The effects of Bt corn pollen on two non-target lepidopteran species, *Danaus plexippus* (Lepidoptera: Danaidae) and *Euchatias egle* (Lepidoptera: Arctiidae)

by

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CHAPTER 1: GENERAL INTRODUCTION

Thesis Organization

Individual papers, and a general summary follow a literature review. The 1st paper presents the results of a study on the levels of deposition of Bt corn pollen on *Asclepias syriaca* (Asclepidaceae), and the effects exposure to a range of Bt corn pollen had on *Danaus plexippus* (Lepidoptera: Danaidae) larvae and adults. In the 2nd paper I present the results of a 2 year field survey assessing the use of *A. syriaca* in and near corn fields by *D. plexippus*. The 3rd paper presents the results of a study in which the survival of experimental cohorts of *D. plexippus* placed on *A. syriaca* in and near Bt and non-Bt corn fields during anthesis were monitored. The 4th paper examines the effect of a high level of Bt corn pollen on another non-target lepidopteran, *Euchatias egle* (Lepidoptera: Arctiidae). The formats of these papers follow the guidelines for the journals they will be submitted to or have been published in.

Literature Review

Transgenic Crops

Transgenic crops are quickly becoming a common pest management tool in many agricultural systems. Transgenic plants fall into three broad categories based upon use; herbicide resistant, crop protection against insects and plant pathogens, and altered product quality (Rogers & Parkes, 1995). Between 1986 and 1992, 31 transgenic plant varieties were approved by regulatory agencies for field releases in 28 countries (Rogers & Parkes, 1995). Over 50 species of transgenic plants have been produced by researchers (Malik et al., 1996);

it is likely that the number of transgenic crops approved for field release will continue to increase.

Benefits of planting insecticidal or herbicide resistant transgenic crops include: 1) reduced environmental impacts from insecticides (Wolfenbarger & Phifer, 2000), 2) increased soil conservation due to reduced cultivation practices (Wolfenbarger & Phifer, 2000), 3) increased or protected yields (Ferber, 1999; Wolfenbarger & Phifer, 2000), and 4) a reduction in fungal infections resulting in reduced fumonisin levels (Munkvold, 1999). Some of the possible risks of planting these transgenic crops include 1) gene flow leading to increased invasiveness of weedy hybrids due to increased competitive ability (Mikkelsen et al., 1996; Rogers & Parkes, 1995; Stewart et al., 1997; James et al., 1998; Hails, 2000; Wolfenbarger & Phifer, 2000), 2) direct negative effects on non-target organisms, such as lepidopterans, and soil organisms (Edwards, 1994; Pilcher et al., 1997; Sims, 1997; Hilbeck et al., 1998; Losey et al., 1999; Saxena et al., 1999; Hails, 2000; Obrycki et al., 2001), 3) indirect effects on non-target organisms, such as a drop in population of host specific natural enemies, or a reduction in the agroecosystem biodiversity (Orr & Landis, 1997; Pilcher, 1999; Hails, 2000; Wolfenbarger & Phifer, 2000; Obrycki et al., 2001), 4) resistance among target pests of insect resistant crops, and herbicide resistant weeds (McGaughey et al, 1998; Wolfenbarger & Phifer, 2000), and 4) new human allergies to the new proteins in food crops (Ferber, 1999; Wolfenbarger & Phifer, 2000). Many of these risks and benefits lack adequate empirical evidence to fully evaluate them (Wolfenbarger & Phifer, 2000).

Transgenic *Bacillus thuringiensis* (Bt) corn, developed to suppress *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), was first field tested in 1992, and by 1995 was registered by the Environmental Protection Agency for commercialization (Carozzi &

Koziel, 1997). In 1998 approximately 9 million acres of Bt corn were planted in the U.S. (Federici, 1998), and during the past 2 years about 30 million acres (1/3 of total U.S. corn acreage) of Bt corn was planted. The speed with which this new technology has become commercially available and widely planted has caused controversy over how to regulate, as well as, how to assess and manage potential risks of transgenic plants (Paoletti & Pimentel, 1996; Miller, 1998). The foundation for regulation of transgenic Bt crops is based on a history of relatively safe use of Bt as a microbial insecticide spray (Beegle & Yamamoto, 1992; Carozzi & Koziel, 1997; Miller, 1998). In this formulation, the rapid breakdown of Bt toxins in the environment was believed to reduce effects on non-target organisms. However, previous studies examining the effect of *Bacillus thuringiensis* insecticide sprays on non-target organisms have found negative non-target effects (Miller 1990; James, et al., 1993; Johnson et al., 1995; Wagner et al., 1996; Herms et al., 1997; Peacock, 1998; Whaley et al., 1998).

Bacillus thuringiensis

Bacillus thuringiensis (Bacillaceae) is a soil bacterium with a long history of use in controlling lepidopteran pests (Beegle & Yamamoto, 1992). *Bacillus thuringiensis* is a sporulating bacterium that produces toxic crystalline inclusions during sporulation (Höfte & Whitely, 1989; Glare & O'Callaghan, 2000). *Bacillus thuringiensis* was first described by Japanese bacteriologist S. Ishiwata around the turn of the 20th century, but the crystal toxin was not identified as the source of the toxicity until the early 1950's (Beegle & Yamamoto, 1992). In 1954, T.A. Agnus reported that in *Bombyx mori* (Lepidoptera: Bombycidae) larvae the spores had no effect when ingested, and only caused septicemia when injected into the

larvae, however, spores accompanied by crystals killed larvae when ingested (Agnus, 1954). *Bacillus thuringiensis* crystalline inclusions are glycoproteins, referred to as Cry proteins, these proteins exhibit toxicity to insects in many orders, but are primarily used to control insects in the orders of Lepidoptera, Coleoptera, and Diptera (Gill et al., 1992; Glare & O'Callaghan, 2000). When ingested by a susceptible insect the crystalline inclusion breaks down into δ-endotoxins, known as Cry toxins (Gill et al., 1992; Glare & O'Callaghan, 2000).

The effect that the Cry toxins have on Lepidoptera has been well studied. All *cry*1 genes that have been sequenced encode 130-140 kDa proteins (Höfte et al., 1988; Höfte & Whiteley, 1989). When a susceptible lepidopteran ingests a Cry1 protoxin, proteases in the presence of the high midgut pH (pH 8 to 9) cause the 130-140 kDa protoxin to be cleaved at specific sites into 60-70 kDa protease resistant active toxins. During processing in the gut there are 7 cleavages of the protoxin starting at the C-terminus, some cleavage does occur at the N-terminus, but for most Cry1 toxins the active toxin is derived mainly from the N-terminal half of the protoxin (Höfte & Whiteley, 1989; Gill et al., 1992). It is the hydrophobicity, not the specific amino acid sequences, of the activated toxin that is conserved in different *B. thuringiensis* toxins and is believed to play a significant role in the toxicity of the protein (Gill et al., 1992).

In the insect's midgut the toxin binds to a receptor on the brush border membrane vesicles (BBMV) on the columnar cells (Hofmann et al., 1988; Gill et al., 1992; Carroll et al., 1997). It is believed that when the Bt toxin binds to the BBMV it disrupts the ion flow in the cell, the cell swells and eventually lyses, killing the insect if enough toxin was ingested (Gill et al., 1992; Carroll et al., 1997). Sensitive insects are grouped into three groups depending on how the Bt toxins kill them; Type I, extremely sensitive to the toxin, mortality results

from the gut contents spilling into the hemolymph, death occurs within 7 hours; Type II, gut is paralyzed, but contents do not spill into the hemolymph, death takes 2-7 days; Type III, death results from septicemia after the Bt spores germinate in the midgut (Höfte & Whiteley, 1989; Beegle & Yamamoto, 1992; Gill et al., 1992; Pietrantonio et al., 1993).

The first field use of *B. thuringiensis* was against the European corn borer, *O. nubilalis*, in 1927 (Glare & O'Callaghan, 2000). However, it was not until the late 1950's that *B. thuringiensis* insecticides began to be marketed; a company called Bioferm was the first to market *B. thuringiensis* insecticide under the name Thuricide. Thuricide was exempt from U.S. Food and Drug Administration residue tolerances on crops based on a history of safety (Beegle & Yamamoto, 1992). Today *B. thuringiensis* sprays are considered to be a safe alternative to chemical insecticides because they are toxic to a narrow array of insect species and therefore are less likely to kill non-target insects (Vadlamudi et al., 1995).

Bt sprays are commonly used in forest systems to control the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae) and the western spruce budworm, *Choristoneura occidentalis* (Lepidoptera: Tortricidae). There is concern about the effects of spraying on non-target Lepidoptera feeding in these forests. For example, *Tyria jacobaeae* (Lepidoptera: Arctiidae), a beneficial lepidopteran introduced into North America for biological control of the weed, Tansy ragwort, often occurs near forests (James et al., 1993). In laboratory bioassays exposure to *B. thuringiensis* caused increased mortality in the fourth and fifth instars of *T. jacobaeae* feeding on the plant (James et al., 1993). Drift from Bt sprays can affect Lepidoptera feeding up to 3000 meters from the spray site (Whaley et al., 1998). It has also been shown that Bt sprays can have an effect on non-target Lepidoptera for up to 30

days after spraying (Johnson et al., 1995). Furthermore, a reduction in the species richness was found two years after forest plots were sprayed with Bt (Miller, 1990).

Bt Corn to Control *Ostrinia nubilalis*

Ostrinia nubilalis costs growers an estimated $1 billion annually in damage and control costs (Ostlie, 1997). In the Midwest, *O. nubilalis* typically has 2 generations each year. *Ostrinia nubilalis* overwinters as larvae in corn stalks. Depending on the weather they pupate in early May and adults emerge in late May and June (Mason et al., 1996). The first generation causes yield loss by physically interrupting nutrient movement (Carozzi & Koziel, 1997). The second generation occurs in late July and August. In addition to physiological damage, the larvae of this generation also feed directly on the corn ears and kernels. Stalk boring by both generations can lead to the ears dropping or the stalk breaking (lodging) making it difficult to harvest (Mason et al., 1996).

Ostrinia nubilalis is difficult to control with insecticides, because the second and third instars move into the corn stalk to feed (Mason et al., 1996). Intensive scouting of fields is necessary to assess *O. nubilalis* populations. Scouting is time consuming, but necessary for any effective control of *O. nubilalis* (Mason et al., 1996). Currently, more farmers in Iowa ignore *O. nubilalis* infestations and accept the losses rather than use insecticides to control them (Pilcher & Rice, 1998). In a survey of farmers in Iowa and Minnesota, 70% of them had never used insecticides to control *O. nubilalis* (Rice & Ostlie, 1997).

Transgenic *Bacillus thuringiensis* (Bt) corn was developed specifically to control *O. nubilalis* (Koziel et al., 1993). When inserting *cry*1 genes into corn DNA, only the activated

toxin portion of the DNA was inserted, which increased toxin expression (Perlak et al., 1991). When the wild type truncated *cry*1Ab gene was inserted into corn, expression of the toxin was very low (Perlak et al., 1991; Koziel et al., 1993). The codons for the toxin protein are not common in plants and led to low expression of the protein (Perlak et al., 1991). Prokaryotes tend to have a higher A-T content than plants, so whenever possible codons in the *cry*1Ab gene were changed to G-C (Perlak et al., 1991; Koziel et al., 1993). The *cry*1Ab inserted into corn was altered from a G-C content of 38% to a G-C content of 65% (Koziel et al., 1993). *Cry*1Ab was first inserted into maize by microprojectile bombardment under the control of a phosphoenolpyruvate carboxylase (PEPC) and pollen promoter (Event 176), or the cauliflower mosaic virus (CaMV) 35s promoter (Event 171) (Koziel et al., 1993). The PEPC promoter is involved in photosynthesis, so expression of Cry1Ab was mainly in the green tissues. The pollen specific promoter was utilized in Event 176 because high expression of the Bt toxin in the pollen helps control early instars of the second generation of *O. nubilalis* that feed on pollen that has accumulated in the axils of the corn leaves (Carozzi & Koziel, 1997). The CaMV promoter results in expression of the Bt protein in most corn cells and is the promoter used in the Bt11 and MON810 Bt corn transformation events.

Non-Target Effects of Transgenic Bt Corn

The expression and dispersal of Bt toxin in the pollen from transgenic crop plants may pose a risk to non-target lepidopterans. When Bt corn pollen lands on the leaves of plants in and around corn fields, it exposes non-target lepidopteran larvae feeding on these plants to Bt toxins. In Iowa, where the landscape is dominated by row-crop agriculture, areas

effected could include a significant portion of the non-cultivated areas, such as remnant prairies, roadside ditches and wetlands.

Corn pollen is one of the largest pollen grains (90 to 100 μ in diameter) that is wind-dispersed from a graminaceous plant (Raynor et al. 1972). The large size of corn pollen makes it easy to identify and reduces its dispersal. Previous studies have shown that the large size of corn pollen prevents it from dispersing great distances (Hodgson, 1949; Raynor et al., 1972; Paterniani et al., 1973). Thus, the greatest effect from a toxin in corn pollen from transgenic plants will most likely occur within 10 meters of the source of the pollen where higher densities of Bt pollen may occur on non-cultivated plants.

Pollen deposition on a leaf surface is a function of the surface's angle to the ground, and leaf surface characteristics that influence the retention of pollen. Thus plants with horizontal leaves covered with fine hairs, e.g., common milkweed, *Asclepias syriaca*, will retain more pollen grains than plants with smooth vertical leaves. The amount of pollen remaining on a leaf over time will also be affected by rainfall and wind (Hodgson, 1949; Fokkema, 1971; Raynor et al., 1972).

There have been relatively few studies on the non-target effects of Bt corn pollen. One possible effect of Bt corn pollen is on insect predators that feed on corn pollen (Pilcher, et al. 1997). No detrimental effects on development and survival of *Coleomegilla maculata* (Coleoptera: Coccinellidae), *Orius insidiosus* (Hemiptera: Anthocoridae), or on *Chrysoperla carnea* (Neuroptera: Chrysopidae) were observed (Pilcher et al., 1997). Wraight et al., (2000) found that exposing the black swallowtail, *Papilio plolyxenes* (Lepidoptera: Papilionidae) to Bt corn pollen under field conditions did not increase larval mortality compared to larvae exposed to non-Bt corn pollen. However, the monarch butterfly, *Danaus*

plexippus L. (Lepidoptera: Danaidae) is sensitive to the Bt toxin expressed in Bt corn pollen (Losey et al., 1999).

Danaus plexippus

The monarch butterfly, *Danaus plexippus*, is a species that occurs temporally and spatially with Bt corn fields during anthesis, and could be negatively affected by the deposition of Bt corn pollen on larval food sources (Urquhart, 1960; Borkin, 1982; Malcolm et al., 1993; Ritchie, et al., 1997; Wassenaar & Hobson, 1998; Losey et al., 1999). *Danaus plexippus* is distributed widely in North America, with the major population occurring east of the Rocky Mountains (Brower & Malcolm, 1991); multiple generations are produced each summer and larvae feed exclusively on *Asclepias* spp. foliage (Urquhart, 1960; Borkin, 1982; Brower & Malcolm, 1991; Malcolm et al., 1993). Immature *D. plexippus* are present on milkweed plants in the midwest from mid-May to mid-Sept so larval stages are present on *Asclepias* plants when corn pollinates; for 7-10 days, generally in mid-July to mid-August (Borkin 1982; Ritchie et al., 1997). The Eastern population of *D. plexippus* overwinters each year in 10 colonies in Mexico's Transvolcanic mountain range. The monarchs aggregate in these boreal *Abies religiosa* forests at approximately 3,000 meters above sea level (Brower & Malcolm, 1991). The low temperature and moist environment in these forests keeps the adults relatively inactive until spring, allowing them to conserve energy for mating and migration north in the spring (Brower & Malcolm, 1991). Fifty percent of the overwintering adults in Mexico originate from the central United States, an area dominated by row crop agriculture (Wassenaar & Hobson, 1998), and most (over 90%) of these adults fed on *Asclepias syriaca*, the common milkweed, as larvae (Malcolm et al., 1993).

Asclepias syriaca, a native perennial, is the most common milkweed species in northeastern North America (Evetts & Burnside, 1972; Bhowmik & Badeen, 1976; Malcolm et al., 1993). *Asclepias syriaca* is a common weed in cultivated fields due to the emergence of new shoots from root fragments, reduced tillage conditions that are conducive to the establishment of seeds, and the suppression of competitive weeds by herbicides that often do not effectively control *A. syriaca* (Evetts & Burnside, 1975; Bhowmik & Badeen, 1976; Cramer, 1977; Burnside, 1977; Minshall, 1977; Bhowmik, 1994; Yenish et al., 1996;Yenish et al., 1997). In a survey of 13 mid-western states, Evetts (1977) estimated that 12 million acres (5 million hectares) of corn and 6.1 million acres (2.5 million hectares) of soybeans are infested with at least 1 *A. syriaca* plant. An average of 36% of corn fields and 51% of roadsides were infested with *A. syriaca* in eastern Nebraska (Cramer & Burnside, 1982). In a recent study in Iowa, 46% percent of corn fields and 71% of roadsides were contained *A. syriaca* plants (Hartzler & Buhler, 2000). Most of the roadsides in rural Iowa, adjacent to agricultural fields, are less than 10m wide (Hartzler & Buhler, 2000), therefore the entire roadside habitat of *A. syriaca* can be affected by agricultural practices.

Danaus plexippus conservation efforts have focused on the vulnerable forested wintering habitats in Mexico (Malcolm & Zalucki, 1993), some estimates indicate that the forests will be lost in the next 10 to 20 years, eliminating the overwintering habitat of the eastern population of the monarch (Brower & Malcolm, 1991). The forests are threatened with commercial cutting and thinning of trees, managed burns to kill young trees for harvesting, and an increasing Mexican population (Brower & Malcolm, 1991). However, conservation of *D. plexippus* also requires a consideration of larval food sources (Taylor et al., 1999). The abundance of milkweeds in and next to corn fields (Evetts, 1977; Cramer &

Burnside, 1982; Hartzler & Buhler, 2000), and the sensitivity of *D. plexippus* to the Cry1Ab toxin in transgenic Bt corn pollen create a situation in which the planting of Bt corn may have negative effect on *D. plexippus* populations (Losey et al., 1999; Hartzler & Buhler, 2000).

Objectives

1) Determine the levels of Bt corn pollen deposited on *A. syriaca* plants in and near Bt corn fields, and to quantify the effects of field deposited pollen on *D. plexippus* larvae.

2) Quantify the effects on *D. plexippus* larvae exposed to a range of Bt corn pollen densities that they would likely encounter in the field.

3) Determine if *D. plexippus* females oviposit on *A. syriaca* plants in and bordering corn fields and if larvae are present on the *A. syriaca*.

4) Determine if under field conditions Bt corn pollen influences *D. plexippus* oviposition and larval survival.

5) Determine if Bt corn pollen increases mortality in *Euchatias egle* (Lepidoptera: Arctiidae), the milkweed tiger moth.

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CHAPTER 2: 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 hrs of *D. plexippus* larvae exposed to 135 pollen grains/cm^2 of transgenic pollen for 48 hrs ranged from 37 to 70%. We found no sub-lethal effects on *D. plexippus* adults reared from larvae that survived a 48-hour exposure to three concentrations of Bt pollen. Based upon 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 meters from transgenic field borders. However, the highest larval mortality will likely occur on *A. syriaca* plants in corn fields or within 3 meters of the edge of a transgenic corn field.

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Based on our study we conclude that the ecological effects of transgenic insecticidal crops need to be evaluated more fully before the widespread planting of these transgenic crops.

Key words:

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

Introduction

Starting in the late 1990's, 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 & Koziel 1997). In 1998 approximately 3.6 million hectares of Bt corn were planted in the U.S., and by 2003 it is predicted that 12 million hectares (1/3 of total U.S. corn acreage) of Bt corn will be planted in the U.S. (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). *Cry*IAb was first inserted into corn by microprojectile bombardment and expression was controlled by the phosphoenolpyruvate carboxylase (PEPC) 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 & Koziel 1997). The CaMV 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 environmental risks of transgenic plants (Paoletti & 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 & Gould 1992, Pilcher et al. 1997a,b, 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 & Palma 1997, Lavigne et 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 *Bacillus thuringiensis* (Wagner et al. 1996, Whaley et al. 1998), which was 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 & Hobson 1998). Most of these adults are from larvae that feed on *Asclepias syriaca*, the common milkweed (Malcolm et al. 1993). At least three overlapping generations of monarchs are observed annually in the central U.S., 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 fed upon by monarch larvae (Cramer & Burnside 1982, Bhowmik 1994, Yenish et al. 1997, Hartzler & Buhler 2000, Hansen, unpublished data). Additionally, corn pollen is produced, depending on planting date, in mid to late summer for 1-2 weeks and is wind-dispersed at least 60 meters (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) determine the levels of transgenic pollen on *A. syriaca* plants placed within and adjacent to plots of transgenic corn, 2) assess mortality of *D. plexippus* larvae exposed to field deposited pollen, and 3) 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 *Asclepias syriaca*

Four corn hybrids were planted in a 2,500 m^2 plot on the Iowa State University campus. Corn was planted in May in 1998 and June in 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* were transplanted from natural populations and potted in 27.5 cm. pots. *A. syriaca* plants used in the field studies were approx. 50-100 cm tall (including the pot). A #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 3 dates from 29 July to 4 August from event 176 and from 12 plants on 3 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 4 dates from 31 July to 9 August from event Bt11 and on 2 dates, 4, 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 it was recorded as 400, 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 were removed. The number of pollen grains on the un-washed leaf disks were counted and each disk was placed in a 5.2 cm d. 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 hours. 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 Aug., 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 hrs, and the pollen was sifted through a sieve (300 um openings), and stored at – 20°C for

9-10 months. *Asclepias 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 0.05ml drop of the 0.01g pollen/10 ml water solution the mean number of pollen grains was 208±12 (n=12), the mean number in a 0.05ml drop of the 0.001g solution was 22±1 (n=12). The number of pollen grains in 0.05ml of the 0.1g solution was estimated to be 1,966. This number was estimated by multiplying the mean number in the 0.01g 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 1300 pollen grains/cm^2. Each leaf disk was then placed on moistened filter paper in a 5.2 cm d. petri dish. One 1^{st} instar *D. plexippus* was placed on the leaf disk and maintained at 21°C; L:D 16:8. Larvae were placed on top of the leaf disk, but they were able to move and feed from underneath the disk. Following a 48 hr. exposure to pollen, each larva was placed in a plastic box (1224 - 1354 cm^3) and fed clean *A. curassavica* leaves daily until pupation. The bioassay was done once with larvae that were less than 12 hours old (n=10 larvae per treatment), and once with larvae

that were 12-36 hours 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 hrs. 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. Crysali were maintained at 21°C, L:D 16:8. Twenty-four hours after eclosion, each adult was placed in a 9.2 x 8.5 cm envelope and placed 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 & Taylor 1999).

After drying adults for 24 hrs 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 hrs in the first replicate and for 24 hrs 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 & 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, IA. Effects of transgenic pollen on *O. nubilalis* larvae were measured by 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). Over 200 first instar *O. nubilalis,* were then fed each type of colored pollen for 5 hrs. Groups of 6 larvae that had blue digestive tracks were then transferred to 10 plastic cups containing 0.01g of non-dyed pollen of the same hybrid dusted on moist filter paper. After 48 hrs 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, 7333 Bt and 4494 using a kit purchased from Agdia Incorporated, Elkhart, Indiana. 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 3 times for approx. 10 sec. at 4 watts in 5 ml of the extraction buffer provided by Agdia using a Fisher Model 60 Sonic Dismembrator. The pollen was then refrigerated for 15 hrs, and then sonicated for approx. 5 sec. before 100 µl of the sample was removed and placed in a test well.

Data analysis

Pollen deposition on *Asclepias 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 hrs. and 12-36 hrs.), each age class was analyzed separately. The number of days that a larva survived after the initial 48 hr 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 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-square 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 expected, assuming 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 *Asclepias syriaca*

The cumulative deposition of transgenic pollen in 1998 was highest within the corn field (74 to 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 to 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, 8 rainfall events of ≥ 0.84 cm occurred in 1998 and 3 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 for event Bt 11 (ANOVA, p=0.0241). For unexplained reasons, more pollen from event Bt11 was observed on the middle and lower leaves. In 1999, event Bt 11 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

Danaus plexippus larvae exposed for 48 hrs 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 to 306 transgenic pollen grains/cm^2. The average number of pollen grains/cm^2 was 74±15 for event 176 and 36±9 for the non-Bt treatment.

Laboratory assessment of mortality and sub-lethal effects

The survival curves for <12 hr. old larvae exposed to 1,300 pollen grains/cm^2 and 135 pollen grains of event 176, event Bt11 or non Bt corn pollen for 48 hours were significantly different (Log-rank & Wilcoxon $p\leq0.007$) (Fig.1). The survival curves of larvae exposed to 14 pollen grains/cm^2 were similar (Log-rank & Wilcoxon $p=0.3$). At 1,300 grains/cm^2 the non-Bt and event Bt11 survival curves were similar; 40% of larvae survived to 120 hrs. (Log-rank & Wilcoxon $p=0.7$). However, both non-Bt and event Bt11 survival curves were significantly higher than the event 176 survival curve (Log-rank & 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 hrs.) was significantly higher than the transgenic pollen survival curves (Log-rank & Wilcoxon $p<0.006$) (Fig. 1b). The survival curves for larvae exposed to event 176 pollen (30% surviving at 120 hrs.) and to event Bt11 pollen (40% surviving) were similar (Log-rank & Wilcoxon, $p=0.6$) (Fig. 1b).

The survival curves of 12-36 hr. old larvae exposed for 48 hrs to 1,300 pollen grains/cm^2 (Log-rank & Wilcoxon, p=0.005), and 135 pollen grains/cm^2 (Log-rank p=0.03, Wilcoxon, p=0.04) were significantly different (Fig 2). Survival curves at 14 pollen grains/cm2 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 hrs.) was significantly higher than the transgenic pollen survival curves (Log-rank & Wilcoxon $p<0.05$). The survival curves for event Bt11 (44% surviving at 120 hrs.) and event 176 (31% surviving 120 hrs.) were similar (Log-rank & 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 hrs.) was significantly higher than the curves for the larvae exposed to event 176 (63% surviving) or event Bt11 (75% surviving) (Log-rank & Wilcoxon $p>0.05$) (Fig 2b).

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 hours 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 $p>.05$, df=7) (Table 2). Similarly, total development time was similar for all treatments of the 12-36 hr. old larvae (ANOVA p=0.074, df=7) (Table 3). Pupal weights, and adult dry weights, lipid 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 & McCurdy 1993). This range is higher than the lipid contents we determined (ranging from 9.7-19.6) (Tables 2 & 3), which is presumably due to the fact that the *D. plexippus* in our study had not fed as adults.

Bt levels in pollen used in laboratory experiment

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 (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 MON810 (0.09 μg/g fresh weight of pollen) (EPA 1999a). This level of Bt toxin may be due to the presence of pieces of anthers in the Bt11 pollen we used in our laboratory tests. There was more anther tissue in the Bt11 pollen (43±2%) than the event 176 pollen (9±1%) or the non-Bt pollen (0%). Percentages are based upon microscopic examination of ten 6 mm^2 samples of a 0.03±0.002 g sample of pollen spread out in a 5.2 cm d. 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 have 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 hours. *Danaus plexippus* larvae developing in late summer are likely to be exposed to transgenic pollen for most of their larval development, thus mortality may be higher due to the cumulative exposure to insecticidal Bt toxin in transgenic pollen. These findings combined with the prediction that a large portion of larvae will be in or near transgenic corn fields during pollination (Urquhart 1960, Borkin 1982, Malcolm et al. 1993, Wassener & 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 of 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 meters of the field edge where pollen densities are highest (Table 1).

It is clear from our study 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 hrs 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 were observed, but no larval mortality in a non-transgenic pollen treatment was 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 (4 times more than 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. The pollen collected from the Bt11 hybrid was collected and handled using the same procedures as the other two hybrids. The presence of anthers in the pollen may indicate that this hybrid may shed 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-hour 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 didn't 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 & 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 (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 & 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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Table 1. Cumulative field deposition and range of deposition of transgenic corn pollen grains (X (SE)/cm^2 leaf area) on potted milkweed plants placed within and adjacent to a test plot.

1998	Location in field Meters from edge				Predicted pollen Deposition	Est. maximum distance of pollen deposition (meters)
Source of Pollen*	Within	0.2	1.0	3.0		
Event 176	217.0	41.6	25.3	6.3		5.6
	(35.3)	(5.1)	(10.4)	(2.7)	$y = -29.56 \text{ LOG}(X) + 22.22$	
Range	0-506	0-99	0-122	0-35	$r^2 = 0.98$	
Event Bt11	74.2	80.5	49.7	20.4		8.3
	(23.8)	(57.5)	(25.1)	(7.6)	$y = -50.54 \text{ LOG}(X) + 46.44$	
Range	0-152	0-427	0-222	0-56	$r^2 = 0.99$	

1999	Location in field Meters from edge						Predicted pollen Deposition	Est. maximum distance of pollen deposition (meters)
Source of Pollen**	Within	0.2	1.0	3.0	5.0	10.0		
Event 176	79.8	54.4	22.4	7.6	1.6	1.1		6.7
	(24.3)	(26.8)	(17.6)	(4.8)	(0.7)	(0.2)	$y = -32.75 \text{ LOG}(X) + 27.1$	
Range	0-177	0-392	0-61	0-32	0-6	0-4	$r^2 = 0.95$	
Event Bt11	115.4	32.5	28.2	5.0	3.0	0.8		ca. 10
	(26.0)	(3.9)	(15.5)	(0.4)	(1.8)	(0.3)	$y = 47.0 * 10^{-0.198X}$	
Range	0-135	0-42	0-82	0-8	0-11	0-4	$r^2 = 0.88$	

* 1998 Pollen from event 176 was sampled on 29 July, 1 Aug, and 4 Aug. Pollen from event Bt 11 was sampled on 11, 14, and 17 Aug. On each sample date, 9 leaf disks were removed from each of 3 potted milkweed plants placed within the field, 0.2, 1 and 3 meters from the edge. A logarithmic or exponential curve was fit to the pollen deposition data. Range is the highest and lowest number of pollen grains/cm^2 deposited on a single leaf disk during entire collection period.

** 1999 Pollen from even 176 was sampled on 4 and 8 Aug.; pollen from event Bt11 was sampled on 31 July, and 3, 6, 9 Aug. On each sample date, 6 leaf disks were removed from each of 3 potted milkweed plants placed with the field, 0.2, 1, 3, 5 and 10 meters from the edge. A logarithmic or exponential curve was fit to the pollen deposition data. Range is the highest and lowest number of pollen grains/cm^2 deposited on a single leaf disk during entire collection period.

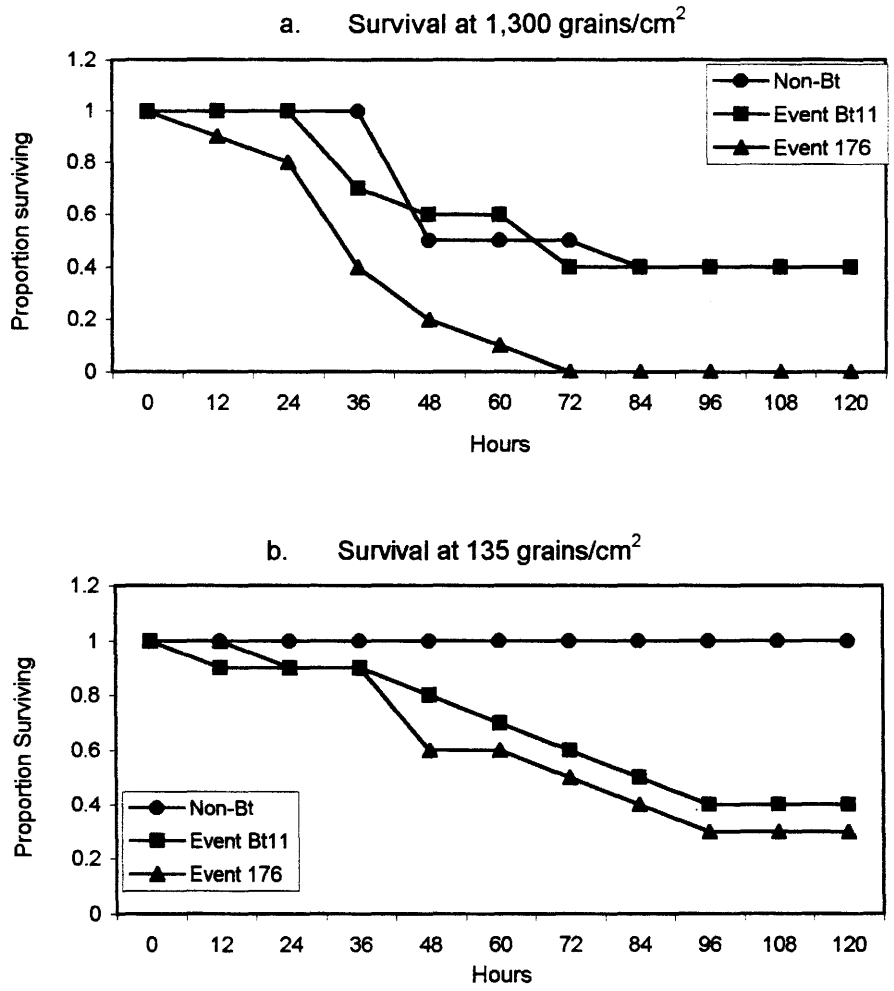

Figure 1: Survival curves for monarch larvae exposed at <12 hrs. old to 1,300 or 135 pollen grains/cm² of non-Bt, event Bt11, and event 176 corn pollen for 48 hours.

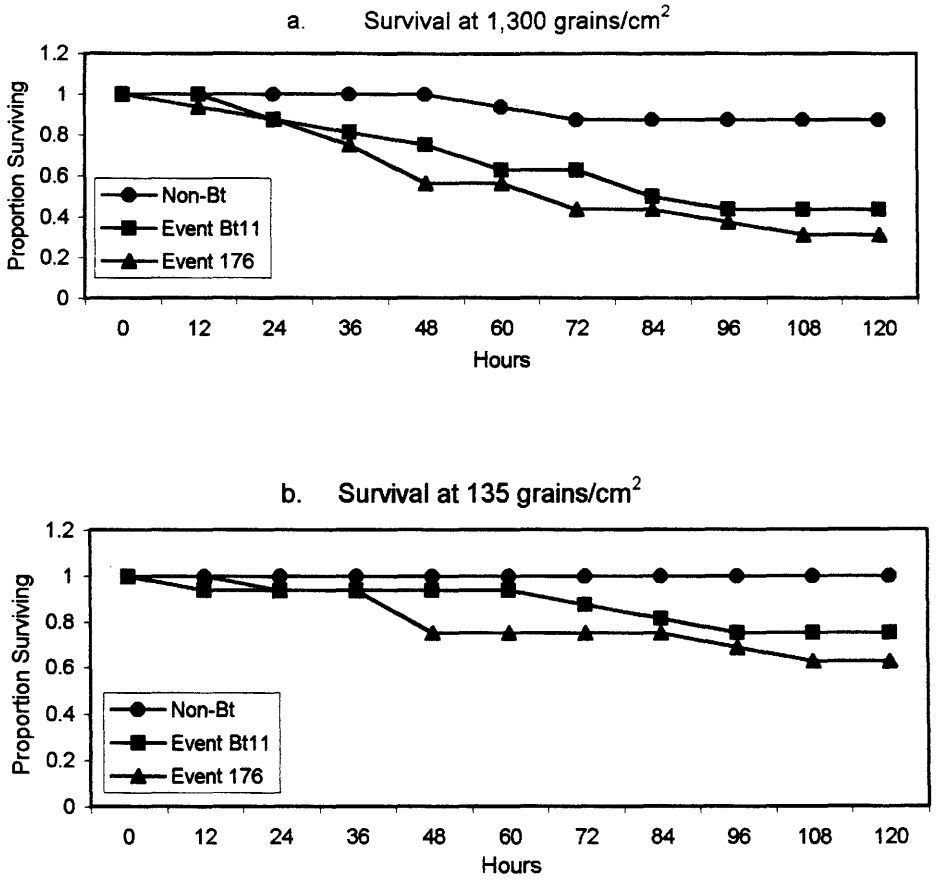

Figure 2: Survival curves for monarch larvae exposed at 12-36 hrs. old to 1,300 or 135 pollen grains/cm² of non-Bt, event Bt11, and event 176 corn pollen for 48 hours.

Table 2: Total developmental (days±SE), pupal weights (grams±SE), wing length (cm±SE) and lipid weight (mg±SE), of larvae less than 12 hrs. old exposed to non-Bt, event Bt11, or event 176 corn pollen, at a concentration of 1300, 135, or 14 pollen grains/cm^2. Data is not presented for event 176 at 1300 pollen grains/cm^2 because there were not enough surviving larvae.

Pollen Type	Concen.	Total devel. time	Pupal weight (g)	Dry weight (g)	Forewing length (cm)*	Lipid weight (mg)
Non Bt						
	1300	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						
	1300	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

* Adults with wings that had not fully expanded before drying were not included in the wing length measurements, but included in the average lipid weight for a treatment.

Table 3: Total developmental time (days±SE), pupal weights grams±SE), wing length (cm±SE) and lipid weight (mg±SE), of larvae 12-36 hrs. old exposed to non-Bt, event Bt11, or event 176 corn pollen, at a concentration of 1300, 135, or 14 pollen grains/cm^2. Data is not presented for event 176 at 1300 pollen grains/cm^2 because there were not enough surviving larvae.

Pollen Concen. Type	Total devel. time	Pupal weight (g)	Dry weight (g)	Forewing length (cm)*	Lipid weight (mg)
Non Bt					
1300	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					
1300	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

* Adults with wings that had not fully expanded before drying were not included in the wing length measurements, but included in the average lipid weight for a treatment.

CHAPTER 3: THE OCCURRENCE AND ABUNDANCE OF *DANAUS PLEXIPPUS* L. (LEPIDOPTERA: DANAIDAE) ON *ASCLEPIAS SYRIACA* (ASCLEPIDACEA) IN CORN AGROECOSYSTEMS

A paper to be submitted to Oecologia

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Abstract

The potential negative effects of Bt corn pollen on the monarch butterfly, *Danaus plexippus,* requires quantification of the presence of *D. plexippus* in corn fields and the effect of Bt corn pollen on female oviposition and larval survival. During a two-year field study, we observed that *D. plexippus* females oviposit on *A. syriaca* in corn fields at similar rates as on *A. syriaca* growing in the roadside habitat (within 10 m of the field edge) parallel to the corn fields. Similar numbers of *D. plexippus* larva were observed on *A. syriaca* in the corn fields compared to the roadside. During 2000, a greater proportion of *A. syriaca* in the corn fields had at least one *D. plexippus* larvae. Similar numbers of eggs were oviposited in transgenic Bt corn fields compared to the non-Bt corn fields, indicating that females do not avoid leaves naturally dusted with densities of Bt pollen from 6-72 pollen grains / cm^2. Similar numbers of *D. plexippus* larvae were found in and near Bt corn fields compared to non-Bt corn fields following corn anthesis. In 2000, no measurable effects of transgenic Event MON810 Bt corn pollen on *D. plexippus* oviposition or larval survival was observed.

Key words: *Bacillus thuringiensis*, Bt corn, oviposition, insect conservation, transgenic crops, risk assessment.

Introduction

Members of the Asclepidaceae are the larval food source for *Danaus plexippus* L. (Lepidoptera: Danaidae), the monarch butterfly (Brower 1969). The common milkweed, *Asclepias syriaca* (Asclepidaceae), is particularly important for the migratory generation of *D. plexippus* in eastern North America; over 90% of the adult monarchs wintering in Mexico fed on *A. syriaca* as larvae (Malcolm et al. 1993). Fifty percent of the wintering adults developed as larvae on *A. syriaca* growing in the upper Midwest, a region with highly concentrated row crop agriculture (Wassenaar & Hobson 1998).

Asclepias syriaca, a native perennial, is the most common milkweed species in northeastern North America (Evetts & Burnside 1972, Bhowmik & Badeen 1976, Malcolm et al. 1993). *Asclepias syriaca* is a common species in cultivated fields due to the emergence of new shoots from root fragments (Cramer 1977, Minshall 1977, Bhowmik & Badeen 1976, Yenish et al. 1997). Additionally, seedling establishment is aided by reduced tillage and cultivation practices that leave seeds near the surface, and the suppression of competitive plant species by herbicides that often do not effectively control *A. syriaca* (Burnside 1977, Evetts & Burnside 1975, Bhomik & Badeen 1976, Bhowmik 1994, Yenish et al. 1996, Yenish et al. 1997). In a survey of 13 mid-western states, Evetts (1977) estimated that 12 million acres (5 million hectares) of corn and 6.1 million acres (2.5 million hectares) of soybeans contained at least one *A. syriaca* plant. An average of 36% of corn fields and 51% of roadsides were infested with *A. syriaca* in eastern Nebraska (Cramer & Burnside 1982). In a recent study in Iowa, 46% percent of corn fields and 71% of roadsides were infested with *A. syriaca* (Hartzler & Buhler 2000). Most of the roadsides in rural Iowa that are

adjacent to agricultural fields are less than 10 m wide, therefore the entire roadside habitat of *A. syriaca* can be affected by agricultural practices (Hartzler & Buhler 2000).

The abundance of milkweeds in and next to corn fields (Cramer & Burnside 1982, Hartzler & Buhler 2000, Evetts 1977), and the sensitivity of *D. plexippus* to the Cry1Ab toxin in transgenic Bt corn pollen create a situation in which the planting of Bt corn may have a negative effect on *D. plexippus* populations (Losey et al. 1999, Hartzler & Buhler 2000, Jesse & Obrycki 2000). It has been hypothesized that *D. plexippus* females will lay fewer eggs on *A. syriaca* growing in corn fields because relatively tall corn plants inhibit visual or olfactory cues females use to detect *A. syriaca* (Tschenn et al. 2001). However, the mechanisms used by lepidopteran females to locate suitable host plants are poorly understood (Calvert & Hanson 1983, Renwick & Chew 1994); and therefore it is difficult to predict which naturally occurring *A. syriaca* plants will be located and found suitable for oviposition by *D. plexippus* females (Tschenn et al. 2001). Previously, defoliation of *A. syriaca* in corn fields by *D. plexippus* larvae was observed in Minnesota, indicating that *D. plexippus* females located host plants within corn fields (Yenish et al. 1997). Examination of the use of *A. syriaca* growing in corn fields by *D. plexippus* is needed to determine the relative importance of *A. syriaca* growing in agroecosystems for *D. plexippus* populations. This was an observational study of the presence of *D. plexippus* in agroecosystems with high densities of *A. syriaca*. The objectives of this study were to 1) compare *D. plexippus* oviposition on *A. syriaca* plants in and bordering corn fields, 2) determine the relative abundance of *D. plexippus* larvae on *A. syriaca* plants within and bordering corn fields, and 3) quantify the effects of Bt corn pollen on *D. plexippus* oviposition and larval survival.

Materials & Methods

Field survey 1999

Objectives 1 and 2 were addressed in 1999. Four field sites with relatively high densities of *A. syriaca* near corn fields were selected from a random survey of sites used to quantify *A. syriaca* distribution in Iowa (Hartzler & Buhler 2000). The four sites were located in 3 counties in north central Iowa (Table 1), sites were 100 m parallel to the roadside and approx. 15 m (15 corn rows and the width of the roadside). For seven weeks, beginning July 11, the number of monarch eggs and larvae in each of the 5 larval stages was counted at each field site by examining the upper and lower surfaces of the leaves and the stem of each *A. syriaca* plant. *Asclepias syriaca* plants were sampled at the following positions at each site in relation to the corn field: 1) in the corn field (50 cm outside the 1st row of corn, and between the 1st and 2nd rows, 4th - 5th and the 14th-15th rows of corn), 2) in the roadside ((ca. 8 m wide) between 50 cm from edge of the corn field and 1 m from the nearest parallel road), and 3) in the road edge (within one m of the edge of the road, typically 8-10 m from the field edge). The number of milkweeds in each position at each field site was counted during the weeks of July 11 and Aug. 22. On July 11 there was an average of 25±14 *A. syriaca* (range; 3-65) in the road edge, 104±74 *A. syriaca* (range; 11-324) in the roadside, and an average of 31±14 *A. syriaca* (range; 3-59) in the corn fields at the 4 field sites. On Aug 22, there was an average of 17±11 *A. syriaca* (range; 1-49) in the road edge, 69±31 *A. syriaca* (range; 27-160) in the roadside, and an average of 22±7 *A. syriaca* (range; 6-40) in the corn fields.

Field Survey 2000

All three objectives were addressed in 2000. In July, 3 field sites in central Iowa were selected so that a Bt and non-Bt corn field were located within 2 miles of each other (Table 1). Each field site had a minimum of 20 *A. syriaca* growing within the first 10 rows of corn, and at least 100 *A. syriaca* growing within a 600 m transect in the roadside parallel to the corn field.

Beginning July 3, the *A. syriaca* located in two positions relative to the corn field were sampled; 1) in the roadside (the 8-10 m between 0.5 m of the edge of the corn field and the nearest parallel road), and 2) in the corn field (within 0.5 m of the first row of corn and within the 1st to the 10th rows of corn). Each field site was surveyed weekly for *D. plexippus* eggs and larvae. The number of *A. syriaca* plants examined and the distance surveyed in each of the three locations on each sampling date was recorded. The number of monarch eggs and larvae in each of 5 instars was counted on each *A. syriaca* by examining the upper and lower surfaces of all the leaves and the stem; the location of the *D. plexippus* life stage (upper or lower leaf surface, top, middle or bottom portion of the plant) was recorded. For each *A. syriaca* with a *D. plexippus* egg or larvae, the presence of predaceous arthropods or aphids, which could serve as prey for predators, was recorded. Also recorded was the height of the plant, and the number of other *A. syriaca* plants within a 1 m dia. circle.

To determine the densities of pollen grains occurring on the *A. syriaca* leaves during corn anthesis, 0.79 cm^2 leaf samples were removed with a #6 cork borer (Jesse & Obrycki 2000) from each *A. syriaca* plant sampled within the corn fields with a monarch life stage. The number of pollen grains was counted under a dissecting scope at 100X. The sample was

removed from a leaf that had an egg(s) or larva(e) present, or if there was insufficient leaf area to remove a sample without disturbing the *D. plexippus,* the leaf disk was removed from the opposite leaf (at approximately the same height and leaf angle). Leaf disk samples were taken during pollen shed (the week of July 10) at the three field sites, and additionally during the week of July 17 at field site 1.

Data Analysis

The occurrence of *D. plexippus* in 1999 and 2000 was analyzed by examining the number of monarch life stages / m. This analysis provides a comparison of the numbers of *D. plexippus* life stages at different field sites and plant locations, regardless of how many *A. syriaca* plants were present. In addition, in 2000, we examined the proportion of plants with *D. plexippus* life stages. In this analysis we determined if the position of a plant in relation to the corn field influenced the probability of an *A. syriaca* having eggs or larvae. All larval instars of *D. plexippus* counted were combined for these analyses.

Data from each year was analyzed separately (PROC MIXED, SAS 8.0) using an analysis of variance for repeated measures. The AR1 (autoregressive) option was used in the repeated measures statement to model the covariant structure (SAS 8.0). Factors and interactions analyzed in the repeated measures design were: field site, plant position (roadside edge, roadside ditch, or within corn field), sampling date, and additionally in 2000, corn type (Bt and non-Bt). The two sources of error used for the data from 1999 were 1) field site*plant position and, 2) week*field site*plant position. The two sources of error used for the data from 2000 were, 1) field site*corn hybrid*plant position and 2) week*field site*corn hybrid*plant position.

A partial life table was constructed for immature *D. plexippus* sampled in 1999, following the methods described by Southwood (1978). We did not construct a life table for data collected in 2000 because our samples did not include the time period following the peak of *D. plexippus* oviposition. The number of eggs, early instars (1 and 2nd instars), and late instars (3-5th instars) entering a stage (lx) in the 100 m transects was estimated by integrating the density estimates for each stage over accumulated degree days and then dividing by the development time in degree days for that life stage (Southwood 1978). The number of individuals dying in a stage (dx) and the percentage mortality in a stage (100qx) was also calculated. Previously, Zalucki (1982) determined *D. plexippus* development times in the field for larvae feeding on *Asclepias fruticosa* L.. Although the *D. plexippus* in our population were feeding on *A. syriaca,* data from Zalucki (1982) are the best development time estimates available. The average of the developmental thresholds for eggs through 5th instars was 11.65^{o}C (Zalucki 1982), so we used this value as our base temperature to calculate accumulated degree days in the field. Developmental accumulations used in our life table analysis were 44.5 degree days for eggs, 58.1 degree days for 1st-2nd instars, and 117.9 degree days for 3rd-5th instars (Zalucki 1982).

Results

Field survey 1999

Danaus plexippus Eggs or Larvae / Meter

The mean number of eggs or larvae / m was similar at the 4 field sites (Table 2). Therefore the data from the 4 field sites was averaged to examine effects of the three plant

positions surveyed (road edge, roadside, and corn field) and time (7 weeks) on the mean number of *D. plexippus* eggs or larvae / m (Fig. 1).

The mean numbers of eggs / m and larvae / m in the road edge, roadside, and within the corn field were similar (Table 2). The average number of eggs / m and larvae / m differed over the 7 week sampling period (Table 2). The highest average number of eggs / m was observed during the third week (July 25) of sampling (ANOVA, df=6, 34, $p \leq 0.02$), particularly on *A. syriaca* growing within the corn field and in the roadside (Fig 1). There was a significant site by week interaction in the numbers of eggs / m (Table 2). For unexplained reasons, field site 4 (Table 1) had highest numbers of eggs during the 4^{th}-7^{th} weeks (Aug 1-28) of sampling in contrast to a peak during the 3^{rd} week at the other three field sites. The highest mean number of larvae / m was observed in the 3^{rd}-6^{th} (July 25-Aug 21) weeks of sampling (ANOVA, df=6, 34, $p \leq .003$) (Fig 1).

Partial Life Table Analysis

In 1999, the mortality rates for eggs in the corn field, roadside and road edge were 69%, 68% and 58%; respectively (Table 3). Mortality rates of the early instars ranged from 76% in the roadside to 94% along the road edge. Mortality for egg through late instars was greater than 90% in all three locations (Table 3).

Field Survey 2000

Eggs or Larvae / Meter

The mean number of *D. plexippus* eggs or larvae / m was similar at the three sites (Table 2), so the site data was combined to examine the effects of the 2 plant positions

surveyed (corn field, roadside), the type of corn (Bt and non-Bt corn), and sampling date (4 weeks) on the mean number of eggs or larvae / m.

We observed similar numbers of eggs / m in and near Bt corn fields compared to non-Bt corn fields (Table 2). The average number of eggs / m within the corn fields was similar to the number in the roadsides (Table 2). The highest number of eggs / m was observed during the 4th week of sampling (July 24-28) (ANOVA, d.f.=3, 6, p=0.002). Of the 46 plants observed with two eggs, almost half (21 *A. syriaca*) were observed during the 4th week; 9 *A. syriaca* plants were observed with 4 eggs, 7 of them in the 4th week of sampling. The maximum number of eggs observed on a single *A. syriaca* was 8 eggs, also observed during the 4th week of sampling.

There was no difference in the average number of larvae / m at the Bt corn sites compared to the non-Bt corn sites (Table 2). Similar numbers of larvae were observed in the roadside compared to within the corn, and during the 4 weeks of sampling (Table 2).

Probability of an *Asclepias syriaca* plant having a *Danaus plexippus* life stage

The probability of observing either egg(s) or larva(e) on an *A. syriaca* plant was similar at the 3 sites (Table 2), so the site data were combined to examine the effects of plant positions surveyed (corn field, roadside), the corn type (Bt and non-Bt corn), and sampling date (4 weeks) on the probability of an *A. syriaca* plant having an egg(s) or larva(e) on it.

There was a similar probability of an egg(s) occurring on an *A. syriaca* growing in or next to a Bt corn field compared to *A. syriaca* growing in or next to non-Bt corn (Table 2). There was also a similar probability of egg(s) on an *A. syriaca* plant growing in the roadside compared to within the corn field (Table 2). The highest probability that an *A. syriaca* would

have at least one egg on it occurred during the 4th week of sampling (July 24-28) (ANOVA, d.f.=3, 6, p=0.03) (Fig 2).

The probability of one or more larvae occurring on a *A. syriaca* plant was similar at the Bt and non-Bt corn sites and over the 4 weeks of sampling (Table 2). *Asclepias syriaca* within a field had a greater probability of larval occurrence compared to *A. syriaca* in the roadside (Table 2, Fig 2).

Location of *Danaus plexippus* life stages: on and among *Asclepias syriaca* plants

Most of the *D. plexippus* eggs (95%) were oviposited on the adaxial surface of the *A. syriaca* leaves. This result is similar to that of Zalucki and Kitching (1982), in which 96.5% of eggs were laid on the underside of leaves, and Borkin (1982) who observed 67% of eggs on the underside of leaves. Forty-six percent of the eggs were laid in the middle portion of the plant, 39% on leaves in the top portion, and 15% in the bottom portion.

Most of the larvae (83%) were observed underneath *A. syriaca* leaves; 17% were observed on the adaxial surface. A similar number of larvae were observed in the top and middle portions of the milkweed plant, 38% and 35%, respectively, and 27% of larvae were observed on leaves in the lower portion of *A. syriaca* plants.

Most of the eggs and larvae (53%) were observed on plants with 1-3 other *A. syriaca* within 0.5 m, 28% of eggs and 36% of larvae were observed on plants with no other *A. syriaca* within 0.5 m. The remaining 19% of eggs and 11% of larvae were observed on *A. syriaca* with more than 4 other *A. syriaca* within 0.5 m.

Predaceous arthropods and pollen densities on *Asclepias syriaca*

We observed Araneae, Formicidae, coccinellid larvae, chrysopid eggs and larvae, Reduviidae, Pentatomidae, Nabidae and Aphididae, that attract predators, on *A. syriaca* plants with *D. plexippus* eggs and larvae. Of the 331 plants with a *D. plexippus* life stage surveyed over the 2nd to 4th weeks of sampling, 9% had aphids and 15% had predaceous arthropods on them.

Pollen densities on *A. syriaca* plants with a *D. plexippus* life stage in the Bt corn fields ranged from 5.9±1.5 pollen grains / cm^2 at site 1 to 72.3±14.5 pollen grains / cm^2 at site 3 (Table 4). In the non-Bt corn fields *A. syriaca* with a *D. plexippus* life stage had from 7.5±3.0 pollen grains / cm^2 at site 1 to 125.6±41.5 pollen grains / cm^2 at site 3 (Table 4).

Discussion

Presence of *Danaus plexippus* in and near corn fields

A previous study reported that *D. plexippus* females oviposit on *A. syriaca* plants growing in corn fields (Yenish et al. 1997). In both 1999 and 2000, we observed similar oviposition rates on *A. syriaca* plants within roadside habitat compared to plants growing in corn fields. This observation indicates that ovipositing *D. plexippus* females do not discriminate between *A. syriaca* growing in these two habitats. During our sampling, corn plants were over 1.5 m, whereas the *A. syriaca* plants were less than 1 m, so *A. syriaca* plants growing in corn fields may not have been visually apparent to females, however, they are likely chemically apparent (Feeny 1975). These field results differ from a greenhouse study that showed that *D. plexippus* females oviposited less frequently on *A. syriaca* visually concealed by corn plants (Tschenn et al. 2001). Many factors, other than the presence of

corn plants, may affect oviposition. For example, ovipositing *D. plexippus* females detect the cardiac glycoside content of *Asclepias* spp. and prefer *Asclepias* plants with moderate levels of glycosides (Oyeyele & Zalucki 1990, Van Hook & Zalucki 1999). Possibly solar irradiation, soil conditions, or nutrient levels in the corn field may influence glycoside content of *A. syriaca* plants. In addition, previous studies have shown that height and patch size of *Asclepias* spp. plants affects the number of eggs oviposited on plants and larval survival (Zalucki & Kitching 1982, Cohen & Brower 1982). Both Zalucki & Kitching (1982) and Cohen & Brower (1982) observed that the number of *D. plexippus* eggs / plant and larval survival were directly related to plant height.

The partial life table analysis in 1999 showed similar rates of *D. plexippus* mortality from eggs to late instars at the road edge (98%), in the roadside (92%) and within the corn field (95%). These levels of mortality are similar to rates reported for *D. plexippus* by Zalucki & Kitching (1982) in Australia (92-97% mortality by the 5th instar).

In 2000, the probability of an *A. syriaca* plant having at least one larva on it was higher if the plant was growing in a corn field. *Danaus plexippus* larvae feeding on *A. syriaca* contend with the combined plant defenses of a high latex content and inducible cardiac glycosides (Zalucki & Malcolm, 1999). Reduced light within the corn fields, fertilizer, or herbicides could influence the latex or cardenolide contents of *A. syriaca* plants, and thus affect *D. plexippus* larval survival, especially 1st instars (Zalucki et al. 1990, Zalucki & Malcolm 1999). In 1999 we observed slightly lower mortality rates of early instar *D. plexippus* in the roadside (76%) compared to early instars within the corn field (85%). Other possible mortality factors affecting *D. plexippus* on *A. syriaca* growing in the roadside and road edge areas include herbicide applications, mowing, and grading of gravel roads.

Seasonal occurrence of *Danaus plexippus* relative to corn anthesis

In 1999, more *D. plexippus* eggs / m were observed during the 3rd week (July 26-30) of sampling. The number of larvae was highest during the 3-6th weeks of sampling (July 26-Aug 19). In 2000, the highest numbers of eggs / m and the greatest proportion of plants with eggs occurred during the 4th week (July 24-28) of sampling; similar to the peak in egg numbers observed in 1999. In a field survey of immature *D. plexippus* in Wisconsin, USA, Borkin (1982) also reported a peak in the numbers of eggs and larvae from mid-July to late August. This time period coincides with corn anthesis in Iowa (Ritchie, et al., 1997), potentially exposing *D. plexippus* larvae to Bt corn pollen.

Effect of Bt corn pollen on the presence of *Danaus plexippus* life stages

Similar numbers of eggs were counted on *A. syriaca* in and near transgenic Bt and non-Bt corn fields after pollen shed (2-3rd week of sampling), indicating that *D. plexippus* females do not avoid *A. syriaca* plants dusted with Bt corn pollen. In caged experiments, Tschenn et al. (2001) observed similar numbers of eggs oviposited on *Asclepias curassavica* plants dusted with Bt and non-Bt corn pollen. However, in a greenhouse experiment, *D. plexippus* females oviposited less frequently on *A. syriaca* dusted with corn pollen (Tschenn et al. 2001). We observed no differences in the number of eggs on *A. syriaca* in the corn fields, where more pollen is deposited on milkweeds (Jesse & Obrycki 2000). Thus, other factors (i.e. cardenolide content, plant height, or nutrient content), may have greater influence on oviposition behavior than Bt corn pollen. In addition, pollen densities we recorded on *A.*

syriaca growing in the corn fields were below the 500 pollen grains / cm^2 used in the previous greenhouse study (Tschenn et al. 2001).

The number of larvae in and near Bt corn fields was similar to non-Bt corn fields following corn anthesis (2^{nd} week of sampling). Pollen densities observed in the Bt corn fields were generally lower than the 135 pollen grains / cm^2 which caused significant mortality in larvae exposed to an Event Bt11 corn hybrid Bt expression in this transgenic pollen is similar to Event MON810 (EPA 2000; Jesse & Obrycki 2000). However, our leaf samples may underestimate pollen densities at the peak of pollen shed because samples were taken from relatively few plants at weekly intervals that were not synchronized with the exact date of peak pollen shed. In addition, *D. plexippus* is a Type A insect herbivore with over 70% mortality by the mid-larval stages (Price 1997, Zalucki & Kitching 1982), thus high levels of mortality may make it difficult to identify mortality from a additional source, i.e. Bt corn pollen, because natural mortality may overshadow it (Carey 1993).

Danaus plexippus conservation efforts have focused on the vulnerable forested wintering habitats in Mexico (Brower & Malcolm 1991, Malcolm & Zalucki 1993), however, conservation of this species also requires a consideration of larval food sources (Taylor et al. 1999). The abundance of *A. syriaca* growing in or in close proximity to agricultural fields makes it necessary to assess the impact of agricultural practices on the survival of *D. plexippus* larvae (Taylor et al. 1998). Crop protection practices that may negatively effect *D. plexippus* include herbicide treatments and cultivation that reduce the above ground parts of *A. syriaca* plants, and pest management tactics that harm *D. plexippus* directly, i.e. insecticide sprays, or the planting of transgenic crops producing Bt toxins in the pollen (Losey et al. 1999, Jesse & Obrycki 2000)

The *A. syriaca* plants growing in corn fields and the immature *D. plexippus* on them will be affected by agricultural practices. Our research shows that these *A. syriaca* are oviposited on by *D. plexippus* and that larval *D. plexippus* are present on them. In the Midwest, where agriculture is the dominant use of land, immature *D. plexippus* will be exposed to a range of agricultural practices (e.g. cultivation, herbicide applications, planting of Bt corn); some may have negligible impact, others will have potentially large negative effects. Conservation of *D. plexippus* larval habitats requires increased scrutiny of the effects of agriculture practices on *D. plexippus* populations.

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Table 1: Location of field sites in 1999 and 2000. Sites were in central and north central Iowa.

Site	Corn	County	Latitude	Longitude	Hybrid	Event
1999						
1	Unknown*	Grundy	N42°27.82'	W92°56.76'	—	—
2	Non-Bt	Webster	N42°34.42'	W94°24.23'	—	—
3	Non-Bt	Pocahontas	N42°49.26'	W94°36.04'	—	—
4	Unknown*†	Pocahontas	N42°50.13'	W94°37.59'	—	—
2000						
1	Bt	Story	N42°08.28'	W93°38.35'	Agropro 9355	MON810
	Non-Bt	Story	N42°08.22'	W93°39.62'	Agropro 9565	—
2	Bt	Hamilton	N42°06.05'	W93°37.27'	Asgrow 730Bt	MON810
	Non-Bt	Hamilton	N42°06.38'	W93°37.45'	Asgrow 730	—
3	Bt†	Story	N42°15.63'	W93°31.24'	Pioneer 35NO5	MON810
	Non-Bt	Story	N42°15.21'	W93°32.41'	Garst hybrids	—

* In 1999 we were unable to determine the type of corn (Bt or non-Bt) at sites 1 and 4 due to absentee land ownership.
† Adjacent road was paved, with approx. 1.5 m of gravel between paving and roadside plants.

Table 2: Partial ANOVA table for *D. plexippus* eggs or larvae / meter and proportion of plants with eggs or larvae; 1999 and 2000. Non-significant interactions between factors are not presented.

Year	Analysis	Effect	*df*	error *df*	F	*p*
1999	Eggs/Meter	Field site	3	6	1.34	0.35
		Plant position	2	6	2.13	0.20
		Week	6	34	4.31	0.003*
		Site*Week	17	34	2.40	0.015*
	Larvae/Meter	Field site	3	6	1.19	0.39
		Plant position	2	6	2.94	0.13
		Week	6	34	2.43	0.046*
2000	Eggs/Meter	Field site	2	2	2.35	0.30
		Corn hybrid	1	2	0.70	0.49
		Plant position	1	2	0.00	0.95
		Week	3	6	8.71	0.01*
	Proportion of Plants w/Eggs	Field site	2	2	3.54	0.22
		Corn hybrid	1	2	0.78	0.47
		Plant position	1	2	9.00	0.10
		Week	3	6	5.71	0.03*
	Larvae/Meter	Field site	2	2	1.64	0.38
		Corn hybrid	1	2	0.03	0.87
		Plant position	1	2	1.51	0.34
		Week	3	6	0.18	0.90
	Proportion of Plants w/Larvae	Field site	2	2	0.19	0.84
		Corn hybrid	1	2	3.57	0.20
		Plant position	1	2	58.9	0.02*
		Week	3	6	0.27	0.85

* Significant effect ($p<0.05$)

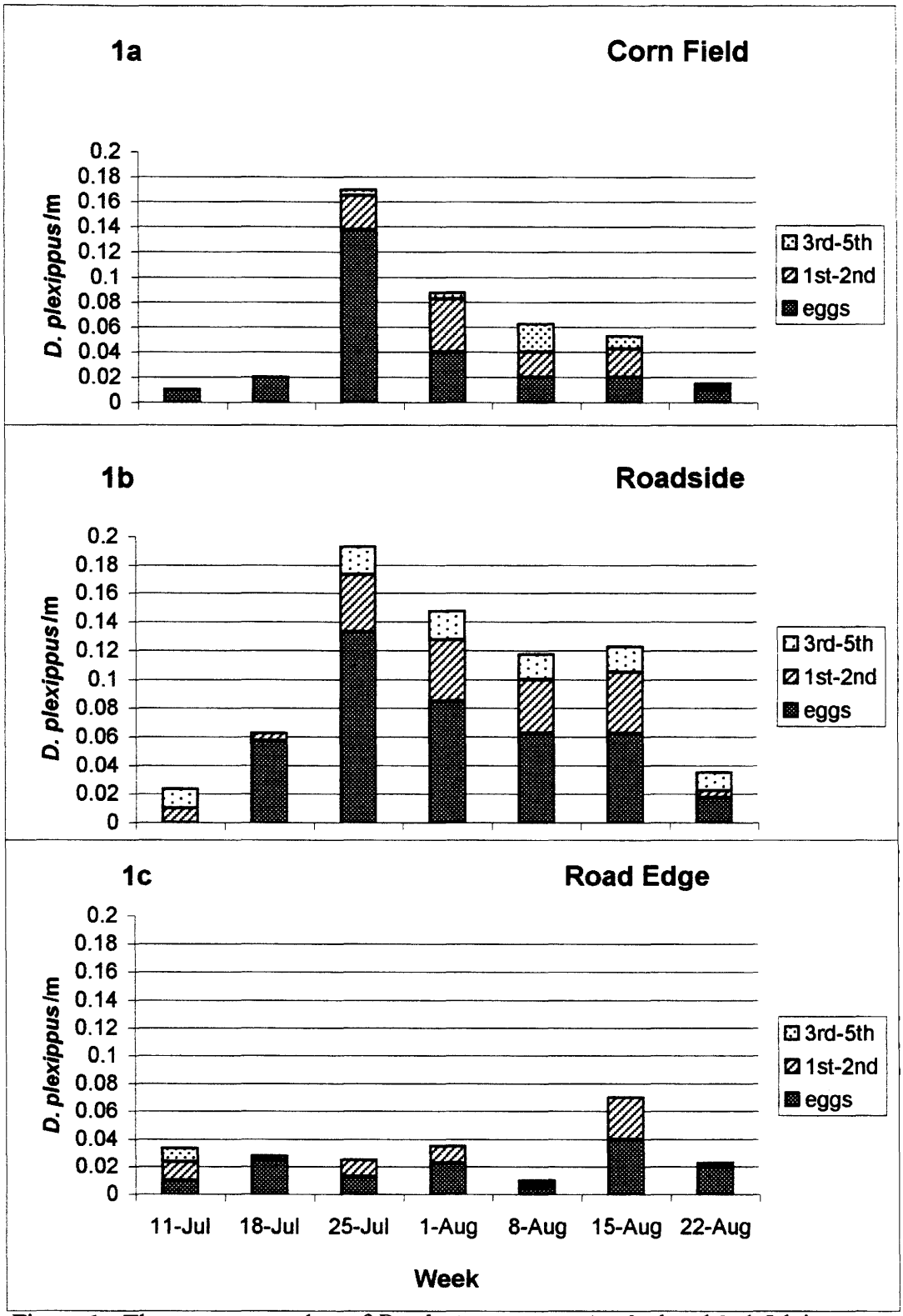

Figure 1: The avarage number of *D. plexippus* eggs, 1st-2nd and 3rd-5th instar larvae / meter in the corn field (a), roadside (b), and road edge (c) in 1999.

Table 3: Partial life table for *D. plexippus* eggs, early instar (1^{st}-2^{nd}) and late instar (3^{rd}-5^{th}) larvae in central Iowa, 1999. Numbers of *D. plexippus* in the corn field, roadside, and road edge were averaged from three field sites.

Location	Stage	lx[a]	dx[b]	100qx[c]
Corn field	Egg	212	147	69
	Early instars	65	55	85
	Late instars	10	—	—
	Egg-Late instars	—	202	95
Roadside	Egg	296	200	68
	Early instars	96	73	76
	Late instars	23	—	—
	Egg-Late instars	—	273	92
Road edge	Egg	85	49	58
	Early instars	36	34	94
	Late instars	2	—	—
	Egg-Late instars	—	150	98

[a]lx – number of individuals entering stage

[b]dx – number of individuals dying in stage

[c]100qx – percent mortality in stage

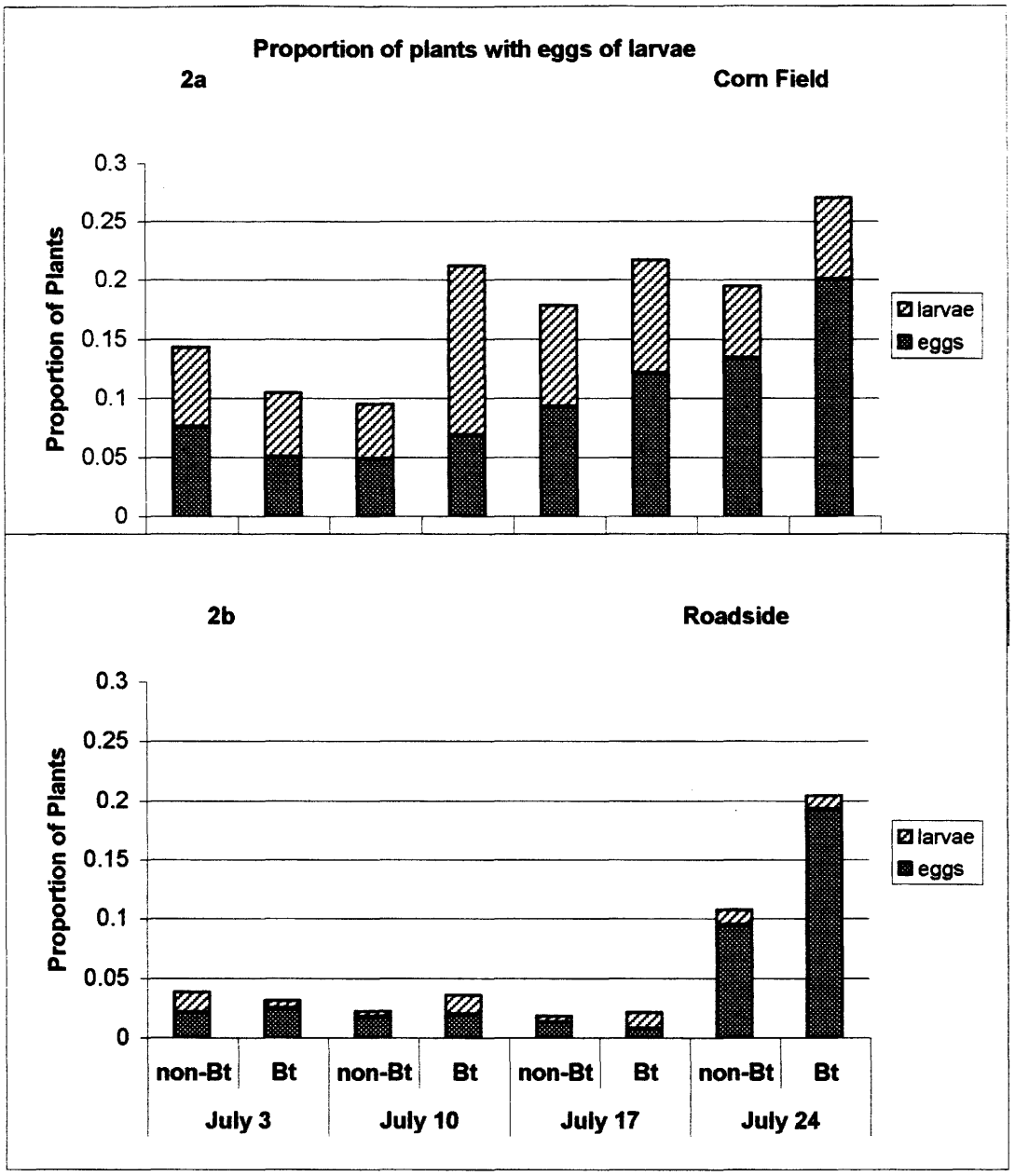

Figure 2: Average proportion *A. syriaca* at the 3 field sites with *D. plexippus* eggs larvae in the corn fields (a) or roadsides (b) of Bt or non-Bt corn in 2000.

Table 4: Pollen samples from *A. syriaca* with a *D. plexippus* life stage; mean number of pollen grains / cm^2 ± SE, number of samples (n) at the three field sites in 2000.

Site	Week	Bt Corn	Non-Bt Corn
1	July 10	35.8±8.8 (15)	7.5±3.0 (11)
	July 17	23.0±8.1 (7)	59.4±17.3 (10)
2	July 10	5.9±1.5 (14)	23.0±10.2 (6)
3	July 10	72.3±14.5 (7)	125.6±41.5 (4)

CHAPTER 4: SURVIVAL OF EXPERIMENTAL COHORTS OF *DANAUS PLEXIPPUS* L. (LEPIDOPTERA: DANAIDAE) LARVAE PLACED IN BT AND NON-BT CORN FIELDS

To be published as part of a larger paper

Laura C. Hansen Jesse & J. J. Obrycki

Introduction

Two recent studies have raised concerns about the effects of transgenic Bt corn pollen on the monarch butterfly, *Danaus plexippus* L. (Lepidoptera: Danaidae) (Losey et al., 1999; Jesse & Obrycki 2000). As corn pollen is shed it settles on nearby plants, including *Asclepias syriaca* (Asclepidacea), a major food source for *D. plexippus* larvae (Malcolm et al. 1993), and a common weed in agricultural fields (Yenish et al., 1997; Bhomik & Badeen, 1976; Cramer, 1977; Evetts & Burnside, 1975; Hartzler & Buhler, 2000). *Danaus plexippus* larvae feeding on *A. syriaca* in and adjacent to Bt corn fields accidentally ingest the Bt corn pollen, exposing them to the *Bacillus thuringiensis* derived toxin, Cry1Ab. Losey et al. (1999) and Jesse & Obrycki (2000) reported increased *D. plexippus* larval mortality when exposed to Bt corn pollen applied in the laboratory to *Asclepias curissavica* leaves, and Jesse & Obrycki (2000) showed increased mortality in *D. plexippus* larvae following exposure to Bt corn pollen naturally deposited in the field on *A. syriaca* plants.

Detailed field studies are needed to assess the effect of naturally deposited Bt corn pollen on *D. plexippus* larvae. The objective of this study was to determine the effect of Bt

corn pollen on cohorts of *D. plexippus* 1st instars placed on *A. syriaca* within and near Bt and non-Bt corn fields during pollen shed.

Materials & Methods

In 2000, 3 one acre sites at Iowa State University research farms (Burkey, Bruner, and Johnson Farms) in Story Co., IA were planted as paired plots of approx. 0.5 acre of Bt corn (N4640Bt, Northrup King, Event Bt11) and 0.5 acre of non-Bt corn (N4640, NK). Two sites were planted on April 28 and the third on May 3. All 3 sites were treated with nitrogen fertilizer and the Johnson Farm site was treated with a soil application of Lorsban for corn rootworm control at planting; the sites were cultivated in late May.

Asclepias syriaca plants were transplanted to the field sites during May and June. Plants were watered as needed and fertilized with Osmocote®, Scotts-Sierra Co. The *A. syriaca* plants were approx. 5 cm to 50 cm tall when transplanted. Multiple plants (<3) were transplanted to a single location because of high levels of plant mortality following transplanting (if more than 1 plant survived, larvae were placed on only 1 of the plants). The *A. syriaca* plants were placed 2 m from the edge of the corn field, at the edge of the corn field, and 4.6 m within the corn fields. Six *A. syriaca* were placed at each location for each corn hybrid, thus we transplanted 36 *A. syriaca* at each site (18 plants / hybrid).

Approximately 7 days after the start of anthesis, five 1st instar *D. plexippus* were placed on each *A. syriaca* plant (36 plants / site x 3 sites x 5 larvae / plant = 540 larvae). Larvae were placed in the field on July 15th at Burkey and Bruner Farms, and July 19th at Johnson Farm. The number and life stage of *D. plexippus* larvae were recorded every 24 hours for 7 days, and then, every 48 hours for another 7 days. Naturally occurring *D.*

plexippus life stages were noted. One 0.79 cm diameter leaf sample was removed with a #6 cork borer from a middle leaf of each plant. Pollen grains were counted under a dissecting scope to assess pollen densities / cm^2 (Jesse & Obrycki 2000). Samples were removed on July 17th at Burkey and Bruner Farms and July 27th at Johnson Farm.

Data Analysis

The number of live larvae over time was analyzed with a GLM procedure (SAS 8.0). The effects of field site and corn hybrid on the numbers of *D. plexippus* larvae were analyzed with farm*hybrid as the error term, and the effect the location of the *A. syriaca* on larval survival as analyzed with field site*hybrid*location as the error term. Survival curves for the larvae were analyzed using LIFETEST (SAS 8.0). Because of the tendency of *D. plexippus* larvae to move off *A. syriaca* when molting (Borkin, 1982), we observed larvae reappearing on later sampling dates. These reappearing larvae were added to the previous sample date count.

Results

The numbers of larvae surviving at the three field sites were similar (d.f.=2, F=0.26, p=0.79). There were similar numbers of larvae alive in the Bt compared to the non-Bt corn (d.f.=1, F=0.01, p=0.93). The location of the *A. syriaca* plants (within the corn, at the edge of the field, or 2 m. outside the field) had no effect on larval survival (d.f.=2, F=3.77, p=0.12).

The survival curves for the *D. plexippus* larvae were similar at the three field sites (log-rank, p=0.60, Wilcoxon p=0.44). Combining the three field sites, the survival curve for

D. plexippus larvae in the Bt corn was similar to the survival curve of larvae in the non-Bt corn (log-rank p=0.84, Wilcoxon p=0.72) (Fig 1a). The survival curves of the *D. plexippus* larvae on *A. syriaca* within the field, at the edge of the field, and outside the field were somewhat different (log-rank P=0.09, Wilcoxon p=0.04). There may be a trend to slightly lower survival of *D. plexippus* on *A. syriaca* at the edge of the field (Fig 1b).

Average pollen densities within the corn field ranged from 154-367 pollen grains / cm^2, densities outside the corn field ranged from 11-116 pollen grains / cm^2, and densities 2 m outside the field ranged from 5-36 pollen grains / cm^2 (Table 2).

Discussion

In the presence of other mortality factors *D. plexippus* larval survival was not reduced by Bt corn pollen relative to non-Bt corn pollen. These results differ from those previously reported by Jesse & Obrycki (2000); 20% mortality in *D. plexippus* larvae exposed in the laboratory to field deposited Bt pollen vs. 0% mortality for larvae exposed to non-Bt corn pollen. This level of mortality was observed when larvae were exposed to an Event 176 corn hybrid. Event 176 utilized a pollen specific promoter and expresses 10 times more of the Bt toxin in the pollen grains than Event Bt11, the transgenic Bt corn event used in the current study (EPA, 2000). Jesse & Obrycki (2000) reported 25-60% mortality in larvae exposed to 135 pollen grains / cm^2 of Bt11 corn pollen. The pollen used in this study contained anther parts that increased the amount of Bt toxin the larvae were exposed to (Jesse & Obrycki 2000). An ELISA showed Bt protein levels were 0.39 µg Bt / g pollen. Based on data presented on the EPA website (2000), we estimate the Bt protein expression in the pollen of Event Bt 11 corn hybrids is similar to expression in Event MON810; 0.09 µg / g pollen

(EPA, 2000). In addition, mortality observed by Jesse & Obrycki (2000) were measured under laboratory conditions, where other sources of mortality were eliminated. Larvae were provided with a single 1.54 cm^2 leaf disk with the Bt corn pollen on it for 48 hrs., so the *D. plexippus* larvae were unable to avoid ingesting pollen, except by not feeding.

Pollen densities that we observed were similar to those reported previously (Jesse & Obrycki, 2000), who reported 74-217 pollen grains / cm^2 in Bt corn fields and 32-80 pollen grains / cm^2 at the edge of the corn field.

To more fully assess the role of Bt pollen in larval mortality, experimental techniques may be needed to separate mortality due to Bt corn pollen from other factors, for example, predation. Experimental elimination of other causes of mortality is necessary to assess the effect of Bt corn pollen relative to other mortality factors (Carey, 1993). An approach such as a multiple decrement life table analysis may be required to assess the effect of Bt corn pollen (Carey, 1993). During this one-year study using experimental cohorts of *D. plexippus* larvae, it appears that in the presence of other mortality factors, the mortality due to Bt corn pollen is negligible.

Acknowledgements

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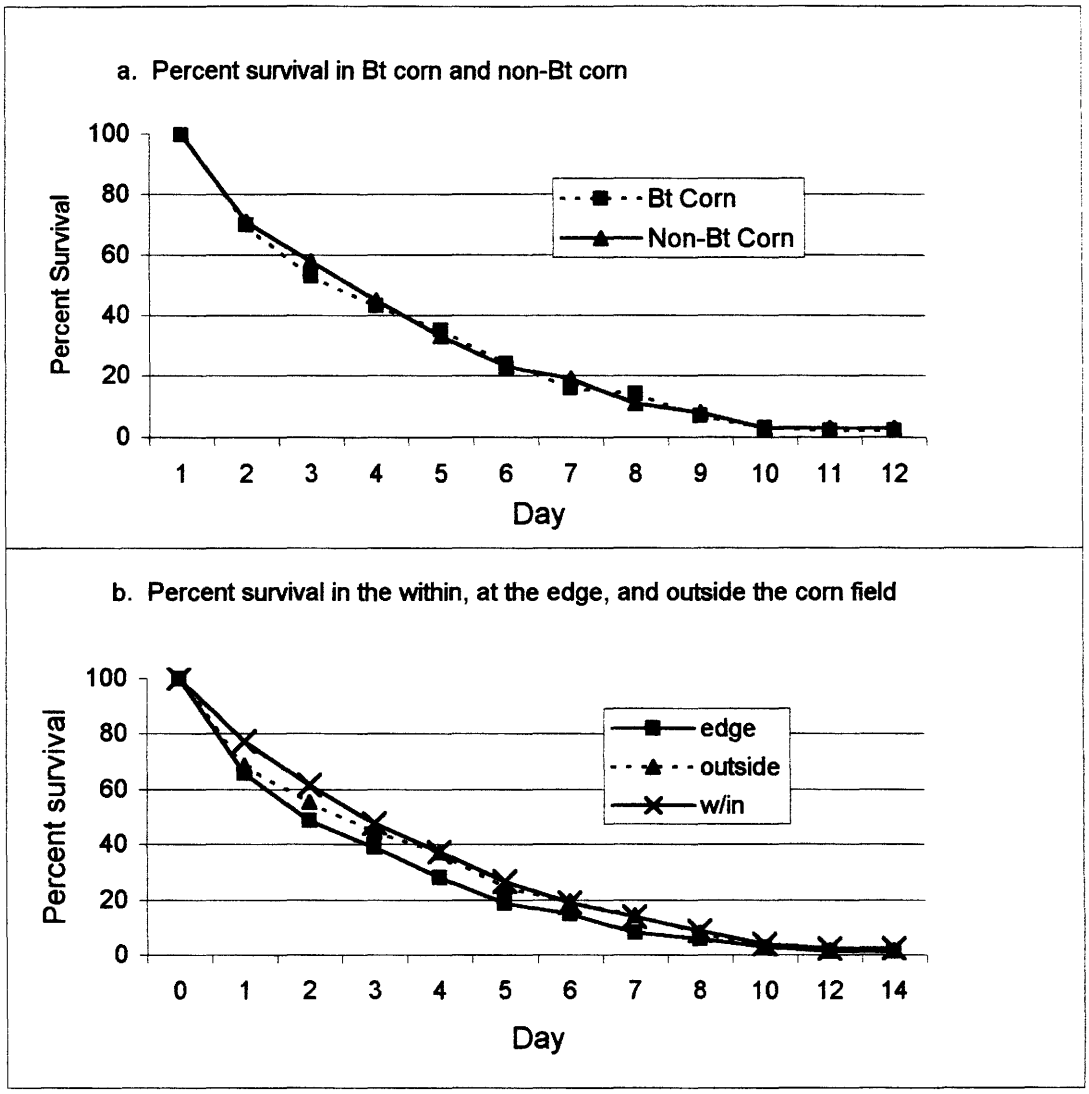

Fig 1: Survival curves for *D. plexippus* larvae placed in and near Bt and non-Bt corn fields (a), larvae were placed on *A. syrica* within the field, at the edge of the field, or 2 m outside the field (b).

Table 1: The average number of pollen grains / cm^2 ±SE on a single pollen sample from each of the 6 *A. syriaca* plants located in the corn field, at the edge of the corn field, and 2 m outside the corn field.

	Corn Hybrid	
Location of *A. syriaca*	**Bt Corn**	**Non-Bt Corn**
Within the corn field		
Burkey Farm	318±45	367±65
Bruner Farm	154±35	185±53
Johnson Farm	252±27	320±84
Edge of the corn field		
Burkey Farm	70±17	116±40*
Bruner Farm	11±7	23±7
Johnson Farm	99±28	92±16
2m outside the corn field		
Burkey Farm	32±12	18±6
Bruner Farm	5±2	6±5
Johnson Farm	36±13*	36±10

* Sampled 5 *A. syriaca* plants because 1 of the plants had died.

CHAPTER 5: ASSESSMENT OF THE NON-TARGET EFFECTS OF BT CORN POLLEN ON THE MILKWEED TIGER MOTH, *EUCHATIAS EGLE* (LEPIDOPTERA: ARCTIIDAE)

A paper to be submitted to the Journal of the Kansas Entomological Society

Laura C. Hansen Jesse & John J. Obrycki

Abstract

Assessment of the effects of transgenic corn pollen expressing a *Bacillus thuringiensis* derived toxin on non-target Lepidoptera species is currently lacking. A laboratory experiment examining the effect of transgenic Bt corn pollen on *Euchatias egle* showed no larval mortality following a 48 hr exposure to Bt corn pollen on its food source, *Asclepias syriaca*. Two Bt corn events were used; 176, which expressed 1.60 μg Bt/g pollen of the Bt toxin and Bt11, which expressed 0.39 μg Bt/g pollen of the Bt toxin. Seven percent of larvae exposed to non-Bt corn pollen died. *Euchatias egle* will probably not be adversely affected by the wide scale planting of Bt corn.

Introduction

Reports of negative effects of Bt corn pollen on *Danaus plexippus* (Lepidoptera: Danaidae), the monarch butterfly, (Losey et al., 1999; Jesse and Obrycki, 2000) have focused attention on assessing the potential non-target effects of the widespread planting of Bt corn. However, less well-known lepidopteran species that feed in and near Bt corn fields during anthesis could be affected by the dispersal of the Bt toxin in corn pollen. To assess the

potential non-target effects of Bt corn it is important to determine the sensitivity of these lepidopteran species to the *Bacillus thuringiensis* derived Cry1Ab toxin that is expressed in the pollen of most commercial Bt corn hybrids (Koziel et al., 1993; Fearing et al., 1997, Losey et al., in press). The Cry1Ab toxin expressed by transgenic corn plants is toxic to larval lepidopterans, the toxin binds to receptors in susceptible lepidopterans midguts and causes the midgut cells to lyse and gut contents to spill into the hemolymph, killing the larvae (Hofte and Whiteley, 1989; Beegle and Yamamoto, 1992; Gill et al., 1992; Pietrantonio et al., 1993).

In a recent field study, Wraight et al. (2000) demonstrated that larvae of *Papilio polyxenes* (Lepidoptera: Papilionidae), the black swallowtail, did not show increased mortality in the presence Bt corn pollen. *Papilio polyxenes* is a non-target species that is likely to be exposed to Bt corn pollen because larvae feed on Apiaceae species, some of which are common plants along roadsides, and near the edges of cultivated fields (Wraight et al. 2000). A second lepidopteran species that feeds on plants growing near corn is *Euchatias egle* (Lepidoptera: Arctiidae), the milkweed tiger moth. Similar to the monarch, *E. egle* larvae feed on *Asclepias* plants. Because of the concern about the effects of Bt corn on *D. plexippus* information has been gathered on the amount of corn pollen deposited on *A. syriaca* leaves, and the distribution of *A. syriaca* plants in agricultural areas (Hartzler and Buhler, 2000; Jesse and Obrycki, 2000, Chapter 3). Transgenic corn pollen was deposited on milkweed plants at least 10m from the field edge; highest densities were observed on plants placed within or within 3 m of corn fields (Jesse and Obrycki, 2000). The common milkweed, *Asclepias syriaca* was observed infesting 46% of corn fields and 71% of roadside areas in Iowa (Hartzler and Buhler, 2000).

The objective of this study was to determine if young *E. egle* larvae (1st or 2nd instars) are sensitive to the Cry1Ab toxin expressed in the pollen of two transgenic Bt corn hybrids, Northrup King (NK) MAX 454 (Event 176) and NK 7333Bt (Event Bt11). Event 176 utilized a pollen specific promoter and has a relatively high expression of Cry1Ab in the pollen grains, the cauliflower mosaic virus 35s promoter controls expression of *cry*1Ab in Event Bt11 pollen resulting in less Cry1Ab expression in the pollen (EPA, 2000a).

Materials and Methods

Pollen was collected from the Bt corn hybrids (MAX 454 and 7333Bt) and a non-Bt corn hybrid genetically similar to MAX 454 (NK 4494) from 29 July to 19 Aug., 1998 by stapling brown paper bags over corn tassels. After 6 - 7 days, the bagged tassels were removed from the corn stalk, dried for 24 hrs, and the pollen was sifted through a sieve (300 μm openings), and stored at – 20°C for 9-10 months. An ELISA was conducted on the pollen used in this experiment 5 months later to determine Cry1Ab levels in the pollen (Jesse and Obrycki, 2000). The ELISA showed that the MAX 454 (Event 176) pollen had 1.60 μg Bt/g pollen, the 7333Bt (Event Bt11) pollen had 0.39 μg Bt/g pollen, and the non-Bt 4494 pollen had 0.09 μg Bt/g pollen. This contamination of the non-Bt pollen likely occurred during the planting of the corn, or during the collection and processing of the pollen. The amount of Bt toxin in the Event Bt11 pollen was higher then reported by the EPA for the similar event MON810 (0.09 μg/g fresh weight of pollen) (EPA 1999a). This level of Bt toxin may be due to the presence of pieces of anthers in the Bt11 pollen we used in our laboratory tests. There was more anther tissue in the Bt11 pollen (43±2%) than the event 176 pollen (9±1%) or the non-Bt pollen (0%) (Jesse & Obrycki, 2000).

Forty-five field collected 1st or 2nd instar *E. egle* larvae were exposed to transgenic (MAX 454, 7333Bt) or non-transgenic (4494) pollen. Fifteen larvae were exposed to 1,300 pollen grains/cm^2 of each type of corn pollen. This density was obtained by suspending 0.1 g of pollen in 10 ml of distilled water, and then a 0.05 ml drop of the suspended pollen solution was placed on a 1.54 cm^2 disk of *A. syriaca*. The mean number of pollen grains per square cm was estimated to be 1,300 (Jesse and Obrycki, 2000). Each leaf disk was placed on moistened filter paper in a 5.2 cm dia. petri dish. One *E. egle* larva was placed on the leaf disk and maintained at 21^{o}C; L:D 16:8. Larvae were placed on top of the leaf disk, but they were able to move and feed from underneath the disk. Following a 48 hr. exposure to pollen, each larva was placed in a plastic box (1224 - 1354 cm^3) and fed clean *A. syriaca* leaves until pupation. Larval survival was recorded until pupation. Larvae were coded as either alive or dead; this data was analyzed with the GLM procedure (SAS 8.0).

Results & Conclusion

There was no difference in the mortality of *E. egle* larvae following a 48 hr. exposure to Cry1Ab toxin from two transgenic corn hybrids and pollen from a non-transgenic hybrid (ANOVA, df= 2, 41, F=1.08, p=0.35). A similar number of larvae survived exposure to pollen from each of the three corn hybrids. All 15 larvae exposed to either the Event 176 pollen or the Bt11 corn pollen survived to pupation. Fourteen of the 15 larvae survived exposure to the non-Bt corn pollen. *Euchatias egle* larvae may lack receptors for Cry1Ab in their midgut, since it is the interaction of the toxin with binding sites in the midguts of insects that is believed to determine the host spectrum of Cry toxins (Hofmann et al., 1988; Hofte and Whiteley, 1989; Vadlamudi et al., 1995; Glare and O'Callaghan, 2000).

Bt sprays have been widely used in forest systems to control the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae) and the western spruce budworm, *Choristoneura occidentalis* (Lepidoptera: Tortricidae) (Wagner et al., 1996; Whaley et al., 1998; Herms et al., 1997). Because of questions about the ecological impacts of spraying these forests, research has focused on the effect of Bt sprays on non-target lepidopterans (Wagner et al., 1996; Whaley et al., 1998; Herms et al., 1997; Johnson et al., 1995; Miller, 1990; Peacock, 1998; James et al., 1993). In North Carolina the possible non-target effects of spraying Bt on moths were investigated by developing a list of moths found within the region where *L. dispar* eradication was occurring and determining the relative risks of Bt spraying for each species (Hall et al., 1999).

Similar types of ecosystem level studies are needed to assess the ecological risks of transgenic Bt corn, and other genetically modified organisms (Hails, 2000; Wolfenbarger and Phifer, 2000). In addition to assessing the effect of Bt corn on lepidopterans, research has been done on the persistence of Bt toxins in root exudates from Bt corn (Saxena et al., 1999), the breakdown of the Bt toxin in the pollen grains (Ohlfest et al., submitted), the effect of Bt corn on natural enemies (Pilcher et al., 1997; Orr and Landis, 1997; Hilbeck et al., 1998), insect pollinators (EPA, 2000a), and micro-organisms (Munkvold et al., 1999). However, additional information is needed for a thorough assessment of the ecological risks of Bt corn (Obrycki et al., 2001). In December 1999, 4 years after the initial approval of transgenic Bt field corn, the United States Environmental Protection Agency issued a call for data on the effects of Bt corn on *D. plexippus* and the endangered Karner blue butterfly, *Lycaeides melissa samuelis* (Lepidoptera: Lycaenidae) (EPA, 2000b). The benefits of Bt corn must be

weighed against the possible negative ecological impacts of the widespread planting of transgenic insecticidal crops (Obrycki et al., 2001).

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CHAPTER 6: GENERAL SUMMARY

Transgenic crop plants expressing insecticidal toxins from the bacterium *Bacillus thuringiensis* express Bt toxins in most of their cells. It was believed that this method of expressing insecticides would prevent non-target effects, however, these crops are grown as part of a larger ecosystem. Transgenic plant residues break down in the soil, and pollen grains are dispersed by the wind beyond field borders. In this manner, other animals that are not pests of the transgenic crops are exposed to Bt toxins.

This thesis examined the non-target effects of Bt corn pollen on two lepidopteran species that do not feed on corn. *Danaus plexippus* and *Euchatias egle* feed on *Asclepias syriaca*, a plant that commonly occurs in agricultural areas. Larvae of lepidopteran species that feed on plants growing in or in close proximity to corn fields are most likely to be affected by the dispersal of Bt corn pollen.

In some agroecosystems, the planting of insecticidal crops will reduce insecticide use, and therefore non-target effects, but field corn in the Midwest is rarely sprayed with broad-spectrum insecticides to control the European corn borer, *Ostrinia nubilalis*. The relatively low economic value of field corn and the difficulty in controlling *O. nubilalis* leads most farmers to ignore losses due to *O. nubilalis* larval feeding, rather than invest in insecticide treatments.

There is already considerable concern about the conservation of *D. plexippus*, mainly because the few areas in Mexico, where the entire eastern population of *D. plexippus* spend the winter, are vulnerable to logging, fires and an increasing human population. The timing of Bt corn anthesis in many areas of the Midwest will expose the larvae of the migratory

generation of *D. plexippus* to an additional mortality factor. Currently, the relative importance of corn fields as a source of *D. plexippus* adults is not known. In addition, the effect of potentially increased mortality due to Bt corn pollen on populations of *D. plexippus* has not been determined.

However, public concern over the effect of Bt corn on *D. plexippus* was in a large part due to the fact that this type of effect should have been predicted and discussed prior to the registering and planting of Bt corn. Further, if this non-target effect, that seems ecologically obvious, was not addressed what other effects are possible, but were not examined. Transgenic crops are not grown in isolation, there are many other organisms that share the same ecosystem and will be affected by agricultural practices. Given the large percentage of the land in the Midwest that is used for agriculture, we must assess the effect of agricultural biotechnology on organisms in agroecosystems.

Chapter 2 of this thesis focused on the amounts of Bt corn pollen deposited on *A. syriaca* plants in and near Bt corn fields and the effects of these pollen densities on *D. plexippus* larval survival and adult characteristics. The highest concentrations of pollen grains were deposited on *A. syriaca* within or near the edge of the corn fields. I observed pollen on *A. syriaca* at 10 m. from the corn field at a rate of 1 pollen grain / cm^2.

In 1998, I documented increased *D. plexippus* larval mortality following exposure to field deposited Event 176 Bt corn pollen. In laboratory experiments, *D. plexippus* 1st instar larvae were exposed for 48 hrs. to three densities of corn pollen that larvae would likely encounter in the field. Significantly higher larval mortality was documented when larvae were exposed to 135 pollen grains / cm^2 of Event Bt11 pollen, a density that is found on *A. syriaca* in or near the edges of corn fields. These results demonstrate that the expression of

an insecticide within the cells of a transgenic crop plant does not completely eliminate non-target effects.

No increase in larval development time, or decreased pupal weights, adult dry weight, forewing length or lipid content was observed in larvae that survived exposure to Bt corn pollen for 48 hrs. as 1st instar larvae. This laboratory study showed no sub-lethal effects following exposure to Bt corn pollen, however, additional experiments are needed to determine if longer exposure to Bt corn pollen influences development time or adult characteristics.

In Chapter 3, I conducted a field assessment of the risk Bt corn poses for *D. plexippus*; determining if *D. plexippus* females oviposit on *A. syriaca* plants that are known to grow within and near corn fields. Additionally I determined if larvae are present on the *A. syriaca* plants growing in these two locations relative to corn fields. I surveyed *A. syriaca* in corn fields, roadsides and road edges in 1999, and *A. syriaca* in corn fields and roadsides in 2000. In both years, I observed that monarch females oviposit on *A. syriaca* within the field at a similar rate as on the plants in the roadsides. Larvae were present on *A. syriaca* in the corn fields and roadsides during the period of corn anthesis.

In 2000, I compared the densities and proportion of plants with a *D. plexippus* life stage in Bt and non-Bt corn fields. Similar numbers of eggs were observed in and near Bt corn fields compared to non-Bt corn fields, indicating that *D. plexippus* females are not avoiding concentrations of Bt corn pollen observed in the field. There were also similar numbers of *D. plexippus* larvae in Bt and non-Bt corn fields, indicating that densities of Bt corn pollen we observed in the field were not causing increased mortality . Most of the pollen densities we observed in the Bt corn fields were below the 135 pollen grains / cm^2 we

observed to cause larval mortality in the laboratory study. In addition, in a life table analysis conducted with the data collected 1999, high rates of larval mortality (92-98% by late larval stages) were quantified.

In Chapter 4, I placed cohorts of *D. plexippus* 1st instars on *A. syriaca* in and near Bt and non-Bt corn fields during corn anthesis to determine if larval mortality increased following exposure to Bt corn pollen in the field. The survival curve for larvae in the Bt corn fields was similar to the survival curve for larvae in the non-Bt corn fields. To more fully assess the possible role of Bt pollen in larval mortality, *A. syriaca* plants might be caged to prevent the *D. plexippus* larvae from wandering off the plants and to reduce mortality from other biotic factors, i.e. natural enemies. Elimination of other causes of mortality is necessary to quantify the effect of Bt corn pollen in comparison to other mortality factors. In this one-year field study, it appears that in the presence of other mortality factors, the effect of Bt corn pollen on *D. plexippus* larval survival is negligible.

In Chapter 5, I examined the effect of Bt corn pollen on a second lepidopteran species that feeds on *A. syriaca*, the milkweed tiger moth, *Euchatias egle*. *Euchatias egle* larvae were exposed to 1,300 pollen grains / cm^2 of Bt (Events 176 and Bt11) and non-Bt corn pollen. These are the same hybrids I exposed *D. plexippus* larvae to in Chapter 1. No mortality of *E. egle* larvae was observed following a 48 hr. exposure to Bt corn pollen, compared to 56-100% mortality in *D. plexippus* larvae exposed to the same pollen density. It is possible that *E. egle* larvae lack the midgut receptors necessary for the Bt toxin to harm lepidopteran larvae, whereas *D. plexippus* larvae are susceptible to the Bt toxin.

The field of genetics has progressed significantly since Watson and Crick first discovered the double helix structure of DNA in 1953. Increasing rates of technological innovation have lead to faster and faster methods of sequencing genes and mapping genetic codes. The genetic codes of several plants and animals, including humans, have been mapped and cloned. The information contained in these genes and the ability to clone these genes is currently revolutionizing many fields, including agriculture.

The biotechnology that enables humans to take genes from one species and insert them into a completely unrelated species - and have those foreign genes expressed in the resulting transgenic organism - is known as recombinant DNA technology. As an application of our expanding knowledge of genetics, recombinant DNA technology has seemingly endless possibilities in such areas as health care, pharmaceuticals, and agriculture.

The use of agricultural biotechnology has become a controversial issue over the last decade. It has become the focus of environmental groups energized by the environmental, ethical and economic questions emerging from the use of this technology. It has polarized trade relations between the United States, which has broadly accepted the use of transgenic crops, and the European Union, which is less certain about its safety and use in agriculture. It has raised concerns, mainly in developing countries, about the influence of large multinational corporations on their agriculture. This technology has been praised, by some, as a technology that is needed to feed the growing human population.

Biotechnology has become the major thrust of agricultural research over the last two decades. The race has been on to create the next generation of transgenic crops. The research is expensive, meaning that private firms conducting this research will attempt to market new transgenic products as quickly as possible. In the U.S. these crops must be

registered with the U.S. Environmental Protection Agency, however transgenic crops were not regulated as though they were novel, resulting in a less rigorous examination of non-target effects. The pace of research and development and the competition to get new cultivars to market may continue to result in a superficial examination of potential non-target effects of transgenic crops.

APPENDIX

This data was collected as part of the field survey described in Chapter 3 of this thesis.

Materials & Methods

During the 2-4th week of sampling for *D. plexippus* life stages we randomly selected *A. syriaca* and recorded their characteristics in order to compare the characteristics of *A. syriaca* plants with and without with out *D. plexippus* stages. We recorded the height and diameter of the plant, plant stage, number of other plants within 1 square meter, the presence of predaceous arthropods and aphids was recorded. Every 15th, 20th, or 25th *A. syriaca* the roadsides was selected, and every 5th or 10th plant was selected in the corn field. How often we selected a plant depended on the density of plants at each field site and each plant position observed during the first week of sampling, locations with lower plant densities were sampled at a higher frequency. If the randomly selected *A. syriaca* had a *D. plexippus* life stage we recorded its characteristics, but these plants were removed from the data set of randomly selected plants prior to analysis, since the plants characteristics were already included in the survey for *D. plexippus*. Pollen samples were also removed from a leaf in the middle of the *A. syriaca* from the 1st and 2nd field sites (Table 1) during the second week of the survey.

Characteristics of *Asclepias syriaca* with and without *Danaus plexippus*

There was no difference in the heights of *A. syriaca* with or without a *D. plexippus* life stage at the three field sites (ANOVA P=0.15), in the Bt or non Bt corn (ANOVA P=0.95), or in the roadside compared to the field (ANOVA P=0.06). There was no

difference in the heights of *A. syriaca* with a *D. plexippus* compared to those without a *D. plexippus* (ANOVA P=0.13). There also was a similar number of *A. syriaca* plants within 50 cm of a plant with a *D. plexippus* compares to plants without a *D. plexippus* (ANOVA P=0.74).

On *A. syriaca* plants with or without a *D. plexippus* life we observed Araneae, ants, Coccinellid larva, Chrysopid eggs and larva, Reduviid bugs Pentatimids and aphids. There was a similar proportion of *A. syriaca* with or without *D. plexippus* having predaceous arthropods or aphids on them (ANOVA $P \geq 0.48$). Of the 294 plants without a *D. plexippus* life stage surveyed over the 2-4th weeks of the survey, 8% had aphids and 12% had predaceous arthropods on them. Of the 331 plants with a *D. plexippus* life stage surveyed in the 2-4th weeks of the survey, 9% had aphids and 15% contained predaceous arthropods.

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