SURVIVAL OF MONARCH BUTTERFLY, *DANAUS PLEXIPPUS* (NYMPHALIDAE), LARVAE ON MILKWEED NEAR BT CORNFIELDS

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ABSTRACT. Pollen from corn plants genetically modified to express endotoxins from *Bacillus thuringiensis* (Berliner) has been identified as a potential hazard to the monarch butterfly, *Danaus plexippus* (L.), developing in and near cornfields. We conducted two field experiments to examine the effect of Bt corn on larval survival. A two-parameter Weibull model was used to perform detailed comparative survivorship analyses. Survival on milkweed plants near Bt corn and non-Bt corn was similar. Larval mortality rates were lower on milkweed plants located 0, 1, and 2 m from Bt corn compared with larvae 8 m from the corn. Cardinal direction from Bt corn did not influence larval survival. Multiple rainfall events likely resulted in the relatively low Bt corn pollen densities on milkweed leaves. We present evidence that late instar movement may bias estimates of survivorship in field studies.

Additional key words: *Bacillus thuringiensis*, risk assessment, transgenic corn, Weibull model.

Corn, *Zea mays* L., has been genetically modified to express a variety of toxins from the soil bacterium, *Bacillus thuringiensis* (Berliner), (Bt corn), to offer protection from the lepidopteran pest *Ostrinia nubilalis* Hübner (Ostlie et al. 1997, Rice & Pilcher 1998). Each successful genetic modification of corn is considered an event. Events (e.g., events Bt11, Mon810 and 176) have different promoters, protein genes and genetic markers, and differ in where this genetic material was inserted. Multiple events can express the same strain of Bt toxin (Ostlie et al. 1997).

Using a laboratory assay, Losey et al. (1999) identified Bt corn pollen (event Bt11) as a potential stressor to larvae of the monarch butterfly *Danaus plexippus* (L.). Jesse and Obrycki (2000) corroborated this finding with laboratory assays using natural and manipulated densities of corn pollen (events 176 and Bt11) on milkweed leaves. U.S. and Canadian researchers set out to perform a detailed ecological risk assessment on the potential adverse effects of Bt corn on monarch butterfly populations and concluded that the risks are negligible (Oberhauser et al. 2001, Pleasants et al. 2001, Hellmich et al. 2001, Stanley-Horn et al. 2001, Sears et al. 2001). In this context, risk is defined as the joint probability of larvae being exposed to Bt corn pollen (i.e., the stressor) and resultant mortality occurring (i.e., the effect) (Environmental Protection Agency 1998). The likelihood of larvae consuming Bt corn pollen represents the probability of exposure (Wolfenbarger & Phifer 2000). Toxicity of Bt corn pollen to larvae represents the probability of an effect occurring.

Bt toxins in corn pollen might increase mortality of larvae in three ways. Larvae may die due to the typical Bt toxin mode of action within the midgut (Federici 1993). Bt toxins may slow larval development (e.g., Pilcher et al. 1997), potentially increasing rates of predation or parasitism (Rawlins & Lederhouse 1981). Bt

pollen might act as a feeding deterrent causing the larvae to leave a pollen-dusted milkweed. After leaving a host plant, larvae have difficulty relocating the same host or finding a new host (Borkin 1982, Urquhart 1960). Due to this difficulty, Bt pollen might indirectly increase larval mortality through starvation after larvae leave a host plant (Borkin 1982).

Comparative survivorship studies that have reported survival at one observation date (e.g., Stanley-Horn et al. 2001) are important, but observation of survivorship over several dates (e.g., Zangerl et al. 2001, Stanley-Horn et al. 2001) is more informative. Studies with multiple measures of survival enable one to examine changes in mortality through time with formal demographic analyses.

The objective of our research was to conduct comparative survivorship analyses for monarch larvae on milkweed near Bt corn versus non-Bt corn and for larvae on milkweed at various distances and directions from Bt corn. None of the studies in Stanley-Horn et al. (2001) report repeated measures of larval survival for multiple distances outside of corn. Our study on the effect of distance and direction from Bt corn is unique from Zangerl et al. (2001) in that we used the commonly grown event Bt11. Event 176, used by Zangerl et al. (2001), has not been grown extensively and is being phased out of production. These studies were designed to directly measure the joint probability of exposure and effect in the field. We hypothesize that larval survivorship should be greater on milkweed near non-Bt cornfields than near Bt fields, and that survivorship should be greater on milkweed located further from Bt cornfields than on plants located near Bt cornfields.

MATERIALS AND METHODS

Larval survival on milkweed near Bt and non-Bt corn. The purpose of this experiment was to exam-

ine monarch larval survival over time near Bt corn compared to non-Bt corn. The experiment was conducted at the Rosemount Research and Outreach Center, University of Minnesota, Rosemount, Minnesota. Four fields of field corn were selected for this study. Two of the fields contained a Bt variety, Pioneer 36F30 (event Mon810). The other two fields contained a non-Bt variety, Pioneer 3751. Ten milkweed ramets, *Asclepias syriaca* L., were randomly selected from natural milkweed patches along the north or east edge of each field. Thus, total replication for each treatment (i.e., Bt corn versus non-Bt corn) was 20. The north or east edges were chosen, in part, to take advantage of prevailing winds (Baker 1983) that may move pollen from the field. The fields began shedding pollen within two days of each other starting on 27 July 1999. Prior to the experiment, each milkweed was thoroughly inspected for wild monarch larvae and eggs, which were removed when found. On 4 August 1999, a small camelhair brush was used to place two monarch larvae on the second or third pair of leaves from the top of each plant. The monarch larvae ranged from late-first instar to early-second instar at the time of infestation. To minimize handling mortality, early first instars were not used. The larvae were laboratory reared on potted *Asclepias curassavica* L. from eggs obtained from a captive colony. Infested plants were visually inspected at 1, 3, and 7 days post-infestation. For each sampling date, the number of larvae remaining and larval instar was recorded. A field guide (Oberhauser & Kuda 1997) was used to identify larvae to instar. Monarch eggs from the wild population, encountered while sampling, were removed.

Effects of direction and distance from Bt corn. The purpose of this experiment was to examine monarch larval survival over time at varying distances from Bt corn (event Bt11), which should result in varying levels of exposure to Bt corn pollen (e.g., Pleasants et al. 2001). In designing the experiment we assumed that corn pollen expressing event Bt11 would show some degree of toxicity to monarch larvae (e.g., Losey et al. 1999, Hellmich et al. 2001). This experiment was conducted in the same location as the 1999 experiment. A 30.5 × 30.5 m plot of N4242 Bt field corn (event Bt11) was planted on 6 May 2000. A fallow buffer of 30.5 m was maintained around the plot. On 5 May 2000, milkweed, *A. syriaca*, seed from a wild population in Rosemount, MN was planted into a field. When the milkweed reached a height of 10–25 cm they were transplanted into 11.4 L pots, with each pot containing three plants. The potted milkweed appeared to express a typical latex response. Corn anthesis began on 8 August 2000. On 11 August 2000, three

pots of milkweed were placed at 0, 1, 2, and 8 m distances from the field edge on each side of the plot (i.e., north, south, east, and west) for a total of 48 pots around the corn. Treatments in this experiment were distance and direction from Bt corn. Total replication for each direction-distance combination was three. Pots at 0 m were placed between corn plants of the first row of corn. The pots at each distance were spaced 1 m apart from each other. On 14 August 2000, two plants in each pot were infested with 2 monarch larvae (4 larvae per pot) ranging from late-first to early-second instar. Again, to avoid handling mortality, early first instars were not used. The larvae were placed on the second or third pair of leaves from the top of each plant using a small camelhair brush. As in 1999, larvae were reared from eggs obtained from a captive colony. The plants were visually inspected at 1, 3, 7, 9, 11, and 14 days post infestation. The number of larvae remaining, larval instar and location was recorded for each pot. Monarch eggs from the wild population, encountered while sampling, were removed.

Collection of milkweed leaves to estimate pollen deposition. On 18 and 22 August 2000, milkweed leaves were collected for pollen counts. One leaf was taken from the middle of one plant in each pot. The leaves were placed on cardboard, wrapped in plastic wrap, pressed, and frozen until they could be processed. Pollen was counted under a dissecting microscope, while viewing through the plastic wrap covering the leaves. Pollen counts were recorded in three to five (depending on leaf size) randomly selected 1 cm² areas on the upper surface of each leaf.

Analysis. In both experiments we observed larvae that remained on milkweed in the field. We understand that the disappearance of larvae over time may be due to a number of causes, including mortality from Bt corn pollen (e.g., Jesse & Obrycki 2000), natural enemies (e.g., Borkin 1982, Zalucki 1981, Zalucki & Kitching 1982), host plant effects (e.g., Zalucki & Brower 1992, Zalucki et al. 2001, Zalucki et al. 2002), and larval movement (e.g., Rawlins & Lederhouse 1981). We assume that disappearance is correlated with mortality and refer to the proportion of larvae remaining as survival.

The number of monarch larvae surviving on milkweed plants near Bt and non-Bt corn fields was analyzed for each sampling date using ANOVA and the Ryan-Einot-Gabriel-Welsch (REGWQ) multiple-range test (SAS 1995). Data were analyzed by date to account for dependence between sampling dates (i.e., repeated measures). The survival through time, near Bt and non-Bt fields, was modeled using a two-parameter version of the Weibull model (Pinder et al.

1978, Hogg & Nordheim 1983). The form of the Weibull model is as follows:

$$S_p(t) = \exp\left[-\left(\frac{t}{b}\right)^c\right]$$

where $S_p(t)$ typically represents the probability at birth of an individual surviving to time t, b represents the rate of mortality, c represents the overall shape of the Weibull model. High b values indicate low rates of larval mortality and low b values indicate high rates of larval mortality. Larval survivorship curves with c values greater than one, equal to one, and less than one reflect type I, II and III, respectively (e.g., Hogg & Nordheim 1983). Mortality is an increasing, constant and decreasing function of age for type I, II and III survivorship curves, respectively. Parameters t, b, and c must all be greater than zero. Parameters b and c were determined by iterative least squares fitting of the data to the model (Proc NLIN, SAS 1995). Welch's unpaired t (Oehlert 2000) was used to create simultaneous 95% confidence intervals for the difference between parameter estimates for each treatment. The confidence intervals were used to test for significant differences in the b and c parameters between treatments (i.e., Bt versus non-Bt). If the confidence interval included zero, parameter estimates were considered to not differ significantly at an error rate of 0.05.

ANOVA and REGWQ (SAS 1995) were used to analyze the effects of direction and distance from field edge on the larval survival and pollen density. Data were analyzed by date to account for dependence between sampling dates. Since no significant differences in the number larvae surviving were found between directions, except for a slight effect on day 9, the directions were pooled for each distance for further analysis. After pooling, total replication for each distance from Bt corn was 12. Survival from 0–14 days at 0, 1, 2, and 8 m from the field edge were modeled using the Weibull model. Welch's unpaired t with a Bonferroni adjustment for multiple comparisons was used to create confidence intervals for difference in b and c parameters between each pairwise combination of
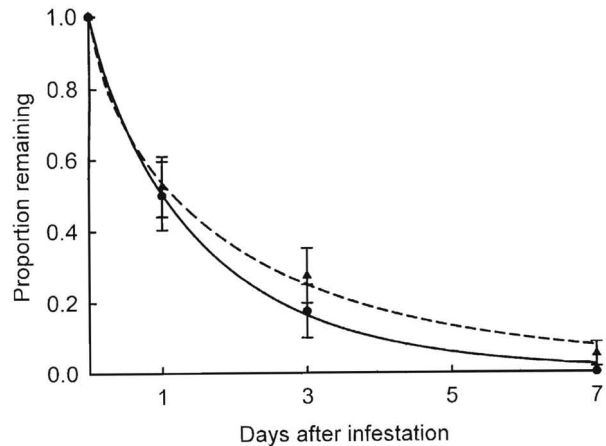

FIG. 1.   Proportion (±SE) of initial monarch larvae remaining on milkweed from 0–7 days post infestation near Bt (Mon810) (triangles) and non-Bt cornfields (circles) in 1999. Predicted lines for larvae near Bt (dashed line) and non-Bt (solid line) corn are based on the Weibull model (n = 30).

treatments (i.e., distances from Bt corn), and were used to test for significant differences between treatments with an error rate of 0.05, as described above. Separate 0–14 day Weibull analyses were performed on larvae observed strictly on plants and for larvae found collectively on the plants, pots, and soil within the pots for each treatment. Again, confidence intervals created using Welch's unpaired t were used to test for significant differences in the b and c parameters between locations (i.e., strictly on plants or collectively on plants, pots, and soil within the pots) within treatments.

RESULTS

Larval survival on milkweed near Bt and non-Bt corn. The Weibull model fit the survivorship data for larvae near Bt corn and non-Bt corn, with r^2 of 0.56 and 0.50, respectively (Table 1). Mortality rate (b) and shape (c) parameters of the Weibull models were not different for larvae near Bt (event Mon810) or non-Bt cornfields (Table 1, Fig. 1). ANOVA indicated that survival was similar for larvae near Bt and non-Bt corn on each sampling date (day 1: df = 1, 38, F = 0.04, p = 0.85; day 3: df = 1, 38, F = 0.87, p = 0.36; day 7: df

TABLE 1.   Weibull model parameters (b = mortality rate and c = shape) for proportion of larvae remaining on wild milkweed plants from 0–7 days post infestation, 1999.

Treatment	b (±SE)	c (±SE)	F	r^2
Bt+	1.91 (±0.35) a	0.73 (±0.19) a	37.48*	0.56
Bt–	1.53 (±0.26) a	0.88 (±0.25) a	28.72*	0.50

For each regression, there were 2 df for the regression and 58 df for residual. Means within a column followed by the same letter are not significantly different; 95% confidence intervals (constructed using Welch's unpaired t) for the difference in parameter estimates between treatments included zero.
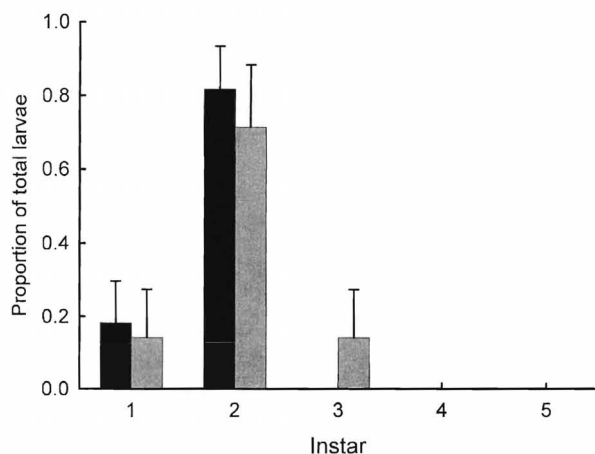
*p < 0.001.

FIG. 2.   Instar distribution (±SD) of monarch larvae remaining on milkweed near Bt (black) and non-Bt (gray) cornfields at 3 days post infestation in 1999.

= 1, 38, F = 2.11, p = 0.15). Larvae appeared to be in similar developmental stages near Bt and non-Bt corn, with the majority of the larvae being in the second instar on day three (Fig. 2), but the sample size was too small to conduct a statistically thorough analysis of developmental effects.

Effects of direction and distance from Bt corn. For all dates, direction of the milkweed plants from the cornfield did not significantly affect larval survival, as measured by the number of larvae remaining collectively on the plants, pots and soil within pots (df = 3, 32; F ranged from 0.51 on day 3 to 2.13 on day 1; p ranged from 0.67 to 0.12). The effect of distance on larval survival was significant at days 3 and 14. On day three, survival of larvae on plants at 0 and 2 m from a field of Bt corn were greater than at 8 m from the Bt field (df = 3, 32; F = 5.47; p = 0.0038). On day 14, survival was greater on plants at 0 m than at 1, 2 or 8 m (df = 3, 32; F = 4.05; p = 0.015).

The Weibull model fit the survivorship data for larvae at various distances from Bt corn, with r^2 ranging from 0.59 to 0.81 (Table 2). For larvae observed collectively on the plants, pots, and soil within the pots, the mortality rate parameters (b) of the 0–14 day Weibull

models were significantly greater at 0, 1, and 2 m compared to 8 m (Table 2, Fig. 3). The shape parameters (c) of the survivorship models were similar for larvae at all distances from the field edge (Table 2, Fig. 3).

Due to high mortality at 3 and 7 days, small sample sizes prevented us from conducting a statistically thorough analysis of developmental effects. Larval development appeared similar for larvae at all distances from the corn at 3 days post infestation; as in 1999, the majority of larvae were in the second instar (Fig. 4). On day 7, larvae observed on plants were predominantly in the second and third instars (Fig. 4). Some larvae, on day 7 and subsequent dates, were found within the pots, but not on the milkweed plants (Fig. 3). Larvae that had left the plants, but remained within the pots, were predominantly in the third and fourth instars (Fig. 4).

Larval movement from potted milkweed plants. Over time, a greater number of larvae moved off of plants onto pots (Fig. 3). Inclusion of larvae on pots as survivors affected Weibull analyses. At 2 m, the mortality rate parameter (b) was significantly lower for larvae strictly on plants compared to larvae within pots (Table 3). Across all other treatments, mortality rate parameters (b) were lower, though not statistically, for larvae strictly on plants compared to larvae within pots (Table 3). Across all treatments, shape parameters (c) were statistically similar for larvae strictly on plants compared to larvae within pots (Table 3). However, for all treatments, shape parameters (c) were numerically greater for larvae strictly on plants compared to those within pots (Table 3). The difference in recording survival as larvae strictly on plants versus larvae collectively on plants, pots, and soil within the pots became apparent between 7–14 days (Fig. 3).

Corn pollen deposition on milkweed leaves. Four days after larval infestation, average pollen densities for each of the direction-distance combinations ranged from 0.15 ± 0.08 to 10.7 ± 0.94 (mean ± SE) grains cm^{-2}. Pollen densities differed significantly (df for error 32; F = 4.77; p = 0.0073) between milkweed north of the corn (3.98 ± 1.26 grains cm^{-2}) compared to

TABLE 2.   Weibull model parameters (b = mortality rate and c = shape) for proportion of larvae remaining collectively on plants, soil, and pots from 0–14 days post infestation, 2000.

Treatment	b (±SEM)	c (±SEM)	F	r^2
0 meters	10.73 (±1.76) a	0.68 (±0.15) a	142.59*	0.80
1 meter	8.42 (±1.33) a	0.71 (±0.17) a	94.81*	0.73
2 meters	8.27 (±0.76) a	1.12 (±0.21) a	147.91*	0.81
8 meters	3.38 (±0.78) b	0.49 (±0.12) a	49.86*	0.59

For each regression, there were 2 df for the regression and 70 df for residual. Means within a column followed by the same letter are not significantly different; 95% confidence intervals (constructed using Welch's unpaired t with a Bonferroni adjustment for multiple comparisons) for the difference in parameter estimates between treatments included zero.
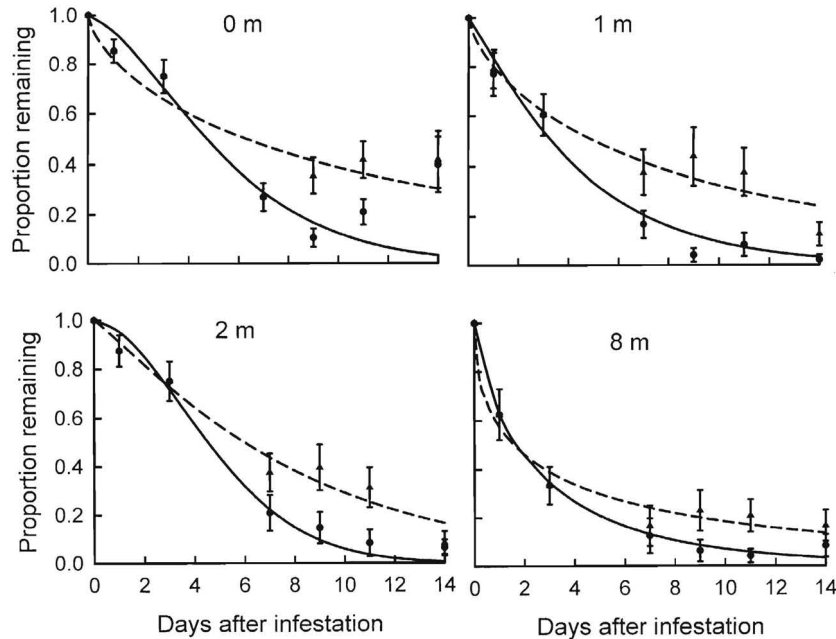
*p < 0.001.

FIG. 3. Proportion (±SE) of initial monarch larvae remaining strictly on potted milkweed plants (circles and solid lines) or within pots (triangles and dashed lines), i.e., collectively on plants, pots, and soil within pots, from 0–14 days post infestation at 0, 1, 2, and 8 m from a Bt corn field (Bt11). Predicted lines are based on the Weibull model.

milkweed east of the corn (1.12 ± 0.28 grains cm^{-2}), with intermediate densities on milkweed south and west of the corn (3.34 ± 1.47 and 1.63 ± 0.28 grains cm^{-2}, respectively), statistically not different from densities on the north and east. Pollen densities differed significantly (df for error = 32; F = 8.14; p = 0.0004) on milkweed 0 and 1 m from corn (4.15 ± 1.28 and 3.86 ± 1.32 grains cm^{-2}, respectively) compared to plants 2 and 8 m from corn (1.58 ± 0.39 and 0.49 ± 0.17 grains cm^{-2}, respectively). Eight days after larval infestation,

the maximum mean pollen density was 2.9 ± 0.74 grains cm^{-2}. Pollen densities differed significantly (df for error = 32; F = 4.50; p = 0.0096) on milkweed north of the corn (1.10 ± 0.38 grains cm^{-2}) compared to milkweed east or west of the corn (0.52 ± 0.10 and 0.34 ± 0.17 grains cm^{-2}, respectively), with milkweed on the south (0.71 ± 0.11 grains cm^{-2}) being indistinguishable from the other directions. Pollen densities differed significantly (df for error = 32; F = 9.88; p = 0.0001) on milkweed at 1 m (1.33 ± 0.34 grains cm^{-2}) compared to

TABLE 3. Weibull model parameters (b = mortality rate and c = shape) for proportion of larvae remaining strictly on potted milkweed plants or collectively from plants, soil, and pots from 0–14 days post infestation, 2000.

Location	b (±SEM)	c (±SEM)	F	r^2
0 meters				
Plants	7.02 (±0.78) a	0.92 (±0.17) a	127.01*	0.78
Plants, soil and pots	10.73 (±1.76) a	0.68 (±0.15) a	142.59*	0.80
1 meter				
Plants	4.45 (±0.41) a	1.19 (±0.17) a	136.95*	0.80
Plants, soil and pots	8.42 (±1.33) a	0.71 (±0.17) a	94.81*	0.73
2 meters				
Plants	5.78 (±0.44) a	1.55 (±0.24) a	170.47*	0.83
Plants, soil and pots	8.27 (±0.76) b	1.12 (±0.21) a	147.91*	0.81
8 meters				
Plants	2.67 (±0.40) a	0.75 (±0.13) a	62.38*	0.64
Plants, soil and pots	3.38 (±0.78) a	0.49 (±0.12) a	49.86*	0.59

For each regression, there were 2 df for the regression and 70 df for residual. Means within a column for each distance followed by the same letter are not significantly different; 95% confidence intervals (constructed using Welch's unpaired t) for the difference in parameter estimates between treatments included zero.
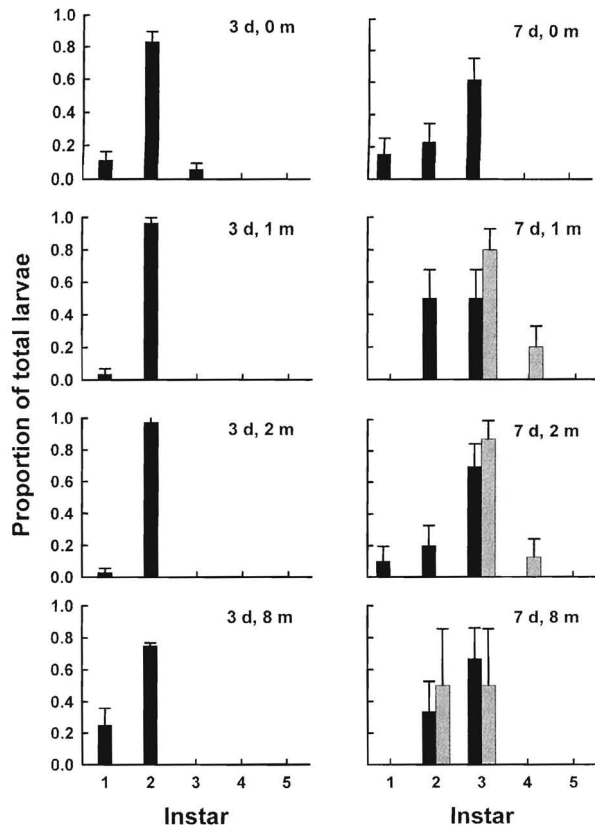*p < 0.001.

FIG. 4. Instar distribution (±SD) of monarch larvae remaining, near Bt corn (Bt11), strictly on potted milkweed plants (black) and larvae that have left milkweed plants, but remained on the pots or on the soil within the pots (gray) at 0, 1, 2 and 8 m on 3 and 7 days post infestation in 2000.

milkweed 0, 2 and 8 m from corn (0.70 ± 0.17, 0.40 ± 0.09 and 0.23 ± 0.08 grains cm^{-2}, respectively).

DISCUSSION

In 1999 and 2000, we were unable to detect an adverse effect of Bt corn on monarch larvae. Survival was similar for larvae near Bt and non-Bt cornfields, and the rate of mortality was lower (i.e., larger Weibull b value) for larvae on milkweed near the edge of a Bt cornfield compared to larvae on milkweed farther from a field. Stanley-Horn et al. (2001) and Zangerl et al. (2001) were also unable to detect any adverse impact of Bt corn on monarch larvae under field conditions. The likely mechanisms by which Bt corn might adversely affect monarchs depend upon larval consumption of corn pollen or other corn tissue, such as anthers (Hellmich et al. 2001). The present study reports a maximum Bt corn pollen density of 10.7 grains cm^{-2} on milkweed leaves. Our observed pollen densities, along with mean pollen densities reported in Jesse and Obrycki (2000), Wraight et al. (2000), Stanley-Horn et al. (2001), Zangerl et al. (2001) and Pleasants

et al. (2001), are far below the least observable effect concentration of 1000 grains cm^{-2} for the commonly grown Bt events Mon810 and Bt11 (Sears et al. 2001).

The lack of an observed adverse effect of Bt corn on monarch larvae in the present studies is similar to results obtained by Wraight et al. (2000) working with black swallowtails, *Papilio polyxenes* Fabricius, near Bt corn. However, not all Lepidoptera are equally susceptible to Bt toxins. For example, Bt sweet corn provides greater control of European corn borer than corn earworm, *Helicoverpa zea* (Boddie) (Burkness et al. 2001). In addition, Wagner et al. (1996) observed differential susceptibilities among non-target forest Lepidoptera to foliar applications of Bt insecticide.

Over time, environmental factors (e.g., rain and wind) may reduce densities of corn pollen (Pleasants et al. 2001) on the relatively smooth upper surface of milkweed leaves (Bhowmik 1994). Multiple rainfall events that occurred during the course of our experiments likely contributed to the low pollen densities we observed. A single rainfall event can remove up to 86% of the corn pollen from a milkweed leaf (Pleasants et al. 2001). The fact that rainfall events occurred and likely reduced the pollen loads on the milkweed leaves does not weaken the significance of our experiments. On the contrary, our results suggest that any potential adverse effect of Bt corn on monarch larvae can be substantially mitigated under field conditions.

Our results from the 2000 study indicate that the rate of larval mortality is lower (i.e., larger Weibull b parameter) on milkweed at the edge of a Bt cornfield compared to milkweed farther from the field. The survival of larvae at different locations relative to the cornfield edge (e.g., on the field edge, within the field or outside the field) has been variable among studies. Lower survival was found at the edges of corn fields in the *Iowa II* and *New York* studies of Stanley-Horn et al. (2001) compared to milkweed at various locations within and outside cornfields. Oberhauser et al. (2001) reported higher larval survival (i.e., larger Weibull b values) on milkweed in cornfields compared to milkweed on cornfield edges. In contrast, in the *Iowa I* study of Stanley-Horn et al. (2001), higher larval survival was found on milkweed at cornfield edges compared to milkweed within cornfields.

The observed increase of larval mortality rate (i.e., decrease in Weibull b parameter) and apparent shift of mortality to a less-strong type III survivorship curve (i.e., increase in Weibull c parameter), due to not counting larvae that have moved off of experimental plants, may lead to biased estimates of mortality. Monarch larvae will spend up to 17.5% of daylight hours off of milkweed plants (Rawlins & Lederhouse

1981). Individual monarch larvae have been difficult to follow for more than one week (Borkin 1982). Larvae, as early as second instars (Borkin 1982), leave what seem to be suitable host plants for no apparent reason (Borkin 1982, Urquhart 1960). We observed that most larval movement from potted milkweed plants began by 7 days post infestation, which was about the third instar and onward. Larvae that were observed off of the potted milkweed plants in this study were predominantly in the third or later instars. Researchers conducting survivorship studies in the field must be aware of the potential for monarch larvae to leave host plants. Biased underestimates of larval survival will result from counting larvae that have disappeared from the host plants as mortality. Conversely, monarch larval movement may also confound results when survivorship studies are conducted in small field cages. Small field cages could preclude normal larval movement from plants and thereby result in overestimates of survivorship compared to open field studies.

ACKNOWLEDGEMENTS

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