

Sperm Precedence in Overwintered Colorado Potato Beetle (Coleoptera: Chrysomelidae) and Its Implications for Insecticide Resistance Management

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ABSTRACT Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is the most important insect defoliator of potatoes and is infamous for its ability to develop insecticide resistance. Sperm precedence is an important consideration in developing resistance management plans. We determined the precedence of sperm from a postdiapause spring mating by using irradiated sterile males. We also investigated whether spring mating (with or without fertilization) affects reproduction of overwintered females. Precedence of sperm from spring matings versus overwintered sperm from fall matings was almost complete. Some fertilization from fall matings did take place in this experiment, but it exceeded 10% in only one of the 22 pairings. Females mated only the previous fall laid fewer eggs than spring-mated females, but spring mating did not seem to improve the hatch rate of deposited eggs. On average, mated females started laying 1.6 d sooner than fall-mated females, but this difference was not significant. Hatch rate did not change with time. Precedence of spring matings over stored sperm can help compensate for assortative summer mating among resistant individuals due to differences in developmental time between refuges and treated areas.

KEY WORDS *Leptinotarsa decemlineata*, sperm precedence, mating behavior, insecticide resistance management

COLORADO POTATO BEETLE, *Leptinotarsa decemlineata* (Say), is the most important insect defoliator of potatoes and also causes significant damage to tomato and eggplant. None of the control techniques developed against this pest during the past 135 yr provided a long-term protection of the crops (Casagrande 1987). Currently, the beetle continues to be a major threat in most potato-growing areas of the world.

A diverse and flexible life history is one of several factors responsible for the Colorado potato beetle's success as a pest. Short- and long-distance migrations, closely connected with diapause, feeding, and reproduction, allow this insect to use "bet-hedging" reproductive strategies, distributing its offspring in both space (within and between fields) and time (within and between years) (Solbreck 1978, Voss and Ferro 1990). Colorado potato beetles overwinter in the soil as adults, with the majority of them aggregating in woody areas adjacent to the fields where they spent the previous summer (Weber and Ferro 1993). After diapause is induced by a short-day photoperiod (de Kort 1990), the beetles engage in a low-altitude flight

directed toward tall vegetation. Upon arrival to wooded sites, they immediately burrow into the soil to diapause (Voss and Ferro 1990). In the spring, males and females terminate their diapause simultaneously and a significant proportion of the population mates within the overwintering sites before moving to host habitats (Ferro et al. 1999). Beetles colonize potato fields both by flight and by walking (Voss and Ferro 1990). Postdiapause females also can lay eggs using sperm from the prediapause mating, however, their fecundity is lower than the fecundity of spring-mated females (Ferro et al. 1991).

Another trait making Colorado potato beetle control so challenging is its remarkable propensity to evolve resistance to virtually every chemical that has been used against it (Forgash 1985, Grafius 1997, Olson et al. 2000). Sometimes, a new insecticide failed after 1 yr (e.g., endrin) or even during the first year of use (e.g., oxamyl) (Forgash 1985). Impressive potential to withstand a variety of poisons may be caused by the coevolution of the beetle and its host plants in the family Solanaceae, which have high concentrations of phytotoxins in their foliage (Ferro 1993). Resistance evolution is also aided by the high fecundity of this species, with a single mutant potentially producing several hundred offspring in just one generation.

The generally recommended resistance management plans rely on the use of refugia to support a

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population of susceptible individuals sufficient to curtail mating between resistant individuals (Whalon and Ferro 1998). In most cases, insecticide resistance in the Colorado potato beetle is incompletely dominant (Argentine et al. 1989, Argentine and Clark 1990, Ioannidis et al. 1992, Rahardja and Whalon 1995, Miyo et al. 1999, Zhao et al. 2000). Therefore, it is assumed that the progeny of resistant \times susceptible crosses, heterozygous at the resistant allele, will not survive high doses of insecticides applied at full label rates. Unfortunately, recent information on beetle biology and behavior is beginning to cast doubt on the efficacy of such approach (Gould 1998, Alyokhin and Ferro 1999a).

Approximately 25% of recently emerged summer generation beetles stay close to the place of their larval development until their reproductive system is mature (Alyokhin and Ferro 1999b). Therefore, a significant proportion of resistant beetles developing to adulthood on treated plants are expected to mate with each other and leave homozygously resistant offspring. Both male and female Colorado potato beetles mate multiple times with different partners (Szentesi 1985, Boiteau 1988). However, when a summer generation female mates to two different males, sperm precedence is incomplete, with the first male still fertilizing 24–48% of the eggs (Boiteau 1988, Alyokhin and Ferro 1999a, Roderick et al. 2003). Therefore, resistant females previously mated to resistant males who then mate to susceptible males will still produce up to half homozygote-resistant offspring. Sperm precedence may increase with the increased number of copulations performed by the second male or succeeding susceptible males, but it still does not reach 100% (Roderick et al. 2003). Thus, it might be argued that a successful resistance prevention program should be directed toward complete curtailment of mating between resistant individuals, a goal very hard to achieve under field conditions.

A general obstacle to refuge success is variation in phenology of refuge and nonrefuge populations resulting from variation in treatments. Variation in treatments that affect generation time may prevent susceptible refuge populations from exchanging genes with resistant individuals in treated areas, which would violate assumptions of random mating refuge-based management (Liu et al. 1999, Gould 1998). In multivoltine populations or species, including Colorado potato beetle, populations in treated areas may develop more slowly (Alyokhin and Ferro 1999c, Costa et al. 2000) or even have fewer generations (Baker et al. 2001) than in adjacent refuge areas. Asynchrony due to variation in treatments may lead to assortative mating within refuges and treated areas, greatly reducing the movement of resistance genes from homozygotes to heterozygotes where they may be eliminated. Beetle tendency to aggregate in overwintering sites outside of the field (Weber and Ferro 1993) might end up being a key factor determining an overall success of the high-dose refuge approach. When refugia are created by leaving strips of untreated potato foliage throughout the field (Whalon

and Ferro 1998), and these strips are oriented more or less parallel to the field borders, resistant beetles from the insecticide-treated crop and susceptible beetles from the refugia overwinter in the same woody areas along the field borders and should mate randomly the following spring. If sperm from the spring mating were to take complete precedence over the overwintered sperm, the next beetle generation would be heterozygous at the resistant locus and display intermediate levels of insecticide resistance.

Before this study, all work on sperm precedence in the Colorado potato beetle focused exclusively on the mating between summer generation, prediapause adults (Boiteau 1988, Alyokhin and Ferro 1999a, Roderick et al. 2003). However, the significant increase in the female reproductive output following spring mating (Ferro et al. 1991) suggests a decline in viability or numerical depletion of stored sperm during the winter diapause. Therefore, the degree of precedence may be much higher between than within seasons. Testing this hypothesis was the major objective of our study. We used the sterile male technique to measure sperm precedence and looked for possible effects of sterilizing radiation on behavioral vigor and mating behavior.

Materials and Methods

Beetle Populations. Overwintering adult Colorado potato beetle were collected on 19 February 2004 from a woodlot adjoining several potato fields in Riverhead, NY. Overwintered beetles were sexed, rinsed in a 10% bleach solution to prevent possible fungal infection, and then placed in plastic containers filled with soil. Males and females were kept in separate containers. Although wild-caught beetles were used to test for sperm precedence of stored sperm, true virgin females were collected from a laboratory colony, held in captivity for 12 generations since initiation from wild-caught beetles in Massachusetts, to confirm radiation doses and the viability of the wild-caught overwintered males' sperm. Virgin beetles used to measure effects of radiation on walking speed or mating frequency were collected from laboratory colonies held in captivity two or three generations after collection from field populations in western Massachusetts.

Effects of Radiation. *Walking Speed.* Twenty-five virgin adult males were collected <24 h after emergence and held in groups of three to six individuals for 1 wk before being irradiated using a Gammacell 3000 Elan gamma irradiator by using cesium 137 as its radiation source. Groups of six or seven males were held in paper cylinders ≈ 60 mm in length and 8 mm in diameter capped with cigarette filters to restrict them to a small enough area to receive a uniform dose. Doses of 3–6 krad were applied to the males. Radiation doses were pooled in analysis because there did not seem to be a quantitative effect of dose on walking speed. After irradiation, males were housed for 24 h with virgin females before being tested for walking speed. Fifty-three male and 52 female virgins were collected from the same laboratory colony at the same

time and were housed in pairs for 1 d without exposure to radiation before testing.

Individual beetles were placed in the center of a paper disc set on a Sybron Thermocline hot-plate. Surface temperature was maintained at 60°C. Assays using locomotor performance often suffer from difficulty distinguishing behavioral motivation from ability. The temperature used in the current study was found to yield optimum walking speeds. Below 60°C, some beetles did not walk or waited before walking, and above that temperature some beetles lost coordination to walk. Beetles were timed as they walked 7 cm from initially crossing a 1-cm-radius circle to an 8-cm-radius circle on the outer edge of the hot-plate. Ln-transformed walking data were analyzed using analysis of variance (ANOVA), first within controls for effect of sex, and then between irradiated and intact groups.

Mating Frequency. Virgin male and female beetles were collected from multiple laboratory colonies as described above. Irradiated males were exposed to 4,500 rad as described above. Each of the 28 virgin females were paired for 24 h with one irradiated and one nonirradiated male marked with a single elytral puncture (Unruh and Chauvin 1993) in cages consisting of an acetate tube 20 cm in diameter and 48 cm in height wrapped around a single 3-wk-old potato plant in a 19-cm-diameter pot with a screened cap. Males were placed on the soil at the base of the cage, and females were placed in the upper foliage. During the 24-h competition period, seven observations were made of the behavior of the female and the two males sharing the cage. The proportion of matings or mounts observed during those seven observations obtained by the nonirradiated male was bimodal, with peaks at 0 and 1, and so were tested for deviation from 0.5 by using a nonparametric (signed rank) test.

Sperm Precedence. Mating Treatments. After being held at 4°C for either 4 d or 3 wk, the beetles were moved to a rearing room maintained at 25 ± 1°C and a photoperiod of 16:8 (L:D) h. Each of the two trials contained each of the first four treatments described below and the data for the two trials were pooled. The beetles were isolated on emergence and moved to individual vented Nalgene boxes (12.5 by 7 by 5.5 cm) maintained in a Percival-I 36VL incubator at 27 ± 1°C and a photoperiod of 16:8 (L:D) h. The beetles were provided with excised potato foliage placed in floral water pics. The foliage was replenished daily.

We tested a total of five treatments detailed below. Sample sizes are those originally placed in different treatments.

1. **Overwintered (OW) Female/Irradiated Male.** Thirty-nine overwintered females were paired 5–7 d after emergence with overwintered males that had been irradiated with 6,000 rad by using a Gammator-50 cesium-137 gamma irradiator at a rate of 6.09 rad/s. This dose was chosen after preliminary trials with this irradiator indicated higher hatch rates at lower doses (3% at 5,500 rad and >5% at doses <5,500 rad). Males were paired with females

<1 h after irradiation, and pairs were held together for 24 h, after which the males were removed. Preliminary experiments (unpublished) indicated that the males irradiated under described conditions are sterile for at least 48 h after exposure, so that the eggs sired by such beetles would not hatch, and all hatching larvae would have been sired by the overwintered sperm from the last-year's mating.

2. **OW Female/Nonirradiated Male.** Sixteen individual overwintered females were paired 5–7 d after emergence with individual intact (not irradiated), overwintered males of the same age for 24 h. This treatment was established to estimate the hatch rate of overwintered females mated to intact overwintered males.
3. **OW Female/No Male.** Twenty-eight overwintered females were kept in isolation to measure hatch rate of eggs fertilized exclusively by overwintered sperm.
4. **Laboratory-Colony Virgin/Irradiated Male.** A group of eight laboratory-reared, virgin (see above), 6-day old females were paired with irradiated overwintered males for 24 h. The treatment was designed to confirm that the radiation indeed prevented fertilized eggs from developing.
5. **Laboratory-Colony Virgin/Nonirradiated Male.** Five overwintered males were paired 5–7 d after emergence with 6-d-old virgin females from the laboratory colony. The treatment was designed to confirm that overwintered males possess viable sperm under the conditions of this experiment.

After being paired for 24 h with the respective males in each treatment, or after 6–8 d in isolation, the females were held in Nalgene boxes with a constant supply of potato foliage for up to 14 d or until they had laid >100 eggs, whichever came first. The eggs were collected and counted daily and maintained at 27°C in an incubator. To reduce cannibalism, hatchlings were counted and removed each day until all the eggs hatched or until 8 d passed since the day they were laid.

Analysis. All analyses were carried out with JMP (SAS Institute 2002).

Sperm Precedence. Sterile male experiments suffer from potential bias due to the lack of development of eggs fertilized by nonirradiated sperm and from any successful development of eggs fertilized by irradiated sperm. To correct for these sources of bias, we used the formula of Boorman and Parker (1976):

$$P_R = \left(1 - \frac{x}{p}\right) + \left(\frac{z}{p} * \frac{1 - (x/p)}{1 - (z/p)}\right)$$

where P_R is the proportion fertilized by the irradiated male, x is the observed hatching rate in a competitive mating, z is the hatching rate after a putatively sterile mating, and p is the hatching rate after mating with an intact male. The group of laboratory-colony virgins mated to irradiated, overwintered males was used to estimate z , and the p was estimated from the proportion of eggs hatching from overwintered females to

nonirradiated, overwintered males. P_R in this case is a measure of sperm precedence of spring matings versus overwintered sperm. A P_R equal to 1 would indicate complete sperm precedence for sperm matings. The distribution of Boorman and Parker (1976) corrected P_R values was tested to see whether it was significantly <1 by using a Wilcoxon's signed rank test.

We also investigated whether spring mating (with or without fertilization) affects reproduction of overwintered females. Treatments 4 and 5, where males were mated to laboratory virgin females, were excluded from the analyses. The proportion of the females that laid eggs was analyzed using a χ^2 test. The rest of the analysis was limited to egg-laying females only.

Daily fecundity was calculated as the total number of eggs collected divided by the number of days during which eggs were collected. Overwintered female fecundity was analyzed using analysis of variance (ANOVA) with two planned orthogonal contrasts: one to test whether radiation affected fecundity within mated females and a second to test whether mating with either irradiated or nonirradiated males influenced fecundity. Female fertility was calculated as the proportion of eggs that hatched after incubation. Hatch rates were arcsine transformed and tested for goodness-of-fit to the normal distribution using the Shapiro-Wilk test. If transformed data were still highly non-normal, nonparametric (Wilcoxon or Kruskal-Wallis) tests were used to compare means.

Temporal Patterns. To see whether there was any difference in sperm precedence over time, a repeated measures multiple ANOVA (MANOVA) was carried out on the arcsine-transformed hatch rates of the first 4 d of egg production by overwintered females, mated either to irradiated or nonirradiated males, or held in isolation. The number of days between diapause termination and initiation of egg laying also was compared between the treatments, with Box-Cox transformation (Box and Cox 1964) to improve the fit of the overall distribution to normality by using JMP to optimize the transformation exponent (λ).

Results

Effects of Radiation. After ln transformation, walking times were not significantly different from normal (Shapiro-Wilk: $W = 0.994$, $N = 130$, $P = 0.867$). There was no effect of sex on walking speed in nonirradiated beetles ($t = -0.056$, $df = 103$, $P = 0.956$), so males and females were pooled into a single nonirradiated group. The mean \pm SE of the ln-transformed time to walk 7 cm was 1.51 ± 0.05 s for irradiated males and 1.51 ± 0.03 s for nonirradiated beetles, and the difference was not significant ($t = -0.061$, $df = 128$, $P = 0.952$). The mean \pm SE proportion of matings or mounts observed with the intact male was 0.53 ± 0.07 , which was not significantly different from 0.5 (signed rank: $N = 20.5$, $df = 27$, $P = 0.625$).

Sperm Precedence. Twenty-two overwintered females mated to irradiated males laid clutches. Irradiated males fertilized almost all eggs in the first treat-

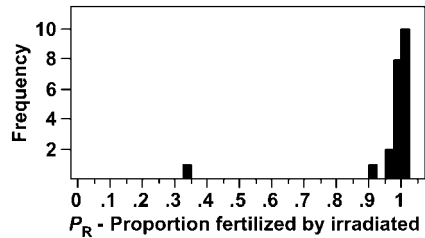


Fig. 1. Distribution of the proportion of eggs fertilized by irradiated males (P_R , Boorman and Parker (1976)) in spring matings.

ment (Fig. 1). Their fertilization success (P_R , Boorman and Parker 1976) was not significantly <1 [Wilcoxon signed rank test: $S = -41.5$, $df = 21$, P (one-tailed) = 0.09], essentially indicating complete precedence of the sperm transferred during the spring mating.

Effects of Mating. Untransformed mean hatch rates and sample sizes of females completing each treatment are presented in Fig. 2. There was no significant difference in the proportion of overwintered females laying eggs in our experiments when classified by mating treatment (χ^2 test: $\chi^2 = 0.456$; $df = 2$, 79; $P = 0.10$). Hatch rates varied significantly among treatments (ANOVA: $F = 29.222$, $df = 4$, 59; $P < 0.0001$). However, hatch rate did not differ between unmated overwintered females and females mated to intact males (unmated versus OW-intact: $F = 1.375$; $df = 1$, 59; $P = 0.246$). Fecundity of overwintered females was significantly affected by spring mating (ANOVA: $F = 11.77$, $df = 2$, 52; $P < 0.0001$). Overwintered females' fecundity did not vary whether they were mated to irradiated or nonirradiated males (irrad versus nonirrad: $F = 0.462$, $df = 1$, 51; $P = 0.499$), so results for both mated-female treatments were pooled and contrasted

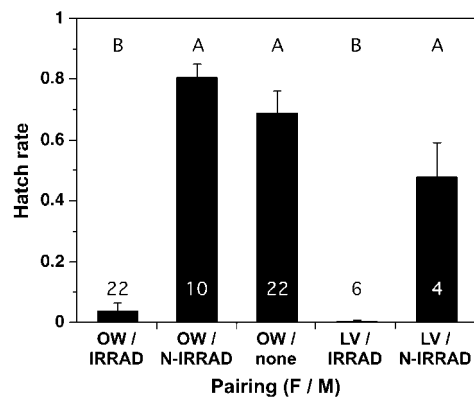


Fig. 2. Hatch rates of different treatments. Females were either OW or laboratory-colony virgins (LV). Males were either absent (none), overwintered and irradiated (OW-IRRAD), or overwintered and nonirradiated (OW-NIRRAD). Sample sizes are indicated at the base of each bar, and means that are not significantly different from each other using Tukey-Kramer honestly significant difference with an α of 0.05 are indicated by a shared letter.

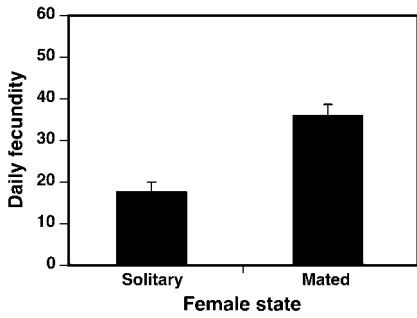


Fig. 3. Daily fecundity as a function of whether females had mated or remained isolated after emergence from winter diapause. Females mated to irradiated or intact males are pooled into a single category.

with those from unmated females. Unmated females laid fewer eggs than mated females (mated versus unmated: $F = 23.24$; $df = 1, 51$; $P < 0.0001$; Fig. 3).

Temporal Patterns. Hatch rate over time was not significantly different for any of the mating combinations (MANOVA: $F = 0.145$; $df = 3, 22$; $P = 0.384$), and the interaction between time and mating treatment also was insignificant (MANOVA: $F = 1.292$; $df = 6, 44$, $P = 0.281$; Fig. 4). On average, mated females started laying 1.6 d sooner than nonmated females, but this difference was not significant. Days of first oviposition since pairing were not distributed normally. After Box-Cox transformation, which improved the normality of the distribution, the difference between means was not significant ($t = 1.545$, $df = 52$, $P = 0.12$) nor were the untransformed means significantly different using a nonparametric test (Wilcoxon: $\chi^2 = 2.33$, $df = 1$, $P = 0.127$).

Discussion

Precedence of sperm from spring matings versus overwintered sperm from fall matings was almost com-

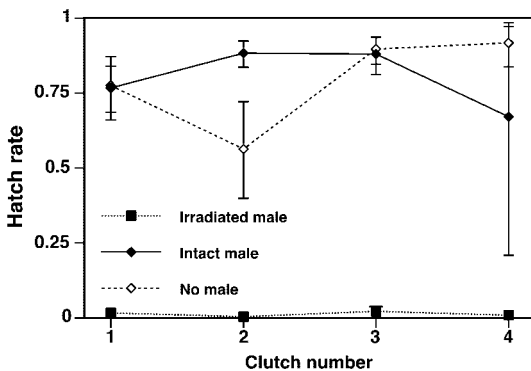


Fig. 4. Least squares mean hatch rates from the repeated measures ANOVA for the first 4 d of egg production for each of three mating treatments involving spring emerged fall mated females. Hatch rates and standard errors are back-transformed from the arcsine-transformed hatch rates used in the ANOVA.

plete. Some sperm persistence from fall matings occurred in this experiment, but only one of the 22 pairings between overwintered females and irradiated males yielded $<90\%$ fertilization by the spring mating. Furthermore, the test of sperm precedence in this experiment was conservative in several ways. First, overwintered females were given access to males for only 24 h, whereas in the field they would presumably mate more frequently and with a number of different males, further diluting sperm from the previous season. Second, hatch rates were measured for only the first few clutches that each female laid. Later clutches can be assumed to include an increasing fraction of sperm from more recent spring matings, as has been observed in other studies of sperm priority in Colorado potato beetle (Roderick et al. 2003). Finally, the use of sterile male technique may have underestimated the precedence of spring matings if radiation reduced the mating competitiveness of males in any way.

This study used radiation and sterile male technique as a marker for sperm from spring matings. Other studies of sperm precedence have used either allozymes (Alyokhin and Ferro 1999a, Roderick et al. 2003) or color morphs (Boiteau 1988). Each technique has strengths and weaknesses, all relating to potential correlated fitness effects of either radiation treatment, reducing population or sample size to create true breeding lines, or removing legs to screen for appropriate combinations of genotypes. Use of radiation allowed us to examine sperm precedence in wild females, with unconstrained mating frequency and number of mates reflecting natural conditions. At the same time, radiation may have affected sperm precedence in ways other than preventing development of fertilized eggs thus biasing the estimates of normal sperm precedence. Ideally, the effect of radiation can be controlled by reciprocally varying treatments (i.e., alternate whether the first or second male to mate is irradiated or intact). In this study, such a control was not possible because wild overwintered females were used and fall matings could not be controlled. However, precedence is typically lower in irradiated males (Simmons 2001). Therefore, if radiation had a negative effect on sperm precedence separate from an effect on hatching after fertilization, then this test would be more conservative, i.e., less likely to show a precedence of spring matings.

The findings of the current study agree with some but not all results of previous studies of overwintered females. Similar to Ferro et al. (1991), unmated overwintered females laid fertile eggs. In contrast to that study, there was no difference in hatching success of eggs laid by mated and nonmated females. This difference may reflect differences in experimental design in regard to mating and egg collection. In the current study, females were exposed to a single male for 24 h, versus two males for 72 h in the previous study. In addition, we collected eggs for up to 14 d, and most often much less if the females quickly laid 100 eggs. In the previous study, eggs were collected for up to 60 d that allowed much more time for sperm depletion

(Boiteau 1988, Roderick et al. 2003), which can account for lower hatch rates in overwintered unmated females. We observed a positive effect of mating on female fecundity, whereas Ferro et al. (1991) found no similar effect. We hypothesize that the differences in mating design also may account for this difference. It is possible that mating can have both positive and negative effects on female fecundity: a positive effect of mating itself on female egg production and a negative effect of multiple mating on female fecundity due to conflicts among ejaculates or accumulation of larger amounts of accessory gland products harmful to the female. Orsetti and Rutowski (2003) observed a significant decrease in hatch rate with an increase in number of matings in summer generation Colorado potato beetles. It is reasonable to suggest that a similar phenomenon also exists in the postdiapause beetles.

Sperm precedence of spring matings might help compensate for challenges to resistance management posed by effects of insecticides on phenology, at least in Colorado potato beetle and species with similar life histories. Liu et al. (1999) showed that *Bacillus thuringiensis* (Bt)-resistant pink bollworm, *Pectinophora gossypiella* (Saunders), larvae developed more slowly on transgenic Bt cotton than did susceptible moths on non-Bt cotton, and a similar result was shown for Colorado potato beetle and Bt (Alyokhin and Ferro 1999c, Costa et al. 2000). When only half a field was treated with imidacloprid at planting, the Colorado potato beetle on the imidacloprid-treated side had only one summer generation, whereas the treated side had two (Baker et al. 2001). Variation in developmental times in alternately treated areas will likely lead to more frequent mating among resistant individuals and more rapid resistance evolution (Liu et al. 1999), but the results here indicate that prediapause mating among resistant Colorado potato beetle will be offset by matings after emergence in the spring. Matings after diapause will take advantage of any higher winter mortality among resistant beetles, but precedence of spring matings makes it imperative that those matings take place between resistant and susceptible individuals, and if mating takes place at overwintering sites, it is important that they accumulate individuals from refuges as well as treated areas.

Ferro et al. (1999) reported that >60% of the beetles observed in their study mated within an overwintering site before leaving in search of a host habitat. They also encountered mating beetles within a field that was rotated out of potatoes. Their experimental protocol involved essentially "snap-shot" observations and did not allow determining the true proportion of mating individuals. Nevertheless, such results indicated that mating within overwintering habitats is rather common for Colorado potato beetle and are likely to underestimate the extent of mating activity taking place at the overwintering sites. Therefore, within-field refugia are likely to be a valuable tool in resistance management. Even if gene flow between the populations of resistant and susceptible summer generation beetles is low, a proper spatial arrangement of refugia will result in their mixing at overwintering

sites and subsequent mating between resistant and susceptible individuals in the following spring. Furthermore, without refugia crop rotation might lead to the export of homozygously resistant beetles to the fields where resistance has not yet evolved, because unfed overwintered beetles are fully capable of flight (Ferro et al. 1991, 1999).

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