

# Effects of Corn Plants and Corn Pollen on Monarch Butterfly (*Lepidoptera: Danaidae*) Oviposition Behavior

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**ABSTRACT** The oviposition behavior of adult insects can have a major impact on the level of exposure of their offspring to natural and artificial toxins. Pollen from Bt-corn hybrids represents a novel toxin and very little is known regarding its detection and possible avoidance by ovipositing females. The effect of corn plant proximity and corn pollen presence on the oviposition behavior of the monarch butterfly, *Danaus plexippus* (L.), was assessed in cage and flight chamber studies. The proportions of monarch eggs oviposited on milkweed plants dusted pollen from with a Bt-corn hybrid, an untransformed hybrid, gravel dust, and undusted control plants were recorded from a cage study. None of the treatments differed significantly in the relative proportion of eggs found. The effect of Bt and untransformed corn plant proximity and corn pollen presence was also assessed in a flight chamber. A significantly higher proportion of eggs (96%) were recovered from patches of milkweed plants not surrounded by corn plants, and a significantly higher proportion of eggs (nearly 70%) were recovered from patches of milkweed plants not dusted with corn pollen. There were no significant differences in the effects of Bt corn plants or corn pollen compared with untransformed plants or pollen. These results may have important implications for the level of exposure of monarch larvae to Bt-corn pollen.

**KEY WORDS** monarch butterfly, *Danaus plexippus*, *Bacillus thuringiensis*, Bt-corn, nontarget effects, transgenic

INSECTS USE A wide array of sensory stimuli to locate oviposition sites that offer the highest probability of survival for their offspring. Stimuli can include visual, olfactory, or tactile cues (Renwick and Chew 1994, Allard and Papaj 1996). Insects use these cues to evaluate the presence of the most suitable larval food (Rausher 1982), for protection from harsh climatic conditions or natural enemies (Singer et al. 1971), and to determine the relative concentration of harmful or toxic substances (Zucker 1982). The ability of insects to sense and avoid toxins during oviposition has important implications for pest management because it could extend to toxins applied for pest control or bred into resistant plants (Khan et al. 1987). Avoidance of insecticides could lead to reduced efficacy toward the target pest and reduced impact on nontarget insect species.

Transgenic insecticidal plants represent a novel method of insecticide delivery and relatively little is known regarding the ability of insects to detect or avoid the toxins produced by these plants (Hellmich et al. 1999). Because the toxins produced by transgenic insecticidal plants are very specific and bound within the plant tissues, they are generally considered to

cause little or no impact on nontarget organisms (Ostlie et al. 1997). However, pollen from corn plants transformed with genetic material from *Bacillus thuringiensis* (Bt) may represent a risk for nontarget organisms because it contains the Bt toxin (Fearing et al. 1997) and is dispersed by the wind (Raynor et al. 1972, Jesse and Obrycki 2000). Many susceptible nontarget Lepidoptera could potentially consume Bt-pollen that has blown on to their host plants from nearby Bt-corn fields. The exposure of these nontarget species to Bt-pollen will be heavily influenced by oviposition behavior. Female preference for oviposition on plants in close proximity to corn or dusted with corn pollen would tend to increase larval exposure to Bt-pollen. This may be the case if females perceive corn pollen as a high-protein food source (Haydak 1970). Conversely, female avoidance of oviposition on plants near corn or dusted with pollen would tend to decrease larval exposure. This could occur if corn plants made other plants harder to detect or if females perceive corn pollen as a foreign and possibly toxic substance.

The monarch butterfly, *Danaus plexippus* (L.), is of particular interest because it is a specialist on milkweed (Asclepiadaceae), a secondary succession plant that frequently occurs in and around the edges of cornfields (Yenish et al. 1997; Bhowmik 1994). Recent work shows that monarch larvae fed leaves of *Asclepias curassavica* L. dusted with Bt-corn pollen suffered

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higher mortality than larvae reared on leaves with untransformed corn pollen or leaves without pollen (Losey et al. 1999, Jesse and Obrycki 2000). Moreover, Wassenar and Hobson (1998) have shown that most of the butterflies in the wintering colonies in Mexico feed as larvae on milkweed growing in the midwestern United States, an important area for corn production. This combination of risk components highlights the importance of quantifying the behavioral and ecological factors that affect the exposure of monarch larvae to Bt-pollen. The oviposition behavior of adult monarchs is one factor that could have a major impact on the level of larval exposure to Bt-pollen. An effect of corn plants or pollen seems likely because oviposition by butterflies is affected by surrounding vegetation (Cromartie 1975) and monarchs specifically are affected by chemical or physical factors which, like pollen, are encountered on the leaf surface (Oyeyele and Zalucki 1990). In this study, we assess the effect of both the proximity of Bt-corn and untransformed corn plants and the presence of corn pollen on the oviposition behavior of monarch butterflies.

### Materials and Methods

**Effect of Corn Pollen on Monarch Oviposition.** To determine the effect of corn pollen on monarch oviposition in a small, confined arena, the numbers of eggs laid on cuttings of *Asclepias currasavica* that were dusted with corn pollen, gravel dust, or misted with deionized water (as a control) were quantified in a cage under controlled conditions. The cages were constructed of wood and nylon mesh and measured 1.3 m tall, 0.9 m wide, and 1.1 m deep. We placed cuttings of *A. currasavica* in small flasks wrapped in paraffin around the base to hold the cutting firmly upright. Each cutting was comprised of five to eight leaves with an area of  $58.1 \pm 2.7$  cm<sup>2</sup>. Plants were randomly assigned to one of four treatments, transgenic pollen (MAX 454), nontransgenic pollen (4494), gravel dust, and no pollen as a control. The hybrid MAX 454 is transformed with Event 176 and marketed as KnockOut by Novartis Seeds, (Wilmington, DE). Hybrid 4494 is the isogenic hybrid to MAX 454 meaning that it differs only by the absence of the Bt genetic material. Event 176 contains a pollen specific promoter (Koziel et al. 1993) and toxin levels expressed in pollen are substantially higher than in Bt11 and MON 810 events. Gravel dust was obtained from a gravel parking lot on the Iowa State University campus. Leaves were misted with water to help pollen stick, and then 0.1 g of the assigned pollen or dust were spread evenly on top surface of leaves with a paint brush for a particle concentration of  $\approx 4,500 \pm 200$  grains/cm<sup>2</sup>. Two or three mated females were placed in each cage. The same adults were used for all replicates but adults were held for 24 h with access to a honey-water solution and no exposure to milkweed in between each of the three replications. Plants were removed 24 h later, and the number of eggs and the leaf areas were determined. The proportion of eggs recorded from each treatment was analyzed with anal-

ysis of variance (ANOVA). Proportions were transformed with an arcsine square-root transformation before analysis to improve homoscedasticity.

To determine the effect of corn pollen on monarch oviposition behavior in a larger mesocosm, the numbers of eggs laid on "patches" of potted milkweed plants, which were either dusted with corn pollen or misted with deionized water (as a control), were quantified in a greenhouse flight chamber. Eight patches of five young common milkweed plants (*Asclepias syriaca* L.), were arranged on metal benches in a greenhouse (6 by 3 by 3 m). The flight chamber provided natural light conditions, ample flying room and was equipped with climate controls.

Four of these patches were dusted with pollen from four different corn hybrids. N4640 Bt (Bt11, Novartis Seeds), N4640 (nontransgenic, isogenic hybrid for N4640 Bt), 36G32 (Mon810, Pioneer) (Des Moines, IA), and 3752 (nontransgenic, isogenic hybrid for 36G32). The four dusted patches of milkweed were arranged alternately with the four control patches on the benches. Eight monarch females from our colony were released and allowed to oviposit for 24 h (from 1000 to 1000 hours). The following day, the butterflies were removed and eggs counted. Plants were visually divided into three sections by height and each section was counted separately.

Monarch butterflies used in the experiment were reared in a greenhouse from larvae taken from our colony that was derived from a long-term colony kept at the Boyce-Thompson Institute. Larvae were reared on *A. syriaca* and after emergence, adult butterflies were transferred in a wood cage equipped with feeders containing a 12% nectar solution and two flowering *A. currasavica*. To ensure independence, new females were released for each replication. All the eggs were removed to avoid cannibalism and to avoid possible rejection of the plants that already contain eggs or larvae (Dixon et al. 1978, Urquhart 1987). All replicates were run in the same flight chamber but patches were rotated between each of the four replications to minimize any effect of position within the chamber.

The pollen for this experiment was from the four hybrids listed above. Plants were dusted with a thin-layer chromatography (TLC) sprayer used at a pressure of 10 PSI. To allow application of a quantified amount of pollen, the relationship between the weight of pollen measured into deionized water and the concentration on leaves was established (Fig. 1). Based on this relationship, a solution of pollen at 0.004 g/liter was used to reach the density of 500 grains/cm<sup>2</sup>, which represents a concentration of pollen that might be encountered on milkweed in or at the very edge of a corn field. Patches not receiving pollen were misted with water from the TLC sprayer at the same rate to minimize differences between treatments. Each treated patch was sprayed at least one m away from all control patches, to avoid pollen contamination.

The proportion of eggs recorded from each treatment was analyzed with ANOVA to determine the effect of being dusted with pollen (control versus dusted), the effect of Bt (Bt versus isogenic) and the

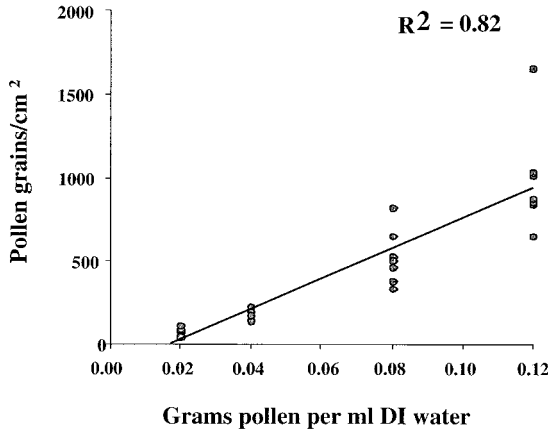


Fig. 1. Relationship between corn pollen mass in water and pollen grain density on milkweed leaves following application of pollen solution to leaves with a thin-layer chromatography sprayer AT 10 psi. Linear regression yields the following best fit equation: pollen concentration (grains/cm<sup>2</sup>) = 12,211.948\*pollen mass + 17.876 (g/ml).

effect of brand (Novartis versus Pioneer). The effect of these same variables on egg position was also analyzed with ANOVA. Proportions were transformed with an arcsine square-root transformation before analysis to improve homoscedascity. Means were separated using Fisher protected least significant difference (LSD).

**Effect of Corn Plants on Monarch Oviposition.** The protocols used for examining the effect of corn plant proximity on monarch oviposition behavior were very similar to those described for testing the effect of corn pollen. In this experiment, none of the milkweed plants was dusted with pollen. Four of the patches of potted milkweed were surrounded by potted corn plants (one for each of the four hybrids) and the other four were “open” patches that were not surrounded by corn plants. Corn plants were 75–100 cm high (measured from the top of the pot) and were not shedding pollen during the experiment. The location of eggs within and among plants was determined as described above. The proportion of eggs recorded from each treatment was analyzed with ANOVA. Proportions were transformed with an arcsine square-root transformation before analysis to improve homoscedascity.

**Results**

**Effect of Corn Pollen on Monarch Oviposition.** In the cage experiments, the greatest proportion of eggs was laid on the leaves coated with Bt-pollen. However, there were no significant differences between any of these treatments (Fig. 2; overall treatment effect,  $F = 0.93$ ,  $df = 3$ ,  $P = 0.5223$ ; for all individual means comparisons,  $P > 0.05$ ). Based on this experiment there is no evidence for monarchs either seeking out or avoiding milkweed leaves coated with either corn pollen or gravel dust.

In the flight chamber, monarch females exhibited a significant preference for ovipositing on milkweed

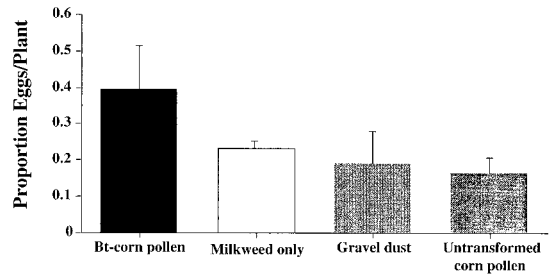


Fig. 2. Mean  $\pm$  SEM proportion of monarch eggs oviposited on four treatments of *A. currasavica* leaves in a cage experiment. Treatment did not have a significant effect on oviposition ( $P = 0.5223$ ) and none of the individual treatments had significantly different proportions of monarch eggs ( $P > 0.05$ ).

plants which were not dusted with corn pollen ( $0.18 \pm 0.02 =$  mean proportion of eggs per patch) compared with those dusted with pollen ( $0.07 \pm 0.01$ ;  $F = 12.91$ ,  $df = 1$ ,  $P = 0.0008$ ). Because there were four patches of each type this translates these values into 72 versus 28% of the total eggs found. There was no evidence for discrimination between milkweed plants dusted with Bt-corn pollen and plants dusted with untransformed pollen ( $F = 0.00$ ,  $df = 1$ ,  $P = 0.9883$ ) or between the two Bt hybrids ( $F = 1.13$ ,  $df = 1$ ,  $P = 0.3004$ ).

There was significant variation in the proportions of eggs laid on the three height positions of the plant (Fig. 3;  $F = 26.02$ ,  $df = 2$ ,  $P < 0.001$ ), and a significant difference between the sides of the leaves (above, below;  $F = 50.68$ ,  $df = 1$ ,  $P < 0.001$ ). Females lay more eggs on the top third of the plant than on the other parts ( $P < 0.001$ ) and have no preference between the middle and the bottom ( $t = 8.84$ ,  $df = 1$ ,  $P = 0.0717$ ). More eggs were laid on the lower side of the milkweed leaves than on the upper side. More than 68% of the eggs were found on the lower surface of leaves in the upper third of the plant. There was no significant effect of pollen presence or source on relative position.

**Effect of Corn Plants on Monarch Oviposition.** Monarchs exhibited a significant preference for ovipositing on patches of milkweed that were not sur-

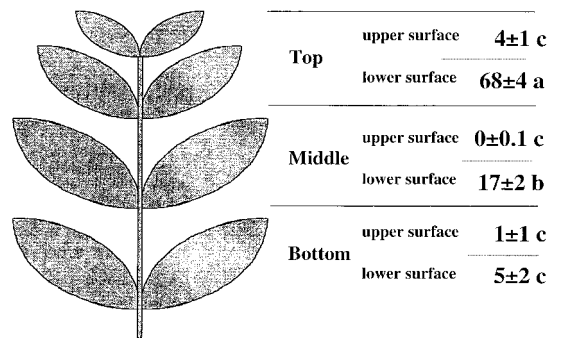


Fig. 3. Mean  $\pm$  SEM percentage of monarch eggs oviposited on different portions of potted *A. syriaca* plants. Means labeled with the same letter are not significantly different at  $P = 0.05$ .

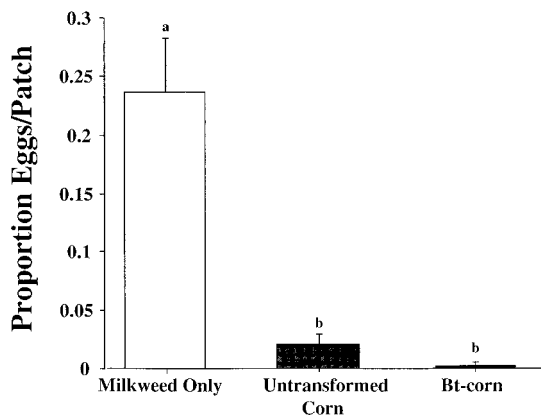


Fig. 4. Mean  $\pm$  SEM proportion of monarch eggs oviposited on patches of potted *A. syriaca* plants surrounded by corn plants (dark bars) or open patches (off-black bars) of *A. syriaca* (white bar). Means labeled with the same letter are not significantly different at  $P = 0.05$ .

rounded by corn plants ( $0.24 \pm 0.05$  mean proportion eggs/patch) compared with milkweeds surrounded by corn plants ( $0.01 \pm 0.004$ ;  $F = 44.26$ ,  $df = 1$ ,  $P = 0.0001$ ; Fig. 4). There were four open patches thus almost 96% of the eggs were laid in open patches and only 4% were laid in patches surrounded by corn. This implies that monarchs may avoid ovipositing in cornfields where they would be exposed to the highest levels of pollen. No difference between the location of a plant within the patch (inside, at the edge) was recorded ( $F = 0.25$ ,  $df = 1$ ,  $P = 0.615$ ), and thus milkweed plants directly adjacent to corn plants in control patches were as acceptable for oviposition as plants farther away from corn.

When the distribution of the few eggs found in patches surrounded by corn plants (21 out of 249) was analyzed separately, the proportion of eggs on milkweed plants surrounded by Bt-corn ( $0.0009 \pm 0.0009$ ) was significantly smaller than the proportion on milkweed plants surrounded by untransformed corn ( $0.021 \pm 0.008$ ;  $F = 5.80$ ,  $df = 1$ ,  $P = 0.0368$ ). There was no significant difference between the two Bt hybrids ( $P = 0.48$ ).

### Discussion

Our results demonstrate the impact of protocols, especially the scale of the experimental arena, on behavioral assays of monarch oviposition behavior. Monarch oviposition behavior was significantly affected by the presence of corn pollen on the milkweed leaves in the flight chamber experiments but it was not affected in the cage experiments. Several factors differed between these two studies in addition to the overall volume of flight area. These factors include using cut leaves versus whole plants, the level of pollen on the leaves, the corn hybrids pollen was collected from, and the lighting conditions. Determining the effect of each of these differences will allow the design

of more effective behavioral assays and possibly provide direct insights for making predictions of monarch egg distribution patterns in the field.

Data from the flight chamber experiments indicates a preference for oviposition on milkweed plants that are not dusted by pollen. A deterrent effect of corn pollen on oviposition certainly seems plausible based on the monarch's sensory physiology. The corn pollen presence might inhibit the recognition of the host plant. Host recognition by monarchs involves the use of the mid-legs, the forelegs and the antennae to assess the suitability of the plant (Baur et al. 1998, Haribal and Renwick 1998). The presence of pollen on the leaves might disturb reception of the oviposition stimulant by the contact chemoreceptors located on the monarch appendages. The sensibility of the female monarch to a density of 500 pollen grains/cm<sup>2</sup> appears to be similar for Bt and non-Bt corn hybrids. Monarch females preferred to lay eggs on a plant without pollen but showed no preference between the two kinds of corn pollen.

Monarch larvae reared on leaves of milkweed dusted with Bt corn pollen show a higher mortality and a lower growth rate than larvae reared on leaves with regular corn pollen or with leaves without corn pollen (Losey et al. 1999). If monarchs avoid milkweed leaves dusted with corn pollen in the field, then the exposure of larvae will be relatively reduced. However, because untransformed corn pollen has been shown to increase the growth rates of monarch larvae (Losey et al. 1999) and adults seem unable to distinguish between the transgenic and nontransgenic pollen, it seems unlikely that the avoidance is based on an assessment of the potential nutritional quality of pollen-dusted leaves as a food source. It seems more likely that ovipositing females are sensing pollen as a general contaminant (a nonmilkweed substance). Many milkweeds, especially along roadsides, are coated with inorganic dust (G. Dively, personal communication), which could also be sensed as a contaminant. Although no effect was found in the cage study, future flight chamber assays and field surveys of oviposition need to incorporate and compare a range of potential contaminants.

In our study on the effect of corn plants, monarch females laid fewer eggs on the milkweeds surrounded by corn than on the patches with only milkweeds. This finding is consistent with those of Cromartie (1975), who reported that the oviposition of pierids on cabbage was influenced by the surrounding vegetation. The role of olfactory and visual cues in host recognition has been documented for several lepidopteran families (Feeny et al. 1989, Allard and Papaj 1996). This finding is also consistent with known monarch behavioral patterns and physiology (Dixon et al. 1978). Specifically, clusters of short pegs on the monarch antennae have been suggested as the site of olfactory sensilla (Haribal and Renwick 1998). The presence of corn plants (180 cm) may inhibit the location (visual cues) or the recognition (olfactory cues) of the milkweed plants (40 cm) by the monarch females.

We also found a significant difference between the oviposition on milkweed surrounded by Bt-corn compared with those surrounded by untransformed corn when eggs within corn patches were analyzed separately. However, very few eggs were laid in those patches (21 of 428), so we caution against over-interpretation of these results. It would be useful to test oviposition on milkweed patches in Bt versus untransformed corn without the presence of open patches to determine if monarchs can discriminate between corn hybrids. We know of no published studies that demonstrate oviposition deterrence caused by the presence of the Bt toxin in a plant. It is unclear how female Lepidoptera would sense the presence of the toxin.

Although the presence of pollen had a significant effect on the distribution of eggs between plants, we found no evidence for an effect of pollen presence or source on the distribution of eggs within plants. Regardless of pollen treatment, significantly more eggs (almost 68%) were laid on the underside of the leaves and on the top third of the plant. The preference for oviposition on the underside of the leaves could be explained by the behavior of monarch females after landing. After landing on the host plant, the female monarch immediately bends her abdomen under the leaf and then assesses the suitability of the leaf with her appendages (Haribal and Renwick 1998). If the plant is accepted a single egg will be laid. By laying their eggs on the lower side of the leaf, the females give higher protection to their eggs against the environmental conditions (rain, wind, UV light) which could harm the larvae. Preference for oviposition on the soft, young leaves at the top of plants has been noted previously (Zalucki and Kitching 1982, Urquhart 1987). There may also be a simple physical location effect. Because monarchs often fly above the height of most milkweed plants they would encounter the top leaves first as they descend.

The distribution of eggs within a plant could have an important effect on the level of pollen exposure. Pollen is deposited primarily on the top surface of the leaf and because neonate larvae do not always chew through the leaves exposure of the most vulnerable lifestage may be minimized. In addition, some recent studies have shown that lower levels of pollen accumulate on the upper third of the plant where the younger leaves are at acute angles to the stem (G. Dively, personal communication). Thus, the lower surface of leaves at the top of plants may be a location that harbors some of the lowest pollen concentrations within a milkweed plant. However, other recent studies have indicated no differences in pollen deposition among plant levels so it is not clear how widely this inference can be drawn (Jesse and Obyrcki 2000).

The next phase of this research will be to determine how accurately data from the cage and flight chamber studies predicts patterns of monarch egg distribution relative to corn in the field. In the flight chamber, oviposition behavior was assayed in potted milkweed patches of uniform density, size and age. Each variable influences monarch oviposition (Dixon et al. 1978, Zalucki and Kitching 1982, Urquhart 1987). In addition,

factors such as the presence of predators and competitors vary widely in the field. Thus, due to multiple sources of variation, if nonrandom oviposition patterns do occur in the field, they are almost certain not to be as pronounced as the patterns exhibited in the flight chamber studies.

Field surveys of monarch oviposition and feeding on milkweeds in and around cornfields as well as other habitats were initiated at both New York and Iowa in 1999. Monarch larvae were found feeding on milkweeds in and around cornfields at both locations (L.H.J. and J.J.O., unpublished data; J.E.L. et al., unpublished data), which confirms earlier reports that milkweed plants in cornfields are colonized and fed on by monarchs (Yenish et al. 1997).

Clearly monarch oviposition preferences will not eliminate the potential for larval exposure to corn pollen. More detailed field work needs to be done at an expanded number of locations to determine if an underlying pattern does exist that is being masked by the variability of field conditions. Combining expanded field surveys with more detailed assays at smaller scales over a wider range of conditions will yield the necessary information to provide an accurate assessment of monarch oviposition behavior and its effect on the exposure of monarch larvae to Bt-pollen.

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