Importance of multiple mating to female reproductive output in *Diaphorina citri*

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Abstract. The importance of multiple mating to female reproductive output in *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is evaluated by grouping individual females with one or three males for 24 h (short duration) or 2 weeks (long duration) and examining oviposition over 18–19 days. For the short-duration treatments, females lay more eggs per day when grouped with multiple males, whereas females in the long-duration treatments lay more eggs when paired with one male. When held for 24 h with one or three males, females show a decline in fecundity beginning 10 and 15 days after mating, respectively. Total fecundity is relatively high for females paired with one male for 2 weeks, but fecundity is low and mortality high when females are held with three males for 2 weeks. In treatments in which females are held with males for 2 weeks, oviposition increases dramatically in the days after removal of males. For females paired with a male for 24 h and re-paired for 24 h, 9 days later, fecundity remains high throughout the 18–19-day observation period. Egg fertility does not differ among treatments, but varies over time in a manner that is similar among treatments. The present study demonstrates that, in *D. citri*, females require multiple matings over time to achieve high reproductive output, but oviposition is constrained by the presence of males.

Key words. Asian citrus psyllid, citrus greening disease, fecundity, huanglongbing, polyandry, reproductive biology.

Introduction

The psyllid *Diaphorina citri* Kuwayama in Florida transmits *Candidatus* Liberibacter asiaticus, a phloem-limited, nonculturable bacterium, which is responsible for citrus greening disease (huanglongbing) (Halbert & Manjunath, 2004; Hung *et al.*, 2004). *Diaphorina citri* was first found in Florida in June 1998 (Tsai *et al.*, 2000) and has subsequently 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 by injection of a feeding toxin and withdrawal of large quantities of plant sap, resulting in distorted, reduced flush growth (Michaud, 2004). However, 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 5–8 years, during which time they produce misshapen, poorly coloured, bitter-tasting, unmarketable fruit (Halbert & Manjunath, 2004; Bové, 2006). Despite the great economic importance of *D. citri* as a vector of greening disease, little is known about the reproductive biology and behaviour of this pest.

Multiple mating in female insects is reported to increase fecundity and egg fertility across a range of taxa (Arnqvist & Nilsson, 2000). Although the ejaculate of male insects often contains products from the accessory reproductive glands that enhance egg production and fertility, the seminal fluid may also contain a suite of substances that function to increase an individual male's relative paternity, but at the expense of female fitness (Chapman *et al.*, 1995; Simmons, 2001; Gillot, 2003). Thus, increased mating frequency generally decreases female longevity (Arnqvist & Nilsson, 2000), caused in part by the negative effects of male accessory gland products (Chapman *et al.*, 1995) and likely by a change in the allocation of nutritional reserves to egg development after

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mating (Proshold *et al.*, 1982). A single mating is rarely sufficient to yield maximum reproductive output in female insects, and thus the optimum mating rate for females of a given species balances the beneficial and deleterious contributions of the male ejaculate (Arnqvist & Nilsson, 2000), as well as possible direct genetic benefits of multiple mating (Jennions & Petrie, 2000). Multiple mating may take the form of mating with two or more different males (polyandry) or remating with the same male. In addition, females may mate multiple times at the onset of reproductive maturity and store sperm for the duration of their lives (eusocial Hymenoptera offer a well-known example; Wilson, 1971), or they may mate intermittently throughout their lives as stores of viable sperm gradually diminish.

Relatively little is known about the mating behaviour of the Psylloidea. Hollis (2004) reports that female psylloids mate usually only once, but published data on mating frequency in this group of insects are scarce. Female reproductive output declines without continuous mating opportunities in the pear psylla, *Cacopsylla pyricola* Foerster (Burts & Fischer, 1967), in the psyllid *Agonoscena pistaciae* Burckhardt & Lauterer (Mehrnejad & Copland, 2006), and in the African psylloid vector of huanglongbing, *Trioza erytreae* (Del Guercio) (Catling, 1973; Van den Berg *et al.*, 1991). By contrast, the fecundity of females is not affected by multiple mating in the psyllid *Tyora tessmanni* (= *Mesohomotoma tessmanni*) (Aulmann) (Igboekwe & Adenuga, 1983).

The present study aims to characterize the effects of multiple mating on reproductive output in female *D. citri*. Specifically, we investigate whether exposure of females to multiple males affects fecundity and egg fertility relative to females that are paired with only one male. Moreover, female/male groupings are maintained over both 24-h and 2-week durations to examine how a brief period of multiple mating at the onset of reproductive maturity affects reproductive output relative to multiple mating over a longer time period.

Materials and methods

Rearing and housing psyllids

Adult psyllids were reared from nymphs housed individually on orange jasmine [*Murraya paniculata* (L.) Jack] seedlings grown in cone-shaped planting containers (Stuewe & Sons, Inc., Corvallis, Oregon). *Murraya paniculata* seedlings were obtained by collecting fresh fruit from plants in the field and planting the extracted seeds. Nymphs were transferred individually to *M. paniculata* seedlings caged in plastic vial containers, as previously described (Wenninger & Hall, 2007). Briefly, each cage consisted of a 52-mm tall vial, modified as an open-ended cylinder with a foam plug used to stopper the top opening and two ventilation holes on the sides; individual cages were slipped over a seedling grown in a cone-shaped planting container. Fifth-instar nymphs were collected from a laboratory colony (as described by Hall *et al.*, 2007) reared on *M. paniculata* and transferred

individually to seedlings in the plastic vial containers. The psyllids were held in an environmental chamber (26 °C, 60% relative humidity, LD 14 : 10 h photoperiod); these conditions yielded 70–80% relative humidity inside the vials based on readings taken with a probe hygrometer. Every 24 h, each nymph was examined to determine the day of adult eclosion and the sex.

Laboratory mating experiments

Female/male pairs or groups of one female with three males were established and maintained for either 24 h ('short duration') or 2 weeks ('long duration'). The treatments comprised: single-male/short-duration; single-male/long-duration; multiple-male/short-duration; and multiple-male/long-duration. To initiate mating treatments, males were gently coaxed onto the bristles of a small paint brush and transferred to a seedling in a vial that housed a female. Mating groups were established just before the onset of photophase. In addition to the treatments listed above, a single-male/'punctuated-mating' treatment was established in which each female was paired with a male for 24 h, and the same male was returned 9 days later for another 24-h period. The mating interval for the punctuated-mating treatment was selected based on the timing of reduced reproductive output in a single-male/short-duration treatment in a pilot experiment. A virgin treatment was also set up in which females remained unmated for the duration of the experiment. All psyllids used in experiments were 4–6 days post-eclosion at the establishment of treatments; by 4 days of age, male and female *D. citri* are reproductively mature, and females typically begin laying eggs on the same day they are mated (Wenninger & Hall, 2007).

Over a period of 18–19 days, at four 4–5-day intervals after initiation of treatments, females were transferred to a new seedling; as far as possible, seedlings at the same two- to three-leaf growth stage were used. The 18–19-day observation period was selected based on timing of reduced fecundity of once-mated females in a pilot experiment. Males in the two long-duration treatments were transferred along with females up until the last transfer. Four such transfers were made, resulting in four egg 'batches,' with the last egg batch being produced in the absence of males for all treatments. Any females that died before the third batch were excluded from analyses, except for those in the multiple-male/long-duration treatment in which the treatment itself appeared to cause high mortality. The initial sample sizes (with number remaining alive at least through the third batch shown in parenthesis) comprised: single-male/short-duration = 16 (n = 16), single-male/long-duration = 16 (n = 16), multiple-male/short-duration = 15 (n = 13), multiple-male/long-duration = 13 (n = 5), punctuated-mating = 15 (n = 13), and virgin = 13 (n = 11).

After transferring adults to new seedlings, the number of eggs and nymphs found on each seedling was counted and any nymphs were removed. Every 2–3 days thereafter for 1 week, any additional nymphs that hatched were counted and removed. Seedlings with eggs were held in environmental

chambers as described above, but the vial enclosure was removed and a ring of petrolatum was added to the base of the stem to ensure that nymphs were isolated on the plant. After the collection of the fourth batch of eggs (18–19 days after mating), females were transferred to a new shoot where they remained for the duration of their lives. Thereafter, each female was examined daily to record longevity.

Statistical analysis

A 2×2 factorial analysis of variance (ANOVA) with repeated measures using unstructured covariance structure was used to examine the effect of the number of males that females were grouped with (one versus three) and grouping duration (24 h versus 2 weeks) on the number of eggs laid per female per day over each of the four times that eggs were collected. The model included the interaction between the two main effects as well as between each main effect and the time factor. The number of eggs laid per day was square-root transformed to achieve normality and equal variance. The effects of treatment and time on egg fertility (arcsine-square-root transformed) were analyzed similarly with repeated measures ANOVA. Repeated measures ANOVA was also used to compare fecundity per female per day among the egg batches collected for the punctuated-mating treatment. One-way ANOVA was used to examine the effect of mating treatment on female longevity (square-root transformed) and on the total number of eggs that females laid over the 18–19-day observation period. The punctuated-mating treatment was included in the analyses on longevity and total fecundity, and the virgin female treatment was included only in the analysis on longevity. Where appropriate, after ANOVA, Fisher's least significant difference test was used to discriminate among treatments. The significance level was set at $\alpha = 0.05$. Results are presented as mean $\pm$ SEM.

Results

The number of eggs that female *D. citri* laid per day did not differ significantly between females grouped with one versus three males or between females grouped with males for 24 h versus 2 weeks, but the effect of the interaction between the main effects was significant (Table 1). For the treatments featuring short grouping durations, females laid significantly more eggs per day when grouped with multiple males ($F_{1,138} = 6.07$, $P = 0.015$; single-male/short-duration: 7.50 ± 0.59, multiple-male/long-duration: 10.31 ± 0.92). For treatments featuring long grouping durations, females laid significantly more eggs per day when paired with one male ($F_{1,116} = 9.93$, $P = 0.002$; single-male/long-duration: 10.20 ± 0.85, multiple-male/long-duration: 6.19 ± 0.93). Fecundity per female per day was not significantly affected by the interaction between number of males and egg batch, but was significantly affected by the interaction between grouping duration and egg batch (Table 1). With respect to the nature of the interaction: in all four treatments, mean fecundity per day tended to increase between the first and second batches of eggs collected; however, reproductive output declined thereafter for the single-male/short-duration treatment, and tended to decline more gradually for the multiple-male/long-duration treatment (Fig. 1). In both the single-male/long-duration and multiple-male/long-duration treatments, reproductive output remained at a similar level between the second and third egg batches and increased dramatically for the last batch (when males were removed; Fig. 1). It should be noted, however, that only three females remained alive in the multiple-male/long-duration treatment

Table 1. Repeated measures analysis of variance comparing number of eggs that female *Diaphorina citri* laid per day (square-root transformed) or egg fertility (arcsine-square-root transformed) over four successive batches of eggs collected among females that were grouped with one or three males for short (24 h) or long (2 weeks) durations.

Source of variation	Numerator d.f.	Denominator d.f.*	F	P
Number of eggs laid per female per day				
Number of males (one versus three)	1	53.6	0.0	0.964
Grouping duration (24 h versus 2 weeks)	1	54.8	0.0	0.970
Number of males × Grouping duration	1	59.1	4.3	0.042
Successive egg batch collected†	3	48.4	3.2	0.030
Number of males × Egg batch	3	48	0.14	0.935
Grouping duration × Egg batch	3	47.2	11.5	< 0.0001
Egg fertility				
Number of males (one versus three)	1	51.1	2.5	0.119
Grouping duration (24 h versus 2 weeks)	1	52.1	0.2	0.685
Number of males × Grouping duration	1	52.4	1.4	0.243
Successive egg batch collected	3	47.1	4.7	0.006
Number of males × Egg batch	3	46.7	0.4	0.758
Grouping duration × Egg batch	3	46.3	1.6	0.208

*PROC MIXED in SAS may use an approximation of the denominator d.f., which may result in d.f. being given with decimal places.
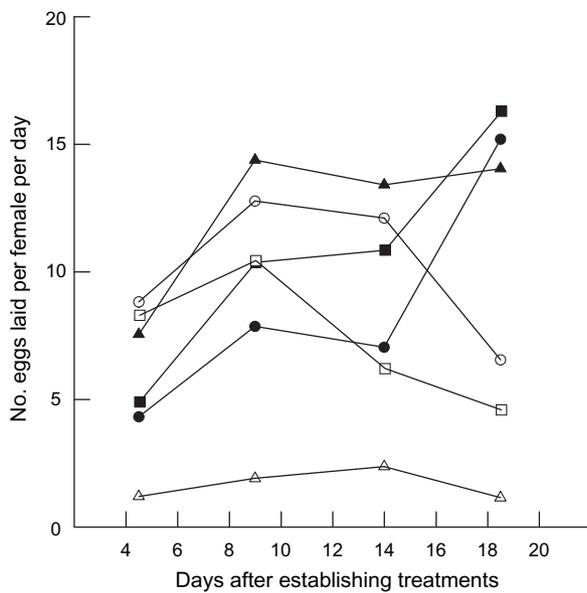†Eggs were collected at four 4–5-day intervals after mating.

Fig. 1. Mean number of eggs laid per female per day for each mating treatment over the four successive egg batches collected. Females were mated at age 4–6 days and eggs were collected at 4–5-day intervals after mating (at either 4 or 5 days after mating for the first egg 'batch' collected, 9 days after for the second, 14 days after for the third, and 18 or 19 days after for the fourth). Males in the punctuated-mating treatment were reintroduced for 24 h, 9 days after establishing treatments; males in the two long-duration treatments were removed 14 days after establishing treatments. Data were square-root transformed for analysis, but untransformed values are shown. Punctuated-mating and virgin treatments are shown for comparison, but were not included in the analysis of variance. Treatments: □, single-male/short-duration; ■, single-male/long-duration; ○, multiple-male/short-duration; ●, multiple-male/long-duration; ▲, single-male/punctuated-mating; △, virgin.

for the fourth egg batch. Although not included in the 2 × 2 factorial ANOVA, females in the punctuated-mating treatment maintained relatively high fecundity per day through egg batches 2–4 (Fig. 1).

Egg fertility was not affected significantly by either of the main effects or any of the interaction terms, but did differ over time (Table 1). Pairwise comparisons among the egg batches (with mating treatment pooled) showed that egg fertility was significantly higher in the second batch ($60.5 \pm 4.1\%$) relative to the third ($42.6 \pm 4.0\%$) and fourth ($43.2 \pm 5.5\%$) batches, which did not differ from each other. Egg fertility in the first batch ($50.5 \pm 4.6\%$) did not differ from the other batches collected.

For females in the punctuated-mating treatment, fecundity per day differed significantly among the four batches of eggs collected ($F_{3,11.5} = 4.92$, $P = 0.020$). The number of eggs laid per day for the first batch was significantly lower than in the remaining batches, which did not differ from each other (Fig. 1).

The total number of eggs laid by females differed significantly among the five mating treatments ($F_{4,70} = 8.95$,

$P < 0.0001$; Table 2). In particular, the fecundity of females in the multiple-male/long-duration treatment was significantly lower than in all other treatments; fecundity in the single-male/short-duration treatment was significantly lower than that in the single-male/long-duration and punctuated-mating treatments, but did not differ from the multiple-male/long-duration treatment. The punctuated-mating, single-male/long-duration, and multiple-male/long-duration treatments did not differ from each other.

Longevity of females differed significantly among treatments ($F_{5,81} = 4.06$, $P = 0.003$; Table 2). The lifespan of females in the multiple-male/long-duration treatment was significantly lower than in all other treatments, except the single-male/long-duration treatment. Longevity of females in the single-male/long-duration treatment was significantly lower than that of virgin females and tended to be lower than in the remaining treatments as well, although the differences were not significant.

Discussion

The results of the present study show clearly that multiple mating in female *D. citri* is required to maintain high reproductive output for periods exceeding 9 days. Moreover, mating is required over the course of the female's reproductive life because the fecundity per day of females in the multiple-male/long-duration treatment began to drop precipitously 2 weeks after mating. Also, given the sharp increase in fecundity after the removal of males in the single-male/long-duration and multiple-male/long-duration treatments, oviposition is constrained apparently by the presence of males. It is not clear whether the punctuated-mating treatment yields the maximum reproductive output for females over our observation period, but it is likely that females generally benefit from mating with one or more males upon reaching reproductive maturity, and then ovipositing for some period of time in the absence of males before remating to replenish sperm stores (and/or accessory gland components that might enhance reproductive output).

Table 2. Total number of eggs laid per female over the experiment (mean ± SEM) and female longevity (mean ± SEM), separated by mating treatment.

Treatment	Total number of eggs laid*	Female longevity (days)*,†
Single-male/short-duration	127.4 ± 13.2^b	49.2 ± 7.4^{ab}
Multiple-male/short-duration	171.9 ± 18.0^{ab}	49.0 ± 8.8^{ab}
Punctuated-mating	214.4 ± 21.2^a	49.4 ± 9.3^{ab}
Single-male/long-duration	180.1 ± 23.0^a	32.1 ± 2.7^{bc}
Multiple-male/long-duration	61.7 ± 17.9^c	17.8 ± 3.8^c
Virgin	—	54.9 ± 7.9^a

*Means within each column with different superscript letters differ significantly based on least significant difference tests.
†Data were square-root transformed for analysis, but untransformed values are shown.

There are limited data available on multiple mating in the Psylloidea with which to compare the present results for *D. citri*. Hollis (2004) reports that female psylloids usually mate only once, but it is not clear from what source this information is derived. Van den Berg *et al.* (1991) report that, in *T. erytreae*, fecundity and fertility does not differ between females paired with one male for 19 days and females held with five males for the same time period; however, their data suggest that once-mated females lay fewer eggs than females paired continuously with a male. Moreover, Catling (1973) show that *T. erytreae* exhibit no difference in fecundity whether held with two or three males for 3 days at the onset of reproductive maturity or with one or two males continuously, but state that females begin to lay infertile eggs 11–16 days after removal of males. Thus, the mating and oviposition patterns for *T. erytreae* and *D. citri* are similar. In both species, females benefit from mating either with multiple males early in their lives or from continuous remating with the same male (although not tested, females would presumably also benefit from remating with a different male). Both species also show a decline in oviposition approximately 2 weeks after isolation from males. Similar results are found in the pear psylla; females that mate only once lay fewer eggs than those that are mated multiple times, and fecundity and egg fertility decline approximately 10 days after removal of males (Burts & Fischer, 1967). Similarly, continuous mating opportunity is necessary for oviposition in *A. pistaciae* (Mehrnejad & Copland, 2006). By contrast, *T. tessmanni* shows no difference in fecundity or fertility among females that are single, double, or triple mated (Igboekwe & Adenuga, 1983).

Female *D. citri* observed on orange jasmine over their lives are found to reach maximum oviposition per day at approximately 21 and 15 days of age when held at 24 or 28 °C, respectively (Fung & Chen, 2006). Thus, the increase in eggs laid per day between days 4–5 and day 9 across all mating treatments evaluated in the present experiment may reflect a time delay between mating and increasing egg production in this species. After the second batch of eggs was collected (9 days after mating), females in the multiple-male/long-duration treatment tend to show a more gradual decline in reproductive output relative to the single-male/short-duration treatment, suggesting that females can almost certainly store sperm from more than one mating, but that their capacity for storage may be restricted to some extent. Blowers & Moran (1967) suggest that the limited capacity of the spermatheca in *T. erytreae* makes multiple mating necessary for the full complement of eggs to be laid. Given the decline in oviposition when isolated from males, *D. citri* might be limited similarly by the size of the spermatheca. Because females lay eggs exclusively on the flush of their host plants (Husain & Nath, 1927; Yasuda *et al.*, 2005) and a lack of flush can limit *D. citri* populations (Hall & Albrigo, 2007), it would be interesting to determine how long mated females can store viable sperm when flush is unavailable and whether they need to remate after long periods without flush.

Oviposition in the multiple-male/long-duration treatment is probably lower than in the other treatments because of frequent mating attempts by males and attempts by females to avoid males by dispersing off of the flush (E. J. Wenninger, personal observation). The markedly higher mortality of multiple-male/long-duration females is perhaps a result of physical harassment from males as well as the presumably high quantities of male accessory gland products that multiple-male/long-duration females acquired from multiple matings (see Introduction). High male to female ratios such as those under investigation in the present study (3 : 1) might be uncommon in the field (Wenninger & Hall, 2007, 2008), but it should be noted that fecundity also rises sharply after males are removed in the single-male/long-duration treatment, and mortality in this treatment tends to be high as well. In the field, females might escape harassment more easily. However, fully gravid females in the field (and in the laboratory colony) are more likely to walk than fly away when disturbed physically (E. J. Wenninger, personal observation), possibly because they are too heavy for sustained flight. Given the clear benefit to females of avoiding males for some time after mating, it would be interesting to determine to what extent dispersal to uninfested trees is driven by the movement of females that are recently mated, but not yet fully gravid. Although female *T. erytreae* do not exhibit a drop in reproductive output due to the presence of multiple males, Van den Berg *et al.* (1991) report the use of a large mating arena (2-L cages) in which females might escape possible harassment by males more easily. In the pear psylla, however, a high male : female ratio does negatively affect oviposition (Burts & Fischer, 1967).

The egg fertility patterns observed in the present study suggest that females are not sperm-limited by restricted access to males. However, although the interaction terms involving egg batch are not significantly related to egg fertility, the examination of an interaction plot (data not shown) reveals that egg fertility for the last batch of eggs drops sharply under the single-male/short-duration and multiple-male/long-duration treatments and rises sharply under the single-male/long-duration and multiple-male/long-duration treatments (egg fertility was similar across treatments for the first three batches). Thus, females that are isolated from males for more than 2 weeks show a tendency toward lower egg fertility, suggesting that females either become sperm-limited over time or sperm viability declines over time, whereas those that are maintained with males for 2 weeks tend to lay more fertile eggs when males are removed, suggesting that fertility as well as fecundity is constrained by the presence of males. Egg fertility values are surprisingly low across all mating treatments in the present study, but it is unlikely that our estimates of egg fertility are affected by nymphs escaping from the seedlings.

Knowledge of the mating behaviour and ecology of insect pests, particularly the factors that limit reproduction, is a key to developing biologically based pest management strategies. The results of the present study show that female reproductive output in *D. citri* is enhanced greatly by multiple mating but limited by continued exposure to males. The need of females to mate multiple times suggests that this species might be amenable to management tactics aimed at limiting

reproduction by exploiting male behaviour (e.g. mating disruption, attract-and-kill). To our knowledge, this is the first investigation into multiple mating in *D. citri*. It remains to be examined to what extent the patterns observed in the present study apply to field populations. For example, the long-term declines in fecundity observed in the laboratory might be less important in field populations if such females exhibit shorter longevity. To what extent mating behaviour influences dispersal in the field also remains to be examined.

Acknowledgements

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