

Influence of Weight of Male and Female Western Corn Rootworm (Coleoptera: Chrysomelidae) on Mating Behaviors

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ABSTRACT We evaluated the effect of weight, collection site, and age upon mating behaviors of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) with multivariate analysis. Our laboratory study of *D. virgifera* revealed that female weight was positively associated with the likelihood of mating, although female weight was not associated with females' receptivity to aedeagus insertion. Furthermore, the relationship between male and female weight on mating was investigated at two field locations in Indiana, and evidence of nonrandom mating was found. The implications of these results for pest management are discussed, with particular focus upon using this information for the construction of robust viable resistance management systems in *Bacillus thuringiensis* corn, *Zea mays* L.

KEY WORDS *Diabrotica virgifera virgifera*, reproductive behavior, mate choice, mating behavior

There is surprisingly little information regarding the reproductive behavior of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), in spite of its importance as a pest of corn, *Zea mays* L., in the United States (Levine and Oloumi-Sadeghi 1991). The mating behavior of *D. v. virgifera* was first investigated extensively by Lew and Ball (1979, 1980). They described the courtship and mating behavior, and described the relationship between copulation time and spermatozoan transfer. In these studies, they used 0- to 2-d-old females and 6- to 8-d-old males confined to petri dishes. In other experiments, Quiring and Timmins (1990) showed that ≈70% of females were mated within the first 24 h after emergence in cornfields. However, other than these relatively narrow fields of investigation, few of the parameters important in the rootworm mating system have been investigated.

Highlighting the need for further research is the fact that corn production is entering a new era of pest management. In the past decade, transgenic plants (primarily corn and cotton, *Gossypium hirsutum* L.) carrying genes for the production of insecticidal *Bacillus thuringiensis* (Bt) *Cry* proteins, and/or herbicide tolerance (e.g., glyphosate-tolerance) have been rapidly adopted by farmers in the United States and elsewhere (Baum et al. 2004). To slow the evolution of potential Bt. resistance, the U.S. Environmental Protection Agency (EPA) mandates a "high-dose/refuge strategy" requiring farmers to grow 20% nontransgenic corn to serve as a "refuge" near transgenic corn (Gould 1998). The purpose of the refuge is to support the development of "susceptible" individuals emerging

from the refuge to mate with putative "resistant" individuals surviving exposure to Bt corn (Tang et al. 2001).

However, without an understanding of what factors may be at play during courtship and mating, models that estimate mating frequency in these environments are entirely based on assumptions, and not empirical data (Crowder et al. 2005; Storer et al. 2006). To date, the roles that male and female weight play in determining mating success have not been investigated in the field or under controlled conditions. In the following experiments, we evaluated this critical parameter in a series of field and laboratory experiments.

Materials and Methods

Experimental Organisms and Protocols. Experiments used only *D. v. virgifera* adults that were collected as larvae or as mating pairs from two plots (each 6.1 by 121.9 m) planted with YieldGard Corn Borer corn (DKC 61-73, Monsanto Co., St. Louis, MO). Both plots were naturally infested and were located in areas of traditionally heavy rootworm pressure in northwestern Indiana within fields that contained late-planted corn the previous year. One plot was at Throckmorton Purdue Agricultural Center (site A) in Tippecanoe Co. and the other at Pinney Agricultural Center (site B) in LaPorte Co. The larvae were obtained from 600 plants per plot that were extracted from the soil between 14 June and 7 July 2007 at approximately the V8 growth stage (Ritchie et al. 1986). Each root system was severed from most of its corn stalk at 5 cm above ground level and kept in soil in 1,025-ml transparent cups (Pro-Kal PK32T-C, Fabrikal Corp., Kalamazoo, MI) that were transported to a Purdue University laboratory that was maintained at

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24°C and 65% RH with a photoperiod of 16:8 (L:D) h (Sylvania/F20T12 fluorescent lights, DaylightExtra, Osram Sylvania, Mississauga, ON, Canada). When it seemed visibly dry, the surface of the soil around the roots was sprayed with water to protect roots and larvae from desiccation. Transparent lids with one 4-cm-diameter screened hole covered each cup to provide ventilation while preventing escape of emerging beetles. The root cups were checked for adult emergence once daily \approx 7–8 h after the initiation of photophase. This method of collecting beetles from naturally infested corn roots is a minor modification of that used by Mabry et al. (2003).

Teneral adults from the root cups generally moved at a slow pace, so they were not chilled for handling. The emerging adults were sexed by characteristics of the apex of the abdomen (White 1977) and segregated into separate rearing cups based on sex, date of emergence, and the place of collection. Throughout our experiment, no mating was observed in the root cups. The adult rearing cups were 1,025-ml cups (Pro-Kal PK32T-C, Fabri-kal Corp., Kalamazoo, MI), with two 4-cm-diameter screened holes and one 2-cm-diameter hole, blocked by a rubber stopper, on the side of the cup. Less than 40 adults were reared in each cup. We placed 8 g of corn silks and 60 g of immature corn ears inside each rearing cup, and we replaced the diet materials daily. The diet materials came from the plants in the collection plots located at site A.

To reduce the influence of the marking and weighing processes upon sexual behaviors of adults, we chose random adults from each of the rearing cups, and then we weighed and marked them 11 h before mating tests took place. Adults were confined in 14.7-ml cups while they were weighed using a digital scale with accuracy within 0.01 mg (Ohaus GA200D, Pine Brook, NJ). For each mating experiment replicate, one 1- to 2-d-old female and two same-aged 6- to 19-d-old males collected from the same location were placed together in mating trios.

To facilitate more robust statistical analyses, we reduced the number of independent variables by placing same-aged males collected from the same location in each mating trio. The heavier of the two males in a mating trio was marked on the right elytron, and the lighter one was marked on the left elytron with a dot of tempura paint (a mixture of water [4 ml] and tempura powder [Rich Art Fresco Powder Tempura orange F-42, Rich Art Color Co. Inc., Northvale, NJ; 20 g]). This mixture was applied to males with a wooden applicator stick. We attached a framed 30-mesh nylon screen (frame: Foam core board White, Uline, Waukegan, IL; screen: Tulle White, Joann, Hudson, OH) to a clipboard and used it to restrain males while marking. An aspirator was used to capture and move adults. After the marking and weighing process, each adult was confined in a 266-ml cup (GFS Clear plastic drinkware, Gordon Food Service, Grand Rapids, MI), which had one 2-cm-diameter screened hole on the side. Food was presented as 0.5 g of fresh corn silks and 15 g of immature corn ears in the cups.



Fig. 1. Mating enclosure used when western corn root-worm mating behavior between two males and one female was observed.

The upper part of each mating enclosure was constructed using a 710-ml plastic cup (GFS 24-oz. Clear Plastic Drinkware, Gordon Food Service), with six 2-cm-diameter screened ventilation holes on the side of the cup, one 2-cm-diameter screened ventilation hole on the top of the cup, and one 2-cm-diameter hole blocked by a cork stopper. The lower part was constructed from a 473-ml container (Kal-Tainer 16S DP, Fabri-kal Corp., Kalamazoo, MI) (Fig. 1). Each mating enclosure contained one YieldGard Corn Borer corn plant at approximately the V2 growth stage (Ritchie et al. 1986) planted in soil collected from site A. The plant served primarily as a perching substrate and provided some security cover for the insects. A fan (Lasko Blower fan model no. 4910, Lasko Products, West Chester, PA) was set up 3 m from mating enclosures to gently circulate air and thereby move any volatile compounds (i.e., pheromones) within the mating enclosures. One female and two males were added to each mating enclosure 90 min after the initiation of the photophase.

Categorization and Description of Male Mating Behaviors. We recorded the mating behaviors of males for 3 h. We observed 17 mating enclosures at once,

test date	date of male emergence	place of male collection	weight of heavier male	weight of lighter male
	date of female emergence	place of female collection	weight of female	

	Heavier male				Lighter male			
1 min	mating attempt***	mounting	aedeagus insertion	interruption	mating attempt	mounting	aedeagus insertion	interruption
2 min	mating attempt	mounting***	aedeagus insertion	interruption	mating attempt	mounting	aedeagus insertion	interruption***
3 min	mating attempt	mounting	aedeagus insertion	interruption	mating attempt	mounting	aedeagus insertion	interruption
4 min	mating attempt	mounting*	aedeagus insertion	interruption	mating attempt	mounting	aedeagus insertion	interruption
5 min	mating attempt	mounting	aedeagus insertion***	interruption	mating attempt	mounting	aedeagus insertion	interruption
6 min	mating attempt	mounting	aedeagus insertion	interruption	mating attempt	mounting	aedeagus insertion	interruption
7 min	mating attempt	mounting	aedeagus insertion*	interruption	mating attempt	mounting	aedeagus insertion	interruption*

Fig. 2. Sample of recording protocol used to evaluate western corn rootworm mating behavior in the laboratory. *, head of arrow (▼) indicates end of an activity. **, tail of arrow (●) indicates initiation of activity. ***, Dash (—) indicates an activity lasting <1 min.

with each enclosure being observed approximately once every 2 min. The mating behaviors of males were assigned to one of the following five categories: 1) no sexual behavior: males show no identifiable response to females; 2) mating attempt: a male quickly approaches a female and attempts to place his forelegs and mid-legs on her elytra; 3) mounting: a male hooks his forelegs and mid-legs on the edge of the female's elytra; the male aedeagus may be everted and be partially and intermittently inserted into the female vagina while the male taps the female's antennae with his antennae; 4) aedeagus insertion (mating): aedeagus is inserted into the female vagina, and the ends of male's antennae consistently point to the rear of his body; and 5) mating interruption: a second male approaches a mounted male with a fast gait and attempts to place his forelegs and mid-legs on the other male's elytra, mounts the other male and taps the other male's antennae with his antennae, or inserts parts of his body between the mating pair.

Mating activities were generally determined by the behavior of males because females showed less overt mating behavior than males. The recording sequence of *D. v. virgifera* mating behaviors is shown in Fig. 2. The upper box of the recording sequence contains records converted into independent variables. Individual behavioral records in the lower box of the recording sequence were converted into dependent variables for statistical analysis. Columns in the lower

box are four categories of the mating behavior, and rows are time sequence.

Field Collection of Mating Pairs. In our field study, mating pairs were collected at two plots, one at site A and the other at site B, the same plots where we sampled corn roots for collecting larvae. We collected 101 pairs of mating pairs at site A between 14 July 2007 and 16 July 2007, and 115 mating pairs from site B between 16 July 2007 and 18 July 2007. Before collecting the individual pairs, we checked whether the male aedeagus was fully inserted in the female's vagina. Mating pairs were collected into vials and weighed 6 h after collection to allow pairs to complete mating and for the associated transfer of sperm and other materials.

Statistical Analyses. SAS software (SAS Institute 2002) was used for all statistical analyses. A probability of 0.05 was considered statistically significant. A null hypothesis of no difference between treatments, slopes, or from an intercept of 0 was assumed. Multiple linear regression analysis described by Affi et al. (2004) was used to analyze the effects of seven independent variables (male age [days], weight of a heavier male in a mating trio [milligrams], weight of a lighter male in a mating trio [milligrams], female age [days], female weight [milligrams], male collection site [[categorical variable]: site A (0), site B (1)], and female collection site [[categorical variable]: site A (0), site B (1)] on a range of dependent variables (the number of mating attempts, time elapsed until the

initial mating attempt, duration of males' mounting of females, number of mating interruptions, duration of the last mounting preceding aedeagus insertion, and the ratio of the number of mountings to the number of aedeagus insertions in the enclosures where aedeagus insertion occurred). Only the male and female collection sites were handled as categorical variables. The duration of male mounting, a dependent variable, was presented as the ratio of the duration of a male's time positioned on the female's elytra to the total duration of mating observation (3 h). Both of these ratios were arcsine-transformed to meet the assumptions for regression analysis. The number of mating attempts, the initial time of the first mating attempt, the number of mating interruptions, and the duration of the last mounting preceding aedeagus insertion were square-root-transformed to meet the assumptions for regression analysis. The sample sizes of the multiple regression analyses varied because not all behavioral events occurred in every replicate. Both forward selection and backward elimination methods were used to find the complete set of independent variables. We used 85% of the F-distributions with given degrees of freedom as the minimum *F*-to-enter, and 70% of the F-distributions with given degrees of freedom as the maximum *F*-to-remove.

The effects of seven independent variables (male age [days], weight of a heavier male in a mating trio [milligrams], weight of a lighter male in a mating trio [milligrams], female age [days], female weight [milligrams], male collection site {[categorical variable]: site A (0), site B (1)}, and female collection site {[categorical variable]: site A (0), site B (1)}) on aedeagus insertion (mating) [categorical variable: no mating (0), mating (1)] were analyzed with multivariate logistic regression analysis described by Afifi et al. (2004). The complete set of independent variables was found using the forward selection and backward elimination method for the multiple linear regression analyses and the logistic regression analysis. Goodness-of-fit was assessed with the Hosmer-Lemeshow χ^2 statistic (Hosmer and Lemeshow 1989).

The differences between the weights of mating females and nonmating females were analyzed using analysis of variance (ANOVA) with a block design, by using female age and place of female collection as the blocks.

Similarly, we used ANOVA with a blocking design using male age and place of male collection as the blocks to analyze differences between the weights of mating males and nonmating males. We used a Tukey-type test (Levy 1976, Zar 1999) to detect the differences between the two correlation coefficients of male weight and female weight in site A and site B without data transformation. We used ANOVA to detect differences among the weights of the mating males and females in site A and site B.

Results

Total Number of Mating Attempts. The complete set of independent variables explaining the variation in the numbers of mating attempts was composed of

female weight and female collection area ($F = 19.19$; $df = 2, 316$; $P < 0.0001$; $r^2 = 0.1083$). The slope of female weight and the slope of the female collection area were positively and negatively associated, respectively, with the number of mating attempts (intercept = 1.64884, $P < 0.0001$; female weight coefficient = 0.05259, $P < 0.0001$; female collection area coefficient = -0.54309, $P < 0.0001$). Thus, males were likely to direct more mating attempts toward heavier females than lighter females, and males were likely to make more mating attempts with females collected from site A than with females from site B.

Time Elapsed until Initial Mating Attempt. The model using female weight as the best single independent variable to predict latency until the first mating attempt was significant ($F = 8.16$; $df = 1, 297$; $P = 0.0046$; $r^2 = 0.0267$). The slope of female weight was negatively associated with the initial time of the first mating attempt (intercept = 4.21965, $P < 0.0001$; female weight coefficient = -0.15279, $P = 0.0046$). Thus, males were likely to attempt mating more quickly with heavier females than with lighter females.

Duration of Male Mounting. The best single independent variable explaining the duration of male mounting was female weight ($F = 9.53$; $df = 1, 314$; $P = 0.0023$; $r^2 = 0.0294$). Female weight was positively associated with the duration of males' mounting of females (intercept = 0.42182, $P = 0.0066$; female weight coefficient = 0.04243, $P = 0.0022$). Thus, males were likely to spend more time mounting upon heavy females' elytra than upon light females' elytra.

Aedeagus Insertion (Mating). Aedeagus insertions were observed in 27.3% (87 of 319) of total trials. No more than one aedeagus insertion was observed in each observation because no mating couples finished mating within our 3-h observation period. The best single independent variable explaining aedeagus insertion was female weight (Hosmer-Lemeshow goodness-of-fit test: $\chi^2 = 4.0695$, $P = 0.8508$; $r^2 = 0.0609$). Female weight was the best single independent variable predicting aedeagus insertions (intercept: maximum likelihood coefficient estimate = -3.9170, Wald $\chi^2 = 30.4159$, $P < 0.0001$; female weight coefficient: maximum likelihood coefficient estimate = 0.2604, Wald $\chi^2 = 18.5012$, $P < 0.0001$) (Fig. 3). Thus, males were more likely to successfully mate with heavy females than with light females (odd-ratio of female weight [milligrams]: point estimate = 1.27, 95% Wald confidence interval = (1.152, 1.461)). ANOVA results indicated that the weight of females in the cages where aedeagus insertion occurred was significantly greater than that of females in the cages where this was not observed ($F = 20.44$; $df = 1, 315$; $P < 0.0001$; the weight of females in the cages where aedeagus insertion occurred: mean \pm SEM = 11.90 \pm 0.24 mg; the weight of females in the cages where aedeagus insertion was not observed: mean \pm SEM = 10.65 \pm 0.14 mg). In contrast, the weight of the males inserting their aedeagus into a female was not different from males that did not ($F = 3.78$; $df = 1, 625$; $P = 0.0522$; the weight of the males inserting aedeagus: mean \pm

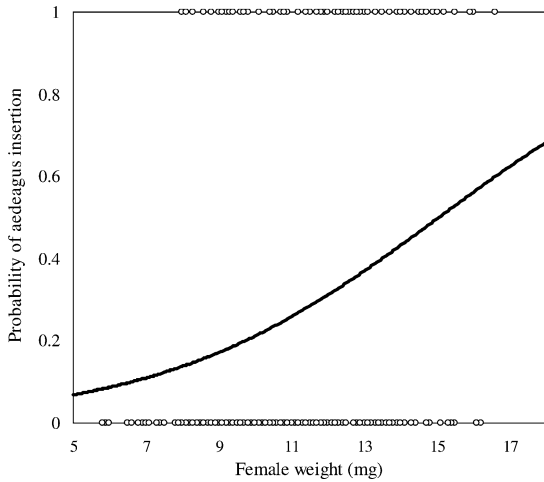


Fig. 3. Probability of aedeagus insertion (mating) predicted by female weight with logistic regression analysis (—) (model: probability of aedeagus insertion (mating) = $(\exp(-3.917 + 0.2604 \times (\text{female weight (mg)})))/(1 + \exp(-3.917 + 0.2604 \times (\text{female weight (mg)})))$) and the raw data (O).

SEM = 11.26 ± 0.18 mg; the weight of the males not inserting aedeagus: mean \pm SEM = 10.88 ± 0.08 mg).

Total Number of Mating Interruptions. The complete set of independent variables explaining the number of mating interruptions was composed of female weight and male age ($F = 15.20$; $df = 2, 316$; $P < 0.0001$; $r^2 = 0.088$). The effect of female weight upon the number of mating interruptions was larger than that of male age (intercept = 0.70135, $P < 0.0001$; female weight coefficient = 0.07658, $P < 0.0001$; and male age coefficient = -0.07909 , $P = 0.0169$). There was a positive slope associated with female weight and a negative slope associated with male age on the number of interruptions. Thus, the mounted male was more likely to be interrupted if mounted on a heavy female. This result also implies that young males initiated more mating interruptions than old males.

Duration of Last Mounting Preceding Aedeagus Insertion. No independent variables significantly explained the duration of the last mounting preceding aedeagus insertion (Full model: $F = 0.64$; $df = 7, 79$;

$P = 0.7209$; $r^2 = 0.0537$). Because female weight also could not explain the duration of the last mounting preceding aedeagus insertion, heavy females were no more likely to quickly accept male aedeagus insertion than light females.

Ratio of Number of Mountings to the Number of Aedeagus Insertions. No independent variables adequately explained the ratio of the number of mountings to the number of aedeagus insertions in the enclosures where aedeagus insertion occurred (Full model: $F = 1.36$; $df = 7, 79$; $P = 0.2340$; $r^2 = 0.1075$). This lack of a relationship between the ratio of the number of aedeagus insertions/the number of mountings in the enclosures where aedeagus insertions occurred and female weight indicated that heavy females did not accept male aedeagus insertion more frequently than light females.

Field-Collected Mating Pairings. Females collected at site A were significantly heavier than those of females at site B ($F = 22.05$, $df = 1, 214$; $P < 0.001$; site A: mean \pm SEM = 10.87 ± 0.19 mg; site B: mean \pm SEM = 9.68 ± 0.16 mg). Similarly, males collected at site A were significantly heavier than males at site B ($F = 7.33$; $df = 1, 82$; $P = 0.0073$; site A: mean \pm SEM = 10.37 ± 0.17 mg; site B: mean \pm SEM = 9.75 ± 0.16 mg). We detected nonrandom mating at site B ($P < 0.0001$, $n = 115$, $\rho = 0.4229$), but not at site A ($P = 0.1036$, $n = 101$, $\rho = -0.1629$). The correlation coefficient of the weights of males and females in copulae differs significantly between site A and site B ($P < 0.001$) (Fig. 4).

Discussion

Our experiment revealed that female weight was a strong predictor of several behaviors in the *D. v. virgifera* mating sequence. A positive relationship between female weight and the number of mating attempts indicates that males directed more mating attempts to heavy females, and males were also quicker to make an initial mating attempt with heavier females.

The attractiveness of these females continued beyond the insertion of the aedeagus. Heavy females already in copulae with a male were more likely to be interrupted by a potential second suitor than lighter

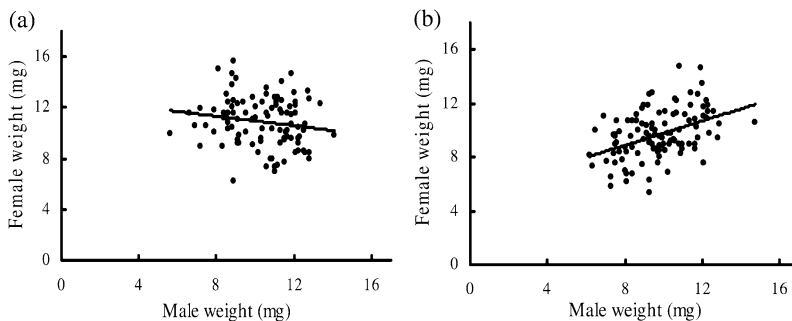


Fig. 4. Association between weights of male and female WCR on mating. (a) Association between the weights of mating pairs at site A. (b) Association between the weights of mating pairs at site B.

females who were also engaged with a male. This preference for heavy females has been demonstrated in several other insect species (Smith 1979; Gwynne 1981; Rutowski 1982; Johnson 1982; Johnson and Hubbell 1984), and it is thought to have an adaptive function. We hypothesize that this may be the case in this species as well, because female *D. v. virgifera* weight has been positively correlated with fecundity (Branson and Sutter 1985).

To place our results in the context of the production agriculture system that this species occupies, it is useful to review the current state of corn pest management. Recent estimates state that 26% of the total area planted with corn (81.6 million acres) is planted with Bt corn (NASS 2005). As stated earlier, the U.S. Environmental Protection Agency requires farmers to grow an area of nontransgenic "refuge" corn equal to 20% of the total acreage of transgenic corn in a given field for the management of Bt resistance (Gould 1998, www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm), based on the premise that western corn rootworm larvae feeding upon Bt corn will have some low level of survivorship to adulthood. In fact, low rates of survival to adulthood is consistently found among field populations of *D. v. virgifera* exposed to Bt corn in the field, and some researchers (Storer et al. 2006; C.H.K., unpublished data) have observed that the body weight of females emerging from Bt corn plots may be significantly greater than that of females emerging from nontransgenic corn plots. Although we performed no direct choice tests by using males exposed to heavy and light females, our results do demonstrate that males mate for longer periods and more readily with heavy females. If this phenomenon is exhibited in a field setting as well, this may make the likelihood of mating with females from a lower density larval habitat (such as Bt corn) higher than that with comparatively smaller females emerging from the more competitive larval habitat represented by nontransgenic corn. The larger females may be more fecund as well (Branson and Sutter 1985), having additional long-term consequences for resistance developmental models. If male preference for heavy females does occur in the field, the random mating assumptions put forward in some current models of resistance development to Bt corn (Crowder et al. 2005; Storer et al. 2006), would be violated. This may influence estimates of the rates of development of WCR resistance to these plant-incorporated protectants.

Some of our other observations of mating behavior may represent new insight into courtship behavior in this species. To lower the likelihood of "forced" mating and allow the females an escape from unsuitable males, the mating enclosure used in our study provided more space and spatial complexity for adults than the petri dishes used by Lew and Ball (1979) to observe mating behavior. We did not observe the western corn rootworm precopulatory behavior, as described by Lew and Ball (1979), during the course of the 320 replicates of our experiment. Tallamy et al. (2002) reported results similar to ours in a related species, observing no precopulatory female mate

choice in the spotted cucumber beetle, *Diabrotica undecimpunctata howardii* Barber. We suspect that the *D. v. virgifera* precopulatory behavior described by Lew and Ball (1979) was not a species-specific ritual courtship behavior but an artifact induced by spatial constraints.

Our observations showed several similarities to *D. undecimpunctata howardii* mating behavior described by Tallamy et al. (2002, 2003). These researchers put individual male and a female in a 4-cm-diameter cup containing two small sticks, whereas we used a slightly larger enclosure containing a corn plant. The *D. v. virgifera* matings we observed in this study demonstrated a similar sequence of mating behavior, including male antennal stroking behavior and a similar female's response to male mounting. However, none of the females in our experiment displayed the calling behavior described by Hammack (1995), who observed calling among females that were derived from a laboratory colony and individually isolated in 5.5-cm-diameter by 1.2-cm-deep petri dishes. Females in the Hammack (1995) experiment, thus, could not interact with males. However, females in our experiment were placed with males within 710-ml mating enclosures. Therefore, it may not be surprising that females in our experiment did not demonstrate calling behavior because males were already within close proximity. Hammack (1995) mentioned that many females that did not exhibit the calling behavior still mated.

These results provide new, crucial information about some key aspects mediating mate selection in this important pest species. These data can be used to help develop future resistance management strategies for *D. v. virgifera* in relation to transgenic corn. Because the mating preferences of adult could have a direct influence upon the likelihood of the union of putative "resistant" gametes, empirical information that sheds light upon the key factors that influence mating dynamics can only improve the practical value of future simulation models predicting insect resistance development under the heavy selection pressure that Bt corn represents.

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