Mating Behavior of *Diabrotica speciosa* (Coleoptera: Chrysomelidae)

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ABSTRACT *Diabrotica speciosa* (Germar) is an economically important pest of Neotropical cultures and represents a quarantine risk for Neartic and Paleartic Regions. Despite its agricultural importance, few studies have been done on mating behavior and chemical communication, which has delayed the development of behavioral techniques for population management, such as the use of pheromone traps. In this study, we determined 1) the age at first mating; 2) diel rhythm of matings; 3) number of matings over 7 d; 4) the sequence of *D. speciosa* activities during premating, mating, and postmating; 5) the duration of each activity; and 6) response to male and female conspecific volatiles in Y-tube olfactometer. The first mating occurred between the third and seventh day after adult emergence and the majority of pairs mated on the fourth day after emergence. Pairs of *D. speciosa* showed a daily rhythm of mating with greater sexual activity between the end of the photophase and the first half of the scotophase. During the 7 d of observation, most pairs mated only once, although 30% mated two, three, or four times. In a Y-tube olfactometer, males were attracted by virgin females as well as by the volatile compounds emitted by females. Neither males nor their volatiles were attractive to either sex. Our observation provide information about mating behavior of *D. speciosa*, which will be useful in future research in chemical communication, such as identification of the pheromone and development of management techniques for this species using pheromone traps.

KEY WORDS copulatory behavior, courtship, pheromones, semiochemicals, daily rhythm

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The genus *Diabrotica* Chevrolat includes some of the most damaging insect pests throughout the Americas (Krysan 1986, Pereira et al. 1997, Walsh 2003). In the last several decades, the importance of *Diabrotica* has increased because of the serious injuries caused by native species on crop plants in the American continent (Krysan 1986, Tollefson 1998). Moreover, some species of this genus have acquired a quarantine status, mainly after the introduction and spread of *D. virgifera virgifera* Le Conte in the European and Mediterranean Region (OEPP/EPPO 2005, Baca 2006, Cornelia et al. 2007).

Considering the importance of *Diabrotica* spp. throughout the world, there has been an important research effort on behavior and ecology of several species in this genus (Guss 1976, Lew and Ball 1979, Hammack 1995, Hibbard et al. 1997, Tallamy et al. 2002, Fernandez and Hilker 2007, Marquardt and Krupke 2009, Spencer et al. 2009). Therefore, the mating behavior and chemical communication of *D. undecimpunctata howardi* Barber, *D. virgifera virgifera* LeConte, and *D. barberi* Smith and Lawrence in the Nearctic Region have been extensively studied. Such research has resulted in understanding of mating mechanisms and pheromone emission, providing the necessary information to develop techniques for managing these species in agricultural crops (Ball and Chaudhury 1973, Guss 1976, Branson et al. 1977, Guss et al. 1983, Lew and Ball 1979, Dobson and Teal 1986, Meinke et al. 1989, Hammack 1995, Spencer et al. 2009). For *D. virgifera virgifera,* pheromone traps have been used for monitoring the abundance and spatial distribution in European areas, to delay its spread (Reynaud 2003, Tóth et al. 2003, Baca 2006, Boriani et al. 2006). Moreover, according to El-Sayed et al. (2006) mass trapping could be an effective method for eradication of invasive species of *Diabrotica.*

Diabrotica speciosa (Germar) is the most abundant and damaging crop pest in Brazil (Christensen 1944, OEPP/EPPO 2005) and presents a risk to the European continent, as well as other species from North America (OEPP/EPPO 2005). It occurs in almost every Brazilian state causing damage to corn, potato, and beans among others (Krysan 1986, Milanez and Parra 2000, Ávila and Milanez 2004). It is a polyphagous insect and the root feeding by larvae causes stunted growth of numerous species of plants. In maize, larvae used to make tunnels in the stem, causing the bend of the first internodes. In groundnut and potato, the pods or tubers may be externally damaged (OEPP/EPPO 2005). Adults cause defoliation and can act as vectors for pathogens (Boff and Gandin 1992, Oliveira et al. 1994, Marques et al. 1999).

Contrary to North American species, research on behavior and chemical ecology for South American

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species, such as *D. speciosa*, are rare. Although some cucumber based attractants have been tested for *D. speciosa* and *Cerotoma arcuata* (Olivier) (Nishida et al. 1986; Ventura et al. 1996, 2000), they have only been used on small crop areas, mainly because of the difficulties to obtain these compounds and use them at a large scale (Stupp et al. 2006). Thus, the lack of basic information about mating behavior and chemical communication delays the improvement of monitoring and control techniques using pheromone traps (Ventura et al. 2001).

The aims of this study were to characterize the following aspects of reproductive behavior of *D. speciosa:* age at first mating, daily periodicity in mating, number of matings per insect pair, and sequence of activities related to sexual behavior. In addition, we investigated the role of pheromones in sexual behavior by examining the attractiveness of males and females to odors released by each sex.

Materials and Methods

Insect Rearing. Individuals of *D. speciosa* were reared in the laboratory based on methodology outlined by Milanez and Parra (2000) and Ávila et al. (2000). Larvae and adults were fed corn roots, *Zea mays* L. (cultivar. Suwan), and leaves of the dry bean, *Phaseolus vulgaris* (L.) (cultivar. Carioca), respectively. Approximately 500 adults were kept in a plastic cage (40 cm length, 30 cm width, 40 cm height) containing bean plants and a substrate for oviposition made up of a circular container (10 cm height, 1.5 cm diameter) lined with a moistened black cheesecloth (Milanez and Parra 2000). Eggs were collected by washing the cheesecloth in running water over a voile-netting sieve. The eggs were treated with a disinfectant solution (1% copper sulfate, $CuSO_4$), and then transferred to petri dishes, where they remained until eclosion. Newly hatched larvae were inserted into corn seedlings kept in plastic containers filled with moist vermiculite (one part vermiculite: two parts water). After 15 d, immatures were transferred to new containers containing the same substrate and new corn seedlings. For rearing and for the behavioral studies, insects were kept in environmentally controlled rooms at a temperature of 22 ± 3°C, a relative humidity of 70 ± 10%, and a photoperiod of 14:10 h L:D.

Collection of Male and Female Natural Extracts. A custom-made dynamic headspace system was used to collected volatile compounds emitted by adults of each sex (Millar and Haynes 1998, Zarbin et al. 1999). The *D. speciosa* holding chambers used with this system, were horizontally oriented glass cylinders (20 cm height, 7 cm diameter) with air intake and outflow openings at opposite ends of the cylinder. At the outflow end, a glass column (7.5 cm height, 0.5 cm diameter) containing 0.150 g of adsorbent polymer (Super Q 80/100 Mesh, Alltech Associates, Deerfield, IL) was connected to trap insect volatiles. The air flow inside the chambers was generated by a vacuum pump and regulated at 800 ml/min by fluxometers. All the connections were installed using Teflon PTFE tubes and the inside air were filtered by a glass column (20 cm height, 3.0 cm diameter) empty with granular activated carbon.

Volatile collections were made between 1900 and 0700 h, during peak of mating activity of *D. speciosa*, according preliminary studies about mating activities. For each replication, 150 virgin males or females, 5 d after their emergence, were used. After each collection, the tubes with adsorbent were removed and the volatile compounds were eluted with 2 ml of hexane. The samples were concentrated to 150 μl using a low flow gaseous N_2 and maintained at -10°C for use in the bioassays.

Mating Age, Diel Rhythm, and Frequency. Seventy mixed-sex pairs were selected on the day of emergence to characterize age at first mating, daily mating rhythm, and number of matings per pair. Each pair was kept in a transparent, circular cage (12 cm height, 10 cm diameter) and fed dry bean leaves that were changed daily. Visual observations were made at intervals of 1 h during 7 d after emergence to verify the number of pairs mating. A mating was considered to have occurred when the male's aedeagus was fully inserted into the female genital chamber. For observations and bioassays, a diode red light emitter was used during the scotophase and a white light was used during the photophase.

Olfactometer Bioassays. The attractiveness of males and females and their released volatiles was evaluated in a Y-tube glass olfactometer consisting of a main tube and two lateral tubular arms (20 cm length, 3 cm diameter) to which chambers containing the test treatments were connected. Airflow through the olfactometer was generated by a vacuum pump that pulled air through the treatment chambers toward the lateral arms and out through the principal tube. Fluxometers at the air intake were used to control a flow of 800 ml/min.

Bioassays were conducted with virgin adults from 1800 to 2200 h, the first period of scotophase, when the peak of mating activities in preliminary studies was observed. Males ($n = 50$) and females ($n = 50$) were released individually into the main tube of the olfactometer, where they could move toward the treatment or control arms during 5 min, as established in preliminary studies. In these bioassays, only the insects that moved and reached toward a lateral arm of olfactometer were considered as responders. The treatments included a group of 15 virgin females or males, each group compared with a clean-air control, and 15 insect-equivalent extract (150 μl) (Zarbin et al. 1999) of volatiles released by females or by males, each compared with a solvent control.

Sequence of Activities Involved in Sexual Behavior. To characterize the sequence of activities involved in the sexual behavior of *D. speciosa*, observations were conducted during the period of greatest sexual activity (1800 to 2200 h) using unmated males and females. Virgin pairs were isolated in transparent, circular containers (10 height, 8 cm diameter) containing dry bean leaves. An ethogram was developed using preliminary

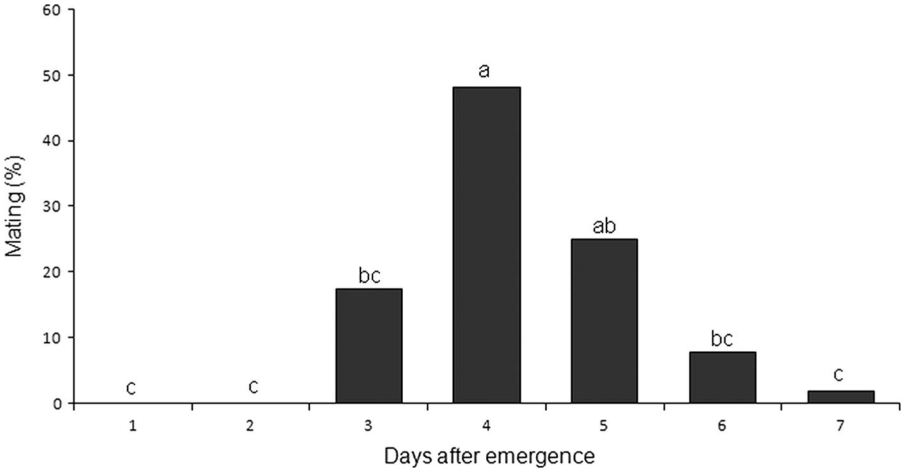

Fig. 1. Percentage of mating observed in *D. speciosa* pairs mating for the first time according to age. Different letters indicate statistically significant differences among the treatments ($n = 53$; Marascuillo; $P \leq 0.1$).

studies of male and female activity ($n = 30$) before, during, and after mating. Once the standard motor activities were known, new pairs ($n = 50$) were observed to confirm the behavioral sequence, quantify the activities, and record their duration. To determine the duration of copula, observations were done at intervals of 1 h. To confirm the calling posture of females, a magnifying glass and a flashlight equipped with a diode red light were used, which was oriented toward the female, in a contrary position to the observer. The behavioral activities were also recorded with a manual digital camera (Canon Power Shot S2 IS) to reevaluate each behavior.

Statistical Analysis. The data for proportion of pairs mating according to the age and time of day were analyzed using χ^2 tests ($P = 0.05$) (Conover 1980) of the homogeneity of the ratio of matings and nonmatings among treatments. For nonhomogeneous ratios, a Marascuillo test of multiple comparisons ($P = 0.1$) was made to compare the proportion of pairs mating during each period (Marascuillo 1966, NIST/SIMANTECH 2002).

Data from the olfactometer bioassays were analyzed using χ^2 tests ($P = 0.05$) using the null hypothesis that equal numbers of individuals moved into treated and control arms ($n = 50$).These Data were analyzed with Statistica software (STATISTICA 1997).

Results

Age, Diel Rhythm, and Number of Matings. Of the 70 pairs observed, ≈76% ($n = 53$) showed mating activity and 80 copulas were observed during the study period. Therefore, 30% ($n = 16$) of the pairs mated from two to four times and 70% ($n = 37$) mated only once.

The age of *D. speciosa* adults influenced the beginning of sexual activity, which occurred between the third and seventh days after adult emergence. The first mating was observed predominantly for 4- to 5-d-old pairs (Marascuillo's test, $P < 0.1$; Fig. 1). Newly emerged, and 1- and 2-d-old pairs did not show mating activity.

D. speciosa adults copulated throughout the day although these activities were intensified between the end of the photophase (1800 to 2000 h) and the first half of the scotophase (2000 to 2400 h) (Marascuillo's test; $P < 0.1$). No matings were observed between 0400 and 0800 h (Fig. 2).

Olfactometer Bioassays. *D. speciosa* males were attracted by virgin females (85%) (χ^2; $P < 0.0001$), as well as by the volatile compounds they emitted (≈75%) (χ^2, $P < 0.001$; Fig. 3A). However, neither males nor females were attracted by conspecifics of their own sex. Moreover, females were not attracted to males or their volatiles (Fig. 3B).

Sequence of Activities Involved in Sexual Behavior. Males and females of *D. speciosa* showed well-defined premating, mating, and postmating behavioral activities (Fig. 4). Several females (52%) displayed the same calling posture observed for *D. virgifera virgifera* (Hammach 1995), which females expose the membrane located between the seventh and eighth-abdominal segments. The calling posture of *D. speciosa* consisted of the intermittent exposure of this structure simultaneously with the distention and retraction of the abdomen. Forty-eight percent of the females observed showed no calling posture even though they had mated.

Regardless of female calling status, premating activity of the male began with walking or leaping toward the female. After approaching the female, male used his antennae and mouthparts (70%) to make exploratory contact with the female's terminal abdominal segments, or mounted her immediately without prior contact (30%). During these activities, unreceptive females avoided the approaching male by moving away rapidly. Once the male had successfully mounted the female, he rubbed his antennae along the

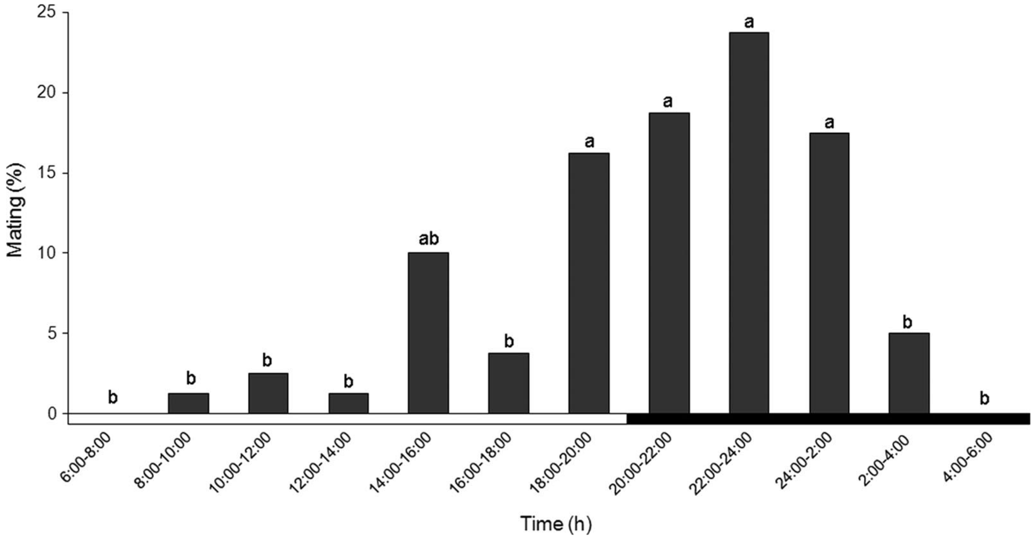

Fig. 2. Percentage *D. speciosa* pairs mating for the first time in relation to time of day. Different letters indicate statistically significant differences among treatments ($n = 53$; Marascuillo; $P \leq 0.1$). White and black bars are photophase and scotophase periods of 14:10 h (L:D) photoperiod, respectively.

whole length of the female's antennae (antennation). The female moved her hind legs toward her partner's body (4%) and/or walked around with the male on top (44%). At the same time, the male intensified courting movements (48%) by tapping his legs and mouthparts against the partner's elytra, turning the antennation faster. For 52% of pairs, females remained motionless, which allowed the initiation of mating without courting behavior. In this case, the male introduced its aedeagus immediately after mounting.

The premating behavior ceased when the male introduced the aedeagus in sharp lateral and frontal

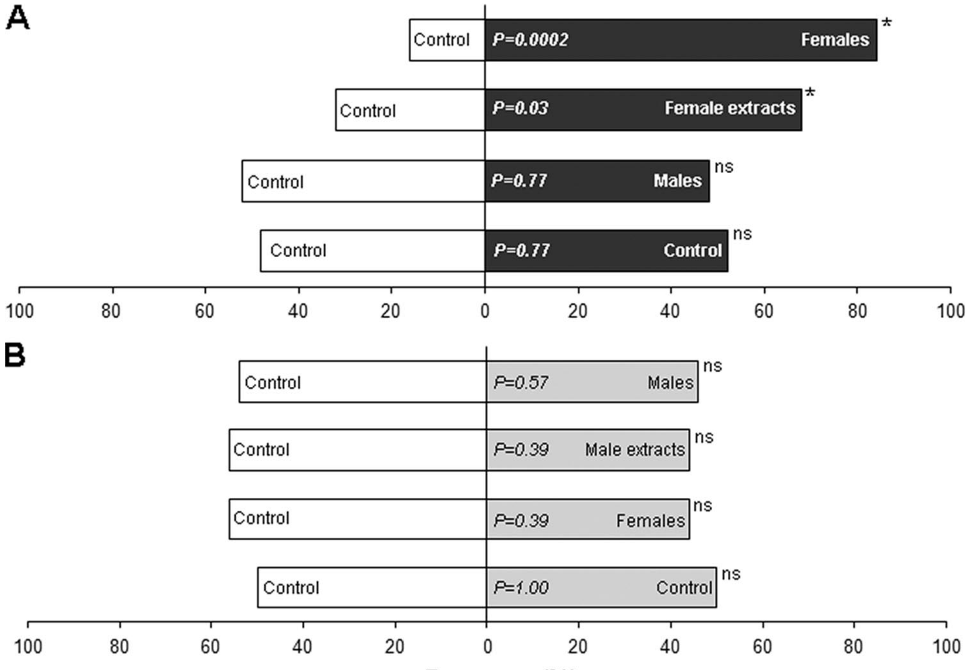

Fig. 3. Response of males (A) and females (B) of *D. speciosa* to conspecific adults, their volatiles obtained by headspace collection and control in a Y-tube olfactometer. "*" Indicate statistically significant differences among treatments within horizontal bars ($n = 50$; χ^2; $P \leq 0.05$).

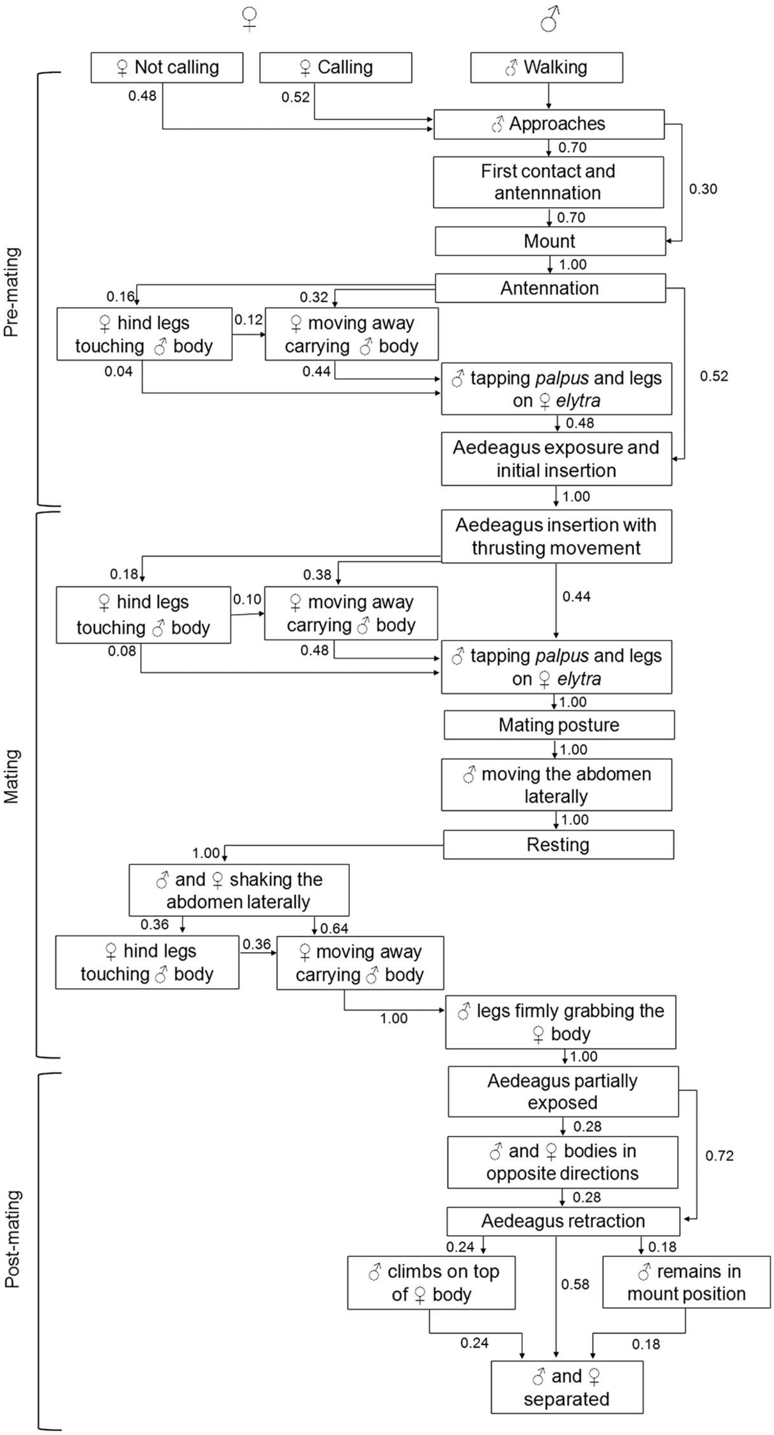

Fig. 4. Sequential activities involved in the mating behavior of *D. speciosa* observations (numbers indicate the proportion of individual/pairs that engaged in each activity; $n = 50$).

abdominal movements. The premating stage lasted 0.05–14.65 min (Table 1).

Mating started when the aedeagus was completely introduced into the female genitalia, when many females (56%) demonstrated a restless behavior, apparently trying to escape. In response of this female activity, male repeated the courting movements with his legs, mouthparts, and antennation movement.

Table 1. Duration of the premating, mating, and postmating behavior of *D. speciosa* ($n = 50$)

Activities	Duration ± SE (min-max) (min)
Premating	2.32 ± 0.68 (0.05–14.65)
Mating	166.20 ± 8.67 (60.00–360.00)
Postmating	0.48 ± 0.09 (0.03–11.73)

Once at rest, the mating pair slowly vibrated their antennae, which in the males were positioned laterally or facing backwards and in the female were facing forwards. Additionally, the male made occasional body movements, turning his body slowly to both sides while keeping the aedeagus locked into the female (100%).

During the final period of mating, the female became restless, moving her abdomen to the sides while walking with the male on top and pushing her hind legs against him (100%). This behavior, observed for all pairs, is related to female attempts to terminate copulation. However, the male binds himself strongly to the partner's body, keeping his front and mid legs on the female's elytra and hind legs bound to female's final sternites. During this activity, the male managed to sustain mating until the aedeagus was finally exposed and retracted. Most pairs (≈40%) remained mating for at least 3 h, although this period varied from 1 to 6 h (Table 1).

Postmating activities began after partial exposure of the aedeagus. In some pairs (28%), the male backed away from the female and the pair kept their bodies in opposite directions with the aedeagus uniting both of them until separation and distancing. For the remaining pairs (72%), the retraction of the aedeagus occurred when the male was still on top of the female, although different sequential behavior patterns were followed. For 58% of the pairs, the male withdrew the aedeagus and quickly dismounted the female. For these pairs, postmating was short, without mate-guarding behavior and lasted an average of 0.48 min. However, 18% of the males remained mounted on the females for up to 11.73 min before moving away. In 24% of the pairs, the male remained on the female's body resting the three pairs of legs on her elytra and touching the female's body with his mouthparts.

Discussion

Pairs of *D. speciosa* showed a circadian rhythmicity in the mating behavior, suggesting synchronization in the emission and reception of sexual stimuli between males and females. Circadian rhythms associated with mating have been found for other Diabrotica species, such as *D. virgifera,* where the adults mate the whole day, although they intensify such behavior at the end of the photophase (Spencer et al. 2009). Considering that *D. virgifera* males are capable of responding to pheromones at any time during the day, it is possible that the emission of these compounds by the females is determining diel rhythms (Dobson and Teal 1986). The results of the current study for *D. speciosa* indicate that sexual communication is concentrated at the end of the photophase and the beginning of the scotophase, although matings were observed at other times. Additionally, field observations made by Nava et al. (2004) demonstrated an increase in male and female activity during the end of the photophase, which may be related to dispersal of these insects to search for individuals of the opposite sex.

The multiple matings recorded for *D. speciosa* could indicate that males and females have multiple partners and multiple matings during their reproductive period, although more research is necessary to confirm this statement. According to Dickinson (1997), such behavior has an important adaptive value because it can increase the reproductive success of the individual and influence various aspects of its behavior and ecology. In addition, the occurrence of multiple matings in insects can significantly decrease the efficiency of monitoring and management of field populations (Sadek 2001), mainly when the female sexual pheromone is used for mass trapping. In this case, a single remaining male in the area could fertilize many females, reducing the success of population control.

The results obtained using Y-tube olfactometer are consistent with those obtained by Ventura et al. (2001), who demonstrate that females are responsible for attracting males. In addition, the attraction of males by female volatiles proves that a pheromone is a mediator of sexual communication in *D. speciosa.* For other species in this genus, the emission of sexual pheromones by the females is common. Among them, 8R-methyl-2R-decanol-propanoate and 10R-methyl-2R-tridecanone were identified as the major components of the pheromones of *D. virgifera virgifera* and *D. undecimpunctata,* respectively (Guss et al. 1983, Dobson and Teal. 1987). These compounds have been synthesized and can be used to improve management of field populations, such as pheromone traps for population monitoring, mass trapping, or mating disruption.

Our observations demonstrated that the sexual behavior of *D. speciosa* males and females is similar to that found for other species in this genus and also for some Coleoptera (Hill 1975, Santolamazza and Cordero 1998, Wang and Davis 2005, Ibeas et al. 2008). Lew and Ball (1979) studied the mating activity of *D. virgifera* and observed 10 distinct behavioral stages from courtship to postmating. For *D. speciosa,* sexual behavior started with calling, which is also common in *D. virgifera* (Lew and Ball 1979, Hammack 1995), where females exposed a membrane located at the tip of the abdomen. According to Lew and Ball (1978), this membrane is composed of secretory epithelial cells that may produce and release sexual pheromones. However, considering that a large proportion (48%) of receptive females of *D. speciosa* did not exhibit calling behavior, it is likely that pheromone emission can happen even without exposure of this membrane. As demonstrated by Hammack (1995), calling posture is not a prerequisite for mating in *D. virgifera* because 35.5% of the females mated even without membrane exposure.

The courtship behavior of male *D. speciosa* was relatively brief and characterized by antennation and leg tapping and mouthpart contact against the female's elytra. According to Tallamy et al. (2003), the contact of the antennae by the male of *D. undecimpunctata* on the dorsal part of the female results in relaxation of the copulatory duct and allows the aedeagus access to the copulatory duct. Furthermore, a rapid series of taps on the dorsal part of the female is associated with immediate acceptance of the partner (Tallamy et al. 2002). A similar behavior also appears to be common in other chrysomelids and cerambycids. In the cerambycid *Monochamus galloprovincialis* Olivier, friction of the mouthparts against the female's elytra determines partner acceptance and accelerates the beginning of mating (Ibeas et al. 2008). For the chrysomelids *Gastrophysa atrocyanea* (Motschulsky), *Leptinotarsa decemlineata* (Say), and various cerambycids, chemical compounds in the females, which act as contact pheromones, are present. These tactile stimuli are recognized by receptors located in the male antennae and palps, and they perform a significant role in the courting behavior (Jermy and Butt 1991, Ginzel and Hanks 2003, Zhang et al. 2003, Sugeno 2006). During our observations, antennation and tapping of the male's mouthparts against the female's elytra was observed for all pairs of *D. speciosa.* Such behavior appears to significantly influence premating, mating, and postmating activities. For other insect species, this behavior is strictly related to a chemical compound present in the female elytra (Ginzel and Hanks 2003, Nojima et al. 2007, Ibeas et al. 2008), such as cuticular compounds, which may be present in *D. speciosa* too. To confirm this hypothesis, the identification of cuticular compounds and evaluation of their biological activity will be required in future studies.

Mating of *D. speciosa* lasted between 1 and 6 h, and the behavioral repertory during postmating was restricted to antennation, rest and lateral movements of the male body while on top of the female. Such activities followed patterns similar to those observed for *D. virgifera,* where a male stayed on top of a female by fixing the meso and metathoracic legs to the lateral edges of the female's body (Lew and Ball 1979). Regarding this aspect, Hammack and French (2007) demonstrated that the males of some species of *Diabrotica* and *Cerotoma* have areas with a differentiated structure on the ventral part of the basitarsus, which likely allow greater fixation and longer periods of mating. Similar structure were verified in *Leptinotarsa* sp., which have sticky tarsal pads that provide increased adherence (Voigt et al. 2008, Geiselhardt et al. 2010) for mating and probably assist with mate guarding (Boiteau 1998). This sexual dimorphism was also observed in *D. speciosa* (C.N., unpublished data), and a more detailed morphological analysis will allow a detailed description of this feature.

This is the first detailed study on the sexual behavior of *D. speciosa,* and it supplies important information as a source for further research on the chemical ecology of this species. Furthermore, these data can be used to identify and synthesize its sexual pheromone, which could allow development of environmentally friendly integrated pest management techniques, such as the monitoring and mass trapping of *D. speciosa* populations.

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