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Laura C. Hansen Jesse · John J. Obrycki

Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly

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Abstract We present the first evidence that transgenic *Bacillus thuringiensis* (Bt) corn pollen naturally deposited on *Asclepias syriaca;* common milkweed, in a corn field causes significant mortality of *Danaus plexippus* L. (Lepidoptera: Danaidae) larvae. Larvae feeding for 48 h on *A. syriaca* plants naturally dusted with pollen from Bt corn plants suffered significantly higher rates of mortality at 48 h (20±3%) compared to larvae feeding on leaves with no pollen (3±3%), or feeding on leaves with non-Bt pollen (0%). Mortality at 120 h of *D. plexippus* larvae exposed to 135 pollen grains/cm^2 of transgenic pollen for 48 h ranged from 37 to 70%. We found no sub-lethal effects on *D. plexippus* adults reared from larvae that survived a 48-h exposure to three concentrations of Bt pollen. Based on our quantification of the wind dispersal of this pollen beyond the edges of agricultural fields, we predict that the effects of transgenic pollen on *D. plexippus* may be observed at least 10 m from transgenic field borders. However, the highest larval mortality will likely occur on *A. syriaca* plants in corn fields or within 3 m of the edge of a transgenic corn field. We conclude that the ecological effects of transgenic insecticidal crops need to be evaluated more fully before they are planted over extensive areas.

Key words *Danaus plexippus · Bacillus thuringiensis ·* Bt corn · Transgenic pollen · Risk assessment

Introduction

Starting in the late 1990s, transgenic crops with insecticidal toxins began to be widely planted in the United States (Gould 1998). *Bacillus thuringiensis* (Bt) corn, developed to suppress the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), was field tested in 1992, and by 1995 was registered by the Environmental Protection Agency for commercial sales (Carozzi and Koziel 1997). In 1998, approximately 3.6 million hectares of Bt corn were planted in the U.S., and the predictions are that by 2003, this area will have extended to 12 million hectares (1/3 of total U.S. corn acreage) (Federici 1998).

To increase toxin expression when transferring the Bt gene into plants, only the genes encoding the active *B. thuringiensis* CryIAb protein toxin were inserted (Perlak et al. 1991). *CryIAb* was first inserted into corn by microprojectile bombardment and expression was controlled by the phosphoenolpyruvate carboxylase and a pollen-specific promoter. This genetic transformation is referred to as event 176 (Koziel et al. 1993). High expression of CryIAb in pollen enhances suppression of *O. nubilalis* because early instars of the second generation often feed on pollen that has accumulated in the axils of corn leaves (Carozzi and Koziel 1997). The cauliflower mosaic virus 35S promoter, used for a second genetic transformation (event Bt11; Walker 1998), was enhanced to produce more stable expression of CryIAb in all corn tissues (Armstrong et al. 1995). Less toxin is expressed in event Bt11 than in event 176 pollen. MON 810, an event that is very similar to event Bt11, expresses 0.9 µg Bt toxin/g fresh weight of pollen (EPA 1999a), compared to event 176 with 7.1 µg Bt toxin/g fresh weight of pollen (EPA 1999b).

The speed with which transgenic crops have become widely planted has caused controversy about the assessment and management of the environmental risks of transgenic plants (Paoletti and Pimentel 1996; Miller 1998; Wraight et al. 2000). Previous examinations of non-target ecological effects of transgenic insecticidal crops have focused on species that comprise crop-based food webs, for example, natural enemies, phytophagous species, or plant pathogens (Johnson and Gould 1992; Pilcher et al. 1997a, 1997b; Hilbeck et al. 1998; Munkvold et al. 1999). Gene flow between genetically modified crops and wild plant relatives due to transgenic pollen dispersal has also been extensively considered (Snow and Palma 1997; Lavigne et

L.C. Hansen Jesse · J.J. Obrycki (✉)
Department of Entomology, Iowa State University, Ames, IA 50011, USA
e-mail: jobrycki@iastate.edu
Tel.: +1-515-294-8622, Fax: +1-515-294-8027

al. 1998). However, unintended effects on species beyond field borders have not been adequately addressed. Consumption of transgenic insecticidal pollen on non-crop plants outside crop fields is one probable environmental risk, because insecticidal toxins are expressed in wind-dispersed pollen (Koziel et al. 1993; Fearing et al. 1997). Assessments outside transgenic fields are particularly relevant because of known detrimental non-target effects of aerial spraying of microbial insecticide formulations of *B. thuringiensis* (Wagner et al. 1996; Whaley et al. 1998), the source of toxin-producing genes inserted into transgenic corn (Koziel et al. 1993).

The non-target species we considered in this study is the monarch butterfly, *Danaus plexippus* L. (Lepidoptera: Danaidae), which is widely distributed in North America. Recently, a high concentration of transgenic Bt corn pollen experimentally applied to *Asclepias curassavica* leaves in the laboratory was shown to cause significant mortality of *D. plexippus* larvae (Losey et al. 1999). Multiple generations of *D. plexippus* are produced in the United States and Canada; eastern populations overwinter as adults in Mexico (Urquhart 1976). Fifty percent of the overwintering adults in Mexico originate from the central United States, an area of concentrated corn production (Wassenaar and Hobson 1998). Most of these adults are from larvae that feed on *A. syriaca*, the common milkweed (Malcolm et al. 1993). At least three overlapping generations of monarchs are observed annually in the central U.S., and larvae are present on *Asclepias* spp. from early June to mid September (Urquhart 1960; Borkin 1982). Milkweed is commonly found in corn fields and adjacent non-cultivated habitats where it is a food plant for monarch larvae (Cramer and Burnside 1982; Bhowmik 1994; Yenish et al. 1997; Hartzler and Buhler 2000; L.C. Hansen, unpublished data). Corn pollen is produced, depending on planting date, in mid to late summer for 1–2 weeks and is wind dispersed at least 60 m (Raynor et al. 1972; Ritchie et al. 1997); thus the monarch, milkweeds, and transgenic pollen are likely to overlap spatially and temporally in the central U.S.

Our study had three objectives: (1) to determine the levels of transgenic pollen on *A. syriaca* plants placed within and adjacent to plots of transgenic corn; (2) to assess the mortality of *D. plexippus* larvae exposed to field-deposited pollen, and (3) to quantify the effects on *D. plexippus* larvae and adults exposed to a range of transgenic pollen densities that they would likely encounter in the field.

Materials and methods

Pollen deposition on *A. syriaca*

Four corn hybrids were planted in a 2,500-m^2 plot on the Iowa State University campus. Corn was planted in May 1998 and June 1999. In 1998, 8–12 rows (77 m) of each hybrid were planted north to south; in 1999, rows (35 m) were planted east to west. The hybrids were: (1) transgenic MAX 454 (KnockOut, Novartis Seeds), event 176; (2) hybrid 4494 (Novartis Seeds), non-transgenic, and genetically similar to MAX 454; (3) transgenic hybrid 7333Bt (YieldGard, Novartis Seeds), event Bt11; and (4) hybrid 7333 (Novartis Seeds), non-transgenic, and genetically similar to hybrid 7333Bt.

In 1998, field deposition of pollen was assessed by placing potted *A. syriaca* plants within the corn plots, 0.2, 1, and 3 m from the field edge. In 1999, distances of 5 and 10 m were added. *A. syriaca* was transplanted from natural populations and potted in 27.5-cm pots. *A. syriaca* plants used in the field studies were approximately 50–100 cm tall (including the pot). A no. 6 cork borer was used to remove 0.79-cm^2 disks from *A. syriaca* leaves. Leaf disks were kept horizontal to minimize pollen loss. In 1998, leaf disks were taken from three positions (tip, middle, and base) of three leaves from the upper, middle, and lower portions of 12 potted plants on three dates from 29 July to 4 August from event 176, and from 12 plants on three dates from 11–17 August from event Bt11. In 1999, leaf disks were taken from two positions (base and tip) of three leaves from the upper, middle, and lower portions of 18 potted plants on four dates from 31 July to 9 August from event Bt11, and on two dates, 4 and 8 August, from event 176. The number of pollen grains on the 0.79-cm^2 leaf disks removed from leaves was counted under a dissecting microscope. If >400 pollen grains were counted on a leaf disk, the value was recorded as 400, and this category was used to indicate a high concentration of pollen. The number of pollen grains deposited on a single leaf sample was reported as pollen grains/cm^2. The cumulative number of pollen grains deposited by each hybrid was described using the curve that best fit the data.

Larvae exposed to field-deposited pollen

To assess mortality of *D. plexippus* larvae exposed to field-deposited transgenic and non-transformed pollen, 143 leaf disks (0.79 cm^2) were removed on 1 and 4 August 1998 from *A. syriaca* plants located within and at the edge of non-Bt (hybrid 4494) and event 176 (MAX 454) corn plots. Pollen was washed off 72 leaf disks. These leaf disks were examined under a dissecting microscope to determine that all pollen grains had been removed. The number of pollen grains on the unwashed leaf disks was counted and each disk was placed in a 5.2-cm-diameter petri dish on moistened filter paper. One first-instar *D. plexippus* was placed on each leaf disk (transgenic n=35, non-transgenic n=36, or washed n=72) for 48 h. Although larvae were placed on top of the leaf disk, their movement was not restricted: they could feed from either leaf surface. The *D. plexippus* larvae were from a 2-month-old laboratory colony started from field-collected individuals.

Laboratory assessment of mortality and sub-lethal effects

Pollen was collected from three of the four corn hybrids from 29 July to 19 August 1998 by stapling brown paper tassel bags (Medico Enterprises, Kirkwood, Mo.) over corn tassels. Pollen was not collected from hybrid 7333 because spring flooding severely reduced pollination. After 6–7 days, the bagged tassels were removed from the corn stalk, dried for 24 h, and the pollen was sifted through a sieve (300-µm openings), and stored at –20°C for 9–10 months. *A. curassavica* was used because it can be grown easily in the greenhouse and is a suitable host plant for *D. plexippus* larvae (Zalucki 1993).

Three densities of transgenic (MAX 454, 7333Bt) and non-transgenic (4494) pollen, representative of observed field densities, were obtained by suspending 0.1, 0.01, or 0.001 g of pollen in 10 ml distilled water. Because corn pollen settled to the bottom of a 10-ml graduated cylinder, the cylinder was inverted twice to mix the pollen and water before each 0.05-ml sample was removed with a pipette. The 0.05-ml drop of the suspended pollen solution was placed on a 1.54-cm^2 disk of *A. curassavica* and allowed to dry. In a 0.05-ml drop of the 0.01 g pollen/10 ml water solution, the mean number of pollen grains was 208±12 (n=12), the mean number in a 0.05-ml drop of the 0.001-g solution was 22±1

(n=12). The number of pollen grains in 0.05 ml of the 0.1-g solution was estimated to be 1,966. This number was estimated by multiplying the mean number in the 0.01-g solution (22) by a scaling factor of 9.45. The scaling factor was calculated as the ratio of pollen grains in the 0.01-g and the 0.001-g solutions (208/22). The number of pollen grains was then divided by 1.54 cm^2 (the area of the leaf disk) to obtain the number of pollen grains/cm^2. Thus, the three densities of pollen used in all tests were 14, 135, and 1,300 pollen grains/cm^2. Each leaf disk was then placed on moistened filter paper in a 5.2-cm-diameter petri dish. One first-instar *D. plexippus* was placed on the leaf disk and maintained at 21°C; L:D 16:8 h. Larvae were placed on top of the leaf disk, but they were able to move and feed from underneath the disk. Following a 48-h exposure to pollen, each larva was placed in a plastic box (1,224–1,354 cm^3) and fed clean *A. curassavica* leaves daily until pupation. The bioassay was done once with larvae that were less than 12 h old (n=10 larvae per treatment), and once with larvae that were 12–36 h old (n=16 larvae per treatment). Larvae were allowed to feed on their chorion and clean *A. curassavica* leaves prior to their transfer onto the leaf disks. *D. plexippus* eggs for this experiment were provided by Monarch Watch, University of Kansas.

During larval development, molting or mortality were noted every 12 h. To assess sub-lethal effects on individuals that survived larval exposure to Bt pollen, we measured pupal weight, adult dry weight, forewing length, and lipid content. Twenty-four hours after pupation, each pupa was weighed and placed in an emergence chamber, a 450-ml inverted plastic cup with two strips of fiberglass window screen glued in an X-pattern (Monarch Watch 2000) to allow the adult to expand its wings. Chrysalides were maintained at 21°C, L:D 16:8 h. Twenty-four hours after eclosion, each adult was placed in a 9.2×8.5 cm envelope and put in a freezer. The right forewing length (cm) was measured from the white spot on the thorax at the base of the wing to the apex (Donham and Taylor 2000).

After drying adults for 24 h at 60°C, weight and lipid content were determined. Lipid content was used as an indication of possible sub-lethal effects of Bt pollen. To extract lipids, the dry adults were ground into a powder in a mortar and pestle with 2 ml of 2:1 chloroform:methanol. Approximately 3 ml of the cloroform:methanol solution was added to the crushed *D. plexippus* and the entire mixture was transferred to a test tube. An additional 2 ml of the cloroform:methanol solution was added to the test tube and the mixture was maintained at 22–24°C for 2 h in the first replicate and for 24 h in the second replicate. The longer time for the second replicate was used to extract higher amounts of lipids from the adults. The liquid portion of the chloroform:methanol/*D. plexippus* mixture was then strained through a 10-ml glass pipette containing glass wool. The chloroform:methanol/lipid mixture was transferred to a pre-weighed test tube; the chloroform:methanol was evaporated under a constant stream of nitrogen at 30–50°C and the lipids weighed (Tuskes and Brower 1978).

To confirm the presence of the insecticidal toxin in the pollen, a bioassay with *O. nubilalis* larvae, a species known to be sensitive to transgenic Bt corn (Koziel et al. 1993), was conducted. *O. nubilalis* larvae were provided by the USDA-ARS Corn Insects and Crop Genetics Research Unit, Ames, Iowa. Effects of transgenic pollen on *O. nubilalis* larvae were measured using a modification of a blue dye droplet assay. Pollen from Max 454, 7333Bt, and 7333 hybrids was dyed blue using a mixture of FDC Blue No. 1 coloring, distilled water, and Tween 80 (Hughes et al. 1986). More than 200 first-instar *O. nubilalis* were then fed each type of colored pollen for 5 h. Groups of six larvae that had blue digestive tracks were then transferred to ten plastic cups containing 0.01 g of non-dyed pollen of the same hybrid dusted on moist filter paper. After 48 h, the number of live and dead larvae was noted for each type of pollen.

An ELISA was conducted to quantify levels of the CryIAb protein in pollen collected in 1998 from hybrids MAX 454, 7333Bt, and 4494 using a kit purchased from Agdia Incorporated (Elkhart, Ind.). The ELISA was conducted on pollen stored at –20°C for 8–9 months after the bioassays were conducted. Approximately 0.1 g of pollen from each hybrid was sonicated three times for approximately 10 s at 4 W in 5 ml of the extraction buffer provided by Agdia using a Fisher Model 60 Sonic Dismembrator. The pollen was then refrigerated for 15 h, and then sonicated for approximately 5 s before 100 µl of the sample was removed and placed in a test well.

Data analysis

Pollen deposition on A. syriaca

The number of pollen grains deposited on *A. syriaca* leaves at various distances was analyzed by analysis of variance (ANOVA; SAS 1999) separately for each corn hybrid each year. The percentage mortality of larvae exposed to Bt event 176, non-Bt, and washed leaves was arcsine transformed and compared by ANOVA (SAS 1999).

Laboratory assessment of mortality and sub-lethal effects

Because significant differences in survival, developmental times, and adult characteristics of *D. plexippus* were observed between the two larval age classes (<12 h and 12–36 h), each age class was analyzed separately. The number of days that a larva survived after the initial 48-h exposure to Bt pollen was analyzed using LIFETEST and ANOVA (SAS 1999). The effect of pollen concentration and the type of pollen varied over time (ANOVA P=0.0001, df=9), so LIFETEST was used to analyze the survival curves, because the number of larvae dead at each time period is dependent on the number dead at the previous time period. LIFETEST is a non-parametric test that makes no assumptions about the distribution of the risk of death over time (Lawless 1982). The test statistics used to compare mortality were the log-rank and Wilcoxon; these tests follow a $\chi 2$ distribution and compare ranked values (Lawless 1982). These test statistics analyze two survival curves by comparing each time interval to determine if the number of deaths differ from an expected assumption that the two curves are identical (Lawless 1982).

Developmental times and adult characteristics of surviving *D. plexippus* larvae were analyzed with ANOVA to determine treatment effects (SAS 1999). Each pollen type at each concentration was analyzed using a one-way ANOVA.

Results

Pollen deposition on *A. syriaca*

The cumulative deposition of transgenic pollen in 1998 was highest within the corn field (74–217 pollen grains/cm^2) and decreased to between 6 and 20 pollen grains/cm^2 at 3 m from the edge of the field (event 176, ANOVA P=0.0001; event Bt11, P=0.0017; Table 1). Similarly in 1999, pollen deposition was highest within the field (80–115 pollen grains/cm^2) and decreased to 5–7 pollen gains/cm^2 at 3 m and 1 pollen grain/cm^2 at 10 m (event 176 and Bt11, ANOVA, P=0.0001). During sampling, eight rainfall events of ≥0.84 cm occurred in 1998 and three rainfall events of ≥1.42 cm were recorded in 1999 (Anon 1999). The varying number of rainfall events and the amount of rain may explain differences in pollen deposition between 1998 and 1999.

In 1998, the amount of pollen deposited on the upper, middle, and lower leaves of potted *A. syriaca* plants was similar for event 176 (ANOVA, P=0.3551), but different

Table 1 Cumulative field deposition and range of deposition of transgenic corn pollen grains [mean (SE)/cm² leaf area) on potted milkweed plants placed within and adjacent to a test plot. In 1998, pollen from event 176 was sampled on 29 July, 1 and 4 August; pollen from event Bt11 was sampled on 11, 14, and 17 Aug. On each sample date, nine leaf disks were removed from each of three potted milkweed plants placed within the field, 0.2, 1, and 3 m from the edge. In 1999, pollen from event 176 was sampled on 4 and 8 August; pollen from event Bt11 was sampled on 31 July, and 3, 6, and 9 August. On each sample date, six leaf disks were removed from each of three potted milkweed plants placed with the field, 0.2, 1, 3, 5 and 10 m from the edge. For both years, a logarithmic or exponential curve was fit to the pollen deposition data. The range is the highest and lowest number of pollen grains/cm² deposited on a single leaf disk during the entire collection period

Source of pollen	Pollen grains/cm² leaf surface						Predicted pollen deposition	Estimated maximum distance of pollen deposition (m)
	Within field	0.2 m from field edge	1.0 m from field edge	3.0 m from field edge	5.0 m from field edge	10.0 m from field edge		
1998								
Event 176	217.0 (35.3)	41.6 (5.1)	25.3 (10.4)	6.3 (2.7)	–	–	y=–29.56log(x)+22.22 r^2=0.98	5.6
Range	0–506	0–99	0–122	0–35	–	–		
Event Bt11	74.2 (23.8)	80.5 (57.5)	49.7 (25.1)	20.4 (7.6)	–	–	y=–50.54log(x)+46.44 r^2=0.99	8.3
Range	0–152	0–427	0–222	0–56	–	–		
1999								
Event 176	79.8) (24.3	54.4 (26.8)	22.4 (17.6)	7.6 (4.8)	1.6 (0.7)	1.1 (0.2)	y=–32.75log(x)+27.1 r^2=0.95	6.7
Range	0–177	0–392	0–61	0–32	0–6	0–4		
Event Bt11	115.4 (26.0)	32.5 (3.9)	28.2 (15.5)	5.0 (0.4)	3.0 (1.8)	0.8 (0.3)	y=47.0×10$^{-0.198x}$	ca 10
Range	0–135	0–42	0–82	0–8	0–11	0–4	r^2=0.88	

for event Bt11 (ANOVA, P=0.0241). For unexplained reasons, more pollen from event Bt11 was observed on the middle and lower leaves. In 1999, event Bt11 had similar amounts on the upper, middle, and lower leaves (ANOVA, P=0.2348), but event 176 had greater deposition on the middle and lower leaves (ANOVA, P=0.0369).

Larvae exposed to field-deposited pollen

D. plexippus larvae exposed for 48 h to event 176 pollen that had accumulated on *A. syriaca* in the field exhibited 20±3% mortality compared to 0% mortality in the non-Bt pollen treatment, and 3±3% on *A. syriaca* leaves washed to remove pollen (ANOVA, P=0.0415). Mortality was not correlated with the number of pollen grains on the leaf disk or the plant location (within the field or edge of field). Mortality was observed on leaf disks with 10–306 transgenic pollen grains/cm². The average number of pollen grains/cm² was 74±15 for event 176 and 36±9 for the non-Bt treatment.

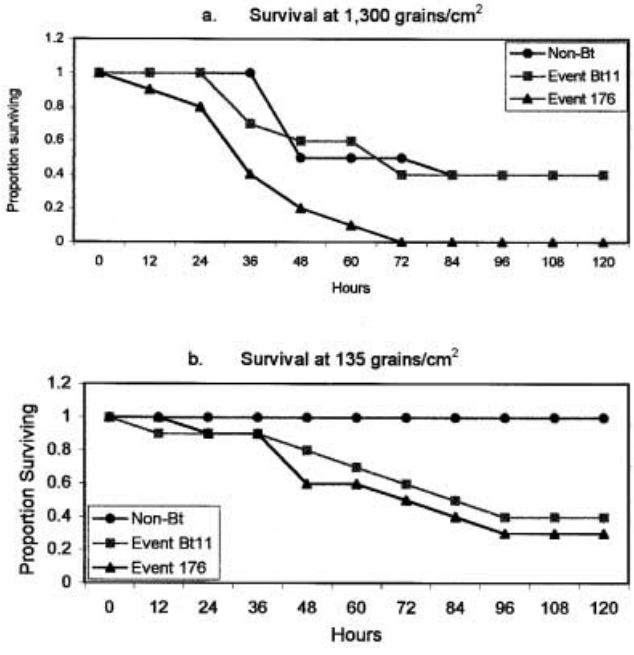

Fig. 1 Survival curves for monarch larvae exposed at <12 h old to 1,300 (**a**) or 135 (**b**) pollen grains/cm² of non-Bt, event Bt11, and event 176 corn pollen for 48 h

Laboratory assessment of mortality and sub-lethal effects

The survival curves for <12-h-old larvae exposed to 1,300 and 135 pollen grains/cm² of event 176, event Bt11, or non-Bt corn pollen for 48 h were significantly different (log-rank and Wilcoxon, P≤0.007; Fig. 1). The survival curves of larvae exposed to 14 pollen grains/cm² were similar (log-rank and Wilcoxon, P=0.3). At 1,300 grains/cm², the non-Bt and event Bt11 survival curves were similar: 40% of larvae survived to 120 h

Table 2 Mean (±SE) developmental time, pupal weight, wing length, and lipid content of larvae less than 12 h old exposed to non-Bt, event Bt11, or event 176 corn pollen, at a concentration of 1,300, 135, or 14 pollen grains/cm^2. Data are not presented for event 176 at 1,300 pollen grains/cm^2 because there were not enough surviving larvae. Adults with wings that had not fully expanded before drying were not included in the wing length measurements, but were included in the average lipid weight for a treatment

Pollen type	Concentration	Total development time (days)	Pupal weight (g)	Dry weight (g)	Forewing length (cm)	Lipid content (g)
Non Bt	1,300	14.2±0.9 *n*=3	1.40±0.2 *n*=3	0.19±0.01 *n*=3	4.7±0.3 *n*=2	14.1±2.4 *n*=3
	135	13.5±0.5 *n*=9	1.27±0.1 *n*=9	0.18±0.01 *n*=8	4.9±0.1 *n*=8	15.7±1.2 *n*=8
	14	13.7±0.5 *n*=9	1.28±0.4 *n*=9	0.17±0.01 *n*=8	4.7±0.1 *n*=7	14.6±1.9 *n*=8
Event Bt11	1,300	15.5±0.3 *n*=3	1.29±0.1 *n*=3	0.18±0.0001 *n*=2	5.1±0.1 *n*=2	15.0±2.3 *n*=2
	135	13.3±0.4 *n*=4	1.47±0.1 *n*=4	0.20±.02 *n*=4	5.1±0.1 *n*=4	19.6±5.8 *n*=4
	14	12.6±0.3 *n*=9	1.26±0.05 *n*=9	0.17±0.01 *n*=6	4.9±0.05 *n*=6	11.1±1.5 *n*=6
Event 176	135	13.5±0.9 *n*=3	1.37±0.1 *n*=3	0.18±0.03 *n*=3	4.6±0.2 *n*=3	9.7±5.2 *n*=3
	14	13.1±0.5 *n*=4	1.35±0.02 *n*=4	0.18±0.002 *n*=4	4.9±0.1 *n*=4	15.1±1.7 *n*=4

(log-rank and Wilcoxon, P=0.7). However, both non-Bt and event Bt11 survival curves were significantly higher than the event 176 survival curve (log-rank and Wilcoxon, P<0.05; Fig. 1a). The survival curve for larvae exposed to 135 grains/cm^2 of non-Bt pollen (no mortality at 120 h) was significantly higher than the transgenic pollen survival curves (log-rank and Wilcoxon, P<0.006; Fig. 1b). The survival curves for larvae exposed to event 176 pollen (30% surviving at 120 h) and to event Bt11 pollen (40% surviving) were similar (log-rank and Wilcoxon, P=0.6; Fig. 1b).

The survival curves of 12– to 36-h-old larvae exposed for 48 h to 1,300 (log-rank and Wilcoxon, P=0.005), and 135 (log-rank, P=0.03, Wilcoxon, P=0.04) pollen grains/cm^2 were significantly different (Fig. 2). Survival curves at 14 pollen grains/cm^2 were similar (log-rank, P=0.98; Wilcoxon, P=0.96). At 1,300 pollen grains/cm^2, the survival curve for the larvae exposed to the non-Bt pollen (88% surviving 120 h) was significantly higher than the transgenic pollen survival curves (log-rank and Wilcoxon, P<0.05). The survival curves for event Bt11 (44% surviving at 120 h) and event 176 (31% surviving 120 h) were similar (log-rank and Wilcoxon, P>0.05; Fig. 2a). Similarly, at 135 pollen grains/cm^2, the survival curve for larvae exposed to non-Bt pollen (100% surviving 120 h) was significantly higher than the curves for the larvae exposed to event 176 (63% surviving) or event Bt11 (75% surviving) (log-rank and Wilcoxon, P<0.05; Fig. 2b).

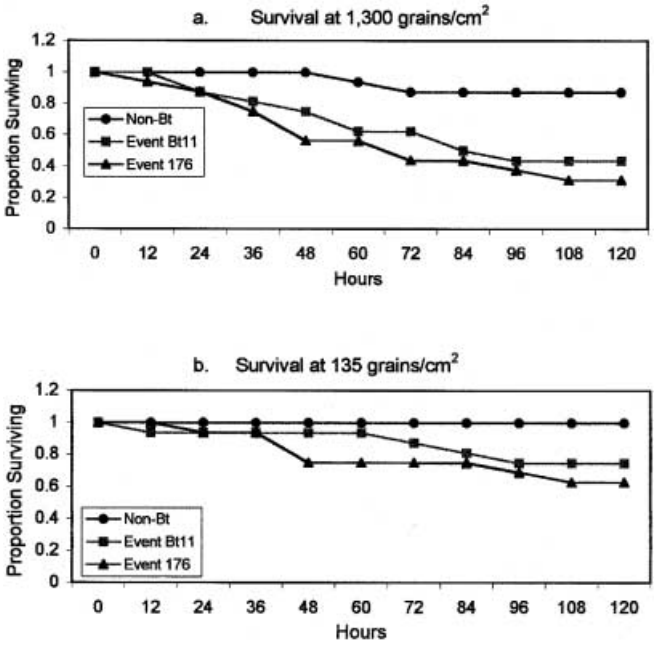

Fig. 2 Survival curves for monarch larvae exposed at 12–36 h old to 1,300 (**a**) or 135 (**b**) pollen grains/cm^2 of non-Bt, event Bt11, and event 176 corn pollen for 48 h

Adult characteristics

Only one larva survived exposure to event 176 at 1,300 pollen grains/cm^2 in either age class, so that treatment was removed from the analysis. Total development time of larvae exposed to Bt pollen when <12 h old was similar for all pollen concentrations and types (ANOVA, P>0.118, df=7; Table 2). Pupal weight, adult dry weight, lipid content, and wing length were also similar for all treatments (ANOVA, P>0.05, df=7) (Table 2). Similarly, total development time was similar for all treatments of the 12- to 36-h-old larvae (ANOVA, P=0.074, df=7) (Table 3). Pupal weights, adult dry weights, li-

Table 3 Mean (±SE) developmental time, pupal weight, wing length, and lipid content of larvae 12–36 h old exposed to non-Bt, event Bt11, or event 176 corn pollen, at a concentration of 1,300, 135, or 14 pollen grains/cm^2. Data are not presented for event 176 at 1,300 pollen grains/cm^2 because there were not enough surviving larvae. Adults with wings that had not fully expanded before drying were not included in the wing length measurements, but were included in the average lipid weight for a treatment

Pollen type	Concentration	Total development time (days)	Pupal weight (g)	Dry weight (g)	Forewing length (cm)	Lipid content (mg)
Non Bt	1,300	15.3±0.6 *n*=10	1.21±0.03 *n*=9	0.15±0.005 *n*=9	4.8±0.05 *n*=9	14.4±1.2 *n*=8
	135	14.7±0.4 *n*=16	1.13±0.03 *n*=15	0.14±0.005 *n*=14	4.6±0.05 *n*=14	14.8±0.8 *n*=14
	14	13.6±0.2 *n*=14	1.16±0.03 *n*=13	0.15±0.006 *n*=13	4.7±0.05 *n*=11	17.9±1.9 *n*=13
Event Bt11	1,300	15.5±1.0 *n*=6	1.16±0.05 *n*=6	0.15±0.009 *n*=5	4.7±0.1 *n*=5	15.8±2.2 *n*=4
	135	14.9±0.5 *n*=11	1.11±0.03 *n*=11	0.14±0.006 *n*=11	4.7±0.06 *n*=10	13.4±0.9 *n*=11
	14	14.4±0.5 *n*=14	1.22±0.03 *n*=14	0.15±0.006 *n*=12	4.7±0.05 *n*=12	16.8±1.7 *n*=12
Event 176	135	15.6±0.9 *n*=6	1.17±0.08 *n*=6	0.14±0.01 *n*=6	4.7±0.09 *n*=6	18.7±3.6 *n*=6
	14	14.1±0.3 *n*=13	1.20±0.03 *n*=13	0.15±0.006 *n*=11	4.7±0.05 *n*=11	15.1±1.2 *n*=10

pid weights, and forewing lengths were also similar (ANOVA, $P>0.05$, $df=7$; Table 3).

Lipid contents of field-collected migrating adult *D. plexippus* range from 30 to 180 mg (Gibo and McCurdy 1993). This range is higher than the lipid contents we determined (range 9.7–19.6; Tables 2, 3), presumably due to the fact that the *D. plexippus* in our study had not fed as adults.

Bt levels in pollen used in laboratory experiments

Forty-eight-hour mortality of *O. nubilalis* larvae was significantly higher on the transgenic pollen from event 176 (50±8%) and event Bt11 (75±8%) compared with non-Bt pollen (3±3%) (data arcsine transformed, ANOVA, $P=0.0001$).

The ELISA showed a low level of Bt toxin in the non-Bt pollen from corn hybrid 4494 (0.052 µg Bt/g pollen). This contamination likely occurred during our collection and sifting of pollen. The ELISA indicated that event 176 pollen contained 1.60 µg Bt/g pollen, which is less than the value reported by the EPA (1999b). The lower concentration in our analysis may be due to differences in sonication extraction method and the length of time pollen was stored at –20°C. Event Bt11 pollen had 0.39 µg Bt/g pollen, higher then reported by the EPA for the similar event MON 810 (0.09 µg/g fresh weight of pollen) (EPA 1999a). This level of Bt toxin may be due to the presence of pieces of anther in the Bt11 pollen we used in our laboratory tests. There was more anther tissue in the Bt11 pollen (43±2%) than in the event 176 (9±1%) or the non-Bt (0%) pollen. Percentages are based upon microscope examination of ten 6-mm^2 samples of a 0.03±0.002 g sample of pollen spread out in a 5.2-cm-diameter petri dish.

Discussion

Based on this study, we predict that transgenic Bt corn pollen will have a negative effect on *D. plexippus* larvae feeding in and adjacent to Bt corn fields, for the following reasons: (1) we demonstrated significant larval mortality resulting from exposure to pollen concentrations representative of field depositions, and (2) our results underestimate the mortality which is likely to be caused by field exposure to transgenic pollen because we exposed larvae to transgenic pollen for only 48 h. *D. plexippus* larvae developing in late summer are likely to be exposed to transgenic pollen for most of their development; thus, mortality may be higher due to cumulative exposure to insecticidal Bt toxin in transgenic pollen. These findings combined with the prediction that a large proportion of larvae will be in or near transgenic corn fields during pollination (Urquhart 1960; Borkin 1982; Malcolm et al. 1993; Wassenaar and Hobson 1998) indicate that the effect of transgenic Bt corn pollen on *D. plexippus* larvae may be substantial.

This raises the question of the extent of the Bt pollen effect outside corn fields. Based upon the cumulative amount of pollen deposited on the milkweed plants over a 6-day period in 1998 and a 9-day period in 1999, we predict that transgenic pollen will be deposited on milkweed plants at least 10 m from the edge of a field. However, the greatest effects on *D. plexippus* will be on those larvae feeding within a Bt corn field or within 3 m of the field edge where pollen densities are highest (Table 1).

Our study shows clearly that concentrations of pollen found on *A. syriaca* within corn fields will cause larval mortality. Exposure to 1,300 pollen grains/cm^2, which might occur on milkweed plants growing within a Bt corn field during periods of low rainfall, and exposure to 135 pollen grains/cm^2, which we observed on *A. syriaca* plants within a corn field, reduced larval survival. Larvae exposed to 1,300 grains/cm^2 when <12 h old experienced high levels of mortality when exposed to the Bt and non-Bt pollen. This observation differs from a previous study examining transgenic Bt pollen-*D. plexippus* interactions, in which 3-day-old larvae were exposed to Bt and non-Bt pollen (Losey et al. 1999). Decreased consumption rates but no larval mortality in a non-transgenic pollen treatment were observed (Losey et al. 1999). This difference is probably due to the presence of some Bt toxin in the non-Bt pollen and to the age of the larvae used.

At 135 pollen grains/cm^2 the two Bt events caused similar mortality, despite the higher levels of Bt toxin in the event 176 pollen (fourfold more than in the event Bt11 pollen). The Bt11 pollen had higher levels of the Bt toxin than previously reported (EPA 1999a), possibly due to the presence of anthers in the pollen. Bt11 hybrid pollen was collected and handled using the same procedures as for the other two hybrids. The presence of anthers in the pollen may indicate that this hybrid sheds these structures in nature; thus the levels of Bt toxins deposited on non-target plants may be higher than Bt toxin levels reported solely from transgenic pollen.

Developmental time and adult characteristics of larvae surviving the 48-h exposure showed no sub-lethal effects of transgenic corn pollen exposure. A reduction in adult lipid levels could indicate that a larva fed less, or did not digest nutrients as efficiently, due to ingestion of Bt toxins. Migratory adult *D. plexippus* rely on lipids for energy (Cenedella 1971); thus a lower level of lipids carried over from the larval stage could reduce their ability to reach Mexico. Similarly, reduced adult weight or smaller wing lengths could decrease the ability of an adult to complete migration. Increased time spent in the vulnerable larval stages, where 92–98% mortality can occur by the last instar (Zalucki and Kitching 1982), would also be a negative impact of Bt pollen exposure. Additional studies are required to determine if continuous larval exposure to Bt pollen influences developmental time or adult characteristics.

Our study quantifies the non-target effects of transgenic Bt corn pollen on one species of phytophagous Lepidoptera, extending the insecticidal effects of a transgenic crop beyond field borders, and demonstrating that this genetically modified crop can influence food webs that are not corn based. Previous studies showing non-target effects following widescale spraying of microbial insecticide formulations of *B. thuringiensis* (Wagner et al. 1996; Whaley et al. 1998) indicate that many Lepidoptera are susceptible. Thus those species whose larval stages are susceptible and occur in habitats near transgenic fields during late summer are potentially at risk from transgenic pollen drift. Wraight et al. (2000) reported that the black swallowtail, *Papilio polyxenes* (Lepidoptera: Papilionidae), was not susceptible to the Bt toxin in corn pollen, and so will face minimal risk from the planting of Bt corn. Transgenic insecticidal crops may be relatively safer compared to broad-spectrum insecticides (Federici 1998), but this comparison may not be appropriate if previous insecticide use against the target pest was low (J.J. Obrycki et al., unpublished data). For example, in 1995, only 2.2% of the corn in Iowa was treated with broad-spectrum insecticides for suppression of the European corn borer, *O. nubilalis* (Wintersteen and Hartzler 1997), the target pest species for transgenic corn in Iowa. Thus, the widespread planting of transgenic corn represents a potentially significant novel mortality factor for non-target species near agricultural fields. Our study and others (Hilbeck et al. 1998; Birch et al. 1999; Saxena et al. 1999; Wraight et al. 2000) indicate that the registration process for transgenic crops may need to take a broader ecological perspective of non-target effects. In December 1999, 4 years after initial registration, the EPA issued a call for data to address the non-target effects of Bt corn pollen on *D. plexippus* and the endangered Karner blue butterfly, *Lycaeides melissa samuelis* (Lepidoptera: Lycaenidae) (EPA 2000).

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