

Likelihood of Multiple Mating in *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)

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ABSTRACT We evaluated the mating ability of male western corn rootworms, *Diabrotica virgifera virgifera* Leconte (Coleoptera: Chrysomelidae), for 20 d after initial mating, using a series of laboratory experiments. Males mated an average of 2.24 times within 10 d after their first mating and averaged 0.15 matings between days 11 and 20 after their first mating. Because estimating the mating frequency in *Bt*/refuge cornfields is critical to developing robust and reliable models predicting *Bt* resistance development in this pest, we discuss how these laboratory findings may influence development and evaluation of current and future insect resistance management plans.

KEY WORDS reproductive behavior, mating behavior, insect resistance management

The western corn rootworm, *Diabrotica virgifera virgifera* Leconte (Coleoptera: Chrysomelidae), is the most serious insect pest of corn, *Zea mays* L., in the United States (Levine and Oloumi-Sadeghi 1991) and has recently invaded southeastern Europe (Edwards et al. 1998). In the past decade, transgenic corn, *Z. mays* L., genetically engineered to contain genes for the production of insecticidal Cry proteins (produced in nature by the bacterium *Bacillus thuringiensis* Berliner [*Bt*]) and/or herbicide tolerance (e.g., glyphosate tolerance), have been rapidly adopted by farmers in the United States and elsewhere (Baum et al. 2004, James 2006). Corn plants engineered to produce the insecticidal toxins are often called *Bt* corn, and different toxins are used to manage larvae of several species of pest insects, including *D. v. virgifera* and several lepidopteran pests (English et al. 2000, Moellenbeck et al. 2001, Ellis et al. 2002, EPA 2002, Baum et al. 2004, Vaughn et al. 2005). To delay evolution of insect resistance to transgenic crops producing *Bt* toxins, nearby “refuges” of host plants not producing *Bt* toxins must be planted in many regions. These refuges are expected to be most effective in slowing resistance when the toxin concentration in *Bt* crops is high enough to kill all or nearly all insects heterozygous for resistance (Gould 1998, EPA 1998, Tabashnik 1994, Tabashnik et al. 2003). However, the efficacy of this refuge strategy relies on several assumptions that have not been empirically tested.

Diabrotica v. virgifera is a univoltine species. Males generally emerge earlier than females (Quiring and Timmins 1990). Unmated females start to release pheromone or “call” for males within 1 d after adult emergence and will continue to release pheromone up to

3 d after adult emergence or until a mate is found (Hammack 1995). Female *D. v. virgifera* are known to mate typically only once (Cates 1968). Males have been shown to mate multiple times in the laboratory (Hill 1975). However, the effect of the operational sex ratio (i.e., what percentage of the males are ready/able to mate?) at the time of female emergence on mating frequency has not been studied. Previous field studies have shown that ≈70% of females are mated in the first 24 h after emergence in cornfields (Quiring and Timmins 1990).

The mating behavior of this species is important for predicting the success of novel management strategies. For example, models that predict *Bt* resistance evolution in *D. v. virgifera* (Crowder et al. 2005, Storer et al. 2006) specify that some males from the refuge must mate with females from transgenic areas of the field to slow resistance development. Some researchers (Storer et al. 2006; C. H. K., unpublished data) have recently shown that adults may emerge earlier from non-*Bt* (i.e., refuge) cornfields than from *Bt* hybrids in the same fields. This asynchronous emergence pattern means that the mating potential of males may influence the success of the refuge system, whereby early emerging, possibly once-mated males from refuge are expected to mate with newly emerged virgin females from the transgenic areas of the cornfield with high frequency. Thus, some measure of the number of matings that male beetles can successfully complete after the initial mating may help in evaluating the robustness of past and future insect resistance management (IRM) (EPA 1998, Gould 1998) plans.

Furthermore, the western corn rootworm beetles found in much of Indiana and Illinois have been shown to be behaviorally resistant to crop rotation (O’Neal et al. 2001, Levine et al. 2002). Females in the rotation-

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resistant population are known to leave natal cornfields to oviposit in other fields, such as soybean (*Glycine max* L. Merr.), causing larval damage in first-year corn (Onstad et al. 2003). Not surprisingly, most research conducted on this behavioral variant to this point has focused on the movement and ovipositional behavior of female beetles (Knolhoff et al. 2006), whereas little is known about male rotation-resistant beetles. For example, the mating potential of variant beetles has never been examined in the field or the laboratory. In addition to exploring this facet of the reproductive biology of a key pest, generating empirical data from the target organism is desirable to improve the robustness and predictive power of IRM models, which typically include estimates of mating frequency (Onstad et al. 2003, Crowder et al. 2005, Storer et al. 2006). With this rationale in mind, we collected and reared larvae from naturally occurring populations at two field locations in "variant rootworm" areas of Indiana, where damage to first-year corn has historically been high, and used the resulting adults for a series of mating experiments.

Materials and Methods

All experiments were conducted using natural infestations in areas of traditionally heavy western corn rootworm pressure to first-year corn in northwestern Indiana. One plot was located at Throckmorton Purdue Agricultural Center in Tippecanoe County, IN, and the other was at Pinney Agricultural Center in LaPorte County, IN. After extraction from the soil, the corn stalks were cut off at 5 cm above ground level and discarded. Between 14 June 2007 and 7 July 2007, 600 YieldGard Corn Borer (DKC 61-73; Monsanto, St. Louis, MO) corn plants, at approximately the V8 growth stage (Ritchie et al. 1986), were removed from the two plots (each plot measured 6.1 by 121.92 m). The roots were kept in soil and placed in 1,025-ml cups (Pro-Kal PK32T-C; Fabrikal, Kalamazoo, MI). The soil around the roots was kept moist to protect roots and larvae from desiccation. We collected the beetle larvae using the method described by Mabry et al. (2003), which includes shaking loose soil from root masses and removing larvae from this soil using small plastic spoons or forceps and placing them into the container with the root mass. We made slight modifications to the container. Transparent lids with one 4-cm-diameter screened hole covered each cup to provide ventilation while preventing emerging beetles from escaping. The cups were transported to a laboratory at Purdue University in West Lafayette, IN. The laboratory was maintained at 24°C and 65% RH. Fluorescent lights (Sylvania/F20T12/DaylightExtra; Osram Sylvania, Mississauga, Ontario, Canada) were set to produce a photoperiod of L 16: D 8. The root cups were checked for adult beetle emergence once daily \approx 7–8 h after the initiation of photophase. The beetle emergence was spread over 20 d, and no mating was observed in the root cups throughout the period. When the soil surface of the root cup appeared visibly dry, it was sprayed with water. The emerging beetles 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. Teneral adults from the root cups generally moved at a slow pace, so they were not chilled for handling. The adult rearing cups were 1,025-ml cups (Pro-Kal PK32T-C; Fabrikal, 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. We placed 8 g of corn silks and 60 g of immature corn ears inside each rearing cup and replaced the diet materials daily. The diet materials came from commercial YieldGard Corn Borer hybrids (DKC 61-73; Monsanto) collected daily while available from field sites at Throckmorton Purdue Agricultural Center.

We used 6- to 19-d-old males and 2- to 5-d-old virgin females for this experiment. Males that were allowed to mate once in a mating enclosure were the source of mated males throughout this experiment. The range of emergence dates of males was the period of 30 June 2007 to 24 July 2007. We used males that initiated their first mating with 2- to 5-d-old virgin females within 5 h. To determine mating status, we checked whether the male aedeagus was fully inserted in the female's vagina. Once a male finished its first mating, it was moved to a new mating cup, with a new 2- to 5-d-old virgin female. Each mating cup consisted of 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. Insects were provided with 0.5 g of corn silks and 15 g of immature corn ears in the mating cups; this diet material was replaced every other day. During our preliminary tests for this experiment, we observed that some mating couples responded to our movements while we were checking their mating status by immediately ceasing mating and seeking shelter. Thus, it is impossible to rule out the possibility that observation itself may affect mating behavior of the insects in our study. In other words, the possibility of interrupting mating may increase as the frequency of checking the mating status increases. It is known that the duration of copulation is usually >2.5 h (Lew and Ball 1980), so we checked mating in each cup at 3-h intervals, or 7 times/24-h period (at 1.5, 4.5, 7.5, 10.5, 13.5, 16.5, and 19.5 h after the start of photophase) until 8 August 2007, at which point we no longer had access to fresh diet material from the field. The duration of mating observations ranged from 10 to 33 d, as dictated by availability of males and diet material. A by-product of not checking the beetles constantly is that they may cease mating and begin remating while they are not being observed, which could lead to inflated estimates of mating times. We used a flashlight with a red filter (Kodak written filter No.29; Eastman Kodak, Rochester, NY) to check mating activity during the dark period. Females in the mating cups were replaced with another 2- to 5-d-old virgin female when they mated or died. If they did neither within 48 h, they were removed and not reused.

We divided our observations into two categories for analysis: number of matings from 0 to 10 d after the initial mating and from 11 to 20 d after the initial mating. Means and SDs were calculated for the following: numbers of mates males had within 10 d after the initial mating, the

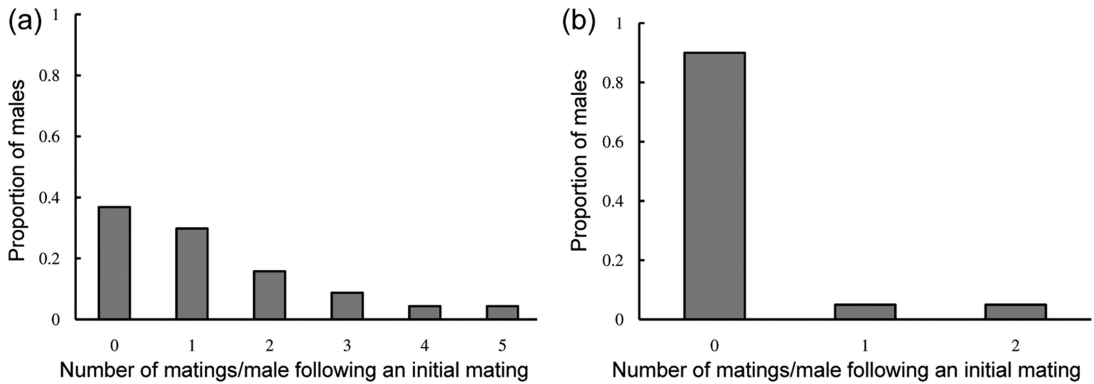


Fig. 1. Number of matings/male *D. v. virgifera* (a) within 10 d after the initial mating ($n = 114$) and (b) 10–20 d after the initial mating ($n = 60$).

number of mates secured by males within between 11 and 20 d after the first mating, time lag between the initiation of sequential matings, and the duration of each mating. Medians of mating durations were estimated. The data from three males that died within 10 d after the initial mating were not used. The mating status of 52.63% (60/114) of males, which had been used to evaluate the number of matings/male within 10 d after the initial mating, was continuously recorded during the period 11–20 d after the initial mating. Matlab 7.0 (Kruskal-Wallis; Math Works 2005) was used to conduct the nonparametric Kruskal-Wallis test (Sokal and Rohlf 1995) to determine whether there were differences between the number of matings/male within 10 d with respect to male age.

Results

Number of Matings/Male Within 10 d After Initial Mating. Overall, *D. v. virgifera* males mated an average 2.24 ± 1.34 times within 10 d after their first mating. A large percentage of males, 36.84% (42/114), did not mate again within 10 d after the initial mating (Fig. 1). Only 17.54% (20/114) of males mated more than twice within 10 d after the first mating. The effect of male age on the number of mating/male within 10 d after the first mating was not significant (Kruskal-Wallis statistic = 1.56, $P = 0.11$).

Number of Matings/Male Within Between 11 and 20 d After Initial Mating. Males mated an average of 0.15 ± 0.48 times 11–20 d after the initial mating. The majority, 90% (54/60), of males did not mate 11–20 d after the initial mating (Fig. 1). No male mated more than twice within 11–20 d after the initial mating.

For 20 d, all average time lags between their initiation of sequential matings were >1.5 d from the initial mating (Table 1), and the arithmetic mean of mating duration was >6 h (Table 2). Because we used males that were allowed to mate once previously throughout this experiment, the mating durations shown in Table 2 represent the subsequent matings of each male.

Discussion

To slow *Bt* resistance development, matings between beetles emerging from *Bt* cornfields should ideally be rare, and matings between those emerging from nontransgenic cornfields and beetles from *Bt* cornfields should be common (EPA 1998, Gould 1998, Tabashnik 1994, Tabashnik et al. 2003). To facilitate this, emergence of adult beetles from *Bt* cornfields and nontransgenic cornfields should be spatially and temporally synchronized as much as possible. Although proposed changes to refuge structures include seed mixes, complete spatial overlap is not possible at the time of this publication. Recent studies have reported that *D. v. virgifera* adults in *Bt* cornfields emerge later than those in nontransgenic cornfields. For example, Storer et al. (2006) reported a 3- to 6-d delay in the early part of the emergence curve for adult beetles emerging from *Cry34/35Ab1* corn plots compared with the control plots (near isoline maize) without the *Cry34Ab1* and *Cry35Ab1* genes at Slater, IA, in 2003. They reported a 7-d delay in similar experiments at Fowler, IN, during 2004. Although the methods used in our study allowed males of all ages equal access to virgin female, older males were not as likely to mate (or remate) as younger ones—a very small fraction of males

Table 1. Time lag between initiation of subsequent matings

Mating lag	First–second mating lag	Second–third mating lag	Third–fourth mating lag	Fourth–fifth mating lag	Fifth–sixth mating lag
N	72	44	27	12	6
Mean (d)	2.52	2.96	2.70	2.70	1.81
±SD (d)	3.82	4.53	4.67	3.07	0.92
Maximum (d)	24.29	27.42	24.67	10.75	3.25
Minimum (d)	0.25	0.25	0.25	0.38	0.63

Table 2. Mating duration of successive matings for male *D. v. virgifera*

Mating duration	First mating duration	Second mating duration	Third mating duration	Fourth mating duration	Fifth mating duration	Sixth mating duration
N	72	72	40	24	12	4
Mean (h)	9.42	10.08	8.25	8.25	8.5	9.75
±SD (h)	7.37	7.20	6.40	5.25	6.62	9.91
Median (h)	6.00	9.00	6.00	6.00	6.00	6.00
Maximum (h)	36.00	33.00	24.00	21.00	24.00	24.00
Minimum (h)	3.00	3.00	3.00	3.00	3.00	3.00

All males were allowed to mate once at the initiation of this experiment. Initial mating data are not presented here.

confined with virgin females mated between 11 and 20 d after their initial mating. Applying these findings to a field situation suggests that the later-emerging females from the transgenic area of the field may be more likely to mate with any young males emerging from *Bt* plants (which may be relatively uncommon, but are nonetheless assumed to be present in current models; Crowder et al. 2005, Storer et al. 2006) than with earlier-emerging old males from non-*Bt* or refuge areas of the field. From the male perspective, our results suggest that males should mate early, perhaps with the first suitable and receptive mate they encounter after their initial mating, because their ability to mate seems to decline with age. This may also represent a reproductive disincentive to long-range movement (e.g., from high beetle density refuge areas into *Bt* areas of cornfields) in search of mates and may result in the majority of refuge males mating near their natal plants—also not an ideal scenario for IRM in a structured refuge system. If the combination of these factors lowers the incidence of mating between refuge and *Bt* emerging beetles, it could also effectively accelerate the evolution of *Bt* resistance development, resulting in actual field evolution of *Bt* resistance development in *D. v. virgifera* that may be faster than predicted by initial models that did not include these parameters (Crowder et al. 2005, Storer et al. 2006).

We stress that these are laboratory results and thus any conclusions about mating in a field situation must be made with some degree of caution. However, in terms of male mating output, the results from our laboratory assays are probably somewhat optimistic from a resistance management standpoint. That is, we probably overestimate male mating by presenting a “best-case scenario” for the likelihood of older males securing mates. By presenting males with virgin females in a confined space, we are removing some significant and important variables in the mate acquisition sequence, including long-range mate location, courtship, and male mate competition. Given these factors, it is quite possible that the rate of mating by old males is lower in a field situation.

Although not the primary focus of our study, we also documented longer mating durations in our study than previously published accounts. *D. v. virgifera* is known to exhibit prolonged mating associations. For example, males mount and “ride” on females’ elytra before copulation (Lew and Ball 1979). Lew and Ball (1980) reported that the duration of *D. v. virgifera* mating exceeded 2.5 h in general. Sherwood and Levine (1993) reported that “short” matings that were ended prema-

turally (i.e., terminated 1 h after initiation) resulted in females that did not contain spermatozoa, suggesting that short copulations are unlikely to result in significant fertilization of eggs. In light of this, we checked mating status 7 times/24 h to minimize disturbance to the insects in the event that observation itself interferes with mating. Thus, the minimum mating duration we recorded in this study was 3 h, meaning that our level of resolution itself may contribute to the fact that the mating duration estimated in our study is greater than that in the study of Lew and Ball (1980). We note also that Lew and Ball (1980) evaluated the duration of the first mating, whereas we estimated that of matings after the first mating. Therefore, the mating durations calculated by the two studies are not directly comparable. However, it is known that the likelihood of transferring a spermatozoon directly increases with duration of copulation, and maximal insemination of a female during a single copulation requires prolonged copulation of 3–4 h (Lew and Ball 1980). Therefore, the longer mean durations found by our study may be a reflection of older/mated male beetles attempts to mobilize and transfer sufficient sperm and associated materials to effectively fertilize multiple females.

Current western corn rootworm *Bt* resistance development models assume that females emerging from the *Bt* area of the field randomly mate with males emerging from both the refuge area of the field and the *Bt* area of the field. However, our findings suggest that these frequency estimates should include a component of male age, because the mating ability of rootworm males declines rapidly, a parameter that is not addressed by current models (Crowder et al. 2005, Storer et al. 2006). Future models should include this information in assessing the likelihood of mating between beetles emerging from various areas of the field and thereby generate more biologically relevant, and therefore reliable, estimates of the evolution of resistance development.

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