Effects of Cry1Ab-Expressing Corn Anthers on the Movement of Monarch Butterfly Larvae

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ABSTRACT Decreased larval feeding and weight of the monarch butterfly, *Danaus plexippus* L., have been detected after 4 d of exposure in the laboratory to a high density of *Bacillus thuringiensis* (*Bt*)-expressing anthers. One hypothesis is that larvae exposed to *Bt* anthers exhibit increased wandering, resulting in less feeding and lower weight gain. To test this hypothesis, 2-d-old monarch butterfly larvae exposed to milkweed leaf disks with no anthers, anthers that express *Bt* (Cry1Ab, event MON810), or other non-*Bt* anthers were observed using a video-tracking system. As had been shown in previous studies, larvae exposed to *Bt* anthers fed less and gained less weight than larvae exposed to non-*Bt* or no anthers, yet there was no evidence of feeding on anthers. Total distance moved, maximum displacement from release point, percentage of time spent moving or near anthers, or mean turn angle did not differ across treatments. However, larvae exposed to *Bt* anthers spent more time off milkweed leaf disks than those exposed to no anthers and were more likely to move off the leaf than larvae exposed to non-*Bt* anthers. Results suggest that larvae exposed to *Bt* anthers behave differently and that ingestion may not be the only way *Bt* can affect nontarget insects like the monarch butterfly.

KEY WORDS transgenic corn, nontargets, *Danaus plexippus*, behavior, EthoVision

During anthesis, pollen and anthers from corn, *Zea mays* L., that express *Bacillus thuringiensis* (*Bt*)-derived protein are naturally deposited onto leaves of common milkweed, *Asclepias syriaca* L., in *Bt* corn fields (Pleasants et al. 2001, Anderson et al. 2004). A laboratory study by Losey et al. (1999) suggested that larvae of the monarch butterfly, *Danaus plexippus* L., may be adversely affected by consuming milkweed leaves dusted with *Bt* corn pollen. There is also evidence that *Bt* anthers may harm monarch butterflies (Jesse and Obrycki 2000, Hellmich et al. 2001, Anderson et al. 2004, 2005). However, subsequent laboratory and field studies have concluded that, because of low exposure to toxic *Bt* doses, the impact of *Bt* corn anthers and pollen on monarch butterfly populations in North America is negligible (Hellmich et al. 2001, Oberhauser et al. 2001, Pleasants et al. 2001, Stanley-Horn et al. 2001, Sears et al. 2001, Zangerl et al. 2001, Anderson et al. 2004, 2005, Dively et al. 2004).

Anderson et al. (2004) have shown that monarch butterfly larvae may be affected by *Bt* anthers without ingestion of the toxin. In two studies, larvae exposed to *Bt* anthers for 4 d showed little evidence of feeding on anthers but weighed and fed less than larvae exposed to non-*Bt* or no anthers. Larval weight was reduced 16–27% and leaf feeding was reduced 21–40%. Before anthers were presented to the larvae in these experiments, they were examined under a dissecting microscope (×6–60) to ensure they were undamaged. After 4 d of larval exposure, they were reexamined for evidence of feeding. In the "multiple anther density bioassay," no evidence of anther feeding was detected in any of the 112 petri dishes with *Bt* anthers (Anderson et al. 2004). In the "single anther density bioassay," only 9 of 107 larvae given *Bt* anthers (8%) showed any detectable amount of feeding (Anderson et al. 2004). The mean area of anther tissue consumed by the nine larvae was small (0.9 mm^2).

To explain the reduced feeding and weight gain, we propose the following hypothesis: larvae exposed to *Bt* anthers exhibit increased wandering behavior and therefore feed less. To test this hypothesis, 2-d-old monarch butterfly larvae exposed to *Bt* anthers, non-*Bt* anthers, or no anthers in petri dish arenas were observed with the EthoVision video-tracking system (Noldus Information Technology 2002, Noldus et al. 2002). EthoVision has been used to examine the behavior of a number of arthropod species (Blanché et al. 1996, Kröber and Guerin 1999, Belmain et al. 2000, Drost et al. 2000, Szentesi et al. 2002, Belgacem and Martin 2002). However, its use to examine the behav-

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ioral response of monarch butterfly larvae to the presence of *Bt* toxin is novel.

Materials and Methods

Insects and Plant Material. Monarch butterfly larvae were from a colony established with ≈1,200 eggs collected from 25 locations in and near Ames, IA, from 21 May to 19 June 2003. Common milkweed leaves, harvested from nonagricultural areas in Ames, IA, were sterilized in a 0.6% solution of sodium hypochlorite for 10 min followed by three 1-min rinses in a salad spinner with tap water. All adults tested negative for the presence of the protozoan parasite *Ophryocystis elektroscirrha* (Altizer et al. 2000). High parasite loads of *O. elektroscirrha* can decrease larval survival (Altizer and Oberhauser 1999). Anthers were collected and processed using the same methods as the Iowa studies in Hellmich et al. (2001). Anthers were from *Bt* hybrid 38G17Bt (Cry1Ab, MON810 event; Pioneer Hi-Bred International, Johnston, IA) or its near non-*Bt* isoline 3893 (Pioneer Hi-Bred International) and were grown in Ames, IA, at the Johnson Research Farm.

Pretest Exposure Protocol. There were 45 replicates of three treatments: common milkweed leaf disks with *Bt* anthers, non-*Bt* anthers, or no anthers. Monarch butterfly neonates (<12 h old) were too small to be detected by the video tracking system; therefore, they were reared for 2 d before their behavior was recorded. Larvae were reared in one of two ways to determine if pre-exposure (conditioning) had an effect on behavior or weight gain. For 20 replicates, one larva was placed in each rearing dish (using a camel's hair brush) and was fed a milkweed leaf disk with no treatment applied for 2 d (referred to as naïve replicates). For the other 25 replicates, one larva was placed into each rearing dish and was fed a milkweed leaf disk with a treatment applied for 2 d (referred to as non-naïve replicates).

For both types of replicates, rearing dishes were prepared as follows. Two layers of solidified agar (2.5% wt:vol, 1.5 and 2.5 mm thickness) were prepared in separate petri dishes (60 by 15-mm Fisherbrand; Fisher, Pittsburgh, PA). A no. 13 cork borer was used to produce one 18-mm-diameter hole in the middle of the 1.5-mm-thick layer of agar. The 1.5-mm layer of agar was removed from its dish and placed over the 2.5-mm-thick layer. The top agar layer was pulled back, and a 31-mm-diameter common milkweed leaf disk (no. 15 cork borer) was centered under the hole in the upper layer of agar. The top layer of agar was repositioned to seal the disk between the agar layers, keep the leaf from dehydrating, and keep the larva on the upper side of the leaf so it could be detected by the video-tracking camera at all times.

For treatments with pre-exposure to anthers (non-naïve replicates), four whole anthers (examined under a dissecting microscope to ensure they were undamaged, ×6–60) were placed on each milkweed leaf disk (1.2 anthers/cm^2 or ≈ 60 anthers per whole common milkweed leaf). This anther density is rare; it occurs on 0.2% of milkweed leaves in and near cornfields but was used because previous studies have shown significant adverse effects on leaf feeding and weight gain at this density (Anderson et al. 2004). Anthers had dehisced; however, a small amount of pollen remained in some anthers. Petri dishes were incubated at 25°C, 8-h scotophase, and 60% RH. After 2 d of development either with or without exposure to anthers, larvae were placed into a recording dish. If there were anthers in the rearing dish, they were checked under a dissecting microscope (×6–60) for evidence of feeding.

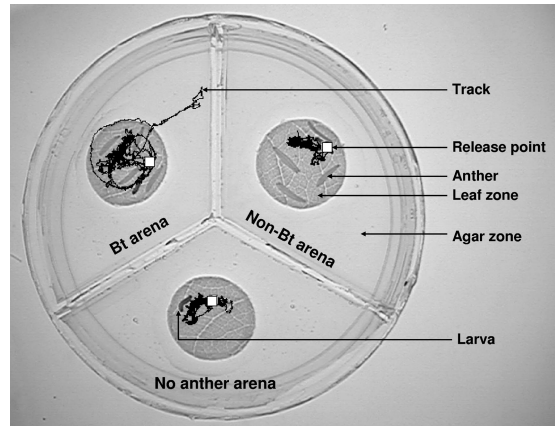

Fig. 1. Recording dish showing arena and zone definitions. Each section of the dish was defined as a separate arena (*Bt*, non-*Bt*, or no anthers). Each well had two primary zones: the milkweed leaf disk zone (leaf zone) and the agar zone. The space occupied by each anther was defined and added together to create a cumulative zone for all anthers in each arena. The track or larval path is a series of connected points representing the location of the larva at each frame capture interval (6 frames/s).

Test Period Protocol. Recording dishes consisted of a 100-mm-diameter by 15-mm-deep divided petri dish with three equal-sized wells (Labware; Tyco/Healthcare, Mansfield, MA). Each well was considered a separate arena (Fig. 1) and was prepared in the same manner as the initial rearing dishes, resulting in a double layer of agar with the same sized hole and leaf disk placed between the agar layers. As described previously, four undamaged anthers were placed on each leaf disk. For the naïve replicates, one 2-d-old larva was randomly selected and placed into each arena using a camel's hair brush. For the non-naïve replicates, an appropriate larva was placed into each arena (e.g., larva previously exposed to *Bt* anthers was placed in the *Bt* arena). A thin layer of Tanglefoot (The Tanglefoot Company, Grand Rapids, MI) was applied to the rim of the petri dish and to the top of plastic pieces, dividing the arenas so larvae could not move between wells. The recording dish was placed on a Plexiglas platform (at ≈1000 hours CST) under a video camera (Panasonic WV-BP330 CCD; Panasonic, Secaucus, NJ) in a walk-in environmental chamber (25°C, 8-h scotophase, and 60% RH). Overhead florescent lights and two infrared LED arrays (Tracksys,

Table 1. Monarch butterfly larval development and behavior after exposure to *Bt*, non-*Bt*, or no anthers

Response variable	Anther treatment			*F*	*P*
	Bt	Non-*Bt*	None		
Leaf feeding (mm^2)[a]	33.0b	40.8a	41.0a	$3.5_{(2,129)}$	0.034
Weight gain (mg)[a]	8.8b	11.1a	11.5a	$7.8_{(2,69)}$	0.001
Total distance moved (mm)[b]	290.5a	291.2a	317.3a	$0.2_{(2,129)}$	0.815
Maximum displacement (mm)[b]	14.6a	13.0a	11.4a	$1.8_{(2,123)}$	0.176
Percent time moving[b,c]	4.6a	4.2a	2.5a	$0.7_{(2,129)}$	0.485
Percent time near anthers[b,c]	7.6a	7.0a	—	$<0.1_{(1,86)}$	0.977
Percent time off the leaf[b,c]	6.9a	2.5ab	0.5b	$3.2_{(2,129)}$	0.044
Mean turn angle[b]	85.9a	86.1a	80.1a	$1.3_{(2,129)}$	0.270

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

Anthers and pollen were from 38G17Bt (MON810 event) and near isoline 3893 (Pioneer Hi-Bred International). Four anthers were placed on each leaf disk (1.2 anthers/cm^2).

[a] Measured at end of 24-h period.

[b] Measured by EthoVision for 4 h.

[c] Data were square root transformed before analysis.

Nottingham, United Kingdom) placed under the dish were used to aid in larval detection. A white plastic sheet was placed under the recording dish to help diffuse the light passing from under the Plexiglas platform.

Each arena had two mutually exclusive zones, the milkweed leaf zone, and the agar zone (Fig. 1). The space occupied by each anther was defined and added together to create a cumulative anther zone overlaid on the milkweed leaf zone. The *X*, *Y* coordinates of the center of gravity of each 2-d-old monarch butterfly larva was recorded for 4 h by EthoVision at a capture rate of six images per second. These coordinates were used to generate a track (two-dimensional path) for each larva using EthoVision version 3.0 (Noldus et al. 2002). From these tracks, seven parameters were calculated: total distance moved (millimeters), maximum displacement from release point (millimeters), number of larvae that crossed the boundary between the leaf and agar zones at least once, percentage of time spent moving, mean turn angle, and percentage of time spent in the agar zone and the cumulative anther zone (Fig. 1). A larva was considered "moving" once its velocity exceeded 0.5 mm/s and continued "moving" until its velocity dropped below 0.2 mm/s (Noldus et al. 2002). Larvae remained in the recording dish for 20 h after the 4-h recording (24 h total). After 24 h, the amount of leaf and anther feeding (square millimeters) was counted using a dissecting microscope (×10) with an eyepiece reticle grid. For the 25 non-naïve replicates, a pre- and postrecording weight were taken to calculate a weight gain.

Data Analysis. EthoVision tracks the center of gravity of each larva. Consequently, small shifts in the center of gravity caused by loss of pixels along the edge of the larva and regular shifting of the body during locomotion (i.e., body wobble, Noldus Information Technology 2002) results in artificial inflation of the total distance moved parameter and distortion of other parameters. Therefore, a down-sampling step of five and a minimum distance moved of 0.4 mm was set to distinguish between real locomotion and body wobble (Noldus Information Techology 2002). Data for percentages of time moving and in anther and agar zones were square root transformed before analysis (SAS Institute 1999). The experiment was a randomized complete block design blocked by pre-exposure versus no pre-exposure to anthers. An analysis of variance (ANOVA) was conducted on the data using the PROC GLM procedure in SAS with least significant difference (LSD) used to separate the means ($P \leq 0.05$; SAS Institute 1999). For the *Bt* and non-*Bt* anther treatments, the number of larvae that crossed the boundary between the leaf and agar zones one or more times was compared using a χ^2 test for differences in probabilities (Conover 1999).

Results

One non-naïve replicate was removed before analysis because one of the three larvae died during recording ($n = 44$). No evidence of anther feeding was detected in any of the 88 rearing dishes with *Bt* or non-*Bt* anthers before the recording or in the 88 recording arenas with *Bt* or non-*Bt* anthers after the recording.

There was no significant block effect (no effect of naïve versus non-naïve larvae) for any of the variables. There were significant differences detected among treatments for leaf feeding and weight gain (Table 1). Larvae exposed to *Bt* anthers fed less and gained less weight than those exposed to non-*Bt* anthers or no anthers. Comparing the *Bt* and non-*Bt* anther treatments, there was a 19% reduction in leaf feeding and a 21% reduction in weight gain when larvae were exposed to *Bt* anthers. Leaf feeding and weight gain were not different in the non-*Bt* anther and no anther treatments.

There were no differences detected among treatments for total distance moved, maximum displacement from the release point, percentage of time spent moving, or mean turn angle (Table 1). There also were no differences detected between the *Bt* and non-*Bt*

anther treatments for percentage of time spent on or near anthers.

There were differences detected among treatments for percentage of time larvae spent off the leaf disk (Table 1). Larvae exposed to *Bt* anthers and non-*Bt* anthers spent similar time of the leaf disk, but the former spent more time off the leaf than larvae exposed to no anthers. The number of larvae that crossed the boundary between the leaf and agar zones in the *Bt*-anther treatment (18) was two times greater than the non-*Bt* anther treatment (9) ($n = 44$, $\chi^2 = 4.328$; df = 1; $P = 0.037$). In the no anther treatment, six larvae crossed the boundary between the leaf and agar zones.

Discussion

As in a previous study by Anderson et al. (2004), larvae exposed to *Bt* anthers fed and weighed less than larvae exposed to non-*Bt* anthers or no anthers (Table 1). One hypothesis to explain these effects is that exposure to *Bt* anthers results in increased wandering, which in turn results in less feeding and reduced weight gain. Results did not support this hypothesis. There were no differences detected among treatments for total distance moved or maximum displacement from the release point (Table 1). Larvae exposed to *Bt* anthers did not move a greater distance or wander further away from their release point than larvae exposed to non-*Bt* anthers or no anthers. Also, larvae did not differ in the percentage of time spent moving, and their movement pattern as represented by mean turn angle did not differ.

Although the data did not support the original hypothesis of increased wandering with exposure to *Bt* anthers, two measures suggest that larvae spend less time in proximity to *Bt* anthers. Larvae exposed to *Bt* anthers spent significantly more time off milkweed leaf disks than larvae exposed to no anthers, whereas larvae exposed to non-*Bt* anthers spent similar time off milkweed leaf disks compared with larvae exposed to no anthers or *Bt* anthers (Table 1). The presence of *Bt* anthers on a leaf disk also increased the probability that the larva would cross the boundary between the leaf and agar zones. Previous research on a variety of other insect species has shown similar effects of exposure to *Bt* toxin (Yendol et al. 1975, Mohd-Salleh and Lewis 1982, Gould et al. 1991, Harris et al. 1997, Gore et al. 2002, 2005).

In other studies, changes in behavior occurred after measurable *Bt* ingestion or exposure to secondary compounds associated with biorational insecticide production. In our study, we found no evidence of ingestion of *Bt* tissue. There are several possible explanations for the change in behavior with exposure to *Bt* anthers: (1) larvae fed on a small amounts of anther tissue that were not detected, (2) larvae were able to detect *Bt* through olfaction or chemoreception without ingestion, (3) some other characteristic of the *Bt* anthers deterred larval feeding, or (4) larvae fed on small amounts of *Bt* pollen. It is possible that anthers were fed on in a manner that did not produce damage visible under the dissecting microscope used (×6–60). For example, larvae could have removed the top layer(s) of anther cells without producing a visible hole in the anther (hereafter referred to as "grazing"). The possibility of olfaction or sensing *Bt* without ingestion seems unlikely because *Bt* proteins are large and would not volatilize and stimulate larval sensory structures (Avé 1995). However, the *Bt* proteins produced by genetically modified corn plants are truncated versions of the naturally occurring *Bt* protoxin. Whether the proteins are small enough to be detected by the larval sensilla is unclear. If larvae were unable to detect *Bt* with olfaction or by grazing on small amounts of anther material, perhaps they were able to detect some other compound or factor that was undesirable (Slansky 1993, Renwick 2001, Vickerman and de Boer 2002). It is possible that transformation of the corn plant to produce *Bt* alters the anther tissue in some way that is detected by the larva. There is also the possibility of pollen rather than anther consumption. Anthers were allowed to dry and dehisce before the experiments; however, small amounts of pollen were present in some anthers. Careful removal of all pollen from the anthers could eliminate the potential compounding effects of *Bt* pollen; however, this would be difficult to do without damaging the anther. Damaging *Bt* anthers (breaking them into smaller pieces) increases the likelihood that larvae will feed on them and experience adverse effects (Hellmich et al. 2001). These effects would be artificial because anther pieces do not commonly occur on milkweed leaves in cornfields (Hellmich et al. 2001, Anderson et al. 2004). Furthermore, the amount of pollen left in anthers was almost certainly below the density associated with observable adverse effects (>1,000 pollen grains/cm^2; Hellmich et al. 2001). Larvae would have had to consume thousands of grains of pollen before the adverse effects on leaf feeding and weight gain would have been seen.

It is unclear whether the changed behavioral measures (increased time spent off leaf disks and increase frequency of larvae moving off leaf disks) would translate into changes in behavior on intact milkweed plants in the field. In our somewhat artificial laboratory experiment, a larva exposed to *Bt* anthers had a choice between spending time on a small area of milkweed leaf with a high density of anthers or spending time on clear agar. Larvae also were forced to feed on the upper side of the leaf and encounter anthers. On a milkweed plant in the field, the larva would have the choice to move to another area of the same leaf with a lower density of anthers, move to the underside of the leaf where there are no anthers, move to another leaf on the same plant, or move to another plant. In the field, early instars tend to feed on the upper third of the milkweed plant where the lowest density of anthers occur (Anderson et al. 2004) and on the underside of leaves where they would avoid any contact with anthers (Rawlins and Lederhouse 1981, Jesse and Obrycki 2003); consequently, reducing the likelihood of the adverse effects and behavioral changes we saw in the laboratory.

The small percentages of time larvae spent off the leaf disk may indicate that larvae did not like walking off of the leaf onto clear agar. On a real plant, where there is greater scope for movement, larva wandering after encountering *Bt* anthers would not be limited to walking off the leaf but could also walk over both sides of the leaf and up and down the stem. If the presence of *Bt* anthers results in larvae moving off the plant, they may have difficulty finding the same plant or a new host plant (Urquhart 1960, Borkin 1982) and may experience increased mortality (Borkin 1982). Behavioral studies on intact plants are necessary to discern whether the changes in behavioral measure seen in the laboratory will translate to the field.

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