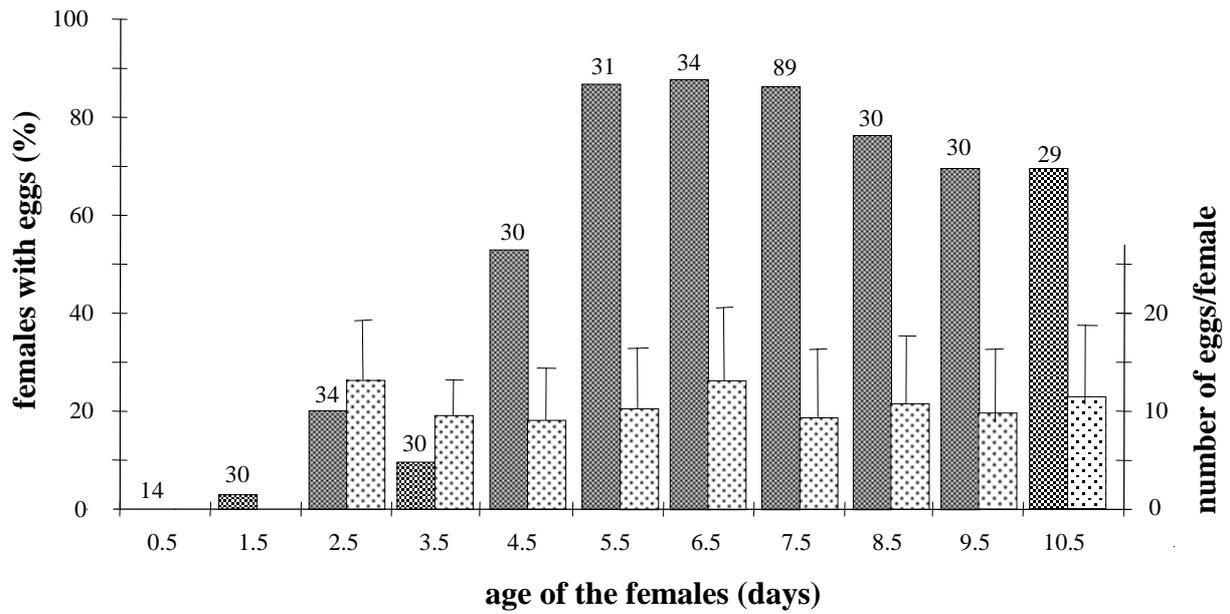


Figure 1. Egg production in virgin female *L. pabulinus*, held at 25 °C with pollen. ■ Percentage of females with eggs. □ Mean (+ s.d.) number of eggs per female with eggs. On top of the bars the number of females dissected is indicated.

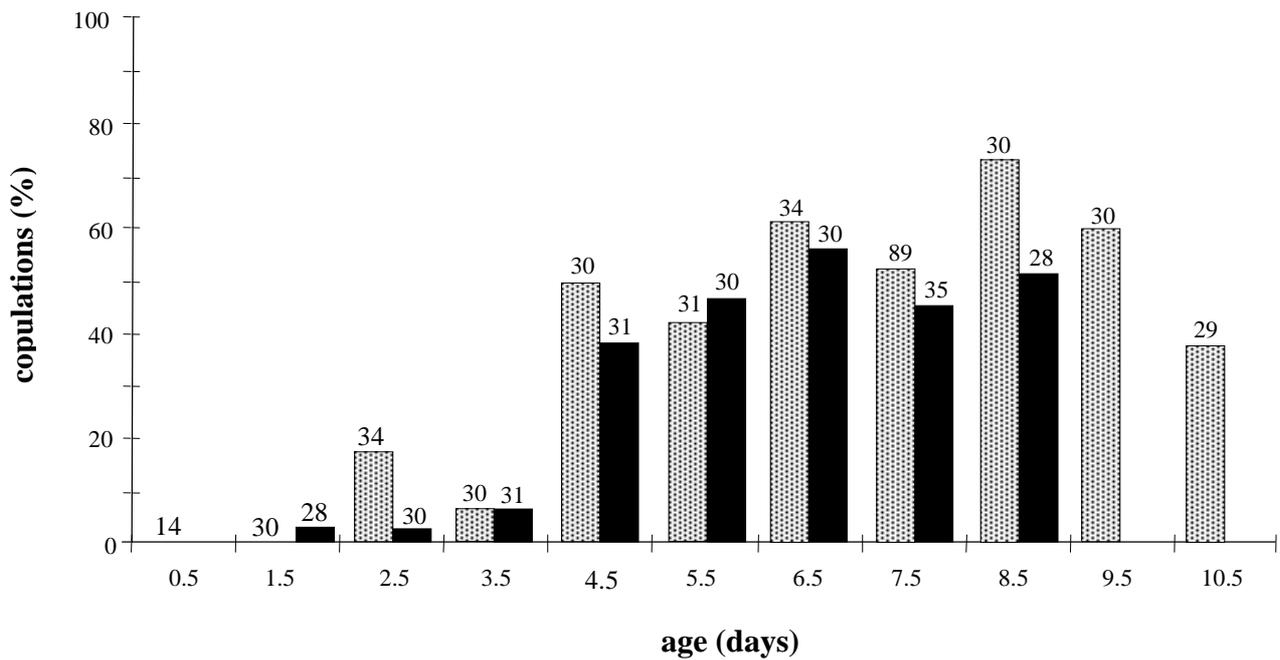


Figure 2. Sexual maturation and copulation frequency of *L. pabulinus*. □ Percentage of females copulating. ■ Percentage of males copulating. On top of the bars the number of bugs observed is indicated.

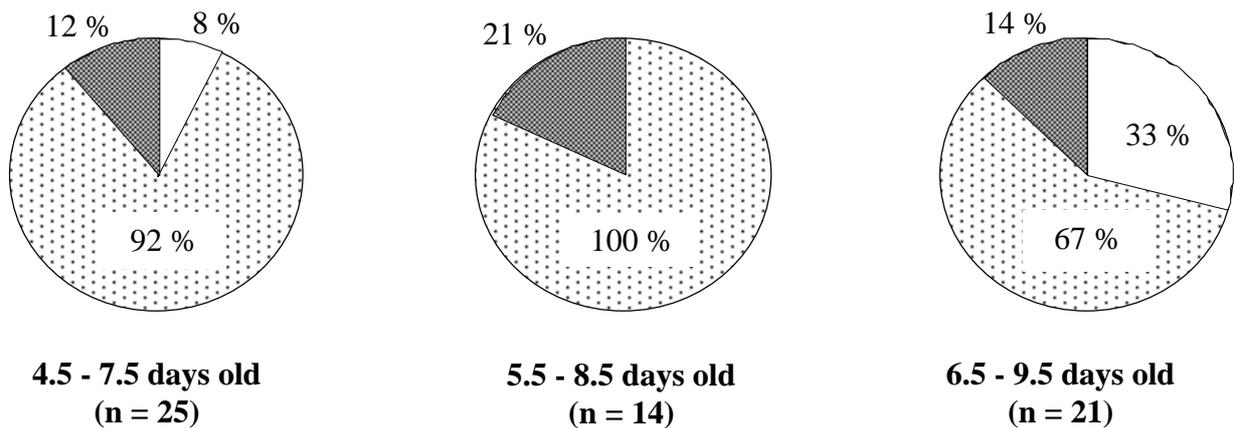


Figure 3. Frequency of copulations in groups of *L. pabulinus* females at different ages. □ No copulations. ◻ Copulated once. ◼ Copulated twice.

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 min, the thorax and abdomen slightly lower than the head, so that the body axis was in an angle of  $\pm 20^\circ$  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).

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  $8 (\pm 4.7 \text{ s.d.})$  times before mat-



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

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
<i>Random behaviour:</i>		
1. Resting	15	15
2. Grooming	9	10
3. walking	15	15
<i>Precopulation behaviour:</i>		
4. Male antennating female	13	9
5. Female antennating male	11	8
6. Male vibrating	14	2

ing, 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  $\pm 200$  Hz. The 8 analysed signals showed a mean duration of  $2.4 (\pm 0.96 \text{ s.d.})$  seconds and a mean number of  $12.4 (\pm 4.8 \text{ s.d.})$  clicks with  $5.2 \pm 0.7$  clicks per second. We strongly suspect that vibration of the male capsid bugs is a sexual signal, as this occurs almost 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 & Cokl, 1991; Ryan & Walter, 1992). In this species both males and females emit specific vibrational signals to locate potential mates on plants (Ota & Cokl, 1991) and to initiate mating (Ryan & 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.

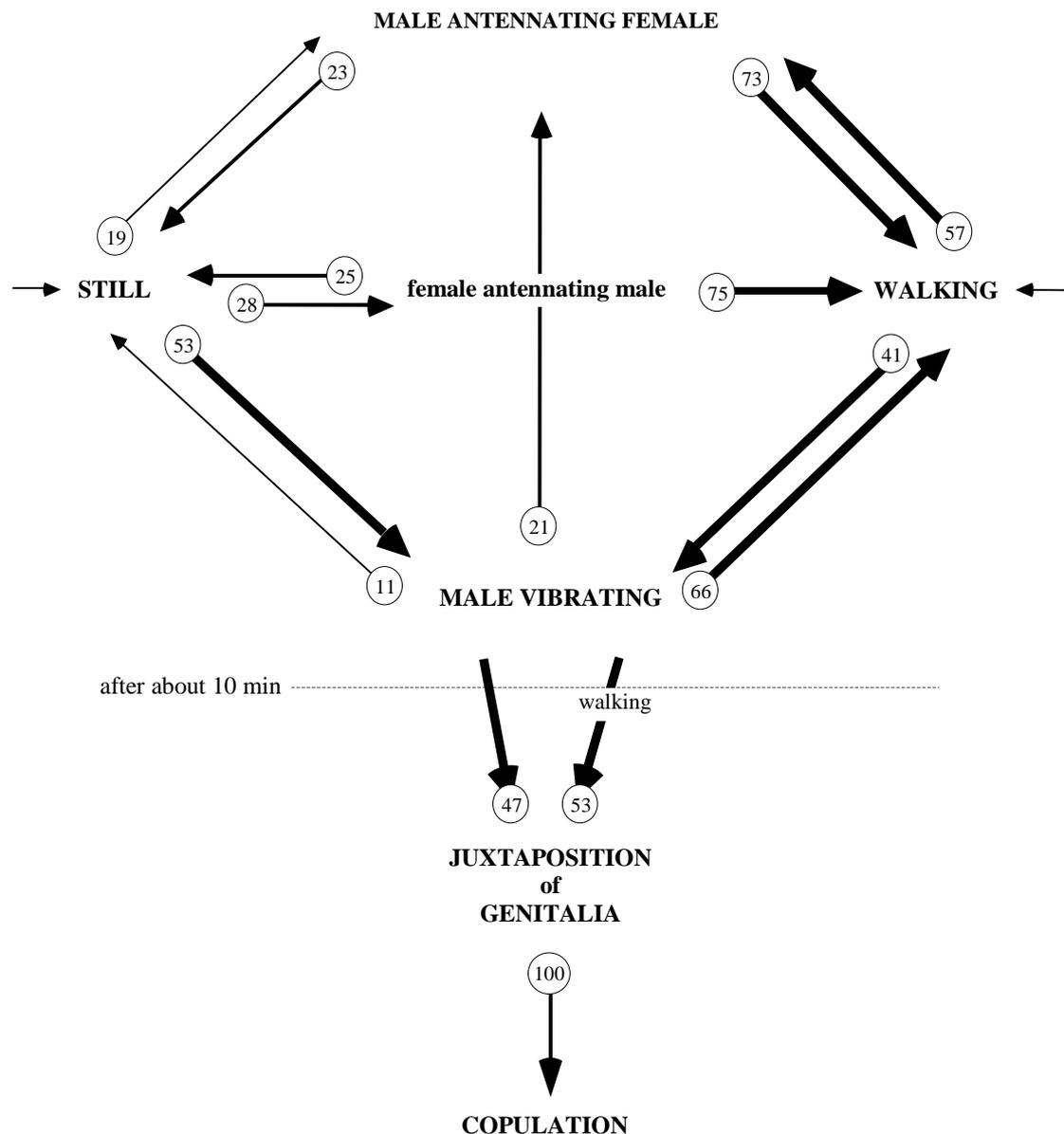


Figure 5. Ethogram of the precopulation behaviour of male *L. pabulinus*, based on the observations of 15 matings. 'Female antennating male' is synonymous to 'male being antennated by the female' from the original dataset. The numbers next to the arrows are relative percentages, calculated from the originating 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.

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  $45^\circ$  to  $90^\circ$  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 motion-

less in a position of about 45° for 1–2 min (see also Petherbridge & Thorpe, 1928; Kullenberg, 1946).

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

## 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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