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Field and Semifield Evaluation of Impacts of Transgenic Canola Pollen on Survival and Development of Worker Honey Bees

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ABSTRACT A 2-yr field trial (2001 and 2002) and 1-yr semifield trial (2002) were conducted to evaluate the effect of transgenic herbicide (glyphosate)-tolerant canola *Brassica napus* L. pollen on larval and adult honey bee, *Apis mellifera* L., workers. In the field trial, colonies of honey bees were moved to transgenic or nontransgenic canola fields (each at least 40 hectares) during bloom and then sampled for larval survival and adult recovery, pupal weight, and hemolymph protein concentrations. No differences in larval survival, adult recovery, and pupal weight were detected between colonies placed in nontransgenic canola fields and those in transgenic canola fields. Colonies placed in the transgenic canola fields in the 2002 field experiment showed significantly higher hemolymph protein in newly emerged bees compared with those placed in nontransgenic canola field; however, this difference was not detected in the 2001 field experiment. In the semifield trial, bee larvae were artificially fed with bee-collected transgenic and nontransgenic canola pollen and returned to their original colonies. Larval survival, pupal survival, pupal weight, and hemolymph protein concentration of newly emerged adults were measured. There were no significant differences in any of the parameters measured between larvae that were fed transgenic canola pollen and those fed nontransgenic corn pollen. Results from this study suggest that transgenic canola pollen does not have adverse effects on honey bee development and that the use of transgenic canola does not pose any threat to honey bees.

KEY WORDS honey bee, transgenic pollen, canola, nontarget impact

THERE HAS BEEN A steady increase in the acreage of transgenic commercial crops worldwide over the past decade. The estimated global area of transgenic crops for 2002 was 58.7 million hectares (145 million acres), grown by \approx 6.0 million farmers in 16 countries (James 2002). The rapid increase in agricultural use of transgenic crops has raised concerns about the potential impact of this technology on current natural and agricultural ecosystems.

The honey bee, *Apis mellifera* L., plays a critical role in providing pollination for many crops, fruits, and vegetables (Morse and Calderone 2001). Thus, possible impacts of transgenic crop-based agricultural ecosystems on bee survival, development, and pollen-foraging behaviors should be an important element of the ecological risk assessment for this novel agricultural technology. In addition, the majority of the beneficial, parasitic insects are in the same order (Hymenoptera) as honey bees. Many of them also forage during adult stage for nectar from plants. Thus, the honey bee may serve as a relevant indicator organism

for evaluating the ecological impact of transgenic crops.

Different approaches have been used to evaluate the potential impact of transgenic plants on honey bees. Malone and Pham-Delègue (2001) reviewed >16 laboratory studies where only purified transgene products (i.e., proteins) were fed to caged adult bees or larvae in hives. More recently, Malone et al. (2002) and Brødsgaard et al. (2003) evaluated the impact of transgenic plants by exposing honey bee larvae to a purified transgene-product (serine proteinase inhibitor) via an in vitro rearing protocol in the laboratory. Although some of the laboratory studies have detected adverse effects of some purified transgene products (e.g., trypsin inhibitor), it is often difficult to extrapolate the ecological impact on honey colonies from results obtained in such in vitro laboratory studies simply because the realistic ecological or apicultural context cannot be included in the study design. For example, the extraction of the purified transgene-product by both honey bee adults and larvae from the in vitro rearing exposure system may be different from the whole plant tissues such as pollen. Also, evaluation of the purified transgene-product ignores the possibility of pleiotropic effects or gene interactions (Ueberlacker et al. 1996, Kohli et al. 1998). Finally, laboratory in vitro studies eliminate social interactions of the

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colonies and thus have limitations for prediction of the impact on colonies under realistic apicultural conditions.

To overcome the shortcoming of laboratory bioassay in assessing the possible adverse effects of transgenic plants on honey bees, some "semifield" studies involving the use of the whole transgene plant tissues, such as pollen, have been conducted by using honey bee colonies under open or caged apicultural conditions (Chaline et al. 1999, Schur et al. 2000, United States Environmental Protection Agency 2000, Hanley et al. 2003). In contrast, relatively few large-scale field studies have been conducted in assessing the possible ecological impact of transgenic crops on honey bee colonies under realistic apicultural conditions (Arpaia 1996, Osborne et al. 2001, Pierre et al. 2003). The current study involves the use of both field and semifield trials in evaluating potential impacts of transgenic herbicide-resistant canola *Brassica napus* L. crops on both adults and larvae of honey bees. In both field and semifield trials, several responsive variables related to the survival, development, and pollen-foraging behaviors of test adult and larval bees were examined upon exposure to pollen or fields of blooming transgenic and conventional (nontransgenic) canola crops grown by Canadian commercial farmers.

Materials and Methods

Field Trial with Bee Colonies. Study Sites. The 2-yr field trials were conducted in commercial fields of canola grown in Saskatchewan, Canada, in 2001 and 2002. In each year, two fields of both conventional (nontransgenic) canola and transgenic herbicide-resistant canola (each ≈ 40 –65 hectares) were selected for the study from the local farms, where both nontransgenic and transgenic canola were grown and managed according to agricultural practices typical of canola-growing regions. In 2001, the two transgenic canola fields were planted with Pioneer 45A54 hybrid variety, which is tolerant to the action of the herbicide glyphosate, whereas the two nontransgenic canola fields were planted with a local nontransgenic canola variety Formost. All the canola fields used for the study in 2001 were located 9 miles east of Saskatoon (52.07° N, 106.38° W), Saskatchewan, Canada. In 2002, different varieties of nontransgenic and transgenic glyphosate-resistant canola were grown near the towns of Kennedy and Langbank (50.05° N, 1020.20° W). The nontransgenic canola varieties grown in the two locations were Dow AgroSciences Q2 and Wheat Pool Armado (Saskatchewan, Canada), whereas transgenic canola varieties grown were glyphosate-resistant varieties SP Admire (Pioneer 45H21) and 811RR (breeder DSV/Brett Young, distributor Federated Coop, Saskatchewan, Canada). All the transgenic canola varieties used in the field trials contain a transgene that encodes a 5-enolpyruvyl-shikimate-3-phosphate synthase protein that confers tolerance to glyphosate (Mazur and Falco 1989). In both years, before the placement of honey bee colonies in these fields, drive by and visual surveys were conducted on

nearby habitats to make sure no transgenic canola fields were found within 2.4 km next to nontransgenic fields and vice versa.

Honey Bee Colonies. In both 2001 and 2002, three study colonies, each with 10 standard Langstroth frames ($\approx 20,000$ workers) and a queen, were placed next to each canola field (10–100 m to the edge of canola fields), with a total of 12 colonies each year. Another one or two colonies at each field were used to monitor the amount and type of incoming pollen. In these colonies, a pollen trap was installed in front of each colony, and pollen was emptied and weighed every 3–4 d. In 2002, pollen pellets were visually inspected and sorted as canola or noncanola and both types weighed to calculate the proportion of canola pollen. Neither *Varroa* nor tracheal mites were present in these populations and thus no mite control treatment was applied to the bee colonies. In both years, colonies were placed near the canola field when 10–15% of the canola plants started blooming. Frames containing large amount of pollen were removed from the colonies before beginning of experiment to encourage more active pollen foraging and to reduce the likelihood of bees using previously stored pollen for producing brood food. An empty frame was washed and put into the center of the hive to encourage the queen to lay eggs.

Measurement and Analysis. Three to 5 d after the colonies were moved near the field, frames containing a patch of ≈ 200 larvae (24–36 h old) were mapped on a transparency. The same patch of larvae residing on the mapped frames was then checked 5 d later when the cells were sealed by workers. Larvae were considered alive if the cell had been capped and dead if it was emptied. About the same time, brood frames were withdrawn from each colony and 100 newly emerged workers were identified (by their slower walking behavior and matted hairs on thoraces) and marked with paint (Testors PLA) on their thoraces. Adult recovery was measured 10 d (2001) or 8 d (2002) later by carefully examining each frame two times for the presence of paint-marked bees, which were vacuumed and counted after being frozen. Two to 3 wk later, 10 pupae (pink-eyed stage) from each colony were sampled, and their fresh weight determined by weighing Eppendorf tubes before and after the addition of each pupa. Hemolymph also was obtained in newly emerged workers ≈ 24 d after the hives were moved into the canola fields. Hemolymph was collected (1 μ l from each bee) from 10 cold-anesthetized, newly emerged bees and added to 65 μ l of phosphate buffer (0.1 M, pH 7.0). These samples were stored frozen (-20°C) until analysis. Protein concentration was determined as described in Hanley et al. (2003), by using a Vmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

Semifield Study. Pollen Collection and Honey Bee Colonies. The semifield study was conducted at the Michigan State University Bee Biology Laboratory by using both nontransgenic and transgenic herbicide-resistant (glyphosate-resistant) canola pollen collected in Saskatchewan, Canada, in 2001. For pollen

collection, honey bee hives, each installed with a pollen trap were placed directly in both transgenic and nontransgenic canola fields. Pollen from the traps was collected every 3 d for 3 wk during the canola blooming time. In total, 600 g of nontransgenic canola pollen and 450 g of transgenic canola pollen were collected. Canola pollen was stored at -20°C until tested in 2002.

The honey bee colonies used in the semifield study originated from five 1.4-kg packages of honey bees purchased from York Bee Company (Jesup, GA) in April 2002. Each colony was medicated with fumidil, terramycin, apistan, and coumophos strips for protection against *Nosema*, American foul brood, and *Varroa* mites, respectively, and provided with pollen patties (3 parts of commercial bee pollen and 1 part of 50% sugar syrup). By the time of the first trial in June, the hives of package bees had grown to an average size of $\approx 30,000$ bees. We termed this study a semifield study because larvae were artificially fed with canola and other pollen and then returned to their natal colonies. Colonies were placed in an open field and workers were free to forage for their own food. No canola was planted in a 16-km radius.

Treatment and Measurements. The five pollen-feeding treatments that were used in the semifield study included not artificially fed control, mixed pollen (containing no canola), diazinon-treated mixed pollen, nontransgenic canola pollen, and transgenic glyphosate-resistant canola pollen. The mixed pollen used in this test also was collected from bee colonies and purchased commercially (Stakich Inc., Bloomfield Hills, MI).

Diazinon, which is toxic to honey bees, was used as a positive control at a dose of $1.07\ \mu\text{g}$ per larva. Five milliliters of acetone containing $888\ \mu\text{g}$ of technical grade diazinon (Sigma-Aldrich, St. Louis, MO) was mixed with 1.25 g of bee pollen. Pollen treatments that did not contain diazinon also were mixed with 5 ml of acetone to control for a possible solvent effect. The acetone was allowed to evaporate and pollen was mixed with 5 ml of 50% sugar syrup. The pollen-sugar solution was vortexed and stored in a refrigerator until use.

In each of the five colonies, a brood patch of ≈ 150 4- to 5-d-old larvae were selected and divided into five sections. A plastic transparency was placed over the brood frame, and an outline of the cells containing larvae was recorded to note their location. Each section was then randomly assigned to one of the five treatments. Each larva was fed $6\ \mu\text{l}$ of the designated treatment solution (containing 1.5 mg pollen) by pipetting the pollen solution directly near the mouth of larvae, using a repeat pipettor (Eppendorf). In total, 30 larvae were tested for each pollen treatment. The frame was then returned to the natal colony, and larvae presumably fed additionally by nurse bees and were then capped with beeswax by wax-workers.

After the larvae had been capped in each of the five colonies, each patch of brood was checked for larval survival. Frames of sealed brood were moved into an incubator (34°C , 60% RH) 3–4 d before emergence. Sections of different treatments were caged separately

to measure the rate of adult emergence. We regarded any mortality between being capped and adult emergence as "pupal mortality," although technically a bee could have died during the larval stage (postcapping but before pupation). Pupal survival was measured in three colonies per treatment. The experiment was repeated for a second trial with the same five colonies, 3 d after the first trial.

In one of the trials, we performed two additional measurements of bee responses to the pollen treatments in three colonies: pupal weight and hemolymph protein concentration. Five pupae at the black-eye stage were collected into preweighed Eppendorf tubes and fresh pupal weights determined.

Statistical Analysis. For the field study, data for each year were analyzed separately using one-way analysis of variance (ANOVA) with type of crops (nontransgenic or transgenic) as the independent variable. Each colony was treated as an experimental unit, and weights of 10 pupae, or hemolymph protein titers of 10 bees within each colony were treated as subsampling. For the intracolony feeding study, data were analyzed as a randomized complete block design, with each hive as a block. Each bee was treated as an experimental unit for pupal weight and hemolymph protein titer. Percentage of larval survival, percentage of pupal survival, percentage of adult survival, pupal weight, and hemolymph protein level were analyzed using the SAS general linear model procedure (PROC GLM, SAS Institute 2000). Data were transformed when necessary (e.g., percent data to arcsine) to meet the normality requirement of ANOVA. Means were compared using Tukey's honestly significant difference (HSD) test, and untransformed means with standard errors were presented.

Results

Field Study. In 2002, $47.6 \pm 5.3\%$ of incoming pollen was canola pollen ($N = 12$ colonies, over a 3-wk period), and there was no difference in percentages of canola pollen between colonies placed near nontransgenic or transgenic canola fields (49.4 ± 6.9 and $45.9 \pm 8.4\%$, respectively; t -test, $P > 0.05$). Similarly, no difference was seen in the amount of trapped pollen between the two groups (59 ± 10 and 58.1 ± 14 g pollen/3 d, respectively; t -test, $P > 0.05$).

In both 2001 and 2002, larval survival was not significantly different between colonies placed at nontransgenic canola and transgenic canola fields ($F = 0.21$; $\text{df} = 1, 10$; $P = 0.66$ for 2001, and $F = 1.69$; $\text{df} = 1, 10$; $P = 0.22$ for 2002; Fig. 1A), nor was adult recovery significantly different between colonies placed at nontransgenic canola and transgenic canola fields (ANOVA: $F = 1.40$; $\text{df} = 1, 10$; $P = 0.26$ for 2001, and $F = 0.00$; $\text{df} = 1, 10$; $P = 0.96$ for 2002; Fig. 1B).

There was no significant difference in pupal weight between colonies placed at nontransgenic canola and transgenic canola fields (Fig. 2A) in both 2001 ($F = 0.75$; $\text{df} = 1, 20$; $P = 0.41$) and 2002 ($F = 0.72$; $\text{df} = 1, 20$; $P = 0.42$). Although there was no significant difference in hemolymph protein titers between colonies

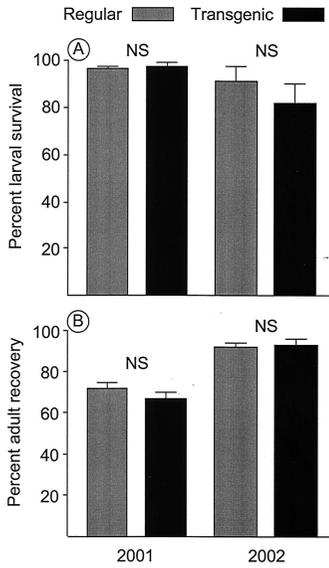


Fig. 1. Mean \pm SE larval survival (A) and adult recovery (B) for colonies placed either at nontransgenic (gray) or transgenic (solid) canola fields. Each bar represents the mean value of six colonies ($N = 200\text{--}300/\text{colony}$), with three colonies at field plot, two field plots for each canola type. Survival was assessed 10 (2001) or 8 (2002) days after newly emerged workers were reintroduced into their natal colonies. Difference was not significant ($P > 0.05$) by ANOVA.

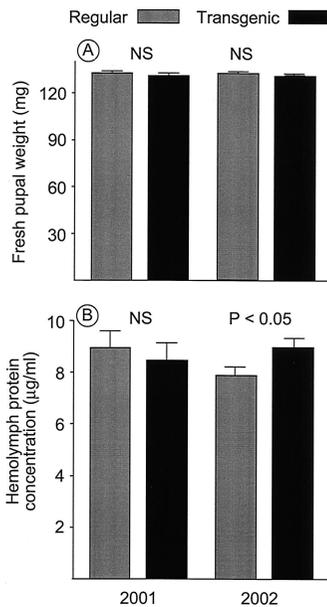


Fig. 2. Mean \pm SE weight of freshly collected black-eyed pupae (A) and total hemolymph protein concentration of newly emerged bees (B), after larvae consumed food secreted from nurse bees, which consumed $\approx 50\%$ canola pollen at nontransgenic (gray) or transgenic (solid) canola fields. Each bar represents the mean value of six colonies ($N = 10$ pupae/colony).

placed at nontransgenic canola and transgenic canola fields in 2001 ($F = 0.21$; $df = 1, 10$; $P = 0.66$), significantly higher protein titers were found for bees placed at the transgenic fields compared with these at the nontransgenic fields in 2002 ($F = 6.12$; $df = 1, 10$; $P = 0.03$; Fig. 2B).

Semifield Study. Larval survival (Fig. 3) was significantly lower in bees fed the diazinon-treated pollen than all other pollen treatments in both trials (Tukey's HSD test, $P < 0.05$; ANOVA: $F = 12.30$; $df = 4, 16$; $P < 0.05$ for trial 1 and $F = 10.08$; $df = 4, 16$; $P < 0.05$ for trial 2). No significant differences in larval mortality were observed among mixed pollen, nontransgenic canola pollen, transgenic canola pollen, or untreated controls (Tukey's HSD test, $P > 0.05$).

No pupa died in other treatments, except in the diazinon treatment in both trials. Pupal survival (Fig. 4) in the diazinon-pollen treatment was significantly lower than that of all other pollen treatments in both trials (Tukey's HSD test, $P < 0.05$; $F = 34.0$; $df = 4, 16$; $P < 0.05$ for trial 1; $F = 10.89$; $df = 4, 16$; $P < 0.05$ for trial 2). There were no significant differences among all other treatments (Tukey's HSD test, $P > 0.05$).

The mean pupal weight of bees fed with diazinon-treated pollen (Fig. 5A) was significantly lower than the mean pupal weight of all other treatments (Tukey's HSD test, $P < 0.05$; ANOVA: $F = 23.99$; $df =$

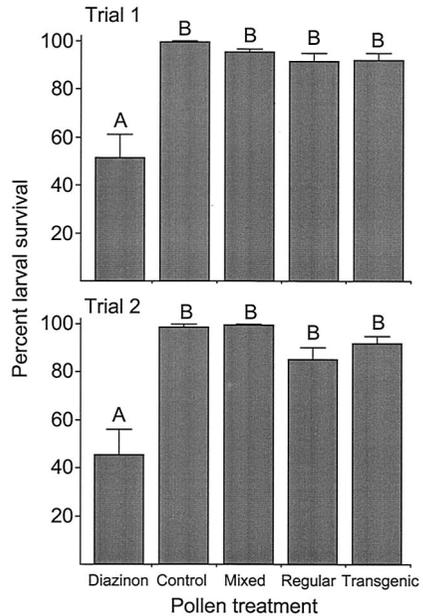


Fig. 3. Mean \pm SE percentage of larval survival in 4- to 5-d-old larvae, artificially fed with various pollen ($N = 30$ larvae/treatment). Bars with different letters indicate significant differences ($P < 0.05$) by Tukey's HSD tests, after an ANOVA indicated a significant treatment effect. Diazinon, nontransgenic bee pollen mixed with diazinon at a dose of $1.07 \mu\text{g}$ per larvae. Control, no artificial feeding. Mixed, mixed pollen. Nontransgenic: nontransgenic canola pollen from "Formost." Transgenic, transgenic canola pollen from "45A54." All pollen were honey bee collected and harvested from pollen traps.

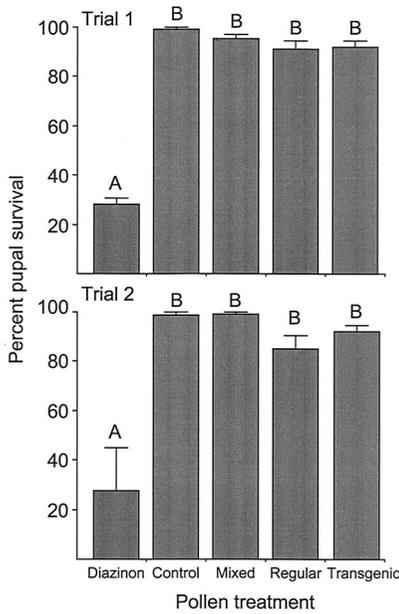


Fig. 4. Mean ± SE percentage of pupal survival ($N = 25$ pupae/treatment), after 4- to 5-d-old larvae that were artificially fed with various pollen. Bars with different letters indicate significant differences ($P < 0.05$) by Tukey's HSD tests, after an ANOVA indicated a significant treatment effect. The survival rates here are combined larval and pupal survival, i.e., the total percentage of bees that survived from last instar larvae until adult emergence. Treatment legends the same as Fig. 3.

4, 68; $P < 0.05$). No significant difference in pupal weight was observed when bees were fed any of the other pollen treatments (Tukey's HSD test, $P > 0.05$).

Figure 5B shows the hemolymph protein concentrations of newly emerged adult bees. There was no significant difference among all the pollen treatments (ANOVA: $F = 1.87$; $df = 3, 54$; $P = 0.15$). Due to high mortality of diazinon-treated pollen fed bees, no hemolymph protein titers were determined in this group.

Discussion

Except for hemolymph protein titers in the 2002 field trial, no significant differences were detected in larval survival, adult recovery, pupal weight, amount of pollen collected, and the proportion of canola pollen collected between the honey bee colonies placed in nontransgenic and transgenic canola fields. In the semifield study, transgenic glyphosate-resistant canola pollen had no significant effects on the survival of larvae and pupae, nor on pupal weight and hemolymph protein concentration of newly emerged bees, compared with the bee larvae fed nontransgenic bee-collected pollen, nontransgenic canola pollen, or even the not artificially fed control. These findings suggest that transgenic glyphosate-resistant canola pollen has no significant deleterious effects on honey bees when fed to them at the immature stage artificially or when fed naturally during larval stage by nurse bees, or

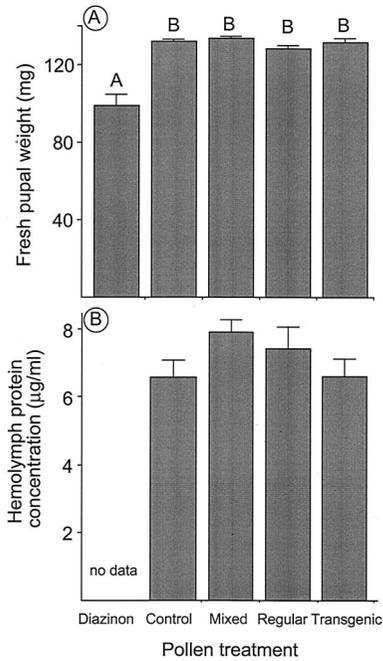


Fig. 5. Mean ± SE weight of freshly collected black-eyed pupae (A) and total hemolymph protein concentration of newly emerged bees (B), after larvae were artificially fed with various pollen (trial 1 only). (A) Bars with different letters indicate significant differences ($P < 0.05$) by Tukey's HSD tests. (B) For hemolymph protein, insufficient larvae survived to adulthood for hemolymph collection in the diazinon-fed group, for the other treatments ANOVA did not show any significant treatment effect ($P > 0.05$). Treatment legends the same as Fig. 3.

when consumed by adult bees during their posteclosion development.

Our data are consistent with Chaline et al. (1999) who observed no mortality difference in brood compared with nontransgenic canola, when colonies were housed in large tents containing both types of plants. Pierre et al. (2003) studied behaviors and preferences of foraging honey bees and also found no difference in honey bees when foraging on the same canola varieties that were either untransformed or transformed.

Findings from our field and semifield studies complemented each other. The field study examined the parameters when colonies were placed in the open field near large fields of either transgenic canola or nontransgenic canola. Approximately 50% of pollen came from canola regardless of whether bees were placed near nontransgenic or transgenic fields. Larvae were supplied with food (secretions from hypopharyngeal glands) that was presumably produced after nurse bees ingested canola pollen. These larvae did not show differences in survival, pupal weight, or adult hemolymph protein concentration (in 2001). Nurse bees ingesting the transgenic and nontransgenic canola pollen did not show any significant differences in mortality because recovery was similar in both years in the field study. In the semifield study, larvae were

directly fed with different types of pollens, which simulated the natural feeding behavior by nurse bees; old larvae receive some pollen before cell capping, whereas young larvae do not (Winston 1987). However, the pollen dose we fed was low compared with the natural dosage (27% of the natural amount provided by nurse bees, Hanley et al. 2003). Nevertheless, our positive control pollen showed a significant lethal effect on test bees even at this low pollen supply level.

Hemolymph protein titer has been used to assess the nutritional quality of various types of pollen fed to adult worker bees (Cremonese et al. 1998). In this study, it was used as a gauge for nutrition received during larval stage. Presumably, higher protein level in hemolymph of newly emerged bees would suggest a better quality of food received during the larval stage. It is not clear why colonies placed at transgenic canola fields in 2002 showed a higher hemolymph protein titer compared with those placed at nontransgenic canola fields. It is likely that the observation of high protein titer in 2002 may be due to some unknown artifact because this same effect was not detected in either the 2001 field trial, nor was in the 2002 semifield study. One possible reason would be misidentification of young bees that were older than 24 h as newly emerged bees, because we did not use an incubator to precisely obtain known aged workers in the field study. Young bees would feed on large amounts of pollen and their hemolymph protein concentrations increase steadily after emergence (Cremonese et al. 1998). Regardless, if this effect was real and not due to an artifact, the higher hemolymph protein titer in the bee colonies placed to the transgenic canola field would point to a positive effect on the nutritional status of the worker bees.

Since the first laboratory study that showed a possible harmful effect of transgenic corn pollen on the monarch butterfly (Losey et al. 1999), many large-scale field studies have been conducted on transgenic Bt crops. Findings from these field trials (Sears et al. 2001) showed that the effect of Bt crops on nontarget organisms, including the monarch butterfly, would be negligible under field conditions. This supports our notion that laboratory and field studies are both important in our full understanding of impacts of transgenic plants on nontarget organisms.

Recently, a large-scale field study was conducted in Great Britain with three different transgenic herbicide-resistant crops: corn, canola, and sugar beet (Hawes et al. 2003, Roy et al. 2003). Results from these farm-scale field studies indicated that many groups of nontarget arthropods, including herbivores, predators, parasitoids, and pollinators, were sensitive to changes in the management systems associated with genetically modified crops. For example, Hawes et al. (2003) showed that the abundance and diversity of herbivores, detritivores, and many of their predators and parasitoids in arable systems varied significantly between transgenic (herbicide-tolerant) and nontransgenic crop (canola, corn, and sugar beet) fields. Similarly, honey bee density was significantly greater in nontransgenic beet and oilseed rape fields com-

pared with the transgenic fields (Haughton et al. 2003) and also was more abundant in margins of nontransgenic beet fields compared with margins of transgenic beet fields, during July (Roy et al. 2003). Perhaps only after we are armed with understanding of impact of transgenic plants on honey bees in semifield and field setting, such as from this study, will we be able to more easily understand the intricate interactions between honey bees and other invertebrates, especially other pollinators, in a more complex design such as these of Roy et al. (2003) and Hawes et al. (2003).

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