

Isolated females and limited males: evolution of insect resistance in structured landscapes

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Abstract

To delay evolution of insect resistance to insecticidal *Bacillus thuringiensis* Berliner (Bt) transgenic crops, USA and Canadian maize growers commonly set aside a portion of each field as a refuge, where susceptible pests can develop without exposure to the toxin(s) expressed in the insecticidal crop. Abundant mate-seeking refuge adults are expected to move into insecticidal crop areas and mate with rare, resistant insects. Production of heterozygous offspring, rather than homozygous offspring reduces the rate of resistance evolution. Expectations about the refuge strategy are predicated on assumptions about when and where pest insects move and mate. Accumulating evidence indicates that the behavior of western corn rootworm beetles, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) (WCR), in refuge and corn rootworm (CRW)-active Bt-transgenic maize does not always conform to assumptions about random mating and individual movement. Contrary to expectations, up to 23.6% of unmated refuge females left refuge and were non-teneral by the time they mated in CRW-active maize. Consequences of protandry, limited male mating capacity, pre-mating male and female movement patterns, skewed sex ratios, and delayed adult emergence from CRW-active maize all may contribute to unexpected or undesirable patterns of WCR reproductive behavior. Modeling suggests that temporal and spatial components of WCR mating that limit interactions in block refuges can be reduced by deploying blended refuges.

Introduction

To delay evolution of insect resistance to transgenic insecticidal *Bacillus thuringiensis* Berliner (Bt) maize, growers commonly set aside an area within or near each Bt maize field as a refuge, where susceptible pests can develop without exposure to the toxin expressed in the insecticidal crop (Onstad et al., 2011). Susceptible insects produced in refuge are expected to enter the insecticidal crop area to mate with the rare, resistant survivors. Mating between susceptible insects from the refuge and homozygous resistant individuals from the insecticidal crop generates heterozygous

offspring that will subsequently die in the insecticidal crop. The refuge strategy is most effective when the level of toxin expression is high enough to kill almost all heterozygotes.

Thus, the success of an insect resistance management (IRM) strategy depends on more than just the dose of toxin; the behavior and ecology of the target pest are also important details that help determine how much refuge is necessary and where it must be deployed (Tabashnik, 1994; Gould, 1998). Simulation models are used to evaluate the efficacy and durability of a particular toxin in a refuge design (Storer, 2003; Onstad, 2006; Pan et al., 2011; Onstad et al., 2011). These models are parameterized with literature-derived assumptions about pest biology; this framework allows complex variation in pest biology to be explored. For example, the expectation that receptive

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males and females mate whenever conspecifics are encountered in the field may be a flawed assumption. The assumption of random mating is the benchmark of many IRM models (Onstad, 2008). In this case, we define 'mating failure' as the failure of resistant individuals to mate with susceptible individuals to produce heterozygotes, which are expected to be killed by high-dose expression of the toxin. If random mating is common in a landscape, the rarer resistant insects are far more likely to encounter and mate with abundant susceptible individuals. However, what happens if some insect species have male adults that are not efficient searchers, do not fly long distances, have limited multiple mating abilities, or that preferentially mate with females of a given phenotype? When the landscape is composed of large, structured patches of crops, the limited abilities of males become more apparent and important. Thus, mating failure is a problem that can be based on both insect behavior and the variety of landscapes that the insects inhabit.

For diabroticite corn rootworms (CRW) (Coleoptera: Chrysomelidae) in North American maize, there is uncertainty about the assumption that adequate mating will occur between individuals of different genotypes emerging from transgenic insecticidal Bt maize ('CRW-active maize') and the non-Bt refuge ('refuge'). In this study, we describe the behavior, population dynamics, and genetics of *Diabrotica virgifera virgifera* LeConte (western corn rootworm, WCR) in a landscape of maize to demonstrate the consequences of mating failures for resistance evolution.

Western corn rootworm biology

Because core assumptions of the refuge strategy hinge upon insect mating and movement, the effectiveness of a refuge strategy also depends on an understanding of pest behavior. The WCR is the most serious insect pest of maize across the 'USA Corn Belt'. Annually, the WCR and the related northern corn rootworm (*Diabrotica barberi* Smith & Lawrence) cost producers nearly 1 billion USD in combined yield losses and management expenditures (Rice, 2004); it may be the world's most expensive pest to control (Cock, 2011).

The WCR is protandrous; adult males emerge ca. 5 days before females (Branson, 1987); yet, 97.8% of the male emergence period overlaps with that of females (Quiring & Timmins, 1990). After males emerge, ca. 5–7 days of development are required for 80% of males to reach sexual maturity and become responsive to female sex pheromone (Guss, 1976). Branson et al. (1977) observed that males could mate on average eight times during a 42-day period when supplied with virgin females in a confined laboratory

environment, and Quiring & Timmins (1990) found that males could mate up to 17 times during their lifetime. In contrast to males, adult females are sexually mature upon emergence (Hammack, 1995). Among newly emerged females, 54% began calling (i.e., release of sex pheromone from glands near the abdominal tip) on their emergence day, 96.4% called during the following day, and by day 3 all had called (Hammack, 1995). Unmated females can release pheromone for at least 3 days (Hammack, 1995). Quiring & Timmins (1990) found 96.6% of mating females were teneral (97.2% of mating males were not teneral and thus not newly emerged). The elytra of teneral females are completely sclerotized ca. 12–24 h after emergence (Cates, 1968). In a study of 30 female WCR, Marquardt & Krupke (2009) reported that although females begin calling soon after they emerge, the earliest responding males were frequently rejected and no matings occurred until 4 h after emergence.

Many mated females fly out of their natal field within several days after mating. Among dispersing females caught when ascending from cornfields, 84% still contained a spermatophore (Spencer et al., 2005); all traces of a spermatophore are degraded by 5–7 days after mating (Lew & Ball, 1980). Historically, female dispersal from their natal continuous cornfields was responsible for the high proportion of females in rotated cornfields (Godfrey & Turpin, 1983).

Western corn rootworm, CRW-active maize hybrids, and refuge

The 2003 commercialization of a first rootworm-protected transgenic Bt hybrid, Cry3Bb1-expressing YieldGard[®] Rootworm (Monsanto), offered growers an alternative to soil insecticide for rootworm management (Rice, 2004). Grower adoption of all insect-resistant and stacked gene Bt maize varieties (expressing both insect resistance and herbicide tolerance) has steadily increased [USDA-ERS (United States Department of Agriculture-Economic Research Service), 2012].

Although Cry3Bb1-expressing maize was not a high-dose event (nor were any of the subsequent CRW-active events), the IRM plan for Cry3Bb1 included a 20% non-Bt refuge (EPA (Environmental Protection Agency) Office of Pesticide Programs, 2003) later commercial rootworm-resistant hybrids expressing different single rootworm-active Cry proteins – i.e., Cry34/35Ab1 (2005) and mCry3A (2007) – inherited the same refuge requirement. Subsequent stacked and pyramided products were granted 10 and 5% block refuges. Later, integrated refuge versions of these products ('refuge in a bag' seed blends) were registered with the same percentage refuge requirements.

An adjacent or within-field block of refuge maize planted on 5–20% of the field is the most common refuge approach in North America. Where and how the refuge is planted influences its effectiveness (Onstad et al., 2011). However, because of constraints imposed by the shape of fields, producer preferences for row strips or block refuges and the year-to-year location of refuge in a field, the configuration of a compliant 5–20% refuge will be highly variable. As adoption of Bt-technology increased, compliance with refuge regulations designed to delay pest resistance experienced a decline (Jaffe, 2009) at the expense of integrated pest management (IPM) (Gray, 2011a,b). Evidence of WCR field resistance to the Cry3Bb1 toxin expressed in the first commercialized hybrids was discovered in 2009 (Gassmann et al., 2011). Laboratory selection for resistance had previously been demonstrated for Cry3Bb1, Cry34/35ab, and mCry3a toxins (Meihls et al., 2008, 2011; Lefko et al., 2008).

Whereas a large and mobile population of mate-seeking WCR males is the anticipated product (the ‘active ingredient’) of refuge, an equally large population of females is also produced (albeit slightly later). Expectations about the dynamics of refuge male movement and mating in transgenic maize are central to expectations about how refuges function. Furthermore, the impact of a large refuge female population on the reproductive dynamics of the males is unexplored as is the dispersal behavior of virgin females within large patches of CRW-active maize. Although Coats et al. (1987) observed no unmated females flying while tethered in flight mills before the 3rd day after emergence, J Spencer (unpubl.) observed 50–60% of unmated, non-teneral females flying at least 5 m (5–10 rows of maize) upon release in a field. Similarly, Marquardt & Krupke (2009) found that 23% of females flew from their natal plant to another plant before mating.

Western corn rootworm mating and modeling

The assumption of random mating for WCR emerging in refuge and CRW-active maize hybrids was used to develop models predicting the likelihood of resistance evolution in WCR (Crowder et al., 2005; Storer et al., 2006; Pan et al., 2011). These predictions are based on a combination of empirical data and several key assumptions where data are lacking. Here, we review the literature regarding mating in this species to assess how the various refuge types may function, including identifying conditions that may lead to mating failures in this system.

Western corn rootworm female size

Kang & Krupke (2009a) explored constraints on WCR mating. They found a positive relationship between female

weight and the number of male mating attempts (both successful and unsuccessful) when males were given a choice between females of different sizes (Kang & Krupke, 2009a). There was also a negative relationship between female weight and time elapsed until the initial mating attempt: males were more likely to attempt to mate with large females quickly. No effects of male weight were observed. Female weight is viewed as a proxy for fitness in many insects, including WCR where it is correlated with fecundity (Branson & Sutter, 1985). The Kang & Krupke (2009a) experiments showed that female weight is an important variable predicting WCR mating rates in the laboratory. Hibbard et al. (2004) documented significant field differences in female weight on natal maize hosts, furthermore, the relationship was found to vary with larval density. Under high population densities, adults emerging from CRW-active maize may be larger due to release from density-dependent competition (Hibbard et al., 2004), whereas at lower population densities, females emerging from CRW-active maize hosts are smaller (Storer et al., 2006; Murphy et al., 2011). From the perspective of refuge function, these data demonstrate that the effects of CRW-active maize upon adult size may vary across landscapes where WCR population densities are not equal.

Male WCR mating capability

Lifetime WCR male mating potential is an important parameter for assessing refuge effectiveness. Although early work (Branson et al., 1977; Quiring & Timmins, 1990) found that males mated between 8 and 17 times over their lifetime in the laboratory, more recent work by Kang & Krupke (2009b) suggests that these totals may have been inflated, likely due to a combination of small mating enclosures and highly female-biased sex ratios in mating arenas.

Kang & Krupke (2009b) confined individual field-collected males and females in enclosures with fresh maize and silks to determine male mating potential. Each male was allowed to mate once, then supplied with successive virgin females daily. Males mated on average 2.24 times within 10 days following their first mating, but only averaged 0.15 matings during days 11–20. Whereas Kang & Krupke (2009b) addressed some key criticisms of the earlier work, factors like long-range mate location, courtship, and male mate competition were not addressed. It is possible that mating success by aging males is even lower in the field. Physiological constraints may also lower male mating potential. Males invest significantly in each mating; they deposit a large spermatophore (equal to 7–9% of male mass) in the female during copulation (Quiring & Timmins, 1990). The role of the spermatophore in predicting fertility/fecundity remains unclear, although

stable isotope studies using N^{15} indicate that the spermatophore is largely composed of water and protein, some of the material makes its way into the eggs of the female (Murphy & Krupke, 2011). The time needed to replenish spermatophore-forming components between matings may further limit mating potential.

Males could be expected to mate early, likely with the first suitable and receptive female they encounter. For refuge males, these females are likely to be more abundant in the refuge parts of fields. Declining male mating ability with age after first mating may limit the impact of refuge males in CRW-active maize parts of the field. This relatively narrow window for mating among males, combined with WCR emergence delays within fields (e.g., CRW-active maize vs. refuge maize) may mean that later-emerging females from CRW-active maize have little chance to encounter any remaining, viable mate-seeking males that originated from refuge areas. Instead, females from CRW-active maize may be more likely to encounter younger mate-seeking males emerging from the same natal area. The low frequency of multiple matings reported by Kang & Krupke (2009b) indicates that if the population of males is low, some females may remain unmated.

Modeling

Pan et al. (2011) created a model of WCR population genetics and dynamics (including a submodel for adult emergence, dispersal, mating, and oviposition) that accounted for some of the work of Krupke and coworkers. The model was intended to compare refuges deployed as seed blends with refuges deployed as single blocks adjacent the blocks of CRW-active maize. Deploying refuge in a seed blend minimizes the scale of landscape heterogeneity and the isolation of females in CRW-active maize from males in refuge.

In Pan et al. (2011), males were allowed to disperse starting on the 1st day of their adulthood with a mean dispersal rate of 15 m day^{-1} , derived from rates published by Bruss (1981) and Spencer et al. (2009). Mean dispersal rate is used to calculate the probability of male movement to a given 1-ha cell in the simulated maize region. Dispersal distance was assumed to follow an exponential distribution, daily dispersal direction was random.

Marquardt & Krupke (2009) observed that most females moved little before mating. In the model, all females are mated as teneral adults in the natal patch. The model assumed a 35-day emergence period for each sex. Males emerged 7 days before females and all emergence from refuge maize occurred 7 days before emergence in CRW-active maize (Pan et al., 2011). Thus, the first female emerging from CRW-active maize emerges 14 days after the first male in the refuge. Kang & Krupke

(2009b) observed few males mating during the later stages of their adulthood, so the model was simulated with both unlimited male mating capability (all 35 days) and male mating restricted to the first 10 days of male adulthood, as well as both blended and block refuges.

A 10% blended refuge with the assumption of unlimited male mating capability was the reference (Pan et al., 2011); it simulated a field that was planted from bags containing a blend of 90% CRW-active maize and 10% refuge maize seed. In a seed blend, refuge plants are distributed randomly throughout the field, rather than grouped into blocks. Refuge blocks could either be relocated within fields every year or replanted in the same location. The reference simulation results in a 12-year delay before the resistance allele frequency for the major gene increases from 0.001 to 0.5 (Table 1). Table 1 demonstrates how shortening male mating period affects the pace of resistance evolution. When males only mated during the first 10 days of adulthood, evolution of resistance occurred faster. Pan et al. (2011) also found that resistance evolved faster as delays between female and male emergence grew longer. For our reference simulation of a 10% blended refuge, a 3-day difference in emergence slowed resistance evolution by 1 year, from 12 to 13 years. Evolution was delayed by a year from 12 to 13 years when the difference in emergence periods was lengthened to 14 days. With a 5% blend, a shorter 3-day delay slowed resistance evolution by 2 years compared with the standard scenario with a 7-day delay. Doubling the emergence delay from 7 to 14 days only decreased the time required by 1 year. There were no changes with block refuges. The results in Table 1

Table 1 Influence of reducing western corn rootworm (WCR) male mating period from 35 days (entire lifespan of adult) to 10 days on number of years to 50% resistance allele frequency for various scenarios. The 10% blend refuge was used as the reference simulation

Scenario	Male mating period	
	10 days	35 days
20% block refuge, location fixed	16	>20
20% block refuge, location changed yearly	8	9
10% block refuge, location fixed	12	18
10% block refuge, location changed yearly	8	8
5% block refuge, location fixed	11	17
5% block refuge, location changed yearly	7	8
20% blend refuge	10	14
10% blend refuge	9	12
5% blend refuge	8	10
No refuge	5	5

Adapted from Table 10 in Pan et al. (2011).

indicate that the duration of male mating period can accentuate the sensitivity of predictions to emergence timing of males and females. For IRM purposes, the more pressing question may be how these assumptions about basic mating behavior play out under field conditions, using mating pairs in large plots.

Materials and methods

Field study

Western corn rootworm abundance, movement, and mating activity in and around interfaces between refuge and CRW-active maize were the focus of the field study (Figure 1). In this study, 20% block refuges were deployed in CRW-active maize as a single in-field strip (2005, 2006) or an edge-of-field block (2010, 2011). In 2007, a 50% refuge was deployed. The goals of the field study were to determine whether WCR moved between refuge and CRW-active maize before mating and to document patterns of abundance and mating relative to refuge and CRW-active maize areas. Western corn rootworm movement between refuge and CRW-active maize was discerned by analyses of ingested maize tissues in WCR gut contents according to the methods of Spencer et al. (2003). By pairing the planting of refuge and CRW-active maize hybrids that expressed different Cry proteins, we could use the presence of maize tissues specific to refuge or CRW-active maize fields to reveal where WCR had been feeding before they were collected. The Cry proteins expressed in Bt-transgenic maize hybrids provide a source of specific ‘markers’ that are detectable with simple, inexpensive tests (e.g., QuickStixTM; EnviroLogix, Portland,

ME, USA). QuickStix yield data about the presence/absence of a specific Cry protein (e.g., Cry3Bb1, Cry1Ab, or Cry1F) present in tissues consumed by WCR adults.

Although Cry proteins expressed in CRW-active maize hybrids are toxic to neonate WCR larvae (Vaughn et al., 2005), adults are unaffected and readily consume the tissue during normal feeding (Spencer et al., 2003). Ingested Cry proteins remain detectable in the gut contents for ca. 1 day (Spencer et al., 2003). Their use for movement studies is a direct way to reveal patterns of male and female WCR movement between refuge and CRW-active maize relevant to expectations about refuge function.

The study site was the 16.2-ha University of Illinois ‘Shaw Farm’, northeast of Urbana. Fields were planted with 0.76-m wide rows aligned on a north–south axis. There were three 0.6-ha replicates deployed in 2005, 2010, and 2011, and four 0.9-ha replicates deployed in 2006 and 2007 (Figure 1). To minimize movement between replicates and nearby fields, they were separated by 7- to 10-m-wide alleyways. Interfield movement from commercial fields within 10 m (via detection of unique Cry proteins) accounted for just 0.8% of all captures in 2010 (S Hughson & J Spencer, unpubl.). Fields were sampled and maize phenology was recorded 2–5 times per week.

Western corn rootworms were collected by walking down a row while knocking live adults from plants into a funnel mounted on a jar containing chips of dry ice. Beetles that tumbled into the jar were immobilized by CO₂ and killed by intense cold. When encountered, mating pairs were collected in the same manner as singleton adults. Western corn rootworms in copula remain coupled after they fall and are frozen together; pairs engaged in

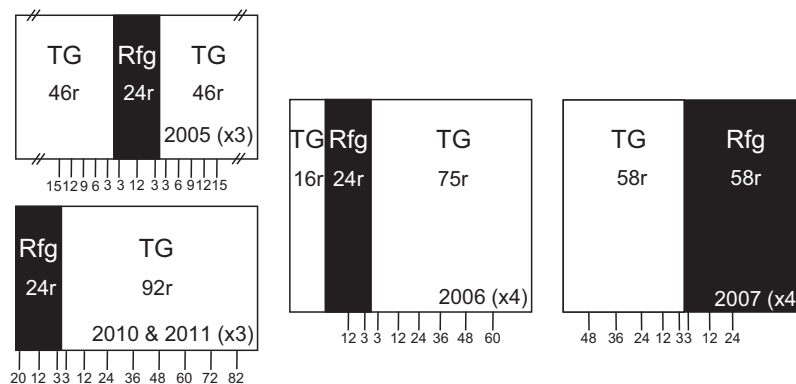


Figure 1 Field layouts used to monitor western corn rootworm (WCR) abundance and mating in refuge (Rfg) and corn rootworm (CRW)-active maize (TG) in 2005–2007 and 2010–2011. WCR live collections were made in the indicated rows (r) within a replicate; row number designations are relative to refuge–TG interface(s). Plot areas were 0.9 ha (2006–2007; four replicates per year) or 0.6 ha (2005, 2010–2011; three replicates per year). Refuge was 20% of field area, except in 2007 when it was 50%. The 2005–2006 TG hybrid was YieldGard RootwormTM (expressing Cry3Bb1) and the Rfg hybrid was YieldGard Corn BorerTM (expressing Cry1Ab). The 2007 TG hybrid was YieldGard PlusTM (expressing Cry1Ab + Cry3Bb1) and the Rfg hybrid was Herculex[®] I (expressing Cry1F). The 2010–2011 TG hybrid was Herculex[®] Xtra (expressing Cry1F + 34/35Ab1) and the Rfg hybrid was Herculex[®] I (expressing Cry1F),

post-mating mate-guarding separate once they fall into the jar. These 'live' collections were timed (e.g., 2–5 min); abundance and encounters with mating pairs were calculated on a 'per min' basis.

A replicate consisted of 8–13 live collections in designated maize rows on both sides of the interface between the refuge and the CRW-active maize in a plot (Figure 1). There were three collection rows in refuge blocks (except in 2006 when there were two); the remaining collections occurred in the adjacent CRW-active maize rows (Figure 1). Collections occurred between 09:00 and 11:00 hours.

After a collection, sample insects were bagged and stored on dry ice. When later examined in the laboratory before storage at -25°C , WCR were counted and sorted by sex. Females were scored as teneral (gray or pale yellow in part or whole; females <24 h post-emergence) or mature (boldly yellow and black; females >24 h post-emergence). No teneral males were discovered in mating pairs. Mating pairs were not physically separated until they were tested for the presence of the two Cry proteins that distinguished between the refuge and CRW-active maize.

Data analysis

Statistical comparisons of the proportion of teneral females in mating pairs and the proportion of mating males and mating females that carried ingested evidence of movement were analyzed with Fisher's exact test (Fisher, 1922). Significance for multiple comparisons was determined using a sequential Bonferroni correction (Holm, 1979).

Results and discussion

Local WCR abundance declined during the field study. Peak WCR abundance in refuge maize was 6.4–13.1 WCR min^{-1} in 2005–2007 vs. 2.8–5.3 WCR min^{-1} in 2010–2011. The rate at which mating pairs were encountered (i.e., mating rate) was also much higher in 2005–2007: 0.16–0.38 vs. 0.07–0.09 matings min^{-1} in 2010–2011. Growing adoption of CRW-active maize hybrids and several consecutive years of saturated soils during critical periods of egg hatch and larval establishment on roots (Sutter et al., 1989) likely contributed to the low population densities.

Analyses of WCR mating and the individual females from mating pairs revealed that female maturity varied between refuge and CRW-active maize areas as well as with maize growth stage (Figure 2). The proportion of teneral females in mating pairs was low during the earliest part of the growing season in CRW-active maize when males were abundant (high sex ratios) as well as late in the season in

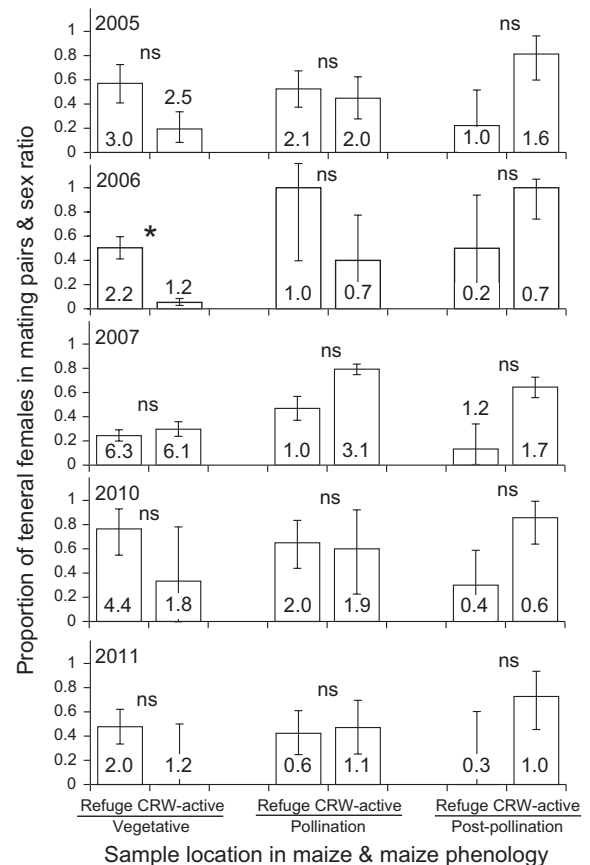


Figure 2 Mean (\pm 95% confidence intervals) yearly proportion of teneral female western corn rootworm (WCR) in mating pairs and male:female sex ratios (i.e., the numbers near the horizontal axes) for sample locations in refuge and corn rootworm (CRW)-active maize rows. Teneral females are newly emerged and pale colored; they are <24 h old and lack a fully sclerotized cuticle. Data are grouped by corn phenology at the time of WCR collection. Comparison of teneral proportions between corn phenology periods was carried out by Fisher's exact test. Significance for multiple comparisons was determined using a sequential Bonferroni correction. ns, not significant, * $P < 0.05$.

refuge maize. Proportions of teneral mating females rose later in the season, particularly in CRW-active maize, when sex ratios were around 1.0 or even female biased (low sex ratios). Overall, greater proportions of teneral females in mating pairs were associated with CRW-active maize during post-pollination and with refuge maize during the vegetative and pollination periods of maize phenology. High proportions of teneral females in mating pairs from CRW-active maize during post-pollination are consistent with documented delays in adult emergence from CRW-active maize (Storer et al., 2006; Murphy et al., 2010).

A linear regression of proportion teneral females in mating pairs on sex ratio during each period of maize phenology revealed a significant positive relationship during pollination ($F_{1,8} = 8.65$, $P = 0.019$; $R^2 = 0.519$). The beginning of the pollination period coincides with the peak adult emergence from refuge maize and an abundance of preferred foods (i.e., maize pollen and silks). However, the analyses also revealed that there was no significant relationship between sex ratio and proportion of teneral females in mating pairs during the vegetative ($F_{1,8} = 0.654$, $P = 0.44$; $R^2 = 0.076$) or post-pollination ($F_{1,8} = 0.707$, $P = 0.43$; $R^2 = 0.081$) periods. This finding suggests that expectations about rapid WCR mating may be unmet during the early season period of emergence from block refuge and later in the season when the (delayed) emergence from CRW-active maize is fully underway. What may explain the failure of high relative male abundance to assure rapid mating of teneral females (Quiring & Timmins, 1990)?

During early female emergence, many of the males that are present will also be newly emerged but not yet be sexually mature (i.e., >5–7 days old), thus the operational sex ratio will be much lower than would be suggested by the observed sex ratio. The net result may be too few sexually mature males to mate with the available females; thus, many females will wait more than a day before they attract a mate (as non-teneral females). Perhaps sexually mature males are present, but because calling females are present in excess, many males are in a post-mating refractory period while they feed to replenish the spermatophore components they provided during their last mating. Males that mate during the vegetative period of maize phenology may be forced to replenish their spermatophore-forming components from maize foliage that is less nutritious than pollen and silks, resulting in longer intervals between matings. During pollination, mating males may feed on pollen and silks and be more rapidly renewed between copulations. During post-pollination, although many males will be present, a significant portion may be past reproductive age – i.e., >10 days after reaching sexual maturity (Kang & Krupke, 2009a) – and food quality/abundance may also be diminished; these factors could also contribute to an even lower operational sex ratio.

Female dynamics may affect the high proportion of mating teneral females in CRW-active maize when sex ratios are equal or female biased. Later in the season, we hypothesize that the operational sex ratio may again become skewed resulting in an operational male bias. At the end of pollination and into post-pollination, many already mated females (originally from refuge or immigrants from neighboring fields) have become distributed across CRW-active maize (S Hughson & J Spencer,

unpubl. 2010 and 2011 field data). The greatest WCR abundance occurs around this time (ca. 1st week of August) as emergence from CRW-active maize is peaking. When females are relatively plentiful, newly emerged calling females will be just a fraction of the female population. From the perspective of the remaining mate-seeking males (which will include a small proportion of males that emerged from CRW-active maize), mated females are irrelevant. The operational sex ratio is thus skewed to favor the rapid discovery of a relatively few calling females by many males. In addition, as the ear develops and the rest of the plant senesces, hungry young and old WCR adults congregate on the dwindling supply of moist, nutritious silks. These circumstances make it likely that mate-seeking males and newly emerged females will be in close proximity.

Analyses of ingested Cry protein within the 1803 mature and teneral mating females from Figure 2 revealed dietary evidence of the proportion of females that moved between refuge and CRW-active maize (or vice versa) before their capture in copula (Figure 3). Most females ($216/230 = 0.94$) that moved were mature females. All but two of the mature moving females were detected during the years of higher WCR abundance (2005–2007). Teneral females were found to have rarely crossed into the other portion of the field before they mated. At low WCR abundance (2010–2011), there was almost no pre-mating intrafield female movement. An insect was considered a ‘non-mover’ if testing revealed that it only contained a Cry protein available in the area where it was collected. Newly emerged (teneral) adults that did not test positive for any Cry protein were also counted as non-movers. Because detection of ingested Cry proteins is limited to ca. 24 h post-ingestion, insects that fed and moved >24 h before capture would not be identifiable as movers. This method documents movement during the 24 h before capture.

Parallel analysis of the male partners from mating pairs revealed a similar, although not identical pattern of movers and non-movers (Figure 4). The pattern of mating male recovery deviated from females (Figure 3) because a pair may include an individual that moved and one that did not, thus partners could be counted in different locations in Figures 3 and 4. The yearly proportion of moving males (2005: 0.285; 2006: 0.474; 2007: 0.216; 2010: 0.090; 2011: 0.168) was significantly greater than the proportion of moving females (teneral + mature) each year (2005: 0.090; 2006: 0.236; 2007: 0.115; 2010: 0.015; 2011: 0.010) with the exception of 2010. When these data were grouped by maize phenology (data not shown), the proportion of mating males that had moved between areas of the field before they mated was significantly higher during the vegetative period (0.38) than during pollination (0.14) which

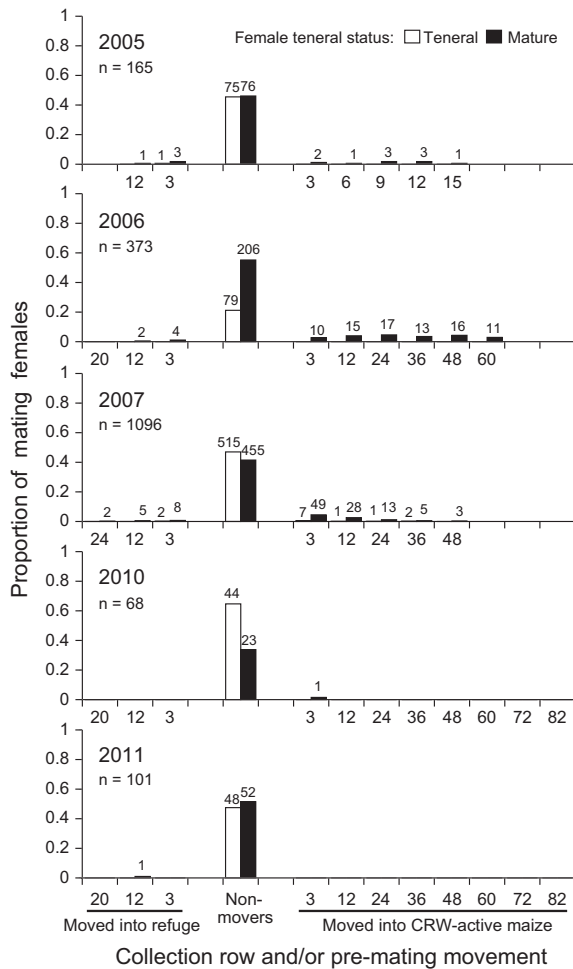


Figure 3 Proportion of western corn rootworm (WCR) females collected in mating pairs containing evidence of pre-mating movement or non-movement. The maturity of mating females in refuge or corn rootworm (CRW)-active maize collection rows was classified as teneral (<24 h old; white bars) or mature (>24 h old; black bars). Movement determination was based on detection of ingested Cry proteins specific to the refuge or the CRW-active maize hybrid in individuals collected in maize in which the specific protein was not expressed. The collection row indicates the number of 0.76-m rows a female had moved before her capture in copula. The number of teneral or mature females in each movement class is depicted above the collection row bar. n is total mating females analyzed.

was significantly greater than that during post-pollination (0.07). The tendency for male movement declined during the season.

Among the 14 teneral females that engaged in pre-mating intrafield movement, 10 (71%) were collected in the sampling row that was nearest to the interface with the other side of the field (i.e., row 3 = 1.5 m). The greatest distance moved from the interface by teneral females

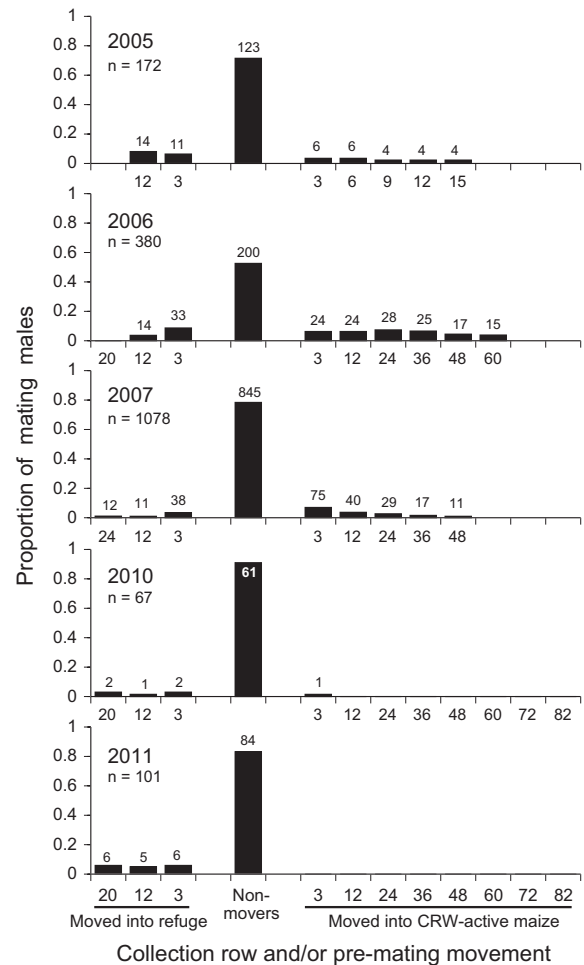


Figure 4 Proportion of western corn rootworm (WCR) males collected in mating pairs containing evidence of pre-mating movement or non-movement. Movement determination was based on detection of ingested Cry proteins specific to the refuge or the corn rootworm (CRW)-active maize hybrid in individuals collected in maize in which the specific protein was not expressed. The collection row indicates the number of 0.76-m rows a male had moved before his capture in copula. The number of males in each movement class is depicted above the collection row bar. n is total mating males analyzed.

before mating was 36 rows (27.4 m) into CRW-active maize; two refuge females were detected at this distance in 2007. Among mature females from mating pairs, individuals that crossed the field interface were found at the farthest ends of the sampling arrays (15, 60, and 48 rows away from the interface, respectively) every year between 2005 and 2007. Given the ca. 1-day detection interval for ingested Cry-expressing tissue (Spencer et al., 2003), maximum pre-mating movement rates for WCR females could be as high as 45.7 m day⁻¹ (i.e., 60 rows day⁻¹*0.76 m per row).

The range of male movement distances is similar (Figure 4), although a greater proportion moved.

Despite evidence for significant pre-mating female movement (i.e., 23.6% in 2006, as noted above), the majority of pre-mating teneral and mature females did not likely move from the refuge area where they developed as larvae. When females moved before mating, the movers were usually mature females. The decline in detection of intrafield movement by pre-mating females in the low-abundance years (2010, 2011) may implicate WCR density as a factor in the likelihood of movement; however, detecting scarce movers would naturally be more challenging at low density.

Gut content analysis also indicated that most males had not moved between refuge and CRW-active maize in the day before they mated; however, the percentage of movers was generally higher (up to 47.4% in 2006, as noted above). We hypothesize that a refuge male population that should have been adequate to mate with the females that emerged in block refuges, dispersed before female emergence was complete and were distributed too thinly across the entire field, leaving a relatively low population to interact with the daily flux of calling females. As females emerged from refuge blocks, the local abundance of mate-seeking males with the capacity to mate was too low to assure a likelihood of rapid mating (perhaps due to exhausted supplies of spermatophore-forming components and/or advancing age, as hypothesized above). The result was a refuge male population that included too few mate-seeking males to mate with all the females as they emerged. These unmated females remained sexually receptive and continued to call for several days (Hammack, 1995) as they fed and moved about. A portion of these females (ca. 9–24% in 2005–2007) dispersed into CRW-active maize areas that were more sparsely inhabited by calling competitors, we hypothesize that these unmated females eventually encountered a mate-seeking male and mated – as mature females.

Conclusions

Over the past 15 years or more, the implementation of science-based IRM has forced applied entomologists to measure a variety of ecological processes that were infrequently studied when the emphasis of integrated pest management research was upon reduction in pest populations and/or damage. Given the importance of assumptions about insect behavior and ecology in IRM, it is necessary that models for assessing the durability of IRM strategies are parameterized with data collected under conditions similar to those to be simulated. Ground-truthing a model helps to explain failed alignment of expectations with

observations and can reveal interactions between factors that would not have been easily predicted. This conclusion echoes the emphasis placed on this topic by Onstad (2008). The study of insect behavior becomes even more challenging when it must be performed in novel structured landscapes.

Revisiting the assumptions about rapid mating for WCR females originating in refuge as well as CRW-active maize suggests that some IRM assumptions about movement and mating may be wrong. When WCR abundance is modest in fields with block refuges, 9–24% of mating females may not rapidly find a mate in the field where they likely emerged. These unexpected patterns of behavior may have other consequences for refuge function. How does the presence of refuge females in CRW-active maize alter the probability that refuge males will mate with resistant females from CRW-active maize? Furthermore, what fraction of refuge males present in blocks of CRW-active maize during female emergence retain the capacity to mate with a female? Is pre-mating female (and male) movement outside of their natal area affected by WCR density? The proportion of teneral females in mating pairs may be an indicator of the local abundance of mate-seeking males; however, the operational sex ratio, crop and insect phenology, and the type of maize (refuge or CRW-active) will also factor into a judgment of whether the refuge is functioning as expected.

Laboratory observations, model outputs, and field data will all contribute to answering the questions we present above. The field study illustrates the importance of assessing refuge function *in vivo*; we observed potential mating failures that may not have otherwise been anticipated. Documenting these patterns will enable refinement of assumptions and improved IRM models.

Given declining levels of grower compliance with government regulations concerning block refuge size and location (Jaffe, 2009; Gray, 2011a,b), blended refuges were evaluated for WCR IRM by Pan et al. (2011) who accounted for larval and adult behavior when simulating seed blends. As indicated in Table 1, the 10–20% blended refuges provide the longest durability for any of the scenarios in which refuge is relocated every year. Although, block refuges maintained at the same location forever extend durability, they are rarely deployed by North American maize growers. Thus, a new era in which seed blends of CRW-active maize are given equal consideration with block refuges has begun (Onstad et al., 2011). Of course the sustainability of any IRM strategy must be evaluated on a case-by-case basis, depending on crop management, insect species, and the particular insecticide/Bt toxin (Onstad, 2008; Onstad et al., 2011).

The agro-ecosystems in which corn rootworms reside are neither static nor homogeneous. As natural selection and pest management occur in the typical North American maize landscape, there are intervals during the season (e.g., early in adult emergence and mating) when individuals of different WCR genotypes may become spatially and temporally isolated near where they emerged. However, there are other intervals (e.g., after post-mating dispersal) when field populations include many WCR that arrived after emergence and mating in nearby and distant fields. Ultimately, the next generation of WCR is derived from the offspring of both resident and immigrant WCR that experienced varied selection pressures as larvae and adults. Understanding and modeling local WCR adaptation at the level of the individual and a field are necessary to appreciate the evolution of resistance on a population scale.

Our observations demonstrate various ways that WCR females can be isolated enough to restrict their opportunities to mate with males in the farm landscape. These opportunities are further restricted by evidence that male mating capabilities decline over time relatively quickly after males become reproductively active. In an unstructured landscape (i.e., a seed blend) without extreme patch-dependent selection pressure and minimal spatial isolation between refuge and CRW-active maize, limitations on male ability would not likely be as significant. The observed and hypothesized circumstances that distort long-held assumptions about WCR mating underscore the need to account for insect behavior in modern maize agriculture, IPM, and IRM. To wit, a review of the literature regarding frequencies of female mating failures by Rhainds (2010) reveals that basic insect behavior has lessons for the relatively applied world of WCR and sustainable maize production. In his review, Rhainds (2010) concluded that mating success of females generally increases with the ratio of males in the population, but the relation between emergence time, sex ratio, and female mating success is variable. Our studies support these conclusions. In commercial maize plantings, emergence times of males and females of various genotypes are inadvertently altered in ways that change temporal sex ratios. These changes can interfere with random mating and have the potential to speed the evolution of resistance. We believe that insight gained from studies of the WCR mating system can shape future investigations of mating failures in genetically diverse populations inhabiting structured landscapes.

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