

ORIGINAL ARTICLE



Effects of dietary transgenic Bt corn pollen on larvae of *Apis mellifera* and *Galleria mellonella*

ANNE V HANLEY,¹ ZACHARY Y HUANG^{1,2} AND WALTER L PETT^{1*}

¹Department of Entomology, Michigan State University, East Lansing, MI 48824, USA

²Ecology, Evolutionary Biology and Behavior Program, Michigan State University, East Lansing, MI 48824, USA

Received 4 April 2003, accepted subject to revision 13 August 2002, accepted for publication 21 August 2003

SUMMARY

The effects of dietary transgenic Bt corn pollen on 4–5-day-old honey bee worker larvae were examined. We measured larval and pupal mortalities, pupal weight, and haemolymph protein concentration of newly emerged adults after they were fed (as larvae) various pollens (mixed bee pollen, non-transgenic corn pollen, Cry1A(b) or Cry1F corn pollen). There were no significant differences in all the parameters tested between larvae fed transgenic Bt corn pollen Cry1A(b) or Cry1F and non-transgenic corn pollen. We also evaluated Bt corn pollen as a potential control for wax moth larvae in a laboratory study. We fed first instar wax moth larvae three types of pollen: non-transgenic corn pollen, Cry1A(b) corn pollen, and Cry1F corn pollen. We found that the mortality of larvae fed Cry1F corn pollen was significantly greater than the mortality of larvae fed Cry1A(b) corn pollen or non-transgenic corn pollen ($P < 0.05$). In each trial Cry1F fed larvae showed 100% mortality. Our studies suggest that transgenic Bt corn pollen does not pose a threat to honey bee larval development and has the potential to serve as an alternative control for wax moth.

Keywords: *Bacillus thuringiensis*, Bt corn pollen, greater wax moth, *Galleria mellonella*, honey bees *Apis mellifera*, transgenic pollen

INTRODUCTION

For the last decade, there has been a steady increase in the global acreage of transgenic commercial crops. In 1998 it was estimated that there were over 28 million ha of transgenic commercial crops grown worldwide, excluding China (James, 1998). Because honey bees (*Apis mellifera*) play critical roles in pollinating fruits and vegetables (Morse & Calderone, 2001), many studies have been conducted to evaluate the effect of transgenic products on honey bees (reviewed by Malone & Pham-Delègue *et al.* 2001).

The majority of studies on the effects of transgenic crops on honey bees have used purified transgene products, i.e. proteins. There have been five laboratory studies done on purified *Bacillus thuringiensis* (Bt) proteins (Sims, 1995; Arpaia, 1996; Malone *et al.*, 1999; US Environmental Protection Agency, 2000; Malone *et al.*, 2001). Nine studies tested the effects of serine protease inhibitors on honey bees (Belzunces *et al.*, 1994; Malone *et al.*, 1995; Burgess *et al.*, 1996; Malone *et al.*, 1998; Girard *et al.*, 1998; Jouanin *et al.*, 1998; Malone *et al.*, 1999; Pham-Delègue *et al.*, 2000; Malone *et al.*, 2001). The effects of other proteins such as chitinase, β -1,3 glucanase and avidin have been tested in laboratory studies (Picard-Nizou *et al.*, 1997; Malone *et al.*, 2002). There have been only two published studies that used intact pollen instead of purified transgene proteins (Jouanin *et al.*, 1998; Schur *et al.*, 2000; cited in Malone & Pham-Delègue, 2001).

While it is important to determine the potential detrimental effects of transgene products on honey bees, it is also important to determine whether pollen from transgenic plants has negative effects on honey bees. This is because there might be other new proteins expressed in the transgenic plants besides the transgene product(s), due to either pleiotropic effects of the inserted genes (Uberlacker *et al.*, 1996), alternative splicing

(Lopato *et al.*, 1999), or other mechanisms such as interactions between host genes and inserted genes (Kohli *et al.*, 1998).

Because some Bt toxins (such as Cry1A(b) and Cry1F) target lepidopteran insects and honey bees store large amounts of pollen in the hive (Winston, 1987), we suspect that Bt corn pollen could be an alternative control method against the greater wax moth (*Galleria mellonella*). The greater wax moth is an important pest of honey bees. Larvae of the greater wax moth cause considerable damage to beeswax combs left unattended by bees. Beeswax combs in weak or dead colonies and those placed in storage are subject to attack (Caron, 1999). Although there are other methods available for controlling this pest (Cantwell, 1981), Bt corn pollen, if effective at killing wax moth, would be economical and unlikely to contaminate honey or beeswax as do current fumigant control methods.

The objectives of this study were two-fold: (1) to determine the effect of Bt corn pollen on honey bee worker larvae, and (2) to evaluate Bt corn pollen as a potential control of the greater wax moth.

MATERIALS AND METHODS

Honey bee pollen-feeding studies

Corn pollen was collected during corn tasseling in late July or early August (2000 and 2001), depending on the variety of corn, from commercial farms (regular corn or Cry1A(b) corn), or Michigan State University (USA) experimental farms (Cry1F corn). Corn tassels were bagged (Lawson No. 402 - Shower-proof^d) in the early morning or in the afternoon when tassels began to shed pollen. On the following day, the bags were inverted, tapped to release the pollen and removed. After the bags

*Corresponding author: pett@msu.edu

were collected, the pollen was sifted using USA Standard Testing sieves (W S Tyler Inc., Mentor, Ohio, USA) number 16 (pore size 0.119 cm) and number 50 (pore size 0.0043 cm) to remove insects, tassels and other debris. Sifted pollen was stored at -20°C until tested. Over 3000 plants were bagged during the two years.

In April of 2002, five three-pound (1.4-kg) packages of honey bees (York Bee Company, Georgia, USA) were established at the Michigan State University Bee Biology Building, East Lansing, Michigan. Each colony was medicated with Fumidil, Terramycin, Apistan and Coumaphos strips for protection against *Nosema*, American foulbrood and varroa, respectively, and provided with pollen patties (3 parts bee-collected pollen : 1 part 50% sugar syrup). By the time of the first trial in June, the hives of package bees had grown to an average size of c. 40 000 bees.

The following six pollen-feeding treatments were used: (1) untreated (larvae not fed), (2) bee-collected pollen, (3) diazinon-treated bee-collected pollen, (4) non-transgenic corn pollen, (5) Cry1A(b) corn pollen (Event Bt11, Syngenta[®]), and (6) Cry1F corn pollen (Event TC1507, Mycogen - Dow AgroSciences).

Diazinon, which is toxic to honey bees and wax moths, was used as a positive control at a dose of $1.07\ \mu\text{g}$ per larva. Five ml acetone containing 888 μg technical grade diazinon (Sigma-Aldrich Co.) was mixed with 1.25 g bee-collected pollen. Pollen treatments that did not contain diazinon were also mixed with 5 ml 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 c. 180 of four- to five-day-old larvae was selected and divided into six sections. A plastic transparency was placed over the brood patch and an outline of the cells containing larvae recorded to note their location. Each section was then randomly assigned to one of the treatments. Each larva ($n = 30$ larvae per treatment) was fed 6 μl of the designated treatment solution (containing 1.5 mg pollen) using a repeat pipettor (Eppendorf).

After the larvae had been capped in each of the five colonies, each brood patch was checked for larval mortality. Larvae were considered alive if the cell had been capped and dead if it was newly emptied. Frames of sealed brood were moved into an incubator (34°C , RH 60%) 3–4 days 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 (post capping but before pupation). Pupal mortality was measured in three colonies per treatment. The experiment was repeated for a second trial with the same five colonies, two weeks after the first trial.

In trial one, we performed two additional measurements, pupal weight and haemolymph protein concentration, in three colonies. Five pupae at the black-eye stage were collected into pre-weighed Eppendorf tubes and fresh pupal weights determined. Haemolymph was collected (1 μl from each bee) from ten cold-anaesthetized newly emerged bees and added to 65 μl phosphate buffer (0.1 M, pH 7.0). These samples were stored frozen (-20°C) until analysis. Protein determination was performed according to instructions by Bio-Rad. Ten μl of sample containing haemolymph was mixed with 80 μl diluted (1 : 4 dye : water) Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad), mixed and allowed to react for 15 min before absorbance was read at 560 nm on a Vmax Kinetic Microplate Reader (Molecular Devices). A standard curve with Bovine Serum Albumen (25 to 500 $\mu\text{g}/\text{ml}$) was run on each plate. Both standards and samples were determined in triplicates.

To determine whether the 1.5 mg of pollen fed to larvae was comparable to the amount of pollen fed naturally to larvae by nurse bees, we determined the amount of pollen in the midguts

and recta of mature larvae that had had their cells recently capped. Larvae were collected from Tucson, Arizona, USA, in December 2002 while bees were foraging actively for canola pollen. We also determined whether pollen artificially applied to larvae was removed by nurse bees; midguts of larvae were removed 24 h after larvae were fed bee-collected canola pollen and recovery rate of canola pollen determined. In both experiments the number of pollen grains was determined by homogenizing the midgut, adding 1 ml of distilled water, and counting the number of total intact pollen grains with a haemocytometer.

Wax moth pollen-feeding studies

Laboratory assays were conducted in the winter of 2000 at Michigan State University. Late instar wax moth larvae were purchased at a local shop (Grand River Bait and Tackle Shop, Lansing, Michigan) and raised to adulthood on an artificial diet consisting of 8 parts wheat bran, 6 parts corn meal, 6 parts whole wheat flour, 2 parts brewer's yeast, 10 parts glycerine and 5 parts distilled water.

This experiment had a completely randomised design comprised of three treatments: (1) non-transgenic corn pollen, (2) Cry1A(b) corn pollen, and (3) Cry1F corn pollen. Each treatment had three experimental units, each of which consisted of small plastic Solo cups containing 2.5 g of the pollen. Five larvae were placed in each cup, which was then placed in the growth chamber at 70% RH, 30°C and complete darkness. After nine days, each cup was inspected for larval mortality. This study was repeated four times ($n = 60$ larvae for the entire experiment).

Statistical analyses

For the honey bee feeding study all data were analysed as a randomised complete block design, with each hive as a block. Percentage larval mortality, percentage pupal mortality, pupal weight, and haemolymph protein level were analysed using the SAS general linear model procedure (Proc GLM; SAS Institute, 1999). Data were transformed when necessary (e.g. percent to arc-sin) to meet requirements of analysis of variance (ANOVA). Means were compared using Tukey's HSD test. For the wax moth study, larval mortality was analysed using the non-parametric Kruskal-Wallis test. After the Kruskal-Wallis test showed significance, we performed Nemenyi tests to compare the different treatment means (Zar, 1998).

RESULTS

Honey bee pollen-feeding studies

Larval mortality was significantly greater in bees fed the diazinon-treated pollen than all other treatments (Tukey's HSD test, $P < 0.05$; ANOVA: $F = 45.45$, $df = 5,50$, $P < 0.05$ for Trial 1 and $F = 26.35$, $df = 5,50$, $P < 0.05$ for Trial 2; fig. 1). No significant differences in larval mortality were observed when bees were fed bee-collected pollen, non-transgenic corn pollen, Bt-transformed pollen or untreated (Tukey's HSD test, $P > 0.05$).

There was significantly higher pupal mortality when honey bee larvae were fed the diazinon-treated pollen compared to all other pollen treatments in both trials (Tukey's HSD test, $P < 0.05$; ANOVA for Trials 1 and 2: $F = 42.90$, $df = 5,29$, $P < 0.05$ and $F = 50.08$, $df = 5,29$, $P < 0.05$, respectively; fig. 2). No significant difference in pupal mortality was observed among bees that were fed bee-collected pollen, non-transgenic corn pollen, Bt-transformed pollen or bees that were untreated (no pollen) (Tukey's HSD test, $P > 0.05$).

The mean pupal weight of bees in Trial 1 fed with diazinon-treated pollen was significantly lower than the mean pupal weight of all other treatments (Tukey's HSD test, $P < 0.05$; ANOVA: $F = 26.98$, $df = 5,82$, $P < 0.05$; fig. 3). No significant differences in pupal weight were observed when bees were fed any of the other pollen treatments (Tukey's HSD test, $P > 0.05$).

Figure 4 shows the haemolymph protein concentrations of newly-emerged adult bees in Trial 1. There was no significant difference among all the pollen treatments (ANOVA: $F = 1.78$, $df = 4,70$, $P > 0.05$; fig. 4). Due to the low number of surviving diazinon-treated pollen-fed bees, we were unable to test their haemolymph protein levels.

Amount of pollen consumed by larvae and recovery of pollen fed to larvae

The six μl of a sugar syrup solution fed to larvae contained $111\,200 \pm 4040$ canola pollen grains ($n = 5$). After 24 h we recovered $89\,200 \pm 2720$ pollen grains per larvae ($n = 5$), representing an 80.22% recovery.

Midguts and recta of mature larvae ($n = 9$) contained an average of $377\,500 \pm 102\,747$ pollen grains, this represented 5881 ± 1729 μg of pollen per larvae, assuming each pollen grain weighed 0.01645 μg (based on bee-collected pollen).

Wax moth pollen-feeding studies

The results of the Kruskal-Wallis test showed a significant pollen treatment effect ($\chi^2 = 26.1$, $df = 2$, $P < 0.0001$). Mortality of wax moth larvae fed Cry1A(b) corn pollen and regular corn pollen did not differ significantly from each other. However, wax moth mortality was significantly higher for larvae fed Cry1F pollen than with larvae from the other two treatments (Nemenyi test, $P < 0.01$) (fig. 5).

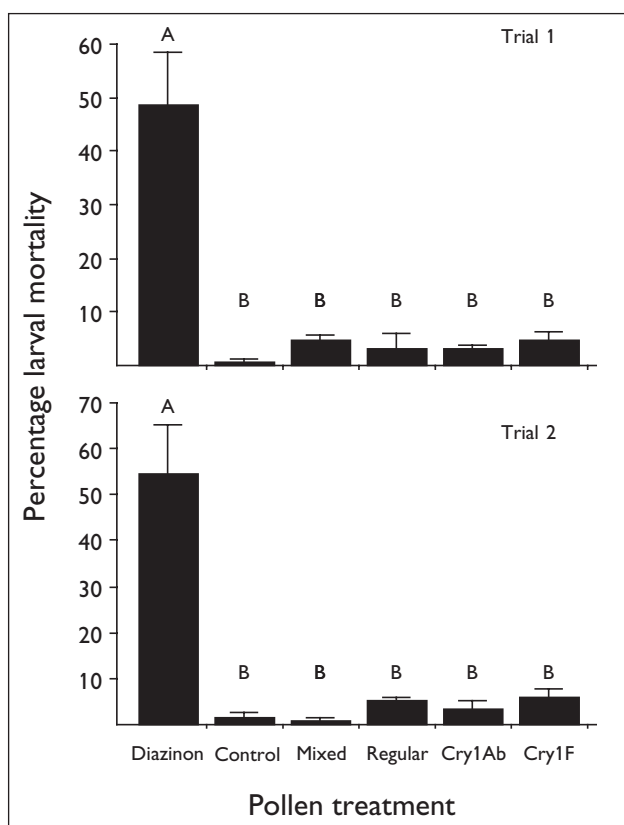


FIG. 1. Mean (\pm s.e.) larval mortality of 4–5-day-old honey bee larvae fed with various pollen ($n = 30$ larvae per treatment). Bars with different letters indicate significant differences ($P < 0.05$) by Tukey's HSD tests, after an ANOVA indicated a significant treatment effect. Diazinon: regular bee pollen mixed with diazinon at a dose of 1.07 μg per larvae. Control: no artificial feeding. Mixed: mixed bee-collected pollen. Regular: non-transgenic corn pollen. Cry1A(b): corn pollen containing Cry1A(b) toxin. Cry1F: corn pollen containing Cry1F toxin.

DISCUSSION

Transgenic corn pollen expressing Cry1A(b) or Cry1F protein had no effect on honey bee larval mortality, pupal mortality, pupal weight or haemolymph protein concentration, when compared with larvae fed regular bee-collected pollen, non-transgenic corn pollen, or the unmanipulated control. This study suggests that transgenic Bt corn pollen, whether Cry1A(b) or Cry1F, has no significant detrimental effects on honey bees when fed to them at the immature stage. The results reported here are consistent with other studies. In a laboratory study, honey

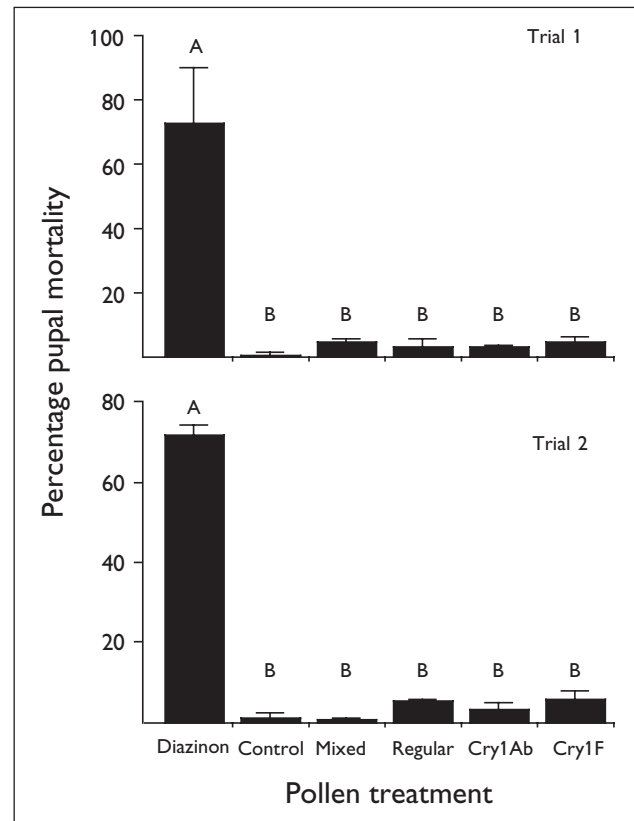


FIG. 2. Mean (\pm s.e.) percentage pupal mortality of honey bees, after larvae were fed with various pollen. Bars with different letters indicate significant differences ($P < 0.05$) by Tukey's HSD tests. Treatments legends same as fig. 1.

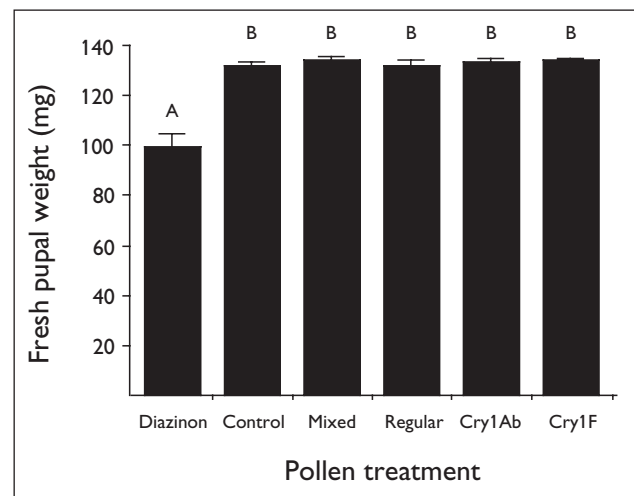


FIG. 3. Mean (\pm s.e.) pupal weight of freshly collected black-eyed honey bee pupae, after larvae were fed with various pollen (Trial 1 only). Bars with different letters indicate significant differences ($P < 0.05$) by Tukey's HSD tests. Treatments legends same as fig. 1.

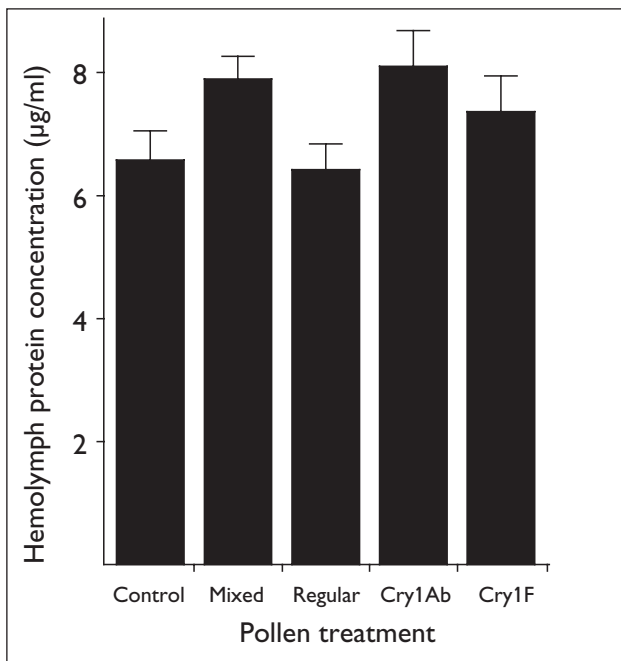


FIG. 4. Mean (\pm s.e.) total haemolymph protein concentration of newly emerged bees, after larvae were fed with various pollen in Trial 1. There was no significant difference among the five treatments ($P > 0.05$) by ANOVA. Insufficient larvae survived to adulthood for haemolymph collection in the diazinon-fed group. Treatments legends same as fig. 1.

bee larvae fed a purified Cry1Ac toxin (20 $\mu\text{g/ml}$) showed no significant increase in larval mortality (Sims, 1995). Arpaia (1996) fed Cry3B protein to whole colonies and found no effect on larval mortality or pupal dry weight. Similarly a semi-field study in which bees were placed inside confined areas covering transgenic rapeseed or Cry1A(b) corn showed no effect on bee mortality or brood development (Jouanin *et al.*, 1998; Schur *et al.*, 2000; cited by Malone & Pham-Delègue, 2001). Our study complements these studies as we fed pollen of both Cry1A(b) and Cry1F to bee larvae (instead of purified Bt toxin), and we measured other parameters in addition to mortality.

There is little doubt that the parameters we measured were capable of detecting a toxic effect, since the positive control using the pesticide diazinon-treated pollen showed consistent effects in all parameters measured. Furthermore, it appears that we were able to measure both acute toxicity and a delayed, more chronic effect. For example, larvae fed diazinon-treated pollen showed not only significantly higher larval mortality, but also reduced pupal weight and increased pupal mortality in bees that survived beyond the larval stage.

It appears that when mixed with pollen, diazinon's effect is about 2700-times less toxic than when applied directly to larvae (LD_{50} 0.00012 $\mu\text{g/larvae}$; Atkins & Kellum, 1986 vs. 1.07 $\mu\text{g/larvae}$, this study). This might be because the pesticide, which is highly fat soluble, became internalised in pollen grains when mixed in acetone in our study. As a result the toxicity became less acute, which is consistent with the delayed effect (reduced pupal weight and increased pupal mortality) we found. This 'pollen containing internalised pesticide' might be a better positive control than direct pesticide feeding because the release of pesticides inside pollen grains might be more comparable to the release of Bt proteins from inside pollen, but this requires further study for confirmation.

Total haemolymph protein concentration in workers has been used to measure the quality of pollen or other diets as a protein source for adult workers (Cremonese *et al.* 1998). They found the measurement to be a rapid and precise method for

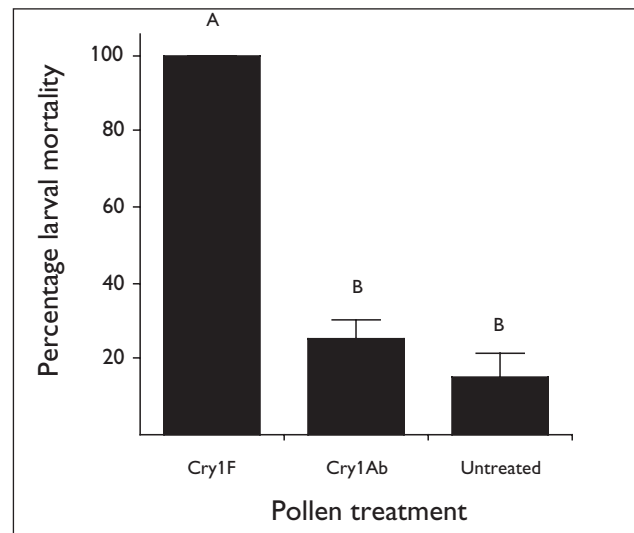


FIG. 5. Mean (\pm s.e.) mortality of wax moth larvae fed with various types of pollen. Bars with different letters indicate significant differences ($P < 0.05$) by Nemenyi tests after treatment effect was found to be significant by the Kruskal-Wallis test.

evaluating alternative food sources for honey bee workers. We suggest that total haemolymph protein concentration in newly emerged adults could also be used to assess the nutritional value of protein diets fed to larvae. Honey bee larvae cannot feed actively, but rather depend completely on adult workers (nurses) for their food (Winston, 1987). The nurses have to digest the forager-collected pollen into proteins and provide the proteins, as well as some undigested pollen grains, to larvae. Therefore, before the newly emerged workers have a chance to eat and digest pollen in the colony, their haemolymph protein levels would reflect the nutrition they received during their larval stage.

We did not detect significant differences in total haemolymph protein concentration among newly emerged adult bees that had received the four pollen treatments as larvae or those which had not been manipulated (fig. 4). This suggests that the transgenic corn pollen was of similar nutritional value to mature larvae as the non-transgenic corn pollen. It is not clear whether haemolymph protein concentration would respond to toxins such as Bt proteins, and if so, whether it would increase or decrease. Total haemolymph protein concentrations in newly emerged workers ranged from 6.44 to 8.1 $\mu\text{g}/\mu\text{l}$ in this study, very close to the value of 4.47 to 7.5 $\mu\text{g}/\mu\text{l}$ reported previously (Cremonese *et al.*, 1998).

While we know how much pollen it takes (i.e. pollen digested by nurses and fed to a larva) to rear a single larva (125 mg; Rosov, 1944), and the concentration of pollen in larval food (2895 ± 541 pollen grains per mg food; Malone *et al.*, 2002), there was no information available prior to this study as to how much pollen is fed directly to 4–6-day-old larvae. Our study suggests that there is a variation in the amount of pollen fed to larvae, with an average of 5.88 mg pollen per larvae. The dose we fed to a larva (1.5 mg) thus represents about 26% of the total pollen a larva would receive naturally. It is not clear whether the artificially added pollen would reduce natural pollen feeding by nurses, resulting in the same total pollen dose as larvae not artificially fed. Our data show that the pollen added artificially was not removed significantly by nurses, as we recovered >80% of the pollen fed to individual larvae. While nurses seem to feed larvae progressively with pollen (Malone *et al.*, 2002), we fed larvae artificially only once to minimize disturbance to the experimental colonies. In future studies, a higher pollen dosage should be used and perhaps over many days, but this might be more easily accomplished if one uses the laboratory larval rearing technique described by Brødsgaard *et al.* (2003).

In the greater wax moth study, we found that mortality in larvae fed Cry1F corn pollen was significantly higher than those fed Cry1A(b) and non-transgenic corn pollen. Even though both Cry1F and Cry1A(b) are toxins targeting lepidopteran insects (Harwood, 1994; Hofte & Whiteley, 1989), only Cry1F corn pollen showed a significant effect. This might be due to the fact that Event TC1507 produces more protein in pollen (31–33 ng Cry1F protein per mg pollen, US Environmental Protection Agency, 2001) than the Cry1A(b) protein produced by Event Bt11 (1.1–7.1 ng/mg pollen, Sears *et al.*, 2001). However, we cannot rule out the possibility that wax moth larvae are more sensitive to Cry1F protein, because Bt proteins are known to be highly species-specific (Knowles, 1994). While field trials are necessary to determine whether Cry1F can be a viable method for controlling the greater wax moth, our data suggest it has promise. Our study suggests that transgenic corn pollen not only poses little threat to honey bee larvae, but may prove to be a good biopesticide against wax moths.

Acknowledgements

This manuscript is part of the dissertation of Anne Hanley, submitted in partial fulfillment of the requirements for a Master's of Science in Entomology, Michigan State University. We thank Sandie Michalek, Ron Lin, Christine DiFonzo and Mike Jewett for their invaluable technical support, Jian Duan for advice on experiments, Diana Sammataro for sampling larvae in Arizona for larval pollen determination, Ed Walker for the use of MicroPlate Reader, George Ayers, Jian Duan and Louise Malone for comments that improved the manuscript. This project was supported by a grant from MSU Project GREEN and a grant from the Michigan Department of Agriculture – Pesticide and Plant Pest Management Division.

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