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Authors: Campbell, L. A., Prasifka, P. L., Storer, N. P., Rule, D. M., and Hendrix, W. H.

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Effects of Bt Corn and Egg Density on Western Corn Rootworm (Coleoptera: Chrysomelidae) Adult Emergence and Estimation of Effective Bt Dose

L. A. Campbell,*[,1] P. L. Prasifka,* N. P. Storer, D. M. Rule, and W. H. Hendrix

Dow AgroSciences, LLC, 9330 Zionsville Rd., Indianapolis, IN 46268 (lacampbell@dow.com; plprasifka@dow.com; nstorer@dow.com; DDRule@dow.com; wmhendrix@dow.com), and [1]Corresponding author, e-mail: lacampbell@dow.com

*These authors contributed equally to this manuscript.

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Abstract

Since 2003, rootworm-protected transgenic corn has been commercially deployed in the United States as a principal method of control of western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Durability of this technology depends partly on larval mortality ("dose") exerted by the traits, but density-dependent mortality can confound calculations of dose. Research reported here examined the effects of density-dependent mortality on adult emergence and estimates of trait dose. At sites in Illinois and Indiana, western corn rootworm eggs were infested at four densities on non-Bt corn and at a single density on corn hybrids with transgenic events MON 88017 (VT Triple PRO), DAS-59122-7 (Herculex Insect Protection), and MON 88017 × DAS-59122-7 (SmartStax corn). Beetles were collected weekly in large emergence cages. Density-dependent mortality and the effect of Bt traits were examined using percent survival from egg to adult, sex ratio, and beetle mass. Beetle emergence from Bt treatments was very low, and percent survival from non-Bt treatments was greatest at the lowest egg density (410 eggs per row-meter). Therefore, emergence from the lowest infestation density on non-Bt corn was used to estimate the effective dose of the Bt treatments. Sex ratio and beetle mass were unaffected by density-dependent effects and were not consistently affected by Bt traits. Dose was estimated at 97.4–99.3% for MON 88017, 98.8–99.9% for DAS-59122-7, and 99.7–100.0% for MON 88017 × DAS-59122-7. This study confirms the need to account for density-dependent mortality when estimating dose of corn rootworm protection events even at relatively low egg infestation densities.

Key words: transgenic corn, *Diabrotica virgifera virgifera*, event MON 88017, event DAS-59122-7, resistance management

Despite several management options over the years, the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), continues to be a significant pest of corn in the United States and Europe (Kiss et al. 2005, Gray et al. 2009). Most injury and yield loss results from the western corn rootworm larvae feeding on corn roots. Intense larval feeding results in roots being shortened or pruned. This type of injury reduces the plant's ability to take up water and nutrients from the soil, leading to decreased photosynthetic activity and lower yields (Spike and Tollefson 1989, Sutter and Gustin 1990, Spike and Tollefson 1991, Dunn and Frommelt 1998). Loss of root structure can also allow plants to lodge under high winds, reducing crop harvest efficiency.

The U.S. Environmental Protection Agency (US EPA) maintains a webpage that lists all of the current and previously registered "plant-incorporated protectants" (US EPA 2016). Transgenic plants that express insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) that are active against corn rootworms have become a widely adopted method of control. Event MON 88017, expressing a modified Cry3Bb1 protein, was combined with event MON 89034, expressing Cry1A.105 and Cry2Ab2, to produce YieldGard VT Triple Pro (Monsanto Company, St Louis, MO; Drury et al. 2008). Event DAS-59122-7, expressing Cry34Ab1 and Cry35Ab1, was combined with event TC1507, expressing Cry1F, to produce Herculex Xtra (Dow AgroSciences LLC, Indianapolis, IN; Ellis et al. 2002, Herman et al. 2002).

Events MON 88017 and MON 89034 and events DAS-59122-7 and TC1507 have been combined through conventional breeding to create SmartStax corn (Dow AgroSciences LLC, Indianapolis, IN, and Monsanto Company, St. Louis, MO), a trait pyramid (plants with simultaneous expression of two or more insecticidal proteins that target the same insect; Gould 1986). Field corn hybrids containing these six Cry proteins (Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, and Cry34Ab1/Cry35Ab1) broaden the spectrum of activity and aid in resistance management of target pest species (McGaughey and Whalon 1992; Tabashnik 1994; Gould 1998; Storer et al. 2006, 2010). Field corn hybrids containing events MON 89034, MON 88017, DAS-59122-7, and TC1507 also contain transgenes for tolerance to glyphosate and glufosinate ammonium herbicides, providing another tool for managing glyphosate-resistant weed species (Green and Owen 2011).

The durability of transgenic traits for insect protection depends, in part, on the level of larval mortality ("dose") exerted by the traits (Gould 1998). If the traits cause very high mortality of susceptible target pest populations such that resistance to the traits is functionally recessive, the "high dose-refuge" strategy can be highly effective at delaying the development of resistant pest populations. In such cases, a small percentage of plants that do not contain the insect protection traits provide a refuge for insects that are susceptible to the traits and are available to mate with any rare resistant insects that may survive in the insect-protected crop, passing the susceptible phenotype onto their progeny and greatly slowing the rate that resistance alleles can spread through the population. For traits that are less than the high dose, resistance may develop more rapidly in the field without the application of additional resistance management tactics, such as larger refuge percentages, or integrated pest management programs (Tabashnik and Gould 2012). Determining the effective dose of transgenic crops, therefore, is important in developing scientifically robust resistance management programs. For corn rootworm larvae, which feed beneath the soil surface, direct measures of mortality under field conditions are impractical. Therefore, researchers have estimated "dose" by comparing adult emergence from plants with and without the insect protection traits when infested with the same number of corn rootworm eggs (Storer et al. 2006; Hibbard et al. 2010b, 2011; Petzold-Maxwell et al. 2013).

As these traits were being developed and their efficacy and dose were being studied, it was common to use artificial infestations of corn rootworm eggs to ensure high insect pressure (Sutter and Branson 1980, Prasifka et al. 2013). The rate of infestation was most commonly 1,000 eggs per 30.5 cm of row. Data from these studies were used to calculate an estimate of dose for each trait and it was assumed that there was not significant density-dependent mortality. Consequently, it is possible that dose could have been underestimated if in fact there was greater density-dependent mortality occurring in control plots than in test plots. Previous studies have shown evidence of crowding and competition for scarce resources in corn rootworms. Based on the analysis of Onstad et al. (2006), density-dependent mortality begins to occur between 300 and 500 eggs per 30.5 cm of row. Branson and Sutter (1985) showed fewer rootworms reached the adult stage at egg densities above 600 eggs per 30.5 cm of row and adults were smaller at egg densities above 300 eggs. In addition to survival and size, studies have shown decreases in fecundity with increased egg density (Branson and Sutter 1985), and that the male: female ratio was skewed in favor of the males as larval densities increase (Weiss et al 1985). Hibbard et al. (2010a) found little or no density-dependent mortality occurred at infestation levels <850 viable eggs per 30.5 cm. However, Hibbard et al. (2010a) also indicated that the level of density-dependent mortality could vary depending on local environmental conditions.

When SmartStax corn was conditionally registered by the US EPA, one of the conditions of this registration was to address uncertainty regarding dose of Cry3Bb1 (MON 88017) and Cry34/35Ab1 (DAS-59122-7) insecticidal proteins from *Bacillus thuringiensis* against corn rootworm larvae. The US EPA identified uncertainty relating to the effect of egg density on adult emergence and based its assessment on emergence numbers that were not adjusted for density-dependent mortality in non-Bt plots (US EPA 2009).

To resolve the uncertainty surrounding the egg density effects, a multilocation field emergence study was conducted in 2010 that used an eightfold range of western corn rootworm egg densities to infest control plots containing plants without traits for corn rootworm protection. Emergence from these plots provided the ability to measure density-dependent mortality of western corn rootworm larvae at each location. Emergence from plots containing event MON 88017 (producing Cry3Bb), event DAS-59122-7 (producing Cry34/35Ab1), or the combination of both (SmartStax) that were infested with the standard density of western corn rootworm eggs corroborated previous estimates of larval mortality caused by each of the rootworm-protection events alone and together.

Materials and Methods

Field studies were conducted at two locations, Burnt Prairie, IL, and Mount Vernon, IN, in 2010 to investigate the effect of Bt corn on western corn rootworm emergence, the degree of density-dependent mortality, and its effect on effective-dose calculations. Both trial locations were in areas where western corn rootworm populations were naturally low, and both were planted on ground that had been previously planted to soybeans with no known history of rotation resistance (Gray et al. 2009). Therefore, the density of western corn rootworms infested into each plot could be controlled to get a clearer understanding of the effect of the Bt corn and egg density on mortality.

Western corn rootworm adult emergence in large emergence tents was measured for Bt and non-Bt corn. Single-event or combined-event Bt plots were infested at 3,280 eggs per row-meter (1,000 eggs per row-foot), and plots containing non-Bt corn were infested at 3,280, 1,640, 820, and 410 eggs per row-meter (Table 1). To minimize any potential effects of corn genotypes, the same near-isogenic corn hybrids (hereafter, isoline) were used at both locations. Treatments are presented in Table 1. All seed was treated with commercial fungicide only and no preventive treatments of soil or foliar insecticides were applied across the test areas. All treatments were replicated four times within each trial location, using a randomized complete block design. Plots were eight rows wide (76-cm row spacing) by 6 m long. All studies were maintained using regionally appropriate agronomic practices (e.g., fertilizer rates and weed control) for optimal productivity.

Plots were artificially infested with western corn rootworm eggs at the V3–V5 corn stage (Sutter and Branson 1980, Ritchie et al. 1993). Plots were infested with eggs suspended in a dilute agar solution using a tractor-mounted injector. Diapausing eggs were obtained from Crop Characteristics Inc., Farmington, MN, and were not rotation resistant.

When western corn rootworm larvae had reached approximately third instar, screen house tents (Eureka Screen House, Eureka, Binghamton, NY; 4.1 m by 2.9 m by 2.2 m, covering three, 3-m rows) were erected over the plots to prevent adult western corn rootworm from escaping or entering from outside the plots. The bottom edges of the cage were placed in a shallow trench and covered firmly with soil to prevent western corn rootworm adult escape. Inside the screen houses, the corn plants were cut to ∼0.5 m above the ground, which reduced above-ground plant material where beetles could hide, but allowed plants to maintain fresh root tissue for larvae to

Table 1. Treatments, events, traits for rootworm control, and egg densities

Treatment	Event(s)	Rootworm trait(s)	Egg density (eggs per row-meter)	Total eggs per cage
SmartStax[a]	MON 88017 + DAS-59122-7 + TC1507 + MON89034	Cry3Bb1 + Cry34Ab1/Cry35Ab1	3,280[e]	40,344
YieldGard VT Triple PRO[b]	MON 88017	Cry3Bb1	3,280	40,344
Herculex Xtra[c]	DAS-59122-7	Cry34Ab1/Cry35Ab1	3,280	40,344
Isoline null	NK603[d]	None	3,280	40,344
Isoline null	NK603	None	1,640	20,172
Isoline null	NK603	None	820	10,086
Isoline null	NK603	None	410	5,043

The near-isoline null had only tolerance to glyphosate. 1,640 eggs per row-meter represents a one-half rate of the full egg density, 820 eggs per row-meter represents a one-quarter rate, and 410 eggs per row-meter represents a one-eighth rate.

[a] Dow AgroSciences, LLC, Indianapolis, IN, and Monsanto Company, St. Louis, MO.

[b] Monsanto Company, St. Louis, MO.

[c] Dow AgroSciences, LLC, Indianapolis, IN.

[d] Monsanto Company, St. Louis, MO.

[e] 3,280 eggs = 1,000 eggs per row-foot.

complete feeding and development. Additionally, plants produced fresh leaf and silk tissue, which provided nutrition for adult beetles between collections. To prevent larval movement to plants outside the tent, the third and seventh rows of each eight-row plot, as well as the excess plants in the three cage rows, were removed. Cage integrity was checked with beetle collections. No adverse weather conditions caused problems with cage integrity at either of the two sites.

Adult western corn rootworm were collected, using a mouth aspirator (1135C, Bioquip, Rancho Dominguez, CA) or battery-powered aspirator (2820A, Bioquip), once per week after emergence began and continuing until late August until two consecutive zero counts were recorded. Beetles were collected in vials, labeled by field plot, and put into alcohol. The beetles were later counted, identified to sex (White 1977), air-dried for at least 24 h, and weighed to the nearest mg.

Statistical Analysis

Statistical analyses were conducted separately for each location. Total beetle emergence was calculated for each plot. The effect of treatment (trait–egg density combination) on beetle emergence, percent survival, percentage of males, and male and female mass was examined using ANOVA with a mixed model function (JMP Pro 2014). A significance level of $P < 0.05$ was used in all analyses, and means were separated using the Student's *t*-test. Because the focus of this experiment was on overall emergence and beetles were only collected weekly, timing and delays of emergence were not included as an effect.

Density-dependent mortality was investigated by examining the emergence rate (expressed as adults emerged per 1,000 infested eggs) from the non-Bt isoline at the four different egg densities. In the absence of density-dependent mortality, the emergence rate would be the same at all densities, whereas if density-dependent mortality was present, the emergence rate would increase with decreasing density. The trends were visualized using the Trendline function in Microsoft Excel 2003 fitting to a two-parameter power curve ($y = ax^b$, where y is the adult emergence rate and x is the egg density), typical of density-dependent functions in insect population ecology (Varley et al. 1973).

Since density-dependent mortality was apparent in the emergence rate data from the isoline throughout the range of densities investigated, a low-end dose estimate for each Bt line at each location was calculated by comparing the emergence rate from the Bt plots with the emergence rate from the non-Bt plots at the lowest egg density (410 eggs per row-meter):

$$D_{Bt} = (1 - (\frac{A_{Bt}}{E_{Bt}} / \frac{A_{iso}}{E_{iso}})) \times 100\% \quad (1)$$

where D_{Bt} is the dose estimate of the Bt line, A_{Bt} is number of adults emerged from the Bt plots, E_{Bt} is the number of eggs infested in the Bt plots, A_{iso} is the number of adults emerged from the isoline plots, and E_{iso} is the number of eggs infested in the isoline plots. The number of eggs per plot was calculated by multiplying the eggs-per-row-meter by the length of the cages (4.1 m) and by the number of rows per cage (three). In this case, $E_{Bt} = 8 \times E_{iso} = 40{,}344$ eggs (Table 1). This approach assumes that there was no density-dependent mortality in non-Bt plots at the lowest egg density, and is therefore, conservative.

Results

Burnt Prairie Emergence

The mean total beetles emerged per plot was greatest from the isoline plots infested at 3,280 and 1,640 eggs per row-meter, and very few beetles emerged from MON 88017 or DAS-59122-7 treatments. No beetles emerged from the MON 88017 × DAS-59122-7 treatment (Table 2). However, the percent survival based on the number of eggs infested was greatest from the isoline at 410 eggs per row-meter. Percent survival from the other isoline treatments was not significantly different, and percent survival from the isoline treatment at 3,280 eggs per row-meter was not significantly different from any of the rootworm-protected treatments (Table 2).

The percentage of males did not differ significantly among any treatments (Table 2). Mean male mass did not differ among isoline treatments, but male mass was significantly greater from isoline treatments than from either rootworm-protected treatment (Table 2). Mean female mass was numerically greater from all isoline treatments than from rootworm-protected treatments, but mean female mass from the higher egg densities (1,640 and 3,280 eggs per row-meter) was not significantly different from mean female mass from MON 88017 or DAS-59122-7 (Table 2).

Table 2. Beetles emerged per plot, percent survival, percentage of males, and mass per beetle for males and females (mg), by site

Site	Treatment	Egg density (eggs per row-meter)	Total beetles emerged per plot, ± SE	Percent survival[a], ± SE	Percent male, ± SE	Male mass per beetle (mg), ± SE	Female mass per beetle (mg), ± SE
Burnt Prairie	MON 88017 × DAS-59122-7	3,280	0.0 ± 16.7c	0.000 ± 0.217c	–	–	–
	MON 88017	3,280	5.0 ± 16.7c	0.016 ± 0.217c	38.5 ± 12.3	1.9 ± 0.2b	2.8 ± 0.3c
	DAS-59122-7	3,280	0.5 ± 16.7c	0.002 ± 0.217c	52.3 ± 18.2	1.2 ± 0.5b	2.2 ± 0.7bc
	Isoline	3,280	166.8 ± 16.7a	0.529 ± 0.217bc	45.3 ± 12.3	2.7 ± 0.2a	3.8 ± 0.3ab
	Isoline	1,640	153.3 ± 16.7a	0.973 ± 0.217b	43.7 ± 12.3	2.8 ± 0. 2a	3.6 ± 0.3abc
	Isoline	820	80.5 ± 16.7b	1.022 ± 0.217b	34.4 ± 12.3	2.9 ± 0. 2a	4.6 ± 0.3a
	Isoline	410	86.5 ± 16.7b	2.197 ± 0.217a	38.5 ± 12.3	2.7 ± 0. 2a	4.5 ± 0.3a
			$F_{6,\ 18} = 18.3$, $P < 0.0001$	$F_{6,\ 18} = 13.76$, $P < 0.0001$	n.s.	$F_{5,\ 12} = 3.0$, $P = 0.03$	$F_{5,\ 12} = 5.1$, $P = 0.01$
Mount Vernon	MON 88017 × DAS-59122-7	3,280	1.5 ± 4.3c	0.005 ± 0.081d	0.2 ± 8.7[b]	–	3.2 ± 0.5
	MON 88017	3,280	12.5 ± 4.3c	0.040 ± 0.081d	25.2 ± 7.4	2.1 ± 0.2	3.1 ± 0.4
	DAS-59122-7	3,280	5.5 ± 4.3c	0.017 ± 0.081d	32.1 ± 7.4	2.2 ± 0.2	2.7 ± 0.4
	Isoline	3,280	100.0 ± 4.3a	0.317 ± 0.081c	39.1 ± 7.4	2.0 ± 0.2	2.9 ± 0.4
	Isoline	1,640	65.8 ± 4.3b	0.417 ± 0.081c	32.3 ± 7.4	2.4 ± 0.2	3.9 ± 0.4
	Isoline	820	67.8 ± 4.3b	0.860 ± 0.081b	38.9 ± 7.4	2.6 ± 0.2	4.0 ± 0.4
	Isoline	410	59.0 ± 4.3b	1.498 ± 0.081a	29.6 ± 7.4	2.3 ± 0. 2	3.6 ± 0.4
			$F_{6,\ 18} = 77.0$, $P < 0.0001$	$F_{6,\ 18} = 46.23$, $P < 0.0001$	n.s.	n.s.	n.s.

All means presented are least-squares means.

Means followed by the same letter within site are not significantly different ($P > 0.05$). All means are based on plot-level comparisons; $N = 4$ replications for all comparisons.

[a] Percent survival based on number of eggs infested per cage and beetles emerged from each plot.

[b] The least squares mean for percentage of males from the MON 88017 × DAS-59122-7 treatment was calculated as 0.2%; the actual mean percent is 0.0%, since no males emerged from that treatment at Mount Vernon.

Mount Vernon Emergence

The total beetles emerged per plot was greatest from the isoline infested at 3,280 eggs per row-meter, similar numbers of beetles emergence from all other isoline treatments, and very few beetles emerged from any rootworm-protected treatment. In contrast to the Burnt Prairie site, beetles were collected from the MON 88017 × DAS-59122-7 treatment, and all six beetles collected were female (Table 2). Percent survival based on the number of eggs infested was significantly greater from the isoline treatment at the lowest egg density, followed by the isoline treatment infested at 820 eggs per row-meter. Percent survival from the isoline plots at the higher egg densities (1,640 and 3,280 eggs per row-meter) was not significantly different, but percent survival from all isoline treatments was significantly greater than percent survival from any rootworm-protected treatments. Percentage of males, mean male mass, and mean female mass did not differ significantly among treatments.

Percent Survival From Non-Bt Plots

Density-dependence was evident when examining the percent survival at the four different infestation levels of the isoline (Fig. 1). The emergence rates follow a power curve typical of density-dependent mortality (e.g., Varley et al. 1973), with the percent survival decreasing with increasing density and with very good agreement between the data and the fitted curve ($R^2 > 0.9$) at both sites. Although the range of infestation levels used was not low enough to capture the level at which density-dependence ceased to be a factor, the data strongly suggest that density-dependent mortality was still occurring at the lowest egg density used.

Dose Estimates

Low-end dose estimates for each Bt treatment at each site (Table 3) were calculated using Equation 1. To obtain the low-end dose estimates, the percent survival at the lowest infestation density was assumed to not be affected by density-dependence. Low-end dose estimates for the single traits ranged from 97.4% to 99.9%, and for the combined events were 100% (Burnt Prairie) and 99.7% (Mount Vernon). These are highly conservative estimate of dose, as the isoline emergence data (Fig. 1) strongly suggest that density-dependent mortality was occurring even at the lowest density used in the isoline. The actual dose in these studies was likely to have been considerably greater than these estimates if indeed density-dependent mortality was significant at 410 eggs per row-meter.

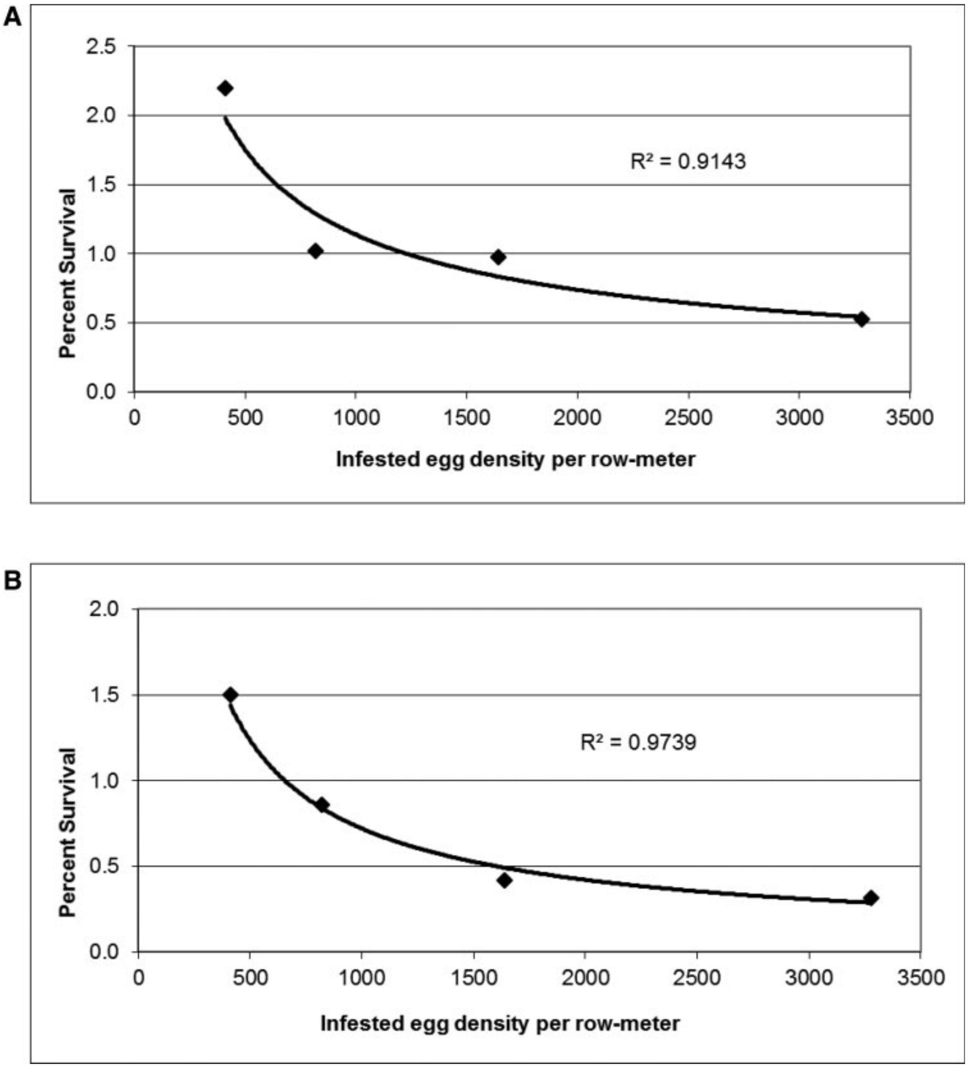

Fig. 1. Percent survival in non-Bt corn plots at a range of egg densities in Burnt Prairie, IL (**A**), and Mount Vernon, IN (**B**). Trend lines are fitted two-parameter power curves indicating a density-dependent decrease in percent survival with increase in egg density. R^2 indicates goodness of fit.

Table 3. Low-end dose estimates for DAS-59122-7, MON 88017, and MON 88017 × DAS-59122-7 against western corn rootworm larvae based on the lowest egg density of the isoline

Treatment	Egg density (eggs per row-meter)	Burnt Prairie, IL		Mount Vernon, IN	
		Adults emerged per cage	Dose estimate (%)	Adults emerged per cage	Dose estimate (%)
Isoline	410	86.50	–	59.0	–
MON 88017	3,280	5.0	99.28	12.5	97.35
DAS-59122-7	3,280	0.5	99.93	5.5	98.83
MON 88017 × DAS-59122-7	3,280	0	100.00	1.5	99.68

Discussion

Percent survival from the lowest egg density in this study (410 eggs per row-meter) was significantly greater than from any other isoline treatment at both sites (Table 2), indicating some density-dependent mortality above 410 eggs per row-meter. However, based on the fitted two-parameter power curves, density-dependent mortality was still present at some level below 410 eggs per row-meter (Fig. 1), since percent survival never plateaued at lower egg densities. Density-independent mortality in this study was ∼98%.

Density-dependent mortality in this study did appear to occur at lower densities than in other studies. Based on their data, Hibbard et al. (2010a) recommended egg infestations below 2,600 eggs per row-meter to avoid density-dependent mortality. Hitchon et al. (2015) found evidence of density-dependent mortality at 1,250 eggs per row-meter; density-dependent mortality may have been a factor even at lower densities in their study, but 625 eggs per row-meter was the lowest egg density they tested. However, density-dependent mortality was documented even in Cry34/35, Cry3Bb1, and pyramided traits between 625 and 1,250 eggs per row-meter (Hitchon et al. 2015), even though larvae would most likely not be crowded given the mortality imposed by the traits. Other studies have documented density-dependent mortality occurring between 1,000 and 1,670 eggs per row-meter (Onstad et al. 2006), between 1,970 and 3,940 eggs per row-meter (Branson and Sutter 1985, Fisher 1985, Sutter et al. 1991), and above 3,940 eggs per row-meter (Branson et al. 1980, Elliott et al. 1989). In an analysis of multiple studies, little or no density-dependent mortality occurred below 330 eggs per row-meter (Onstad et al. 2006).

Other studies have shown conflicting and sometimes substantial density-dependent effects on western corn rootworm biology. No significant effect of egg density on sex ratio was found in this study, similar to the results of Sutter et al. (1991), but sex ratio has been shown to become more male-biased at increased egg densities (Weiss et al. 1985). In this study, egg densities had no significant effect on beetle size as measured by mass (Table 2), but lower larval density has been shown to negatively impact larval weight gain (Robert et al. 2012), and greater densities can impact female (but not male) beetle weight (Weiss et al. 1985). Greater egg densities can also reduce head capsule size (Branson et al. 1980, Branson and Sutter 1985, Robert et al. 2012). Female fecundity and viable eggs per female can be reduced at greater egg densities (Branson and Sutter 1985), and emergence delays can occur at very high egg densities (Elliott et al. 1989, Sutter et al. 1991).

It is also important to note that the environmental conditions could affect density-dependent mortality. The sites in the present study were chosen based on a lack of natural western corn rootworm populations, which may suggest that the habitat may not be ideal for corn rootworm development and may have increased density-independent mortality overall. Competition for food and feeding sites (Branson and Sutter 1985, Weiss et al. 1985) is most likely the major factor in density-dependent mortality in western corn rootworms. Feeding competition would mean that root biomass and growth during the period of larval feeding would affect the intensity of competition. Therefore, density-dependent mortality is expected to be affected by timing of egg hatch relative to corn plant development, the corn hybrid studied, and the environmental conditions that affect plant growth (temperature, moisture, soil structure, soil fertility, etc.). The timing of competition may also be important. Density-dependent mortality has been assumed not to occur during larval establishment (Storer 2003), which was supported by data from Hibbard et al. (2004). Hibbard et al. (2010a) proposed that most density-dependent mortality occurs in the second or third instars, as larger larvae compete for food, but Hitchon et al. (2015) suggested that neonate larvae may suffer the greatest density-dependent effects as they establish on corn roots, because of evidence of density-dependent mortality on Bt traits. At least some density-dependent mortality during establishment has been observed based on the number of infestation point sources and available paths to corn roots (Wilson et al. 2006). The actual timing of competition and degree of competition at that timing may greatly impact western corn rootworm mortality and life history traits.

Bt traits can significantly affect percent survival and life history parameters. Percent survival from the Bt traits was very low (Table 2) and similar or less than results from other studies (Storer et al. 2006, Binning et al. 2010, Clark et al. 2012, Petzold-Maxwell et al. 2013, Hitchon et al. 2015). Although not significantly different, percent survival from MON 88017 × DAS-59122-7 was less than from either of the single Bt traits (Table 2). Sex ratio was not significantly impacted by MON 88017, DAS-59122-7, or the combination (Table 2). Under lab conditions, Cry34/35 can bias the sex ratio toward males (Binning et al. 2010). However, under field conditions, Hitchon et al. (2015) found no male bias in DAS-59122-7 and a slightly female-biased sex ratio from MON 88017 and MON 88017 × DAS-59122-7. In this study, at the Burnt Prairie site, traits significantly impacted male mass, and there was a trend for smaller females emerging from traits (Table 2). At the Mount Vernon site, neither male nor female mass was affected by the trait treatments. Bt traits can significantly affect beetle size, as measured by smaller mass (Binning et al. 2010, Petzold-Maxwell et al. 2013) and smaller head capsule width (Murphy et al. 2011, Petzold-Maxwell et al. 2013). However, Cry34/35Ab1 has had no effect on beetle size in other studies (Storer et al. 2006).

Dose estimated in this study validates results from previous studies and modeling. Event MON 88017 dose was estimated at 97.4–99.3% (Table 3), which is comparable with doses used in resistance evolution simulation modeling (96.2–99.96%; US EPA 2009). Dose of DAS-59122-7 was estimated as 98.8–99.9% (Table 3), which is comparable with results from lifetime larval exposure in the laboratory (99.5%; Binning et al. 2010) and from field data (99.14–99.98%; Storer et al. 2006) and on the high end of dose estimates used in modeling (94.20–99.18%; US EPA 2009). Dose of MON 88017 × DAS59122-7 in SmartStax corn was estimated as 99.68–100.00%, which is at the high end of the dose estimates used in modeling (98.22–99.97%; US EPA 2009). In this study, the actual dose was likely to be greater than the estimated dose values (Table 3), since density-dependent mortality was most likely still a factor at the lowest egg density (Fig. 1). Hitchon et al. (2015) suggested that some density-dependent mortality could be occurring with Bt traits, which could result in the potential overestimation of Bt trait dose. However, because of the mortality already imposed by Bt traits, density-dependent mortality in Bt traits is most likely a minor factor, and the range of dose parameter values previously used in simulation models (US EPA 2009) was likely conservative. This study confirms the need to account for density-dependent mortality when estimating dose of corn rootworm protection events even at relatively low egg infestation densities. Furthermore, the dose estimates, using the approach taken here to account for density-dependence, validate the dose estimates previously obtained for DAS-59122-7 using literature estimates of density-dependence (Storer et al. 2006). Because small changes in assumption of density-dependent mortality can lead to relatively large changes in dose estimates (Hibbard et al. 2010a), field validation of dose is important for understanding the effects of density-dependent mortality.

Density-dependent mortality is a significant factor at the egg densities typically used, and lower egg densities may need to be considered to avoid density-dependent mortality, based on the data presented here and elsewhere (Branson and Sutter 1985; Fisher 1985; Sutter et al. 1991; Hibbard et al. 2010a, Hitchon et al. 2015). However, accounting for density-dependent mortality is challenging when testing traits because egg densities in non-Bt plots need to be relatively low to minimize density-dependent mortality, but egg densities in Bt plots must be greater (around 3,300 eggs per row-meter) to get measurable survival, particularly for pyramided traits. Lower egg densities could be used, but researchers would need to increase replication substantially to account for low measured survival in Bt plots. Since density-dependent mortality can be present at relatively low infestations, it needs to be taken into account when estimating trait dose, but this will be important at each trial and location. The contribution of density-dependent effects to overall rootworm mortality can vary substantially, even within the same environment (Hibbard et al. 2010a), and should vary among growing conditions and environments, which will impact the calculations of effective dose.

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References Cited

Binning, R. R., S. A. Lefko, A. Y. Millsap, S. D. Thompson, and T. M. Nowatzki. 2010. Estimating western corn rootworm (Coleoptera: Chrysomelidae) larval susceptibility to event DAS-59122-7 maize. J. Appl. Entomol. 134: 551–561.

Branson, T. F., and G. R. Sutter. 1985. Influence of population density of immatures on size, longevity, and fecundity of adult *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). Environ. Entomol. 14: 687–690.

Branson, T. F., G. R. Sutter, and J. R. Fisher. 1980. Plant response to stress induced by artificial infestations of western corn rootworm. Environ. Entomol. 9: 253–257.

Clark, T. L., D. L. Frank, B. W. French, L. J. Meinke, D. Moellenbeck, T. T. Vaughn, and B. E. Hibbard. 2012. Mortality impact of MON863 transgenic maize roots on western corn rootworm larvae in the field. J. Appl. Entomol. 136: 721–729.

Drury, S. M., T. L. Reynolds, W. P. Ridley, N. Bogdanova, S. Riordan, M. A. Nemeth, R. Sorbet, W. A. Trujillo, and M. L. Breeze. 2008. Composition of forage and grain from second-generation insect-protected corn MON 89034 is equivalent to that of conventional corn (*Zea mays* L.). J. Agric. Food Chem. 56: 4623–4630.

Dunn, J. P., and K. Frommelt. 1998. Effects of below-ground herbivory by *Diabrotica virgifera virgifera* (Coleoptera) on biomass allocation and carbohydrate storage of maize. Appl. Soil Ecol. 7: 213–218.

Elliott, N. C., G. R. Sutter, T. F. Branson, and J. R. Fisher. 1989. Effect of population density of immatures on survival and development of the western corn rootworm (Coleoptera: Chrysomelidae). J. Entomol. Sci. 24: 209–213.

Ellis, R. T., B. A. Stockhoff, L. Stamp, H. E. Schnepf, G. E. Schwab, M. Knuth, J. Russell, G. A. Cardineau, and K. E. Narva. 2002. Novel *Bacillus thuringiensis* binary insecticidal crystal proteins active on western corn rootworm, *Diabrotica virgifera virgifera* LeConte. App. Environ. Microbiol. 68: 1137–1145.

Fisher, J. R. 1985. Comparison of controlled infestations of *Diabrotica virgifera virgifera* and *Diabrotica barberi* (Coleoptera: Chrysomelidae) on corn. J. Econ. Entomol. 78: 1406–1408.

Gould, F. 1986. Simulation models for predicting durability of insect-resistant germplasm: A deterministic diploid, two-locus model. Environ. Entomol. 15: 1–10.

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: Integrating pest genetics and ecology. Annu. Rev. Entomol. 43: 701–726.

Gray, M. E., T. W. Sappington, N. J. Miller, J. Moeser, and M. O. Bohn. 2009. Adaptation and invasiveness of western corn rootworm: Intensifying research on a worsening pest. Annu. Rev. Entomol. 54: 303–321.

Green, J. M., and M.D.K. Owen. 2011. Herbicide-resistant crops: Utilities and limitations for herbicide resistant weed management. J. Agric. Food Chem. 59: 5819–5829.

Herman, R. A., P. N. Scherer, D. L. Young, C. A. Mihaliak, T. Meade, A. T. Woodsworth, B. A. Stockhoff, and K. E. Narva. 2002. Binary insecticidal crystal protein from *Bacillus thuringiensis* strain PS149B1: Effect of individual protein components and mixtures in laboratory bioassays. J. Econ. Entomol. 95: 635–639.

Hibbard, B. E., M. L. Higdon, D. P. Duran, Y. M. Schweikert, and M. R. Ellersieck. 2004. Role of egg density on establishment and plant-to-plant movement by western corn rootworm larvae (Coleoptera: Chrysomelidae). J. Econ. Entomol. 97: 871–882.

Hibbard, B. E., L. N. Meihls, M. R. Ellersieck, and D. W. Onstad. 2010a. Density-dependent and density-independent mortality of western corn rootworm: Impact on dose calculations of rootworm-resistant Bt corn. J. Econ. Entomol. 103: 77–84.

Hibbard, B. E., T. L. Clark, M. R. Ellersieck, L. N. Meihls, A. A. El Khishen, V. Kaster, H. Y. Steiner, and R. Kurtz. 2010b. Mortality of western corn rootworm larvae on MIR604 transgenic maize roots: Field survivorship has no significant impact on survivorship of F1 progeny on MIR604. J. Econ. Entomol. 103: 2187–2196.

Hibbard, B. E., D. L. Frank, R. Kurtz, E. Boudreau, M. R. Ellersieck, and J. F. Odhiambo. 2011. Mortality impact of Bt transgenic maize roots expressing eCry3.1Ab, mCry3A, and eCry3.1Ab plus mCry3A on western corn rootworm larvae in the field. J. Econ. Entomol. 104: 1584–1591.

Hitchon, A. J., J. L. Smith, B. W. French, and A. W. Schaafsma. 2015. Impact of the Bt corn proteins Cry34/35Ab1 and Cry3Bb1, alone or pyramided, on western corn rootworm (Coleoptera: Chrysomelidae) beetle emergence in the field. J. Econ. Entomol. 108: 1986–1993.

JMP Pro. 2014. version 11.1. SAS Institute, Inc, Cary, NC.

Kiss, J., C. R. Edwards, H. K. Berger, P. Cate, M. Cean, S. Cheek, J. Derron, H. Festić, L. Furlan, J. Igrc-Barčić, et al. 2005. Monitoring of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) in Europe 1992–2003, pp. 29–39. *In* S. Vidal, U. Kulhmann, and C. R. Edwards (eds.), Western corn rootworm ecology and management. CABI Publishing, Wallingford, United Kingdom.

McGaughey, W. H., and M. E. Whalon. 1992. Managing insect resistance to *Bacillus thuringiensis* toxins. Science 258: 1451–1455.

Murphy, A. F., N. J. Seiter, and C. H. Krupke. 2011. The impact of Bt maize as a natal host on adult head capsule width in field populations of western corn rootworm. Entomol. Exp. Appl. 139: 8–16.

Onstad, D. W., B. E. Hibbard, T. L. Clark, D. W. Crowder, and K. G. Carter. 2006. Analysis of density-dependent survival of *Diabrotica* (Coleoptera: Chrysomelidae) in cornfields. Environ. Entomol. 35: 1272–1278.

Petzold-Maxwell, J. L., L. J. Meinke, M. E. Gray, R. E. Estes, and A. J. Gassmann. 2013. Effect of Bt maize and soil insecticides on yield, injury, and rootworm survival: Implications for resistance management. J. Econ. Entomol. 106: 1941–1951.

Prasifka, P. L., D. M. Rule, N. P. Storer, S. P. Nolting, and W. H. Hendrix, III. 2013. Evaluation of corn hybrids expressing Cry34Ab1/Cry35Ab1 and Cry3Bb1 against the western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 106: 823–829.

Ritchie, S. W., J. J. Hanaway, and G. O. Benson.1993. How a corn plant develops, pp. 21. Iowa State Univ. Coop. Ext. Serv. Spec. Rep No. 48, Ames, IA.

Robert, C.A.M., M. Erb, B. E. Hibbard, B. W. French, C. Zwahlen, and T.C.J. Turlings. 2012. A specialist root herbivore reduces plant resistance and uses an induced plant volatile to aggregate in a density-dependent manner. Func. Ecol. 26: 1429–1440.

Spike, B. P., and J. J. Tollefson. 1989. Relationship of root ratings, root size, and root regrowth to yield of corn injured by western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 82: 1760–1763.

Spike, B. P., and J. J. Tollefson. 1991. Yield response of corn subjected to western corn rootworm (Coleoptera: Chrysomelidae) infestation and lodging. J. Econ. Entomol. 84: 1585–1590.

Storer, N. P. 2003. A spatially explicit model simulating western corn rootworm (Coleoptera: Chrysomelidae) adaptation to insect-resistant maize. J. Econ. Entomol. 96: 1530–1547.

Storer, N. P., J. M. Babcock, and J. M. Edwards. 2006. Field measures of western corn rootworm (Coleoptera: Chrysomelidae) mortality caused by Cry34/35Ab1 proteins expressed in corn event 59122 and implications for trait durability. J. Econ. Entomol. 99: 1381–1387.

Storer, N. P., J. M. Babcock, M. Schlenz, T. Meade, G. D. Thompson, J. W. Bing, and R. M. Huckaba. 2010. Discovery and characterization of field resistance to Bt Maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J. Econ. Entomol. 103: 1031–1038.

Sutter, G. R., and T. F. Branson. 1980. A procedure for artificially infesting field plots with corn rootworm eggs. J. Econ. Entomol. 73: 135–137.

Sutter, G. R., and R. D. Gustin. 1990. Effect of insecticide treatments on root lodging and yields of maize in controlled infestations of western corn rootworms (Coleoptera: Chrysomelidae). J. Econ. Entomol. 83: 2414–2420.

Sutter, G. R., T. F. Branson, J. R. Fisher, and N. C. Elliott. 1991. Effect of insecticides on survival, development, fecundity, and sex ratio in controlled infestations of western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 84: 1905–1912.

Tabashnik, B. E. 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 39: 47–49.

Tabashnik, B. E., and F. Gould. 2012. Delaying corn rootworm resistance to Bt corn. J. Econ. Entomol. 105: 767–776.

U.S. Environmental Protection Agency. 2009. Pesticide Fact Sheet for MON 89034 × TC1507× MON 88017 × DAS-59122-7. (http://www3.epa.gov/pesticides/chem_search/reg_actions/pip/smartstax-factsheet.pdf, accessed 3 March 2016).

U.S. Environmental Protection Agency. 2016. Current & previously registered Section 3 Plant-Incorporated Protectant (PIP) registrations. (https://www.epa.gov/ingredients-used-pesticide-products/current-previously-registered-section-3-plant-incorporated, accessed 3 March 2016).

Varley, G. C., G. R. Gradwell, and M. P. Hassell. 1973. Insect population ecology. Blackwell Scientific Publication, Oxford, United Kingdom.

Weiss, M. J., K. P. Seevers, and Z. B. Mayo. 1985. Influence of western corn rootworm larval densities and damage on corn rootworm survival, development time, size and sex ratio (Coleoptera: Chrysomelidae). J. Kans. Entomol. Soc. 58: 397–402.

White, R. 1977. Sexual characters of species of *Diabrotica* (Chrysomelidae: Coleoptera). Ann. Entomol. Soc. Am. 70: 168.

Wilson, T. A., T. L. Clark, and B. E. Hibbard. 2006. Number of point sources of western corn rootworm (Coleoptera: Chrysomelidae) eggs in artificial infestations affects larval establishment and plant damage. J. Kans. Entomol. Soc. 79: 119–128.