

Toxicological, Biochemical, and Histopathological Analyses Demonstrating That Cry1C and Cry2A Are Not Toxic to Larvae of the Honeybee, *Apis mellifera*

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ABSTRACT: The honey bee, *Apis mellifera*, is commonly used as a test species for the regulatory risk assessment of insect-resistant genetically engineered (IRGE) plants. In the current study, a dietary exposure assay was developed, validated, and used to assess the potential toxicity of Cry1C and Cry2A proteins from *Bacillus thuringiensis* (*Bt*) to *A. mellifera* larvae; Cry1C and Cry2A are produced by different IRGE crops. The assay, which uses the soybean trypsin inhibitor (SBTI) as a positive control and bovine serum albumin (BSA) as a negative control, was used to measure the responses of *A. mellifera* larvae to high concentrations of Cry1C and Cry2A. Survival was reduced and development was delayed when larvae were fed SBTI (1 mg/g diet) but were unaffected when larvae were fed BSA (400 μg/g), Cry1C (50 μg/g), or Cry2A (400 μg/g). The enzymatic activities of *A. mellifera* larvae were not altered and their midgut brush border membranes (BBMs) were not damaged after being fed with diets containing BSA, Cry1C, or Cry2A; however, enzymatic activities were increased and BBMs were damaged when diets contained SBTI. The study confirms that Cry1C and Cry2A have no acute toxicity to *A. mellifera* larvae at concentrations >10 times higher than those detected in pollen from *Bt* plants.

KEYWORDS: nontarget effects, in vitro, tier 1 study, environmental risk assessment, SBTI

■ INTRODUCTION

Because genetically engineered (GE) crops can increase yield and reduce pesticide application, their use has increased rapidly and steadily since their first commercialization in the United States in 1996.¹ In 2014, over 181.5 million hectares of GE crops were grown in 28 countries, and 43% of these plantings had insect-resistance (IR) traits.¹ Despite their benefits, GE crops may pose risks to human health and the environment. Therefore, a rigorous risk assessment must be conducted before the commercial cultivation of a new GE plant variety. An important component of such assessment is the evaluation of potential negative effects on valued nontarget organisms (NTOs).^{2–4} Such an assessment is especially relevant for insect-resistant genetically engineered (IRGE) plants that produce insecticidal proteins.

The western honeybee, *Apis mellifera* L. (Hymenoptera: Apidae), is the most important pollinator for many fruits, vegetables, and wild plants and also produces honey and beeswax.^{5,6} *A. mellifera* adults collect pollen as the main protein source for their colonies and also collect nectar from plants and honeydew secreted by aphids or other sucking insects as food.⁷

A. mellifera exposure to insecticidal proteins expressed by IRGM crops is likely to be limited to the pollen, which is their main protein source.^{7–11} Although *A. mellifera* also feed on plant nectar, to our knowledge, no insecticidal protein has been detected in plant nectar so far. The adults of *A. mellifera* use

pollen as a food source, while the larvae are initially fed jelly produced by hypopharyngeal and mandibular glands of adult workers; larvae that are destined to become workers are then fed a diet that contains more pollen and honey and less jelly until pupation.^{8,11,12} During the entire larval stage, each larva can consume 1.52–2.04 mg of maize pollen.⁸ Given that *A. mellifera* is economically very important and that both larvae and adults have the potential to be exposed to transgenic products and are known to be sensitive to toxins and pollutants, the species has traditionally been selected as a surrogate species in the regulatory risk assessment of pesticides and IRGE crops.¹³

Many studies have assessed the potential risk of IRGE crops to *A. mellifera* under laboratory and field conditions. However, the majority of studies have focused on adult workers, and only a few studies have been conducted with worker bee larvae. To date, no negative effect has been documented when worker bee larvae or adults consume insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*) or pollen from *Bt* plants.^{7,9,14–21}

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Recently, several artificial diets and in vitro rearing techniques have been developed for *A. mellifera* larvae.^{18,22–25} On the basis of these new diets and rearing methods, we here describe a dietary exposure assay for assessing the direct toxicity of insecticidal compounds to *A. mellifera* larvae. We then used the dietary exposure assay to evaluate the potential toxicity of Cry1C and Cry2A to *A. mellifera* larvae. These Cry proteins have been expressed in rice and other plants for the control of lepidopteran rice pests, such as *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae) and *Scirpophaga incertulas* (Walker) (Lepidoptera: Pyralidae).^{26,27} In addition, biochemical and histopathological experiments were conducted to further evaluate the potential toxicity of the two Cry proteins on this species.

MATERIALS AND METHODS

Insects. Worker larvae of *A. mellifera* were obtained from three colonies at the Institute of Plant Protection, CAAS. The queens were individually confined in cages (45 × 55 × 65 cm) with an empty comb, where they were permitted to lay eggs for 24 h. On the second day after the eggs hatched, the combs were transferred to the laboratory, and the 1.5–2.0 day old larvae were used for experiments.

Larvae of a *Bt*-susceptible strain of *C. suppressalis* were obtained from a colony that had been maintained on an artificial diet for over 50 generations in our laboratory. The *C. suppressalis* larvae were used to test the bioactivity of the Cry proteins (as described later).

Chemical Compounds. Commercial royal jelly and yeast extract were purchased from China-Bee Science & Technology Development Co., Ltd. (Beijing, People's Republic of China) and Oxoid Ltd. (Hampshire, England), respectively. D-(+)-glucose, D-(+)-fructose, bovine serum albumin (BSA), and soybean trypsin inhibitor (SBTI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cry1C and Cry2A proteins were purchased from Envirotect-China (agent for Envirolig Inc., Portland, MA, USA; www.envirotectchina.com). The *Bt* proteins were produced and purified at the Department of Biochemistry, Case Western Reserve University (Cleveland, OH, USA; contact person Dr. Marianne Pusztai-Carey). The protoxins from *Bt* had been expressed as single-gene products in *Escherichia coli*. The *E. coli* expressed protoxin inclusion bodies were dissolved and trypsinized and then isolated and purified by ion exchange HPLC; the pure fractions were finally desalted and lyophilized. Purity was about 94–96%. The bioactivity of the Cry proteins was verified in a sensitive-insect bioassay using neonate larvae of *C. suppressalis* that were reared for 7 days with artificial diet containing a range of Cry protein concentrations (see Bioactivity of Cry Protein in Diets). The EC₅₀ (toxin concentration resulting in 50% weight reduction in comparison to the control) values of our protein batches were estimated to be 18.1 and 1310 ng/mL for Cry1C and Cry2A, respectively.⁵⁰

Artificial Diet and Rearing Technique for *A. mellifera* Larvae. Diet making and rearing techniques have been described in more detail elsewhere.²⁵ The artificial diet for *A. mellifera* larvae contained 50% royal jelly, 4% glucose, 8% fructose, 37% double-distilled water, and 1% yeast extract. The sugars and the yeast extract were dissolved in distilled water, and freshly thawed royal jelly was added to the mixture and mixed thoroughly by hand. The prepared diets were added to 5 mL centrifuge tubes and stored at –20 °C until they were used. Before they were provided to *A. mellifera* larvae, the diets were thawed and brought to 34 °C in a water bath.

A plastic grafting tool for bee larva was used to gently and individually transfer *A. mellifera* larvae (1.5–2.0 days old) to cells (diameter 1.6 cm) of 24-well cell culture plates (Corning Inc., Corning, NY, USA). After they were transferred, the larvae were examined with a stereo microscope to confirm that they were alive. An Eppendorf pipet was used to carefully add artificial diet to each cell such that the bee larvae floated on the food. The plates were covered with lids and placed in a desiccator containing 10% sulfuric acid (resulting in 90% RH) and incubated in a climatic chamber at 34 ± 1 °C in darkness. New diet was added daily after unconsumed diet in

each cell was gently removed with a vacuum pump. Each larva was provided with 100 μL of diet per day for the first 2 days and then with 200 μL per day until defecation. Defecation was determined to have occurred when yellow, stringy material or uric acid crystals were observed. Once defecation occurred (i.e., once the larvae were mature), each larva was removed from the cell, gently blotted on tissue papers to remove the food on the body surface, weighed, and then transferred to a new 24-cell plate, the cells of which were lined with tissue paper on the bottoms and walls. The plates were then placed in a desiccator containing a saturated NaCl–water solution (resulting in 70% RH) in a climatic chamber at 34 ± 1 °C in darkness until adult emergence. Larvae were observed every 12 h (9:00 am and 9:00 pm) for development and mortality.

Response of *A. mellifera* Larvae to SBTI in the Dietary Assay.

Previous studies have confirmed that SBTI is toxic to *A. mellifera*.^{7,9,23,28} Therefore, SBTI was used as a positive control to verify that the artificial diet and our dietary exposure assay could be used to detect toxicity.

A stock solution of SBTI was diluted with distilled water and mixed into the *A. mellifera* larval artificial diet to give concentrations of 0, 0.1, 1, and 10 mg/g FW of diet. The experimental system and the provision of diets were the same as those described in the previous section. There were 3 replicates per treatment, and each replicate contained 24 larvae. The larvae were examined for development and mortality every 12 h. When the larvae matured (as indicated by the occurrence of defecation), they were weighed.

Toxicity of *Bt* Proteins to *A. mellifera* Larvae. Effects on Life Table Parameters. Larvae were fed one of five diets, which were prepared by treating the artificial diet with (1) Cry1C (50 μg/g FW of diet), (2) Cry2A (400 μg/g FW of diet), (3) BSA (400 μg/g FW of diet; negative control), (4) SBTI (1 mg/g FW diet; positive control), or (5) no added protein (blank control). The experimental conditions and the feeding system were the same as those described earlier. Diets were prepared 3 days before initiation of this experiment and were stored at –20 °C until used. BSA was used here as a nontoxic protein control, because a previous study showed that feeding of BSA does not affect the survival and development of honey bees and a parasitoid Hymenoptera.^{23,29} There were 5 replicates pre treatment, and each replicate contained 15 larvae. Insect development and mortality were recorded twice per day (9:00 am and 9:00 pm), and the mature larvae were weighed.

Uptake of Cry Protein by *A. mellifera* Larvae. *A. mellifera* larvae were fed artificial diet containing no toxin (the control), Cry1C at 50 μg/g FW of diet, or Cry2A at 400 μg/g FW of diet as described in the previous section. After 2 and 4 days of feeding, six larvae were collected per treatment. Additional larvae were allowed to pupate before six pupae were collected per treatment. The larvae and pupae were stored at –20 °C until Cry concentrations were measured.

The concentrations of Cry proteins in individual larvae and pupae were measured by double-antibody sandwich enzyme-link immunosorbent assay (DAS-ELISA) using Cry1C and Cry2A detection kits from Envirolig (Portland, MA, USA). The insects were washed in phosphate buffered saline Tween-20 (PBST) (provided with the kit) to remove Cry proteins from body surfaces before analysis. Cry protein was extracted and ELISA measurements were performed following the manufacturer's instructions. Optical density (OD) values were read with a microplate spectrophotometer (PowerWave XS2, BioTek, Winooski, VT, USA). The concentrations of Cry proteins were calculated by calibrating the OD values to a range of concentrations of Cry protein standards provided with the kit.

Bioactivity of Cry Protein in Diets. The bioactivity of Cry proteins in *A. mellifera* larval diets was assessed in three subsamples that were collected from the pure diet (untreated with Cry protein) or diets containing Cry1C (at 50 μg/g FW of diet) or Cry2A (at 400 μg/g FW of diet) that had been exposed to bee larvae for 1 day. The bioactivity of Cry proteins was determined with a sensitive-insect bioassay that used *C. suppressalis* larvae. For Cry protein extraction, a 50 mg sample of each of the three diets was homogenized with 2 mL of PBST buffer (provided with the ELISA kit as described above). After the preparations were centrifuged at 15.8g, the supernatants were

appropriately diluted and thoroughly incorporated into the artificial diet for *C. suppressalis* larvae.³⁰ The artificial diets were cut into slices and placed in Petri dishes (6 cm diameter, 1 cm height) with neonate larvae of *C. suppressalis* (one slice and one larva per dish). The Petri dishes were then sealed with Parafilm. Each treatment was represented by 30 replicate dishes. After 7 days, the *C. suppressalis* larvae were weighed.

Enzyme Activity Analysis. *A. mellifera* worker larvae (1.5–2.0 days old) were fed pure diets or diets containing BSA (at 400 $\mu\text{g/g}$ FW of diet), Cry1C (at 50 $\mu\text{g/g}$ FW of diet), Cry2A (at 400 $\mu\text{g/g}$ FW of diet), or SBTI (at 1 mg/g FW of diet) as described earlier. After 4 days of feeding exposure, seven larvae were collected per treatment and stored at -20°C before the activity levels of four gut enzymes (the total protease, trypsin, aminopeptidase, and α -naphthyl acetate esterase (α -NAE)) were measured using ELISA kits from Beijing Luyuan Bode Biotechnology Co., Ltd. (Beijing, People's Republic of China). Samples were homogenized at 4°C in 300 μL of 0.15 mol/L NaCl.³¹ The homogenates were then centrifuged at 3000g for 10 min at 4°C , and the resulting supernatants were used for analysis of the enzymatic activities following the manufacturer's instructions. The optical density (OD) values were read with a microplate spectrophotometer (PowerWave XS2, BioTek, Winooski, VT, USA). The activity levels of test enzymes were calculated by calibrating the OD values to a range of concentrations of standards provided with the kits.

Histopathology. *A. mellifera* larvae (1.5–2.0 days old) were fed artificial diet containing no added protein, BSA at 400 $\mu\text{g/g}$ FW of diet, Cry1C at 50 $\mu\text{g/g}$ FW of diet, Cry2A at 400 $\mu\text{g/g}$ FW of diet, or SBTI at 1 mg/g FW of diet. After 4 days of feeding exposure, five larvae were collected per treatment. The midguts of the larvae were fixed in a 10% (v/v) paraformaldehyde solution for at least 24 h at 4°C . Tissues were sectioned at 4 μm thickness with a microtome (Leica, R2235, Wetzlar, Germany). The sections were stained with hematoxylin-eosin stain, and images were captured with a digital camera (Olympus, DP73, Japan) mounted on a microscope (Olympus, BX63, Japan).

Data Analysis. In the bioassays with SBTI or Cry proteins, statistical comparisons were made between each treatment and the control (pure artificial diet) by Dunnett's tests after one-way ANOVAs showed a significant effect for any life table parameter. Student's *t* tests were used to compare the weights of *C. suppressalis* larvae that were fed artificial diets containing the extract from untreated diet vs extracts from diets containing Cry proteins. One-way ANOVAs were used to compare enzyme activities among treatments, and Dunnett's tests were used to separate the means between each toxin treatment and the control.

All statistical analyses were conducted using the software package SPSS (version 13 for Windows, 2004).

RESULTS

Response of *A. mellifera* Larvae to SBTI. In the diet-only control treatment, >95% of the worker larvae developed to pupae (Figure 1A), and >90% developed to adults (Figure 1B). With an increasing concentration of SBTI in the diet, the survival rates of *A. mellifera* were steadily reduced (Figure 1), and no larvae survived to the pupal stage at the highest SBTI concentration of 10 mg/g fresh weight (FW) of diet (data not shown). Pupal and adult development and larval weight were not significantly affected by SBTI at 0.1 mg/g FW diet (Dunnett's test; $P > 0.05$) (Figure 1A–D), but SBTI at 1.0 mg/g FW diet significantly decreased the pupation rate (Dunnett's test; $P = 0.006$) (Figure 1A), eclosion rate ($P = 0.012$) (Figure 1B), and mature larvae fresh weight ($P = 0.003$) (Figure 1D) and significantly prolonged larval development time (days to defecation) (Dunnett's test; $P = 0.023$) (Figure 1C).

Toxicity of Bt Proteins to *A. mellifera* Larvae. Effects on Life Table Parameters. *A. mellifera* pupation rate, eclosion rate,

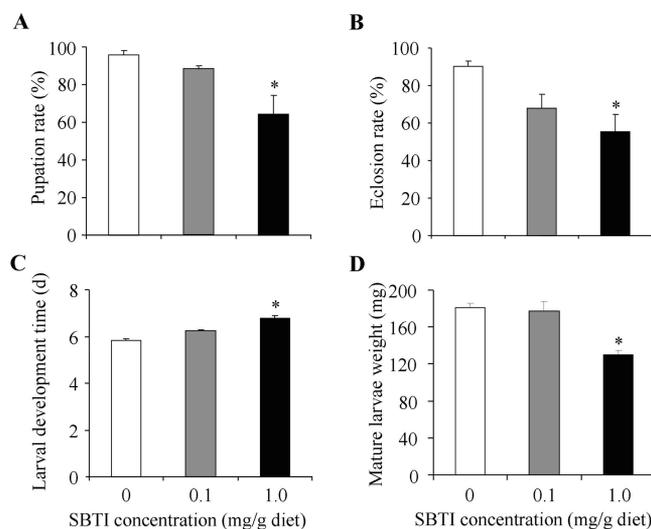


Figure 1. Life-table parameters of *Apis mellifera* larvae fed an artificial diet containing different concentrations of SBTI. An asterisk denotes a significant difference between the treatment and the control ($P < 0.05$). No larva reached the pupal stage at the highest SBTI concentration of 10 mg/g diet (data not shown). Values are means \pm SE, $n = 3$.

larval development time (days to defecation), days to the adult stage, mature larval weight, and adult weight did not significantly differ among the untreated control diet and diets containing BSA, Cry1C, or Cry2A proteins (Dunnett's test: BSA, $P = 0.999, 0.837, 0.711, 1.000, 0.999,$ and 0.998 , respectively; Cry1C, $P = 0.999, 1.000, 0.351, 0.421, 0.998,$ and 0.983 , respectively; Cry2A, $P = 0.999, 0.999, 0.405, 0.699,$ and 0.788 , respectively) (Table 1). *A. mellifera* pupation rate and eclosion rate were decreased by feeding the diet containing SBTI in comparison to those fed control diet (by 13.3 and 17.3%, respectively), although the differences were not significant (Dunnett's test; $P = 0.058$ and 0.054 for pupation rate and eclosion rate, respectively). The developmental time of *A. mellifera* larvae (days to defecation and to adult) were also not significantly affected by the diet containing SBTI (Dunnett's test: $P = 0.12$ for larval development time, $P = 0.68$ for days to adult) (Table 1). In contrast, the mean weights of mature larvae and of newly emerged adults were significantly reduced by SBTI (Dunnett's test, $P < 0.001$) (Table 1).

Uptake of Bt Proteins by *A. mellifera* Worker Larvae. ELISA measurements revealed that all *A. mellifera* larvae contained considerable amounts of Cry1C or Cry2A, but no Cry protein was detected in pupae when they were fed diets containing the corresponding Bt proteins. After 2 and 4 days of feeding, the mean \pm SE concentrations of Cry1C in *A. mellifera* larvae were 0.61 ± 0.04 and 0.82 ± 0.14 $\mu\text{g/g}$ of FW insect, respectively. For Cry2A, the concentrations detected after 2 and 4 days of feeding were 29.11 ± 2.01 and 18.70 ± 1.02 $\mu\text{g/g}$ FW insects, respectively. No Cry protein was detected in larvae or pupae fed with control diet.

Bioactivity of Bt Proteins. After *C. suppressalis* larvae were fed for 7 days in the sensitive-insect bioassay, the mean \pm SE weights per larva were $1.71 \pm 0.12, 0.92 \pm 0.11,$ and 0.19 ± 0.11 mg when the artificial diet contained extract from the control bee diet, extract from the bee diet containing Cry1C protein, and extract from the bee diet containing Cry2A, respectively (Figure 2). According to the Student's *t* test, the difference relative to the diet containing control extract was

Table 1. Life Table Parameters of *Apis mellifera* Worker Larvae Fed Pure Artificial Diet (Untreated Control) or Artificial Diet Containing the Protein BSA (400 $\mu\text{g/g}$ Diet; Negative Control), Cry2A (400 $\mu\text{g/g}$ Diet), Cry1C (50 $\mu\text{g/g}$ Diet), or SBTI (1 mg/g Diet; Positive Control)^a

| parameter | untreated control | BSA | Cry2A | Cry1C | SBTI |
|---------------------------------|-------------------|-------------------|-------------------|-------------------|--------------------|
| pupation rate (%) | 90.67 \pm 2.67 | 92.00 \pm 3.27 | 92.00 \pm 3.89 | 89.33 \pm 1.63 | 77.33 \pm 5.09 |
| eclosion rate (%) | 85.33 \pm 3.89 | 90.67 \pm 3.40 | 86.67 \pm 4.71 | 85.33 \pm 3.27 | 68.00 \pm 7.12 |
| larval development time (days) | 5.79 \pm 0.15 | 5.98 \pm 0.20 | 6.06 \pm 0.07 | 6.08 \pm 0.05 | 6.20 \pm 0.12 |
| time to adult (days) | 18.68 \pm 0.21 | 18.66 \pm 0.28 | 18.59 \pm 0.05 | 18.46 \pm 0.10 | 18.96 \pm 0.20 |
| mature larval weight (mg) | 199.02 \pm 3.35 | 200.34 \pm 4.98 | 190.70 \pm 5.39 | 197.15 \pm 3.65 | 151.43 \pm 9.31* |
| emergence weight of adults (mg) | 137.06 \pm 4.73 | 138.56 \pm 5.11 | 131.17 \pm 4.96 | 134.39 \pm 2.84 | 101.80 \pm 5.41* |

^aEach protein treatment was compared to the control. Dunnett's test was used for all comparisons. An asterisk denotes a significant difference between a treatment and the untreated control ($P < 0.001$). Values are means \pm SE, $n = 5$.

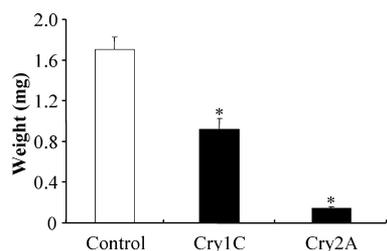


Figure 2. Weight of *Chilo suppressalis* larvae fed for 2 days on an artificial diet that contained an extract from pure bee artificial diet (control) or extracts from bee diets containing Cry1C or Cry2A. An asterisk denotes a significant difference between the treatment and the control ($P < 0.05$). Values are means \pm SE, $n = 30$.

significant for the diet containing Cry1C extract ($t = 4.91$, $df = 57$, $P < 0.001$) and for the diet containing Cry2A extract ($t = 11.91$, $df = 54$, $P < 0.001$) (Figure 2).

Enzyme Activity in *A. mellifera* Worker Larvae. The activities of the total protease, trypsinase, aminopeptidase, and α -naphthyl acetate esterase (α -NAE) were similar when *A. mellifera* larvae were fed the untreated control diet or diets containing BSA, Cry1C, or Cry2A protein (Dunnett's test; $P > 0.1$) (Table 2). When *A. mellifera* larvae were fed a diet containing SBTI, the activity levels of all test enzymes were significantly increased relative to the control (Dunnett's test; all $P < 0.001$).

Histopathology of *A. mellifera* Larvae after Ingestion of Cry Proteins. Midgut sections from *A. mellifera* larvae that had ingested BSA, Cry1C, Cry2A, or SBTI, as well as midguts from control larvae that had not been exposed to the toxins, were examined microscopically for histopathological effects. Midguts of *A. mellifera* larvae had a monolayer epithelial cell structure (Figure 3). For larvae fed pure diet or diet containing BSA or

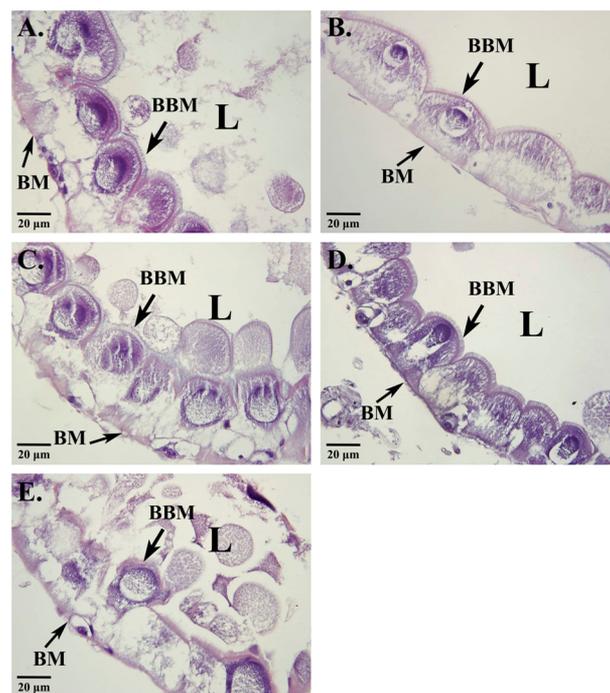


Figure 3. Light micrographs of midgut sections of *Apis mellifera* larvae fed for 4 days on artificial diets containing no toxin (A), BSA at 400 $\mu\text{g/g}$ diet (B), Cry1C at 50 $\mu\text{g/g}$ diet (C), Cry2A at 400 $\mu\text{g/g}$ diet (D), or SBTI at 1 mg/g diet (E). Note that the brush border membranes (BBM) of the epithelial cells (ECs) are clear and that the ECs are homogeneous and intact in the midguts of larvae that consumed a diet without toxin or a diet with BSA, Cry1C, or Cry2A. For larvae that consumed a diet with SBTI, however, the BBMs are lysed and the ECs showed signs of degeneration. BM = base membrane, L = lumen.

Table 2. Enzyme Activity Levels in *Apis mellifera* Worker Larvae Fed Pure Artificial Diet (Untreated Control) or Artificial Diet Containing the Protein BSA (400 $\mu\text{g/g}$ Diet; Negative Control), Cry2A (400 $\mu\text{g/g}$ Diet), Cry1C (50 $\mu\text{g/g}$ Diet), or SBTI (1 mg/g Diet; Positive Control)^a

| treatment | enzyme activity level | | | |
|-------------------|-------------------------------|----------------------------|-------------------------------|--------------------------------|
| | total protease (U/L \pm SE) | trypsinase (U/mL \pm SE) | aminopeptidase (U/L \pm SE) | α -NAE (IU/mL \pm SE) |
| untreated control | 131.79 \pm 2.67 | 104.59 \pm 5.47 | 5.73 \pm 0.21 | 31.57 \pm 0.76 |
| BSA | 151.48 \pm 10.93 | 113.68 \pm 3.47 | 6.18 \pm 0.23 | 33.03 \pm 0.61 |
| Cry2A | 154.55 \pm 6.49 | 115.85 \pm 5.05 | 5.98 \pm 0.14 | 33.04 \pm 1.33 |
| Cry1C | 140.28 \pm 17.03 | 102.10 \pm 3.46 | 5.92 \pm 0.20 | 31.15 \pm 0.98 |
| SBTI | 229.76 \pm 5.04* | 137.93 \pm 5.02* | 7.11 \pm 0.24* | 40.77 \pm 1.36* |

^aEach protein treatment was compared to the control, and an asterisk denotes a significant difference between a treatment and the untreated control ($P < 0.05$). The Dunnett's test was used for all comparisons. Values are means \pm SE, $n = 8-10$.

Cry proteins, the epithelial cells (ECs) were intact and aligned, and their brush border membranes (BBMs) were clearly visible (Figure 3A–D). There was no apparent difference between midgut sections from control larvae and from larvae fed Cry1C or Cry2A protein. For the larvae fed on a diet containing SBTI, however, the ECs showed signs of degeneration and had separated from the basal membrane (BM) and the BBM of ECs lysed (Figure 3E).

DISCUSSION

Evaluating the potential adverse effects of IRGE plants on valued nontarget organisms typically starts with laboratory experiments that measure the potential toxicity of the insecticidal proteins produced by IRGE plants.^{4,32–34} These experiments are referred to as tier 1 studies and are conducted under controlled, worst-case exposure conditions. In the tier 1 assays, purified insecticidal proteins are normally delivered directly to test species by incorporating them into artificial diets at high concentrations. These concentrations are much higher than those encountered in the field and thus yield conservative results: i.e., they tend to overestimate adverse effects.^{33–35} Tier 1 assays require a suitable method for delivering insecticidal compounds to the test organism and arriving at conclusive results.^{33,34,36}

Artificial diets and in vitro rearing techniques for *A. mellifera* larvae have been developed, and these are useful for conducting dietary exposure assays that assess the potential toxicity of insecticidal compounds.^{18–20,22–25,37–39} The artificial diet and the procedure for feeding *A. mellifera* larvae used in the present study were the same as those described by Huang.²⁵ The >90% survival rate with this diet satisfies the basic criterion of >80% survival for diets used for tier 1 assays.^{32–34} In addition to the untreated pure diet control, diet containing the nontoxic protein BSA was included as a negative control treatment in our experiments for further evaluation of how the test system and test conditions, including the diet used in the study, affect the mortality and development of *A. mellifera* larvae. This negative control treatment could ensure nutritional equivalence between dietary treatments, and it assisted in determining whether observed effects are related to the test compounds.³³ Likewise, the survival rate of *A. mellifera* larvae in this negative control treatment was >90%, suggesting that there was no apparent background effect with the diet used in the current dietary testing system.

Positive control treatments are particularly useful in dietary exposure assays with the purposes of indicating whether the test compound is actually ingested and confirming the sensitivity of the testing system. In addition, positive controls may function as useful references to permit comparison to other test results.^{33,34} Compounds used as a positive control should be readily accepted and toxic to the test organism and have properties similar to those of the test compounds in terms of their route of toxicity.^{33,34} SBTI was used to determine the ability of our dietary exposure assay because it is known to be readily accepted and toxic to bees²³ and is an orally active compound, as are the tested Cry proteins. Dose-dependent responses to dietary SBTI were observed for all of the measured life table parameters for worker larvae. This demonstrated that the test system used in the current study was capable of efficiently detecting dietary effects of insecticidal compounds. In addition, these results indicated that artificial diet containing SBTI at 1 mg/g FW of diet is a useful positive control.

After validating the dietary exposure assay described in the present study, we evaluated the potential toxicities of purified Cry1C at 50 $\mu\text{g/g}$ diet and Cry2A at 400 $\mu\text{g/g}$ diet to *A. mellifera* worker larvae. These can be regarded as worst-case exposure scenarios, because these concentrations in the diet are >10 times higher than the Cry protein concentrations in pollen of the current *Bt* rice lines (mean concentrations of Cry1C and Cry2A are 2.6 $\mu\text{g/g}$ dry weight of T1C-19B pollen and 33.5 $\mu\text{g/g}$ dry weight in T2A-1 pollen, respectively^{40,41}). Pollen is the only route by which honeybee larvae would be exposed to Cry proteins, because rice does not produce nectar. Considering that *A. mellifera* larvae ingest only small amounts of plant pollen,⁸ the concentrations of Cry proteins used in this study are much higher than the Cry protein levels likely encountered by *A. mellifera* under natural conditions. Our results indicate that *A. mellifera* larvae were not negatively affected when fed a diet containing Cry1C or Cry2A proteins. Feeding on diets containing SBTI at 1 mg/g FW, in contrast, significantly reduced the survival and weight of *A. mellifera* larvae. The results also demonstrated that *A. mellifera* larvae did ingest the insecticidal compounds that were incorporated in the diet of our assay and that our experimental system was capable of detecting the adverse dietary effects caused by toxic compounds.

To further determine the quantity of Cry proteins ingested by *A. mellifera* worker larvae, the contents of Cry proteins were measured by ELISA in larvae fed for 2 or 4 days on diets that contained Cry protein. High concentrations of both Cry proteins were detected in *A. mellifera* larvae, confirming that the larvae did ingest the Cry proteins. Interestingly, no Cry protein was detected in pupae that had developed from larvae that had fed on both Cry protein treatments. The likely reason for the absence of Cry protein in the pupae is the fact that the bee larvae empty their gut before pupation. The absence of Cry proteins in pupae developing from larvae that had consumed Cry protein containing diet was also documented for the ladybird beetles *Stethorus punctillum* and *Propylea japonica* (Coleoptera: Coccinellidae),^{42,43} suggesting that those insects also empty their guts before pupation. This is not the case for lacewings (Neuroptera: Chrysopidae), however, because insecticidal proteins such as *Galanthus nivalis* agglutinin and Cry proteins could still be detected in lacewing pupae or adults that developed from larvae that ingested these compounds.^{30,44,45}

To have confidence in the findings that Cry1C and Cry2A do not affect larval *A. mellifera*, the bioactivity of the test compounds during the feeding assay needs to be confirmed.^{33,34} A bioassay with the *Bt* protein sensitive species *C. suppressalis* was conducted to test the bioactivity of the Cry1C and Cry2A proteins in the artificial bee diet. The 7 day larval weight was selected as the measurement end point because it has been proven to be a credible and sensitive parameter for assessing the possible impact of *Bt* toxins on this sensitive test insect.^{30,42,46} The results demonstrated that *A. mellifera* larvae had ingested large amounts of bioactive Cry1C or Cry2A protein. We therefore conclude that *A. mellifera* larvae are not sensitive to Cry1C or Cry2A at concentrations that are much higher than those potentially encountered under natural conditions.

The activities of four gut enzymes (the total protease, trypsin, aminopeptidase, and α -naphthyl acetate esterase) have been widely used to indicate the potential toxicity of insecticidal compounds.^{21,28} Our results showed that consumption of

Cry1C or Cry2A did not affect the activity of these enzymes in *A. mellifera* larvae, while the activity levels of all analyzed enzymes were significantly increased in *A. mellifera* larvae fed on a diet containing SBTI in comparison to those fed control diet. A similar pattern of change in the activity of these enzymes has been observed for *Folsomia candida* (Collembola: Isotomidae) fed purified Cry1C and Cry2A.⁴⁷ These results further demonstrate that Cry1C and Cry2A do not affect *A. mellifera* larvae. In addition, microscopy revealed that the midgut brush border membranes of *A. mellifera* larvae were not damaged after ingestion of Cry1C or Cry2A protein. Because the BBM is the locus of Cry protein activity in sensitive organisms,^{28,31} this provided additional evidence that neither Cry protein is toxic to *A. mellifera* larvae.

In summary, we used toxicological, biochemical, and histopathological techniques to assess the toxicity of Cry1C and Cry2A proteins to *A. mellifera* worker larvae. These techniques have been widely used in previous studies to evaluate the potential toxicity of insecticidal compounds on arthropods.^{21,28,48} With the integrated application of these techniques, a convincing conclusion can be drawn that Cry1C and Cry2A have no acute toxicity to *A. mellifera* larvae at concentrations that are more than 10 times higher than those detected in pollen from *Bt*-transgenic plants. The results are consistent with previous studies in which the purified *Bt* proteins Cry1Ac, Cry1Ab, Cry1A.105, Cry2A, and Cry3Bb1 were found to be nontoxic to *A. mellifera* larvae or adults.^{14,17,20,49} Thus, we conclude that the planting of GE crops including *Bt* rice expressing such *Bt* proteins poses a negligible risk to *A. mellifera*.

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Notes

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