DAILY TIMING OF MATING AND AGE AT REPRODUCTIVE MATURITY IN DIAPHORINA CITRI (HEMIPTERA: PSYLLIDAE)

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ABSTRACT

The psyllid Diaphorina citri Kuwayama vectors a bacterium, Candidatus Liberibacter asiaticus, in Florida which is responsible for citrus greening disease (huanglongbing), one of the most serious diseases of citrus. Despite the great economic importance of D. citri to citrus production, little is known about the reproductive biology and behavior of this insect. We conducted studies to determine the copula duration, the age at which both males and females reach reproductive maturity, the pre-oviposition period, the daily timing of mating activity in a laboratory colony, and the temporal dynamics in the sex ratio of a cohort of newly eclosed adults. The emergence patterns of males and females were strikingly similar, with no evidence for protandry or protogyny. Both sexes reached reproductive maturity by 2-3 d post-eclosion. Oviposition generally began within 1 d after mating, but was longer when females were mated at 2 d of age. Mean ± SEM copula duration was 48.3 ± 8.4 minutes (range: 15.2-98.0). Mating on orange jasmine was observed almost exclusively on flush shoots during daylight hours, with no obvious peak of daily mating activity. Mating activity may be constrained during scotophase in part by cooler temperatures and lack of light. Methods for carrying out mating experiments with this species are described.

Key Words: Asian citrus psyllid, mating behavior, reproductive biology, huanglongbing, citrus greening disease

RESUMEN

El psílido Diaphorina citri Kuwayama es un vector del bacterium, Candidatus Liberibacter asiaticus, en la Florida la cual es responsable de la enfermedad del enverdecimiento de los cítricos (huanglongbing), considerada una de las enfermedades mas serias en cítricos. A pesar de la gran importancia economica de D. citri en la producción de los cítricos, poca es lo conocido sobre la biologia reproductiva y el comportamiento de este insecto. Realizamos estudios para determinar la duración de la cópula, la edad en que los machos y las hembras alcanzan su madurez reproductiva, el periodo de pre-oviposición, la hora diaria que sucede el apareamiento, y la dinámica temporal en cuanto a la proporción de hembras y machos adultos recién emergidos del cohorte. Los patrones de la emergencia de los machos y hembras fueron notablemente similares, sin evidencia de protandria o protoginia. Ambos sexos alcanzaron la madurez reproductiva a los 2-3 días después de la eclosión. La oviposición generalmente empezó dentro de 1 día después del apareamiento, pero fue más larga cuando las hembras aparearon a los 2 días de edad. El promedio ± SEM de la duración de la cópula fue 48.3 ± 8.4 minutos (rango: 15.2-98.0). El apareamiento sobre Murraya paniculata fue observado casi exclusivamente sobre los brotes de nuevas hojas durante las horas de luz del día, sin tener un pico obvio en la actividad diaria del apareamiento. La actividad de apareamiento puede ser restringida durante el acotofase en parte por las temperaturas mas frescas y por la falta de luz. Se describen los métodos para realizar experimentos de apareamiento para esta especie.

The Asian citrus psyllid, Diaphorina citri Kuwayama, in Florida vectors Candidatus Liberibacter asiaticus, a phloem-limited, nonculturable bacterium responsible for citrus greening disease (huanglongbing) (Halbert & Manjunath 2004; Hung et al. 2004). D. citri was first found in Florida in June 1998 (Tsai et al. 2000) and has since spread throughout the state’s citrus-growing regions (Michaud 2004). Huanglongbing was first found in southern Florida in August 2005 (Bové 2006). Infestations of D. citri may cause direct feeding damage to citrus, resulting in distorted, reduced flush growth (Michaud 2004). The primary economic importance of the psyllid is transmission of huanglongbing, which is one of the world’s most serious diseases of citrus (Bové 2006). Citrus trees infected by this disease may live only 5 to 8 years, during which time they produce misshapen, poorly-colored, bitter-tasting, inedible fruit (Bové 2006; Halbert & Manjunath 2004). Despite the great economic importance of D. citri as a vector of greening disease, little is known about the reproductive biology and behavior of this pest.
Skelley & Hoy (2004) stated that both male and female *D. citri* reach reproductive maturity approximately 20 d after emerging as adults. However, these authors equated reproductive maturity with a change in abdomen color from green to orange, and we recently confirmed that females with blue/green abdomens mate and lay fertile eggs, even when much younger than 20 d old (E.J.W., unpublished data). Moreover, although earlier reports of the pre-mating interval are imprecise and lack details regarding methods and sample sizes, they suggest that adults mate within hours to a few days after emergence (Husain & Nath 1927; Pande 1971). Similarly, little or nothing has been reported regarding the pre-oviposition period or the daily timing and location of mating activity.

The goal of the studies presented here was to develop a more detailed understanding of the reproductive biology of *D. citri*. In particular, we investigated the duration of copulation, the age at which males and females reach reproductive maturity, the daily timing of mating activity, and the temporal patterns in the emergence of males and females in a cohort of newly emerging adults. A more thorough understanding of the biology and behavior of *D. citri*, especially regarding reproduction, may facilitate the development of more effective monitoring and management strategies.

**MATERIALS AND METHODS**

**Daily Timing of Mating**

We photographed *D. citri* on potted, mature orange jasmine (*Murraya paniculata* (L.) Jack; about 25 cm tall) plants in a laboratory colony (as described by Hall et al. 2007) maintained in a greenhouse under natural light. Photographs were taken over 24-h periods with Nikon Coolpix 5700 digital cameras (Nikon Corporation, Tokyo, Japan) and DigiSnap 2000 intervalometers (Harbortronics, Gig Harbor, WA, USA) set to record an image every 15 min. For each 24-h series of photographs, we focused a camera on a young flush shoot (immature leaves as described by Hall & Albrigo 2007), and in some instances we simultaneously focused a second camera on a more mature shoot that branched from a common point with the flush shoot. A camera flash was used to capture images in darkness. We examined each digital image and counted the total number of psyllids on each shoot as well as the number of mating pairs. We recorded a 24-h series of photographs on 5 different occasions: 7-8 Sep 2006, 20-21 Sep 2006, 22-23 Mar 2007, 29-30 Mar 2007, and 6-7 Jun 2007.

**Rearing and Housing Psyllids**

Psyllids were housed individually on *M. paniculata* seedlings grown in 53 mm-long plastic cone-shaped planting containers (cut from 25 mm wide, 160 mm long “cone-tainers”; Stuewe & Sons, Inc., Corvallis, OR, USA). Cones containing a seedling at the 2- to 3-leaf stage were fitted individually into one end of a plastic tube, modified from a 21-mm inner diameter, 52-mm tall poly styrene vial (BioQuip Products, Inc., Rancho Dominguez, CA, USA) by cutting off the bottom of the vial. The original opening of the vial was slipped over a seedling and around the cone, and a foam plug was used to stopper the upper, cut opening of the vial. Unventilated vials yielded undesirably high humidities, and attempts to ventilate via the upper opening of the vial were unsuccessful. Therefore, to allow ventilation, two holes (9 mm diameter) were drilled opposite each other in the sides =5 mm from the edge of the original opening and covered with mesh screen. *M. paniculata* seedlings were obtained by collecting fresh fruit from plants in the field and planting them (after extracting the seed from the fruit) as soon as possible, as stored seeds lost viability within a few weeks, even when refrigerated. Attempts to house psyllids on cuttings of flush were not successful, as the cuttings apparently provided inadequate nutrients to the psyllids and needed to be replaced frequently. Experimentation with different sized vials demonstrated the need to make the mating arena as small as possible relative to the plant in order to encourage psyllids to locate the plant and remain on it.

We collected fifth instars reared on *M. paniculata* in our laboratory colony and transferred them individually to seedlings in the plastic vial containers. We held psyllids in an environmental chamber (26°C, 60% RH, 14:10 h L:D photoperiod); these conditions yielded 70-80% RH inside the vials based on readings taken with a probe hygrometer. Every 24 h we examined each nymph to determine the day of adult eclosion and the sex.

**Laboratory Mating Experiments**

A pilot experiment suggested that both males and females were reproductively mature no later than 3 d post-eclosion. We established pairs of males and females with individuals at ages 1, 2, 3, and 4 d post-eclosion paired with an individual of the opposite sex at age 5-7 d post-eclosion (*n* = 10 per treatment). To initiate a pairing, we gently coaxed a male onto the bristles of a small paint brush and transferred him to a seedling in a vial that housed a female. We paired males and females just before the onset of photophase; after 24 h, we removed the male and returned him to his original vial. All psyllids were held in environmental chambers as described above under “Rearing and housing psyllids.” Every 24 h after pairings were initiated, we examined each vial to determine the pre-oviposition period (the number of days after pairing before eggs were laid). Begin-
ning 3 d after eggs were found, we examined vials daily to determine whether any eggs hatched. Females were assumed to have mated only if they laid fertile eggs.

To follow up on results from the observations on the daily timing of mating, we paired 5- to 7-d-old males and females and held them in environmental chambers under either complete darkness \((n = 13)\) or constant cool temperature \((20°C; n = 13)\) for the duration of 24-h pairings. Conditions were otherwise identical to those described for the mating pairs above, and all psyllids were moved back to chambers at 26°C with 14:10 h L:D photoperiod following the termination of pairings.

To determine copula duration, we paired 3- to 8-d-old males and females \((n = 10 \text{ pairs})\) in environmental chambers (as above under “Rearing and housing psyllids”) and maintained constant vigil to record the time at onset and termination of copulation. We then held females separately until it could be determined whether they laid fertile eggs.

Comparison of Adult Emergence Patterns between the Sexes

To examine whether the timing of adult eclosion in a cohort of \(D. \text{ citri}\) differed by sex, we introduced ca. 300 adult psyllids to a BugDorm-2 cage (MegaView Science Education Services Co., Ltd., Taichung, Taiwan) with a potted, mature \(M. \text{ paniculata}\) plant (about 30 cm tall) and allowed females to oviposit for 3 d before removing all adults. Beginning 2 weeks later, we carefully examined the plant daily for the presence of newly emerged adults. Adults were removed with an aspirator every 24 h until no new adults were found; each day we counted the number of individuals of each sex collected. We compared between the sexes the distributions of adult emergence patterns (i.e., the number of newly emerged adults collected on each day) using the following quantiles: 1, 5, 10, 25, 50, 75, 90, 95, and 99. We ran a linear regression of the quantiles for female emergence as a function of the quantiles for male emergence.

RESULTS

Daily Timing of Mating

In the laboratory colony, \(D. \text{ citri}\) was observed to mate almost exclusively on flush shoots and predominantly during photophase. Typically, the earliest mating pairs were observed within 1 h before or after sunrise, and the latest mating pairs were observed no more than 1 h after sunset. Results were similar among all observation dates, so only 2 dates are shown (Figs. 1-2). Mating was observed throughout daylight hours, but there was no obvious peak of mating activity. We tended to observe more individuals on flush shoots (mating or not) during photophase, with a gradual increase and decline in the total number of individuals observed on shoots that roughly correspond with the rise and fall of temperature over each day (Figs. 1-2). Often the number of individuals on flush decreased around the onset of scotophase (Fig. 1), but not always (Fig. 2). Over the two 24-h observations on mature shoots, few total psyllids were observed and only two mating pairs (data not shown).

Mating Experiments

\(D. \text{ citri}\) adults of each sex reached reproductive maturity at 2-3 d post-eclosion. When paired for 24 h with a 5- to 7-d-old male, nearly all of the 2-, 3-, and 4-d-old female psyllids subsequently laid fertile eggs (Table 1). Similarly, nearly all of the 5- to 7-d-old females that were paired with a 2-, 3-, or 4-d-old male laid fertile eggs (Table 1). Only one of the 1-d-old females laid fertile eggs, and none of the 5- to 7-d-old females that were paired with 1-d-old males laid fertile eggs.

For pairings in which males and females were 3- or 4-d-old, the pre-oviposition period was roughly 1 d, with most females beginning oviposition on the same day that they were mated (Table 1). The pre-oviposition period was significantly longer when the female in a pairing was only 2 d old at mating, and also tended to be longer for females paired with 2-d-old males (Table 1).

Roughly half of the female psyllids paired with males for 24 h under complete darkness \((5 \text{ of } 13)\) or in \(20°C\) chambers \((6 \text{ of } 13)\) laid fertile eggs. Mean ± SEM copula duration for 10 mating pairs was 48.3 ± 8.4 min (range: 15.2-98.0). In the 2 shortest copulations \((15.2 \text{ and } 20.3 \text{ min})\) the female did not lay fertile eggs, although 1 female that mated for 21.4 min did lay fertile eggs.

Comparison of Adult Emergence Patterns between the Sexes

The temporal patterns of adult emergence of a cohort of psyllids were similar between males and females (Fig. 3). The first day of emergence occurred on the same day for both sexes, and the median of the total number of individuals collected fell on the fourth day of emergence for both sexes. The quantiles of day of female versus male emergence were positively correlated \((F_{1,17} = 289.5, \ P < 0.0001, \ r^2 = 0.976; y = 0.996x – 0.081)\), and the slope of 0.996 for the regression equation indicated that there was no evidence for protandry or protogyny.

DISCUSSION

We observed \(D. \text{ citri}\) to mate almost exclusively on young flush shoots and predominantly during photophase. We tended to observe more psyllids on
flush shoots during photophase, but this was not always the case. On mature shoots, we observed relatively low psyllid numbers and very few mating pairs (data not shown). Throughout photophase, generally about one quarter of all psyllids observed on flush at a given time were mating, with no obvious mating peak observed. In contrast, an African psyllid vector of huanglongbing, *Trioza erytreae* (Del Guercio), may mate at almost any time of the day or night, with peaks just after sunrise and before sunset (Van den Berg et al. 1990).

Given that female *D. citri* lay eggs exclusively on flush (Husain & Nath 1927; Yasuda 1995), that greater light intensity and duration enhance oviposition (Yubin 1989), and that females do not oviposit during scotophase (E.J.W., unpublished data), it is perhaps not surprising that most encounters between the sexes would occur on flush during photophase. However, it is unclear whether males are attracted by females, by the flush itself, or perhaps by some interaction of stimuli from both females and flush. A study by Yasuda (1995) suggests that females are attracted to flush, while males might tend to settle on flush through positive phototaxis and/or negative geotaxis. In any event, adults of both sexes may prefer flush in part because it might yield a better food source than more mature parts of the plant,

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**Fig. 1.** Number of *Diaphorina citri* adults observed (total and mating) on flush shoots of a potted *Murraya paniculata* plant in a laboratory colony at 15-min intervals over 20-21 Sep. 2006 (A). Temperature and relative humidity within the plant canopy (B).
as suggested by the fact that young shoots are required by nymphs for development (Husain & Nath 1927). The preponderance of *D. citri* adults, nymphs, and eggs on the terminal growth of their host suggests that, during a flush, good spray coverage of a foliar pesticide for psyllid control may be needed primarily on the exterior portions of the canopy where most flush shoots develop.

Mating rates were reduced when psyllids were paired under complete darkness or in 20°C chambers, suggesting that mating activity during scotophase is apparently not entirely constrained either by lack of visual cues or by lower temperatures. Ultimately, the near absence of mating behavior during scotophase may well be modulated by a circadian rhythm that is in turn regulated by photoperiod. Interestingly, male mating frequency in the pear psylla *Cacopsylla pyricola* ( Förster ) is enhanced by longer photoperiodic experience (Krysan & Higbee 1990), and mating rates were near zero when pear psyllas were paired in total darkness for 24 h (Krysan 1990). Not only was mating in *D. citri* rarely observed during the night, but movement in general was greatly reduced during scotophase, with psyllids apparently in a sleep-like, inactive state. We frequently observed the same individuals at the same location on a plant over several consecutive 15-min inter-

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**Fig. 2.** Number of *Diaphorina citri* adults observed (total and mating) on flush shoots of a potted *Murraya paniculata* plant in a laboratory colony at 15-min intervals over 29-30 Mar 2007 (A). Temperature and relative humidity within the plant canopy (B).
vals during the night, whereas individuals could rarely be tracked over consecutive images that were recorded during the day. Arai (1993) reported similar sleep-like behavior during scotophase for the mulberry sucker Anomoneura mori Schwartz.

Hollis (2004) reported that, in general, psyllids may mate within a few h of emergence but males usually wait a few d before beginning to mate; the pear psylla fits this pattern, with females able to mate within a few h after molting and males taking 5 d to reach reproductive maturity (Burts & Fischer 1967). However, in T. erytreae, males are sexually mature on the same day they become adults, and females reach sexual maturity at 2-3 d post-eclosion (Van den Berg et al. 1991). In the psyllids Pachypsylla celtidis-gemma Riley (Walton 1960), Schedotrioza multitudinea (Maskell) (Taylor 1985), and Pauropsylla depressa Crawford (Negi & Bisht 1989), males and females may be reproductively mature on the same day of adult eclosion. Our data for D. citri show that both sexes may reach reproductive maturity as early as 2 d post-eclosion; however, oviposition was delayed slightly when females mated with males that were only 2 d old and delayed significantly when females themselves mated at only 2 d old. These data suggest that some individuals may mate at 2 d even though they have not reached full reproductive potential. It may be that the reproductive organs have yet to fully sclerotize in 2-d-old females, resulting in a limited ability to accept and process a spermatophore; males, on the other hand, might have limited ejaculate stores at only 2 d. In our experiments, we observed a single 1-d-old female to mate (E.J.W., personal observation), but she failed to lay any fertile eggs; this was presumably forced copulation. Another female laid fertile eggs when paired at 1 d old, indicating that successful mating is possible before 2-3 d, but apparently rare. Data from Husain & Nath (1927) and Hoffman (1936) suggest that in very warm weather, mating and oviposition may occur within 1-2 d after adult emergence.

For matings involving 3- to 4-d-old psyllids, oviposition generally began within 1 d after mating. This is more or less consistent with Hollis's (2004) statement that psyllids generally begin to oviposit within hours after mating. In contrast, at 24-26°C the pre-oviposition period for T. erytreae was 3-5 d (Catling 1973).

Pande (1971) reported the copula duration of D. citri to average 18 min (range: 10-30 min), but did not state the temperature at which these matings were observed. Given that Pande also observed D. citri to mate as early as 12 h after emer-

### Table 1. Number of Females Laying Fertile Eggs and Duration of the Pre-oviposition Period (Mean ± SEM) for Females Paired with Males in Different Age Combinations.

<table>
<thead>
<tr>
<th>Female age (d)</th>
<th>Male age (d)</th>
<th>No. laying fertile eggs</th>
<th>Pre-oviposition period (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5-7</td>
<td>1</td>
<td>(4.4 ± 1.1)</td>
</tr>
<tr>
<td>2</td>
<td>5-7</td>
<td>7</td>
<td>3.0 ± 0.7 a</td>
</tr>
<tr>
<td>3</td>
<td>5-7</td>
<td>9</td>
<td>0.9 ± 0.3 ab</td>
</tr>
<tr>
<td>4</td>
<td>5-7</td>
<td>10</td>
<td>0.1 ± 0.1 b</td>
</tr>
<tr>
<td>5-7</td>
<td>1</td>
<td>0</td>
<td>(3.8 ± 0.6)</td>
</tr>
<tr>
<td>5-7</td>
<td>2</td>
<td>6</td>
<td>1.3 ± 0.7 ab</td>
</tr>
<tr>
<td>5-7</td>
<td>3</td>
<td>7</td>
<td>0.1 ± 0.1 b</td>
</tr>
<tr>
<td>5-7</td>
<td>4</td>
<td>10</td>
<td>0.6 ± 0.4 b</td>
</tr>
</tbody>
</table>

Kruskal-Wallis statistic: $\chi^2 = 20.7$

P-value: 0.0009

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\(^{1}n = 10\) females for each treatment.

\(^{2}\) Determined by the first laying of any fertile eggs, not infertile ova. Data for the 1 * 5-7 and 5-7 * 1 are shown for comparison but were not included in analysis; 2 females in each of these 2 treatments failed to lay any ova. Means followed by the same letter do not differ significantly based on Dunn's test ($\alpha = 0.05$).

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Fig. 3. Number of newly emerged D. citri adults collected every 24 h from a potted Murraya paniculata plant. The cohort was established by allowing females to oviposit on the plant for 3 d before removing all adults; d 1 represents the first day that new adults were observed.
gence, the observations likely occurred at warmer temperatures than used in our experiments; this would explain the typically longer copula durations that we observed. Copula durations of roughly 30–40 min have been reported in *Ctenarytaina thysanura* Ferris and Klyver (Mensah & Madden 1993) and the pear psylla (*Burts* & *Fischer* 1967), whereas mean copula duration in *T. erytreae* was only about 5.5 min (Van den Berg et al. 1991) and that of *P. depressa* about 10 min (Negis & Bisht 1989). Thus, the mean duration of copulation of nearly 50 min that we observed for *D. citri* may be relatively long for a psyllid.

*Burts* & *Fischer* (1967) and White (1970) reported protandry in the pear psylla and *Cardia-spinia densitexta* Taylor, respectively, but for *D. citri* we observed emergence patterns that were strikingly similar between the sexes. Protandry is often found in species for which there is a strong selective advantage to males that mate with virgin females. Female *D. citri* apparently require multiple matings to achieve optimal fecundity (E.J.W., unpublished data), so there may be limited benefit to mating a virgin female. The sex ratio in the cohort of *D. citri* used in our study was male biased (0.561), which may simply reflect our relatively small sample size (*n* = 408 adults).

Of the 2 known vectors of huanglongbing, considerably more is known about the reproductive biology of *T. erytreae* than *D. citri*. The research presented here contributes to a more detailed understanding of the reproductive biology and behavior of *D. citri*, which may facilitate the development of more effective monitoring and management strategies. For example, the results of our investigation may help to optimize spraying rates (in conjunction with field scouting) and to model population growth rates of this pest. Knowledge of the basic mating behavior also can be used to improve future experiments aimed at further clarifying the reproductive biology and ecology of this species.

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