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Effects of Cry1Ab-Expressing Corn Anthers on Monarch Butterfly Larvae

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Abstract
Previous studies suggest that exposure to corn, *Zea mays* L., anthers expressing *Bacillus thuringiensis* (Bt)-derived protein may have adverse effects on the larvae of monarch butterfly, *Danaus plexippus* (L.). To examine the potential effects of Bt anthers on monarch butterflies, studies were designed to test toxicity in the laboratory; examine anther distribution in space and time; compare distributions of anthers, pollen, and larval feeding; and measure effects of long-term exposure in the field. In the laboratory, monarch butterfly larvae fed on whole corn anthers, but anther feeding was sporadic. Larvae exposed to 0.3 anther/cm^2 fed and weighed less after 4 d compared with larvae exposed to non-Bt anthers. Adverse effects increased with increasing anther density. Monarch butterfly larvae exposed to 0.9 anther/cm^2had reduced feeding, weight, and survival and increased developmental time compared with larvae exposed to non-Bt anthers. Later instars were more tolerant of Bt toxin. For all studies, laboratory testing probably magnified effects because larvae were confined to petri dishes. Field studies showed toxic anther densities are uncommon on milkweed (*Asclepias*) leaves in and near cornfields during anthesis. Mean anther densities on milkweed leaves in cornfields during peak anthesis were between 0.06 and 0.1 anther/cm^2 ($\approx$ 3–5 anthers per leaf). When exposure to a density of five anthers per leaf was tested in field-cage studies, no effects on growth, development, or survival were detected. Based on probability of exposure to toxic densities, Bt anthers alone are not likely to pose a significant risk to monarch butterflies in Iowa.

Keywords
transgenic corn, nontargets, risk assessment, Danaus plexippus

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Effects of Cry1Ab-Expressing Corn Anthers on Monarch Butterfly Larvae

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ABSTRACT Previous studies suggest that exposure to corn, *Zea mays* L., anthers expressing *Bacillus thuringiensis* (Bt)-derived protein may have adverse effects on the larvae of monarch butterfly, *Danaus plexippus* (L.). To examine the potential effects of Bt anthers on monarch butterflies, studies were designed to test toxicity in the laboratory; examine anther distribution in space and time; compare distributions of anthers, pollen, and larval feeding; and measure effects of long-term exposure in the field. In the laboratory, monarch butterfly larvae fed on whole corn anthers, but anther feeding was sporadic. Larvae exposed to 0.3 anther/cm^2 fed and weighed less after 4 d compared with larvae exposed to non-Bt anthers. Adverse effects increased with increasing anther density. Monarch butterfly larvae exposed to 0.9 anther/cm^2 had reduced feeding, weight, and survival and increased developmental time compared with larvae exposed to non-Bt anthers. Later instars were more tolerant of Bt toxin. For all studies, laboratory testing probably magnified effects because larvae were confined to petri dishes. Field studies showed toxic anther densities are uncommon on milkweed (*Asclepias*) leaves in and near cornfields during anthesis. Mean anther densities on milkweed leaves in cornfields during peak anthesis were between 0.06 and 0.1 anther/cm^2 ($\approx$ 3–5 anthers per leaf). When exposure to a density of five anthers per leaf was tested in field-cage studies, no effects on growth, development, or survival were detected. Based on probability of exposure to toxic densities, Bt anthers alone are not likely to pose a significant risk to monarch butterflies in Iowa.

KEY WORDS transgenic corn, nontargets, risk assessment, *Danaus plexippus*

A LABORATORY STUDY BY Losey et al. (1999) suggested that the larvae of monarch butterfly, *Danaus plexippus* (L.), may be adversely affected by consuming corn, *Zea mays* L., pollen expressing *Bacillus thuringiensis* (Bt) protein that falls onto the leaves of common milkweed, *Asclepias syriaca* L., in Bt cornfields. Although corn pollen is naturally deposited onto milkweed plants in or near cornfields, a risk assessment by Sears et al. (2001) concluded that the impact of Bt corn pollen from current commercial hybrids on monarch butterfly populations is negligible (Hellmich et al. 2001, Oberhauser et al. 2001, Pleasants et al. 2001, Stanley-Horn et al. 2001, Zangerl et al. 2001). Recent studies also suggest that Bt corn anthers could be a hazard to monarch butterfly larvae (Jesse and Obrycki 2000, Hellmich et al. 2001). Adverse effects of anther ingestion have been documented in the laboratory, but only when larvae ate pulverized anthers, an artifact of pollen processing (Hellmich et al. 2001). An examination of anthers in and near cornfields showed no evidence that crushed anther pieces occur naturally (Hellmich et al. 2001). Although whole corn anthers do commonly occur on milkweed leaves in cornfields (Jesse and Obrycki 2000, Pleasants et al. 2001), specific data on the spatial and temporal distribution of anthers on milkweed plants in and near cornfields is lacking. It is also unknown whether monarch larvae will feed on whole Bt corn anthers or whether this feeding has adverse effects.

To explore the risk of Bt anthers to monarch larvae, studies were designed to 1) measure effects of short- and long-term exposure in the laboratory; 2) examine anther distribution in space and time; 3) compare distributions of anthers, pollen, and larval feeding; and 4) measure effects of long-term exposure in the field.

Materials and Methods

General Protocol. For all experiments, laboratory and field, monarch butterfly larvae used were from a colony established from eggs collected near Ames, IA, during the spring of each respective year. Larvae were maintained on fresh milkweed leaves. All bioassays used the same petri dish arenas and protocols for surface sterilizing milkweed leaves and assessing leaf consumption as the Iowa studies in Hellmich et al. (2001) unless otherwise noted. The top, inner surface of each petri dish (60 by 15-mm Fisherbrand, Fisher,

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Pittsburgh, PA) also was coated with a thin layer of agar (≈1 mm) to reduce static electricity and keep anthers randomly distributed on the surface of milkweed leaf disks (2.1 cm in diameter). Using a camel's-hair brush, one monarch butterfly larva was placed in each dish. After 4 d, larvae were transferred to larger petri dishes (100 by 15-mm Fisherbrand, Fisher) coated with agar on the inner surfaces. A milkweed leaf disk (7.8 cm^2 in diameter) was placed in each petri dish with sufficient anthers added to maintain the same anther densities as the smaller dishes. Anthers were collected and processed using the same methods that Hellmich et al. (2001) used for pollen collection. Anthers had dehisced; however, some pollen remained in the anthers. Leaf and anther material were replaced every other day. Using a Nikon Stereo-Zoom dissecting microscope with an eyepiece reticle grid, anthers were checked every other day for feeding until day 10. At this time, larvae were transferred to inverted 236 ml (8-oz.) clear plastic cups (Waddington North America Inc., Chlemsford, MA) placed on a large petri dish lid and fed milkweed leaves with no anthers until pupation. Bioassays were incubated at 25°C, 8-h scotophase, and 60% RH.

Single Anther Density Bioassay. This bioassay was conducted twice with greenhouse-grown tropical milkweed, *Asclepias curassavica* L., and twice with field-collected common milkweed. Treatments included milkweed leaves with Bt, non-Bt, or no anthers. Anthers were collected from Bt hybrid N79-L3 (Bt11 event, Syngenta Seeds, Golden Valley, MN) and its near isoline N79-P4 (Syngenta Seeds) and were surface sterilized in a 5% solution of bleach (6% sodium hypochlorite) for 10 min. For treatments with anthers, three anthers were placed on each 2.1-cm-diameter leaf disk (0.9 anther/cm^2). This density was equivalent to 45 anthers per whole common milkweed leaf assuming a mean leaf size of 50 cm^2 (based on measurements of common milkweed leaves inside cornfields in the anther distribution study). This density was maintained throughout the experiment. Treatments were replicated 25 times in each of the tropical milkweed trials and 27 and 30 times for the two common milkweed trials. Data recorded included 4-d leaf feeding (square millimeters), 4- and 10-d larval weight (milligrams), number of days to pupation and eclosion, pupal weight (milligrams), percentage of survival to pupation and eclosion, and total anther feeding (square millimeters). Larvae were checked once daily for pupation and eclosion. Tropical milkweed was used during the winter when common milkweed was not available. These experiments were not designed to test the effects of different milkweed types. To account for potential effects on the measured variables, each trial was considered a random block in the combined analysis. Trial and trial by anther treatment were treated as random effects in the analysis of variance (ANOVA) (Littell et al. 1996).

Multiple Anther Density Bioassay. In experiment 1, neonates were exposed to treatments through the fifth instar. In experiment 2, third instars (previously fed a normal diet of surface sterilized common milkweed leaves) were exposed to treatments through the fifth instar. Treatments for both experiments included the following densities on common milkweed: 0.3, 0.6, 0.9, and 1.2 Bt anthers/cm^2, 1.2 non-Bt anthers/cm^2, and no anthers (anther densities equivalent to 15, 30, 45, and 60 anthers per whole common milkweed leaf based on mean leaf size of 50 cm^2). Anthers were collected from Bt hybrid N79-L3 and its near isoline N79-P4 and were surface sterilized as described previously. Each treatment was replicated 28 times in the first experiment and 25 times in the second. Data recorded included 4-d leaf feeding (experiment 1 only), 4- and 10-d larval weight (experiment 1 only), or larval weight gain (experiment 2 only), number of days to pupation and eclosion, pupal weight, adult weight (experiment 2 only) and total anther feeding. For each experiment, a one-way ANOVA was conducted (Littell et al. 1996).

Data Transformations. For all studies, normality and homogeneity of variance were assessed by examination of normal probability and residual plots. Based on these examinations, larval weights were log transformed before analysis. PROC MIXED was used to calculate restricted maximum likelihood estimate for *F* values in each ANOVA (Littell et al. 1996). For all studies, Tukey's studentized range test was used to separate means ($P \leq 0.05$; Littell et al. 1996).

Anther Distribution in Space and Time. *Iowa.* In 2001, before corn anthesis, naturally occurring common milkweed plants were located inside two nontransgenic cornfields near Ames (18 and 20 plants, respectively). In 2002, four nontransgenic fields were selected near Ames. Before corn anthesis in 2002, eight potted common milkweed plants from locally collected rhizomes were placed at five distances: 5 m inside the field; at the field edge (0 m); and 1, 3, and 5 m away from the cornfield. For both years, each milkweed plant was divided into an upper, middle, and lower third by marking the stem with a permanent marker. One leaf in each of the upper, middle, and lower sections of the plant was marked. The length and width of each leaf were taken to estimate area. Every other day, counts were taken on number of anthers in the upper, middle, and lower third of the plant, and on the number of anthers per marked leaf until no anthers remained on the plants.

Ontario. Field-cage studies were conducted during anthesis in 14 cornfields in 2001 and 18 fields in 2002 in Wellington County, Ontario, Canada. Cage design and experimental protocols are described in Dively et al. (2004); data on pollen and anther densities and larval feeding patterns are presented here. Consumption of leaf material and pollen and anther densities were estimated by removing all leaves from each plant after exposure to larvae, noting their position on the plant and bringing them back to the laboratory for analysis. To minimize loss of pollen and anthers, all leaves were encased in strips of contact paper (ConTact7 Brand, Decora Manufacturing, North Ridgeville, OH).

Consumption was measured by creating a digital image of the leaf (XC-75CE black-and-white video-

Table 1. Effects on growth, development, and survival of monarch butterfly larvae exposed to a density of 0.9 anther/cm^2 in the laboratory

Response variable	Treatment			$F_{(df)}$	P
	Bt anthers	Non-Bt anthers	No anthers		
Leaf feeding 4 d (mm^2)	155.0b	259.4a	275.7a	$16.6_{(2,6)}$	0.004
Log larval wt 4 d	2.0b	2.8a	2.8a	$16.4_{(2,6)}$	0.004
Log larval wt 10 d	2.4b	2.7a	2.7a	$10.7_{(2,6)}$	0.010
Days to pupation	14.5a	13.4b	13.3b	$7.1_{(2,6)}$	0.025
Pupal wt (mg)	1064.8	1107.1	1044.2	$1.4_{(2,6)}$	0.320
Days to eclosion	26.3a	25.2b	25.1b	$6.0_{(2,6)}$	0.037
% Survival to pupation	61.5b	91.4a	85.0ab	$7.5_{(2,6)}$	0.023
% Survival to eclosion	52.8b	80.6a	81.2a	$7.4_{(2,6)}$	0.024
Anther feeding (mm^2)	1.5b	7.9a		$106.9_{(1,2)}$	0.009

Means in a row with the same letter are not significantly different ($P \leq 0.05$).

Anthers were from N79-L3 (Bt11 event) and near isoline N79-P4 (Syngenta Seeds). Anther density equivalent to 45 anthers per leaf based on a mean common milkweed size of 50 cm^2.

camera module and a Cosmicar/Pentax 16-mm TV lens) and using image analysis software (Northern Exposure 2.9e, Empix Imaging, Inc., Mississauga, ON, Canada). Pollen and anther densities also were determined for each leaf. Pollen adhering to the contact paper strips after they were removed from the leaves was stained with acid fuchsin (Sigma-Aldrich, Oakville, Ontario, Canada) to facilitate counting. Pollen was counted within five or three (2001 and 2002, respectively) 1-cm^2 areas on the top and bottom strips and on the top and bottom of the leaf itself. Pollen counts for leaves and strips were added to estimate total pollen density in grains per square centimeter on the top and bottom of each leaf. All anthers on each leaf were counted to determine the anther density per leaf.

Anther Exposure in the Field. Field-cage studies were conducted at three times, 23 July and 1 August 2002 and 22 July 2003 in Ames. Each field cage study was considered a block for the analysis. The fields were ≈1.5 ha and were planted with nontransgenic field corn. Cages, placed in a 30 by 30-m section of detassled corn, consisted of a 191 (5-gal) pot (Nursery Supplies, Fairless Hills, PA) containing one common milkweed plant (≈50 cm in height) with a wire tomato cage placed into the soil in the pot. To exclude predators, a mesh bag made of no-see-um netting (Arrowhead Fabric Outlet, Duluth, MN) was used to enclose the cage. There were 10 replications of three treatments in each study: 1) Bt anthers, 2) non-Bt anthers, and 3) no anthers. For anther treatment cages, five anthers were placed on each leaf. Based on a mean leaf size of 50 cm^2, the mean anther density per leaf was ≈0.1 anther/cm^2. Anthers were Bt hybrid N58-D1 (Bt11 event, Syngenta Seeds) or its near isoline N58-F4 (Syngenta Seeds). Five monarch butterfly neonates were placed in each cage.

On day 6 of each experiment, surviving larvae were transferred to a new plant with the appropriate treatment applied. Larvae stayed on the second plant for 5 d, after which, they were removed, brought back to the laboratory, weighed, and fed common milkweed leaves until pupation. Data recorded included 11-d larval weight, number of days to pupation and eclosion, pupal and adult weight, and percentage of survival to pupation and eclosion. Analyses were run on cage means. Block and block by anther treatment were treated as random effects in the ANOVA (Littell et al. 1996).

Results

Single Anther Density Bioassay. Larvae exposed to a Bt anther density of 0.9 anther/cm^2 had a 40% reduction in 4-d leaf feeding; 27 and 11% reduction in 4- and 10-d larval weights, respectively (based on log transformed data); 30 and 28% reduction in survival to pupation and eclosion, respectively; and a 1.1-d delay in development compared with larvae exposed to non-Bt anthers (Table 1). There were no differences detected among treatments for pupal weight. Larvae exposed to non-Bt anthers fed on significantly more anther material than larvae exposed to Bt anthers. Larvae exposed to no anthers and those exposed to non-Bt anthers did not differ in any variables measured.

Multiple Anther Density Bioassay. In experiment 1, larvae exposed to 0.3, 0.9, or 1.2 anthers/cm^2 had reduced leaf feeding at 4 d compared with larvae exposed to 1.2 non-Bt anthers/cm^2 or no anthers (Table 2). For all Bt anther treatments, larvae weighed less at 4 d compared with larvae exposed to 1.2 non-Bt anthers/cm^2 or no anthers. Larvae exposed to 1.2 Bt anthers/cm^2 weighed less at 10 d than larvae in all other treatments. There were no differences detected among treatments for days to pupation or pupal weight. Larvae exposed to 0.9 or more Bt anthers/cm^2 took 0.7–1.8 d longer to eclose compared with larvae exposed to non-Bt anthers (Table 2). Larvae exposed to 1.2 Bt anthers/cm^2 consumed less anther material than larvae exposed to 1.2 non-Bt anthers/cm^2.

In experiment 2, only larvae exposed to 1.2 Bt anthers/cm^2 had reduced weight gain compared with those exposed to non-Bt anthers or no anthers (Table 2). No significant differences were detected among treatments for days to pupation or eclosion or pupal or adult weight. Anther feeding by larvae exposed to 1.2 Bt or non-Bt anthes/cm^2 was not significantly different (Table 2).

Table 2. Effects on growth and development of monarch butterfly larvae exposed to multiple anther densities on common milkweed in the laboratory

Response variable	Experiment 1: Exposure first–fifth instar							
	Treatment (anthers/cm^2)							
	0.3 Bt	0.6 Bt	0.9 Bt	1.2 Bt	1.2 Non-Bt	None	$F_{(df)}$	P
Leaf feeding 4 d (mm^2)	188.0b	195.8ab	183.2b	163.0b	239.4a	235.4a	$3.9_{(5,159)}$	0.002
Log larval wt 4 d	2.4b	2.6b	2.5b	2.5b	3.1a	2.8a	$3.3_{(5,159)}$	0.007
Log larval wt 10 d	6.3a	6.1a	6.2a	5.6b	6.3a	6.3a	$4.0_{(5,132)}$	0.002
Days to pupation	12.4	12.7	13.0	13.2	12.6	13.2	$1.3_{(5,126)}$	0.263
Pupal wt (mg)	1113.3	1141.8	1168.7	1192.9	1160.1	1150.0	$0.8_{(5,123)}$	0.557
Days to eclosion	23.7d	24.4bc	24.7b	25.8a	24.0cd	23.7d	$10.1_{(5,108)}$	<0.001
Anther feeding (mm^2)	0.6c	1.5b	0.6c	1.4bc	4.4a		$6.5_{(5,133)}$	<0.001
	Experiment 2: Exposure third–fifth instar							
Log larval wt gain	6.0a	6.0a	5.9a	5.5b	6.1a	6.0a	$2.4_{(5,145)}$	0.038
Days to pupation	15.5	15.6	15.7	15.7	15.4	15.6	$0.9_{(5,137)}$	0.470
Pupal wt (mg)	1174.1	1198.7	1196.0	1176.5	1171.2	1176.6	$0.2_{(5,136)}$	0.947
Days to eclosion	27.1	27.4	27.6	27.4	27.4	27.2	$0.9_{(5,111)}$	0.476
Adult wt (mg)	437.1	458.0	453.2	423.6	432.2	457.7	$0.3_{(5,110)}$	0.934
Anther feeding (mm^2)	0.7b	0.8b	2.0ab	2.3a	3.2a	—	$4.1_{(5,121)}$	0.004

Means within a row followed by the same letter are not significantly different ($P \leq 0.05$).

Anthers were from N79-L3 (Bt11 event) and near isoline N79-P4 (Syngenta Seeds). Anther densities equivalent to 15, 30, 45, and 60 anthers per leaf based on a mean common milkweed size of 50 cm^2.

Anther Distribution in Space and Time. *Iowa.* Anther densities decreased rapidly with increased distance outside the cornfield (Table 3). In 2001, anthers remained on milkweed leaves inside cornfields for 25 d, and in 2002, for 21 d (Fig. 1). At peak anther shed, the mean number of anthers per square centimeter inside the cornfield in 2001 was 0.06 anther, and in 2002, 0.09 anther. Peak anther densities occurred 7 d after initiation of anther shed in 2001, 9 d after initiation in 2002 (Fig. 1). Most anthers, 54%, were found in the middle third of the plant, whereas 16 and 30% were found in the upper and lower sections, respectively.

Ontario. Most larval feeding, 54.2%, occurred in the upper eight leaves of milkweed plants where only 3.6% of anthers and 16.8% of pollen were deposited. Most anthers and pollen were found in the middle section of the plant (leaves 9–24), 67.0 and 50.4%, respectively, where 35.4% of larval feeding occurred. The lower section of the plant (leaves 25–36) had 29.4 and 32.8% of anthers and pollen, respectively, and 10.4% of larval feeding.

Anther Exposure in the Field. When monarch butterfly larvae were exposed to five anthers per leaf (≈0.1 anther/cm^2) in the field, no differences were detected among treatments for larval, pupal, or adult weights; days to pupation or eclosion; or survival to pupation or eclosion (Table 4).

Table 3. Frequency distribution of anther densities on milkweed leaves inside cornfields (2001 and 2002) and near cornfields (0, 1, 3, and 5 m away, 2002), Ames, IA

Anthers/cm^2	Inside cornfield	From edge of cornfield (m)			
		0	1	3	5
0	0.499	0.886	0.934	0.988	1.000
0.01–0.10	0.303	0.097	0.065	0.012	
0.11–0.29	0.157	0.017			
0.30–0.59	0.034	0.001			
0.60–0.89	0.003				
0.90–1.20	0.002				
≥1.20	0.002				

Samples size (n): inside cornfield, 3,591; 0, 1, 3, and 5 m, 1,200.

Discussion

Laboratory studies showed that monarch butterfly larvae will feed on whole corn anthers on milkweed leaves, but such feeding is sporadic. Placing the same number of anthers per leaf in each trial did not guarantee that larvae would eat equal amounts of anther material. Anther feeding seemed to be inadvertent, usually not occurring until larvae were third instars or older, when milkweed leaf consumption was high. When comparing Bt and non-Bt anther treatments with the same anther densities, larvae exposed from first through fifth instar showed significant differences in anther feeding, whereas larvae exposed from third through fifth instar did not (Tables 1 and 2). The decrease in anther feeding with earlier exposure may be a result of more selective feeding by early instars or it may be a function of reduced leaf feeding from Bt intoxication. The fact that the same effect was not seen when exposure was from third through fifth instar indicates that later instars are more tolerant of Bt toxin.

Monarch butterfly larvae exposed to a single, high density of 0.9 Bt anther/cm^2 in the laboratory from first through fifth instar fed and weighed less, took longer to develop, and had reduced survival compared with larvae exposed to non-Bt anthers. Effects on larval fitness were seen as early as 4 d after exposure. At 4 d, anther feeding was only detected in 8% of the Bt petri dishes. No clear evidence of anther feeding was seen in the other 92%. Consequently, the effects seen at 4 d (reduced leaf feeding and reduced larval weight) were probably not caused by direct effects of

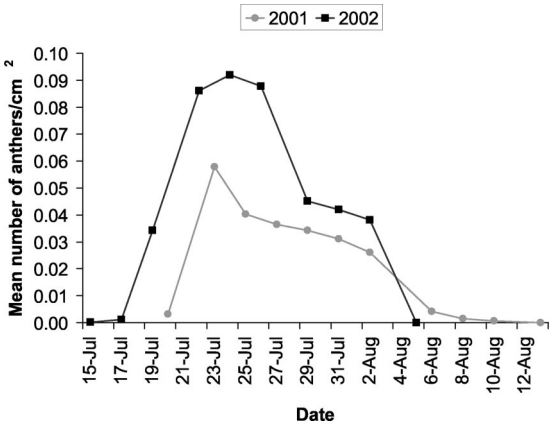

Fig. 1. Mean number of anthers per square centimeter on milkweed leaves inside cornfields for 2001 and 2002, Ames.

Bt ingestion but perhaps by indirect effects such as increased searching to avoid Bt ingestion.

In the first multiple anther density study with exposure from first through fifth instar, the most severe effects were at densities of 0.9 anther/cm^2 or greater. Some effects were seen early in development (4 d) at densities of 0.3 or 0.6 anther/cm^2; however, later measurements of fitness were not affected. Effects on larval weight were seen at all Bt anther densities after 4 d of exposure. Similar to the single density bioassays, no anther feeding was detected at 4 d. This further reinforces the possibility that larvae are being affected indirectly, without ingestion, by the presence of Bt anthers. Effects on larvae without ingestion may only occur in the laboratory where larvae cannot avoid anthers by moving to another area to feed. If increased searching behavior in the presence of Bt anthers occurs, this could indicate that monarch butterfly larvae can detect and attempt to avoid Bt. In the field, increased searching would probably only result in a fitness cost such as reduced feeding or reduced larval weight if all leaves on a milkweed plant had high densities of anthers. If this phenomenon affected larval movement off the plant, it could have implications on larval survival (Rawlins and Lederhouse 1981, Borkin 1982, Zangerl et al. 2001). More studies on larval behavior are necessary to determine how larvae are affected by Bt anthers without actual ingestion. In the second multiple anther density study with exposure from third through fifth instar, effects were only detected at a density of 1.2 Bt anthers/cm^2. These data show that later instars are more tolerant of Bt toxin and are consistent with previous studies that used purified toxin (Hellmich et al. 2001).

It is important to note that laboratory testing probably magnified effects because larvae were confined to petri dishes, which restricted their movement and natural behaviors and may have caused larvae to encounter more anthers than they would have in the field. Our data and previous studies show that monarch butterfly larvae are most sensitive to Bt during the first 4 d of development, when larvae are first and second instars (Zalucki 1982, Hellmich et al. 2001). When no anthers or pollen were present on milkweed leaf disks in laboratory experiments, larvae consumed an average of 2.5 cm^2 of leaf material during the first 4 d. Using the average common milkweed leaf size of 50 cm^2, larvae consumed 5% of a whole milkweed leaf during their first 4 d of development. This slow feeding rate decreases the chances of a first or second instar encountering an anther. The natural feeding behavior of first and second instars also decreases their chances of encountering an anther. Based on our field observations and previous studies, anthers typically are not randomly distributed on a leaf but are grouped around the midrib on the top of the leaf (Pleasants et al. 2001). Most early instars feed on the underside of the leaf and avoid the midrib, thereby avoiding anthers (Rawlins and Lederhouse 1981, Pleasants et al. 2001, Jesse and Obrycki 2003). During the first 4 d of our laboratory experiments, due to confined conditions, all anthers were encountered at least once, but, on average, only 11.9% of anthers showed evidence of feeding. Thus, because of low feeding rates and natural feeding behavior, encounters with anthers are probably low and encounters that result in feeding are probably even lower in the field.

Although laboratory studies probably magnified the effects of Bt anthers, they indicate that Bt anthers pose a potential hazard to monarch butterfly larvae and begin to assess the range of toxic levels. Anther distribution studies showed that toxic anther densities

Table 4. Effects on growth, development, and survival (mean ± SE) of monarch butterfly larvae exposed to five anthers per leaf on common milkweed in field-cage studies, Ames, IA, 2002 and 2003

Response variable	Treatment			$F_{(df)}$	P
	Bt anthers	Non-Bt anthers	No anthers		
Log larval wt 11 d	2.8 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	$2.4_{(2,4)}$	0.207
Days to pupation	14.6 ± 0.8	14.4 ± 0.8	14.5 ± 0.8	$0.4_{(2,4)}$	0.677
Pupal wt (mg)	1220.6 ± 42.0	1275.7 ± 41.8	1283.6 ± 41.2	$1.8_{(2,4)}$	0.280
Days to eclosion	26.6 ± 1.0	26.6 ± 1.0	26.7 ± 1.0	$0.1_{(2,4)}$	0.872
Adult wt (mg)	487.8 ± 28.5	513.0 ± 28.5	525.6 ± 27.9	$1.2_{(2,2)}$	0.449
% Survival to pupation	86.7 ± 3.5	95.4 ± 3.4	99.1 ± 3.4	$4.4_{(2,4)}$	0.100
% Survival to eclosion	80.8 ± 3.9	94.6 ± 3.7	93.4 ± 3.7	$4.0_{(2,4)}$	0.111

Means in a row with the same letter are not significantly different ($P \leq 0.05$).

Anthers were from N58-D1 (Bt11 event) and near isoline N58-F4 (Syngenta Seeds). Five anthers per leaf ≈0.1 anther/cm^2 based on a mean common milkweed size of 50 cm^2.

were rare in and near cornfields during anthesis. Anther densities dropped rapidly with increased distance from the field. At 5 m away from the field, none of the milkweed leaves examined had anthers. At the field edge, only one leaf (0.1% of the leaves examined) had a density ≥0.3 anther/cm^2. Milkweed plants inside cornfields have the most potential to contain anther densities that were shown to be potentially toxic in the laboratory. Densities of 0.9 anther/cm^2 or greater were rare on milkweed leaves inside cornfields, occurring on 0.4% of leaves examined. Densities of 0.3 anther/cm^2 or greater were observed on 4.1% of milkweed leaves examined inside the fields. Although this density is not as rare as 0.9 anther/cm^2, the effects seen in the laboratory when larvae were exposed to 0.3 or 0.6 anther/cm^2 were only seen early in development, were not apparently caused by direct anther feeding, and may have been magnified because larvae were confined to petri dishes, which restricted their movement and natural behaviors and may have caused larvae to encounter more anthers than they would have naturally in the field.

At peak anther shed, larvae were more likely to encounter mean densities of 0.06–0.10 anther/cm^2 (three to five anthers per leaf). When densities of five anthers per leaf were tested in Iowa field-cage trials, no adverse effects on growth, development, or survival were detected. Despite the presence of anthers on every leaf, it is possible that larvae had more opportunity to avoid anthers in the field than in the laboratory. When anthers are deposited naturally on milkweed leaves, they are not distributed as they were in the cage studies (five anthers on every leaf, randomly, but fairly evenly, distributed on each leaf). Anthers tend to gather in the midrib, and the leaves in the middle of the plant canopy tend to have the largest deposits of anthers. Small larvae, which are most susceptible to Bt toxin, tend to avoid the midrib and feed on the underside of the leaf, reducing their chances of encountering toxic levels of Bt anthers (Rawlins and Lederhouse 1981, Pleasants et al. 2001, Jesse and Obrycki 2003). Field-cage studies in Ontario indicated a clear separation of feeding activity from areas with the heaviest deposits of pollen and anthers, effectively reducing exposure to toxic Bt levels. Our laboratory studies suggest that monarch butterfly larvae may be able to detect and avoid Bt anthers, potentially reducing their exposure. Also, rain, wind, and larval behavior, such as vein clipping, removed some anthers on milkweeds in the field.

Although laboratory studies indicated that Bt anthers are a potential hazard to monarch butterfly larvae, field studies showed that toxic anther densities are rare in and near cornfields during anthesis. Field-cage studies testing common anther densities did not show significant effects on larvae. Based on the probability of exposure to toxic densities, Bt anthers alone are not prone to pose a significant risk to monarch butterflies in Iowa.

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