ECOLOGY, BEHAVIOR AND BIONOMICS

Towards the Identification and Synthesis of the Sex Pheromone of the Citrus Leafminer, *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae)

Ana L. Parra-Pedrazzoli[1], Allard Cossé[2], Yasuhiro Murata[3], José M.S. Bento[1], Evaldo F. Vilela[4] and Walter S. Leal[5]

[1]Depto. Entomologia, Fitopatologia e Zoologia Agrícola, ESALQ/USP 13418-900, Piracicaba, SP, Brazil
[2]USDA/ARS, National Center for Agricultural Utilization Research, 1815 N. University Street, Peoria IL 61604, USA
[3]Fuji Flavor Co. Ltd, 3-5-8 Midorigaoka, Hamura-city, Tokyo 190-11, Japan
[4]Depto. Biologia Animal, Univ. Federal de Viçosa, 36571-000, Viçosa, MG, Brazil
[5]Honorary Maeda-Duffey Lab, Dept. Entomology, Univ. California, Davis, CA 95616 USA

Neotropical Entomology 35(1):012-018 (2006)

Bases Comportamentais para a Identificação e Síntese do Feromônio Sexual do Minador-dos Citros, *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae)

RESUMO - O objetivo desta pesquisa foi caracterizar o comportamento sexual do minador-dos-citros, *Phyllocnistis citrella* Stainton, como suporte para o isolamento, identificação e síntese do seu feromônio sexual. O acasalamento de *P. citrella* ocorre no intervalo entre 1h antes do início da fotofase e 1h após o término da escotofase, entre adultos de um e dois dias de idade. O comportamento de corte e cópula foi descrito para essa espécie, sendo a duração média da cópula de 49,6 min. Estudos conduzidos em olfatômetro tipo Y demonstraram que os machos foram fortemente atraídos pelas fêmeas virgens bem como pelo extrato natural de glândulas de feromônio sexual das fêmeas.

PALAVRAS-CHAVE: Comportamento sexual, comportamento de corte e cópula, olfatômetro tipo Y

ABSTRACT - The objective of this work was to characterize the sexual behavior of the citrus leafminer, *Phyllocnistis citrella* Stainton, as the foundation for the isolation, identification, and synthesis of the complete sex pheromone of this species. Mating occurred in a time window of 2h, starting 1h before the onset of photophase. The large majority of tested insects mated in the first two days after emergence, with no significant difference between mating at day 1 and day 2. A stereotypical courtship and copulation behavior were described for this species. When mating was successful, the copulation was recorded in average for 49.6 min. In Y-olfactometer tests conducted at the time of mating activity, males were strongly attracted to caged virgin females as well as to extracts from putative pheromone glands.

KEY WORDS: Sexual behavior, courtship behavior, Y-olfactometer

The citrus leafminer *Phyllocnistis citrella* Stainton is a major pest of *Citrus* spp. throughout the world. Caterpillar feeding affects not only photosynthesis and development of young plants (Badawy 1967, Heppner 1993, Peña & Duncan 1993), but also opens the door for the citrus canker-causing bacterium, *Xanthomonuas axonopodis* pv *citri* (Venkateswarlu & Ramapandu 1992, Cook 1988, Chagas *et al.* 2001) and other pathogens. *P. citrella* was originally described from Calcutta, India and is now established in five continents (Heppner 1993, Argov & Rössler 1996, Prates *et al.* 1996). In Brazil, it was first detected in 1996 in Campinas, Sao Paulo and soon became a major problem associated with the dramatic increase of the citrus canker disease (Gimenes-Fernandes *et al.* 2000).

Attraction of male *P. citrella* to (Z,Z)-7,11-hexadecadienal was accidentally discovered by Ando and collaborators while conducting field screenings of potential attractants for species in the Gelechiidae family, and caught males of the Japanese populations of the citrus leafminer (Ando *et al.* 1985). Attempts to monitoring adult populations with traps baited with synthetic attractant Z7Z11-16Ald, failed in field tests in China, Italy, Spain, USA, Turkey, and Brazil (reviewed in Tongyuan *et al.* 1989, Jacas & Pena 2002, Sant'Ana 2003). Intriguingly, Du and collaborators captured a related species, *P. wampella,* with the same attractant (Du *et al.* 1989). These findings suggest that there could be geographic variations in the pheromone system and/or there might be missing constituents of the natural sex pheromone. Largely, moth sex pheromones comprise of a bouquet of multiple constituents. For example, a single constituent of the sex pheromone of the navel orangeworm, *Ameyolis transitella* Walker (Lepidoptera: Pyralidae), (Z,Z)-11,13-hexadecadienal, was known for over two decades (Coffelt *et al.* 1979) but failed to trap males. Recently, the full pheromone system was identified as a mixture of as many as nine constituents (Leal *et al.* 2005). We launched a collaborative project, which aimed at the isolation, identification, and synthesis of the complete pheromone system of the Brazilian population of the citrus leafminer (Leal *et al.* 2006). This work aimed at characterizing the sexual behavior of the citrus leafminer as the foundation for the full characterization of sex pheromone system.

Material and Methods

Insects. *P. citrella* adults were reared according to the methodology of Chagas & Parra (2000), at the Laboratório de Biologia de Insetos, at ESALQ/USP at 25 ± 2°C, 65 ± 10% relative humidity, and a photoperiod of 14h photophase, starting at 6 AM local time. In order to obtain virgin insects, citrus leaves hosting *P. citrella* pupae were cut out and individualized in glass vials (8 cm long; 1 cm diameter). Small pieces of moistened filter papers were added and the vials were sealed with plastic PVC film (Magipack ®). After emergence, the adults were sexed, according to Jacas & Garrido (1996), and fed on honey.

Copulation. To study mating behavior in *P. citrella*, pairs were transferred to separated cups (clear plastic; 4.5 cm height, 2.5 cm diameter). Five cups were inverted and placed on a petri dish (15 cm) with a damp filter paper on the bottom. In preliminary assays, 10 pairs were observed every 20 min for 24h to determine the approximate time of sexual activity. For detailed observations, six groups of 10 pairs were observed daily for eight days at the time established in the previous assays. The following parameters were recorded for each pair: age, time and duration of the first copulation, and number of copulations per pair. Observations during the scotophase were made with a hand flashlight (Maglite ® with a red filter). The light source was maintained at ca. 60 cm from the arena so as to prevent possible interference with the insect's behavior. A completely randomized experimental design was adopted. The results of age at the first copulation and time of mating were subjected to analysis of variance, and the means were compared by Tukey test at a 5% probability level. The means for age and time of mating were transformed to $\sqrt{(x+5)}$ and log (x+5), respectively.

Sexual Behavior. Attraction was observed with a glass Y-olfactometer (3 cm internal diameter) with three arms of the same length (20 cm). An air current (1 L/min) was produced by vacuum suction at one arm of the olfactometer so that the air entered through the opposite arms, which were used to place treatment and control. To minimize possible stress, test insects were transferred to glass "olfactomer extensions" (3 cm i.d; 6 cm in length) at least 12h prior to behavioral observations. One olfactometer extension was attached to each arm and sealed with Magipack® plastic PVC film strip. Based on preliminary data, the time of behavioral observations was limited to 3 min after the arms and the vacuum line were connected.

To confirm the occurrence of a female-emitted sex pheromone, eight virgin females were tested as the stimulus, whereas the opposite arm side was used as a control (empty extension). Test males (15) were placed in the downstream arm of the olfactometer. To confine females, both sides of the olfactometer extension were covered with voile fabric. To test possible male-mediated attraction, the same procedure was performed with caged males as the stimulus and females as test insects. Once it was determined that attraction was mediated only by females (see below), blank tests were conducted with test males to eliminate possible false positives attraction due to anemotaxis.

Pheromonal activity was tested in the Y-olfactometer described above by comparing male attraction to the arms containing filter papers impregnated either with hexane only or with hexane extracts of the pheromone gland. The natural pheromone was obtained by dissecting at the time of mating 48 virgin female abdominal tips and extracting with 300 µl of hexane. After 30 min, glands were removed, and the extracts were used immediately or kept at -20°C until use. A piece of filter paper (1 x 1 cm) was loaded with an aliquot of eight female-equivalent (based on preliminary tests) and placed in treatment arm of the olfactometer after solvent evaporation (ca. 2 min). For control, a similar filter paper was impregnated with solvent only. To test possible solvent effect, filter paper only was tested against filter paper impregnated with hexane. Fifteen virgin males were released from the test arm of the olfactometer in each of these experiments. These experiments were conducted on a completely randomized fashion with six replicates. Attraction data (in percentage) was subjected to analysis of variance and means were compared for significance by the Tukey test at a 5% probability level after being transformed to.

Behavioral observations. To determine the courtship and copulation behavior, 1- or 2-day-old virgin females and unmated males were placed in pairs in separated plastic cups (4.5 height; 2.5 i.d.) and observed (n = 30) during the time of mating activity. Calling, courtship, and copulation were recorded daily; copulating pairs were identified and separated.

Results and Discussion

Age, time, duration, and number of copulations in *P. citrella*. The large majority (≈80%) of mating occurred in the first two days after emergence, but mating was also observed with small frequency throughout the adult's lifetime. There was no significant difference between mating at day 1 and day 2 (Fig. 1A). In these studies (n = 60), 78% of the observed insects mated within the first 8 days after emergence. Although multiple mating was also observed, the majority (79%) of mating occurred only once (Fig. 1B). Preliminary experiments indicated that mating took place within a limited time window from 1h before the photophase to one hour after the end of the scotophase. Detailed observation at this time window indicated that there was no significant peak of mating activity (Fig. 1C). The mean duration of the copulation was 49.6 ± 2.9 min, with a minimum of 35 min and a maximum of 90 min.

Although this is the first systematic study of the citrus leafminer sexual behavior, an early report indicated that mating occurred in the first day of adult emergence and lasted for 30 to 150 min, with maximum mating noticed at sunset and few individuals copulating during morning hours (Batra *et al.* 1988). On the other hand, Huang *et al.* (1989) suggested that the time of copulation was from 5h to 9h. These discrepancies might be due to the fact that these and other early studies (Pandey & Pandey 1964, Ba-Angood

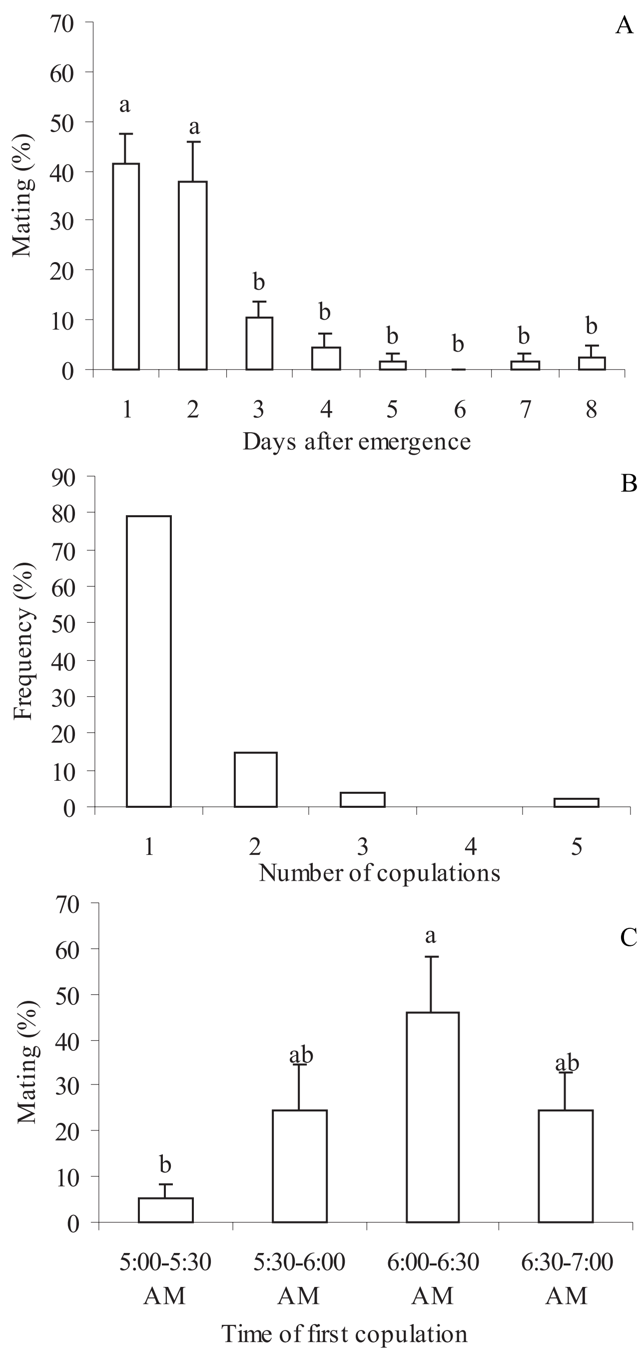

Figure 1. Percentage of citrus leafminer *P. citrella* mating as a function of age (A), time of day (B), and number of copulations per pair (C). Means followed by the same letters are not significantly different (5%, Tukey test).

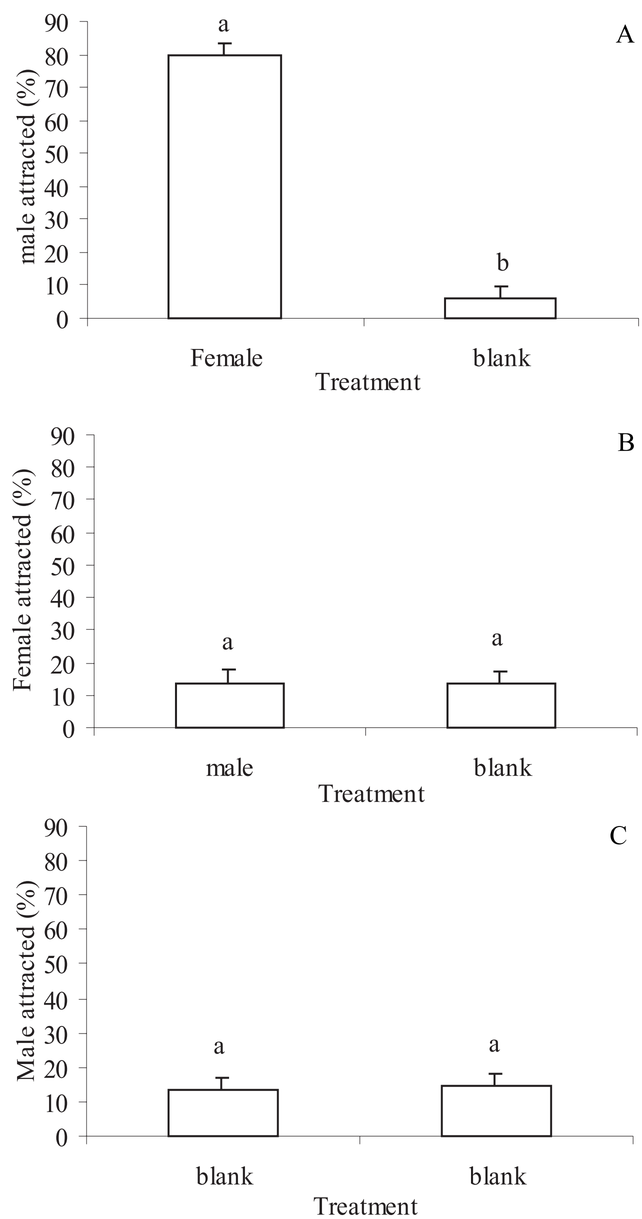

Figure 2. Behavioral responses of males to females (A), females to males (B), and males to blank-blank control (C) in a Y-olfactometer. Means followed by the same letters are not significantly different (5%, Tukey test).

1977, Alba 1996) were not designed to get a better understanding of mating behavior but rather focused on the biology of the citrus leafminer.

Mating activity in the citrus leafminer restricted to a short time window near dawn agrees with observations from several other mining species in the family Gracillariidae, such as *Phyllonorycter ringoniella* (Matsumura), (Jung & Boo 1997); *Phyllonorycter acerifoliella* (Z.) [=*P. sylvella* (Hw.)]; *Phyllonorycter heegerella* (Z.) and *Phyllonorycter ulmifoliella* (HBN), (Mozuraitis *et al.* 2000). The long copulation (avg. 49.6 min) in the citrus leafminer seems to allow sufficient sperm transfer (Benz 1991) given that 80% of the observed females mated only once in eight days.

Sexual behavior and pheromone evidence. Behavioral assays (Fig. 2A) (in a Y-olfactometer) indicated significant male attraction to the side arm with virgin females (80%) than to control (6%). On the other hand, males failed to attract females (treatment, 13.5%; control, 13.7%) (Fig. 2B). There was no significant preference for any arm side in blank-blank tests (Fig. 2C). Altogether, these data suggest the existence of a female-emitted sex pheromone. Indeed, extracts of a putative pheromone gland (abdominal tip) attracted significantly more males (64%) than control (Fig. 3A), whereas tests of filter paper only versus filter paper plus solvent showed no significant difference (Fig. 3B). The attraction of males by females in *P. citrella* also occurs in the other species already studied in the family Gracillariidae, such as *Conopomorpha cramerella* (Snellen) (Beevor *et al.* 1993), *Phyllonorycter elmaella* Doganlar & Mutura (Shearer & Riedl 1994), *P. ringoniella* (Jung & Boo 1997), *Phyllonorycter blancardella* (Fabr.) (Mozuraitis *et al.* 1999), *P. acerifoliella*, *P. heegerella*, *P. ulmifoliella*, (Mozuraitis *et al.* 2000), *Phyllonorycter crataegella* (Clemens) (Ferrao *et al.* 1998), and *Cameraria ohridella* Deschka & Dimic (Svatos *et al.* 1999).

Description of calling, courtship and copulation behavior. During the time of mating activity, females displayed a stereotype calling behavior characterized by raising the

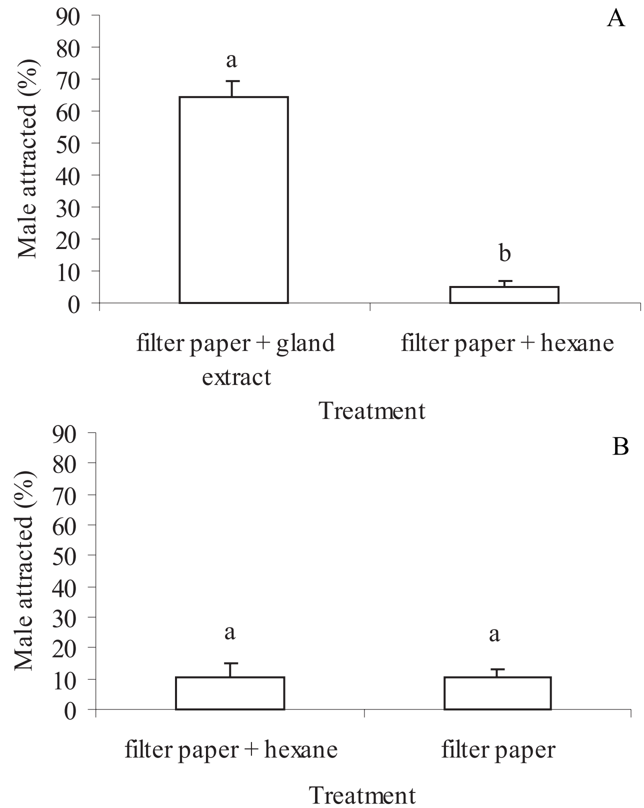

Figure 3. Percentage of males attracted to the natural extract of the sex pheromone produced by females of the citrus leafminer (A) and response to control (solvent) (B). Means followed by the same letters are not significantly different (5%, Tukey test).

wings, keeping the antennae directed backward along the body, extruding and slightly raising the abdominal tip (Fig. 4A). Although calling females remained motionless most of the time, they sporadically moved the abdomen up and down. Males responded by walking fast in several directions, jumping (short-distance flights) and displaying vigorous antennal movements. They approached calling females by

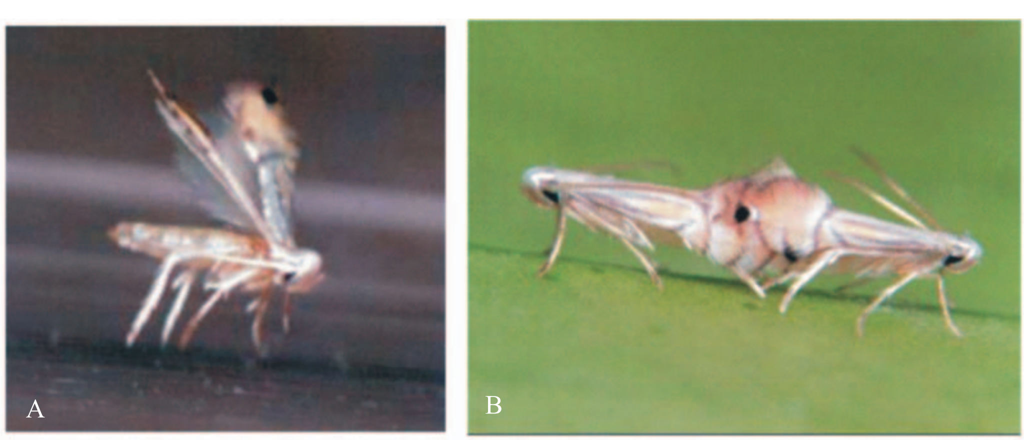

Figure 4. Typical calling behavior of the citrus leafminer *P. citrella* female, with wings raised and open, antennae pointing backwards, abdomen slightly raised, and the abdominal tip exposed (highlighted) (A). Copulation in an end-to-end position (B).

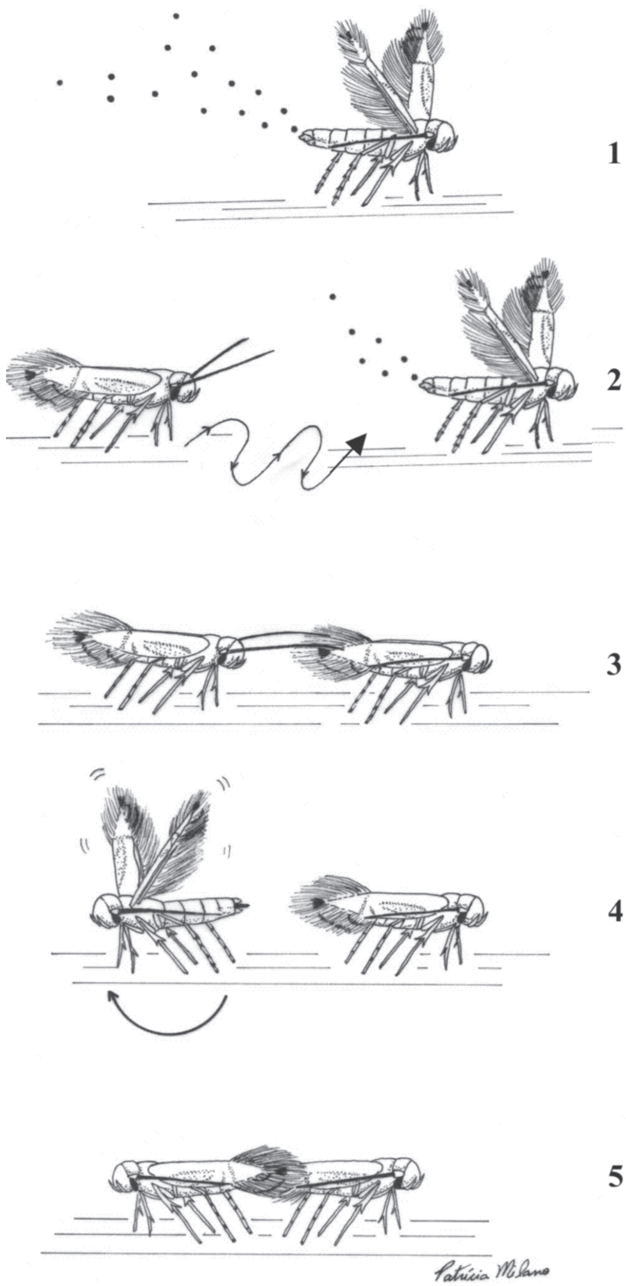

Figure 5. Courtship behavior in *P. citrella*.. 1 - Calling female with wing raised and exposed abdominal tip. 2 - Male approaching a calling female and walking in a zigzag pattern, 3 - Male making the first direct contact with female by antennation on her abdomen. 4 - Male raising his wings, exposing the aedeagus, rotating 180º, and walking backwards towards the calling female. 5 - Copulation.

walking in a zigzag pattern and made the first contact with the female by antennation at her abdominal tip. Touched females responded by lowering the wings and when receptive she remained still, whereas the non-receptive females walked away from the males. Next, a male rotated its body at 180º angle to a receptive female, lifted up the wings, and exposed the aedeagus while staying in the direction opposite to the female. While fanning the wings, the male walked backwards towards the receptive female, introduced the aedeagus and soon after stopped wing movements (Fig. 5). During copulation, male and female remained in opposite directions, with their antennae turned backwards away from the body and the male's wings resting on the female's abdomen (Fig. 4B).

Some features of the calling and courtship behavior in *P. citrella* females, particularly wing movements, were previously observed in *Lymantria dispar* (L.) (Charlton & Cardé 1990), *Mamestra configurata* Walker (Howlander & Gerber 1986), and *Bucculatrix thurberiella* Busck (Lingren *et al.* 1980). Male wings overlapping over female wings during copulation have been previously observed in *B. thurberiella* (Lingren *et al.* 1980).

In summary, we have gained a better understanding of the mating behavior of the citrus leafminer and clarified some discrepancies in the literature. The large majority of mating occurs at day 1 and day 2, with a broad peak of mating activity from the last hour of the scotophase to the first hour of the photophase. Thus, for pheromone isolation and identification, pheromone glands of 1- or 2-day-old virgin females should be extracted within 2h, starting 1h before the end of the scotophase. Behavioral assays should be conducted at this time of mating activity.

Acknowledgments

The authors thank FAPESP and Fundecitrus for financial support, and CNPq and CAPES for the scholarship granted to A.L. Parra-Pedrazzoli in the USA and Brazil, respectively.

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Received 17/VI/05. Accepted 29/VII/05.