Assessing Nontarget Impacts of *Bt* Corn Resistant to Corn Rootworms: Tier-1 Testing with Larvae of *Poecilus chalcites* (Coleoptera: Carabidae)

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ABSTRACT A dietary exposure bioassay with larvae of the ground beetle, *Poecilus chalcites* (Say) (Coleoptera: Carabidae), was developed to assess potential nontarget impacts of corn event MON 863 expressing the Cry3Bb1 protein for control of corn rootworms, *Diabrotica* spp. (Coleoptera: Chrysomelidae). The assay involved 28 d of continuous exposure of *P. chalcites* larvae to an artificial diet treated with a maximum hazard dose (930 μg/g of diet) of the Cry3Bb1 protein. Results from this study showed that the Cry3Bb1 protein at a concentration of 930 μg/g of diet had no adverse effect on the survival, development, and growth (biomass) of *P. chalcites* larvae. Furthermore, statistical power analysis indicated that at levels of 80% power and 5% type I error rate, the study design would have been able to detect a minimum 14 (at day 7) to 19% (at days 14, 21, and 28) reduction in survival and a 21 (at day 14) to 18% (at day 28) reduction in biomass of test larvae relative to the negative buffer control groups. Based on the maximum level (93 μg/g) of the Cry3Bb1 protein expressed in MON 863 corn tissues including leaves, roots, and pollen, findings from this study indicate that corn hybrids containing the MON 863 event have a minimum 10 times safety factor for larvae of *P. chalcites*, and thus pose little risk to this nontarget beneficial insect.

KEY WORDS *Bacillus thuringiensis* crops, Cry3Bb1, *Diabrotica* spp., nontarget impact, *Poecilus chalcites*

CORN ROOTWORMS (*Diabrotica* spp.) are the most destructive insect pests of corn in North America and are frequently the targets of soil insecticides in many corn-growing regions in the United States (Davis and Coleman 1997, Kuhar et al. 1997, Pilcher and Rice 1998). A major concern for growers is that corn rootworms have developed physiological resistance to several classes of insecticides and behavioral resistance to crop rotations (Krysan et al. 1984, Sammons et al. 1997, O'Neal et al. 1999). New technologies, such as genetically modified plants expressing insecticidal traits, are an important source for additional tools on which growers can rely to manage corn rootworms.

Using molecular techniques, Monsanto Company recently developed and commercialized a genetically modified corn event (MON 863), which effectively controls corn rootworms (Vaughn et al. 2005). MON 863 expresses a gene encoding a variant of the wild-type Cry3Bb1 protein derived from *Bacillus thuringiensis* (*Bt*) subspecies *kumamotoensis.* The variant Cry3Bb1 protein has insecticidal activity against Coleopteran species such as corn rootworms and the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Donovan et al. 1992). Recent field trials have shown that MON 863 is highly efficacious against larvae of *Diabrotica* species and may provide corn growers an effective alternative to soil insecticides for managing corn rootworms (Ward et al. 2004, Vaughn et al. 2005). Although MON 863 has been commercially available since 2003 (U.S. EPA 2003), additional maize cultivars that produce different coleopteran-active *Bt* proteins (e.g., binary delta-endotoxins) are under development by other biotechnology companies (e.g., Moellenbeck et al. 2001, Ward et al. 2004).

An important step in the commercialization of genetically modified crops is evaluation of the potential toxicity of plant incorporated protectants (PIPs) to nontarget organisms in the laboratory under worst-case exposure scenarios (U.S. EPA 1989, 1994). *Bt* Cry proteins have no contact toxicity and must be ingested by a susceptible organism to be effective. Thus, dietary exposure assays are required to assess the toxicity or hazard of the PIPs against test nontarget organisms. The potential toxicity of the plant-incorporated Cry3Bb1 protein has been evaluated for a number of

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nontarget organisms, including honey bees, ladybird beetles, lacewings, springtails, and parasitic wasps through laboratory dietary exposure assays (U.S. EPA 2003, Ward et al. 2004), as well as in small-scale field studies (Al-Deeb and Wilde 2003, Al-Deeb et al. 2004).

One group of nontarget organisms that has been largely ignored in nontarget testing with PIPs is ground beetles (Coleoptera: Carabidae) (e.g., O'Callaghan et al. 2005). In particular, the larval stage of Carabidae has not been tested for potential nontarget impacts of genetically modified plants expressing a coleopteran-active insecticidal protein such as Cry3Bb1, in part because of the difficulty associated with maintaining most species of ground beetles in laboratory cultures (Tomlin 1975, Goulet 1976). Ground beetles are an abundant and diverse group of insect predators that consume both insect pests and weed seeds (Lövei and Sunderland 1996, Kromp 1999, Tooley and Brust 2002, Lundgren et al. 2005b). In addition to their role in pest management, ground beetle communities are useful bio-indicators of farm and pest management practices (Lövei and Sunderland 1996, Kromp 1999, Perner 2003, Lundgren et al. 2005b). Recent advancements in carabid rearing based on an artificial diet (Lundgren et al. 2005a) have made it possible to evaluate the potential effects of *Bt* Cry proteins through dietary exposure assays.

Poecilus chalcites (Say) is a carnivorous carabid commonly encountered in crop habitats throughout North America east of the Rocky Mountains (Bousquet and Larochelle 1993). This predator consumes a number of crop pests, including western corn rootworm (*Diabrotica virgifera virgifera* LeCounte; Kirk 1975), corn earworm [*Helicoverpa zea* (Boddie); Lesiewicz et al. 1982], black cutworm [*Agrotis ipsilon* (Hufnagel); Lund and Turpin 1977], and armyworm [*Pseudaletia unipuncta* (Haworth); Clark et al. 1994]. *P. chalcites* currently is being considered for use as a surrogate insect predator species in tier-I testing for plant-incorporated insecticidal proteins that have activity against coleopteran pests (such as Cry3Bb1 protein in *Bt* corn for control of corn rootworms; U.S. EPA 2003). In this study, a dietary exposure assay was developed to evaluate the potential hazard of the Cry3Bb1 protein expressed in MON 863 corn to larvae of the agriculturally important ground beetle, *P. chalcites.*

Materials and Methods

Insects. Larvae of *P. chalcites* originated from eggs laid by adults collected between April and September in agricultural habitats (corn, soybean, and organic vegetable) in Champaign, IL, 2001–2003. On collection, adults of *P. chalcites* were identified using morphological features (Lindroth 1966), and voucher specimens were placed in the arthropod collection of the Illinois Natural History Survey, Champaign, IL (accession nos. 44366–44370). Field-collected *P. chalcites* adults were cultured in the laboratory according to procedures described by Lundgren et al. (2005a).

Table 1. Recipe for meridic diet used for rearing *P. chalcites* larvae in dietary assays (adapted from Lundgren et al. 2005a)

Ingredients[a] blended for 3 min	Amount
Cat food (Iam's adult maintenance formula)	46.6 g
Distilled water (soak the cat food in this water until soft)[b]	93 ml
Raw chicken egg (one)	53 g
Vitamin solution[c]	1.5 ml
Sorbic acid	1 g
Tetracycline	0.5 g
Ingredients blend for an additional 1 min	
Agar	3 g
Boiling water[b]	70 ml

[a] This recipe makes ≈300 ml of diet.

[b] The amount of water may be adjusted to compensate for the amount of dosing solution needed to achieve the target concn of the test substance.

[c] Vitamin solution was a mixture of the following ingredients: distilled water (100 ml), niacinimide hydrochloride (100 mg), calcium pantothenate (100 mg), riboflavin (50 mg), thiamine hydrochloride (25 mg), pyridoxine hydrochloride (25 mg), folic acid (25 mg), biotin (2 mg), and vitamin B_{12} (0.2 mg).

Females oviposited eggs directly into potting soil, which were incubated in a growth chamber (Percival Scientific, Boone, IA) at 27 ± 1.5°C until larvae hatched.

Dietary Assay. Larvae of *P. chalcites* were exposed to a maximum Cry3Bb1 hazard dose of 930 μg/g of diet. This maximum hazard dose was ≈10 times greater than the highest expected environmental concentration of Cry3Bb1 in MON 863 corn plant tissues, as reported by Dudin et al. (2001). The concentration of Cry3Bb1 in MON 863 corn plant tissue was used as a conservative estimate of the potential environmental exposure concentration because there is no information currently available on the concentration of Cry3Bb1 in insect prey, the predominant food source for *P. chalcites.*

Four agar-based artificial diet (Lundgren et al. 2005a) treatments were used, including (1) test substance, Cry3Bb1 at 930 μg/g of diet; (2) control substance, 10 mM sodium carbonate/bicarbonate buffer (pH 10) at 0.147 ml/g of diet; (3) reference substance, potassium arsenate at 200 μg/g of diet; and (4) water assay control, i.e., diet only. The Cry3Bb1 protein was produced in a heterologous *Escherichia coli* fermentation system and prepared in 10 mM sodium carbonate/bicarbonate buffer (the control substance) for storage and dosing. When not in use, the Cry3Bb1 solution was stored in a −80°C freezer or on dry ice. Potassium arsenate (KH_2AsO_4) is a known stomach poison to many insects (Metcalf et al. 1962), including *P. chalcites* (J.G.L., unpublished data), and was used as a positive control to evaluate the efficacy of the dietary exposure system for detecting stomach toxins. Ten to 12 larvae (<3 d old) were tested for each of the diet treatments, and the experiment was replicated six times. Larvae within a replicate originated from the same group of mated females (≈60 individuals).

Artificial diet was prepared by adapting the recipe (Table 1) from Lundgren et al. (2005a). Appropriate amounts of the test, control, and reference substances

were incorporated into the diet suspension with a blender (Waring Commercial Blender, New Hartford, CT) to reach the target concentrations. The diet suspension for each treatment was allowed to solidify in petri dishes or cells of bioassay trays (C-D International, Pitman, NJ) at room temperature. Aliquots of each batch of diet for each treatment were sealed and stored at −20°C for a maximum of 28 d until use.

For testing, individual larvae were placed into 32-ml plastic cups, each filled with ≈15 ml of soil (unsterilized Fer-Til soil; Green-Gro Products, Jackson, WI). For each treatment group, ≈0.2 g of the respective diet was provided to each larva every 48–72 h. The larvae were allowed to feed ad libitum in the test arena. The soil served as critical microhabitat for the test larvae (Lundgren et al. 2005) and was replaced on a weekly basis.

Test larvae were monitored weekly for survival and developmental stage (individual larval instar, pupae, and adults), and once every 2 wk for biomass (weight). Tests were terminated after 28 d of continuous dietary exposure. This 28-d testing period was sufficiently long to encompass all developmental stages of *P. chalcites.* The numbers of larvae that reached the pupal and adult stages were recorded.

Cry3Bb1 Protein Analysis. For each batch (replicate) of the test and control diet, ≈2-ml subsamples were taken from the top, middle, and bottom of the diet container (150-ml blender cup). One set of the subsamples was immediately frozen at −80°C to confirm the Cry3Bb1 dietary concentration and for homogeneity analysis (i.e., day 0 samples). Another set was immediately placed in a −20°C freezer for 28 d to analyze the stability of the Cry3Bb1 protein over the duration of the assay. Levels of Cry3Bb1 in 0- and 28-d-old diet samples were estimated using immunodiagnostics (Western blot) analyses.

For the immunodiagnostic analysis, Cry3Bb1 was first extracted from diet samples using Tris-borate buffer (pH 7.8) containing 100 mM Tris-base, 100 mM $Na_2B_4O_7{\cdot}10\ H_2O$, 10 mM $MgCl_2$, and 0.05% (vol:vol) Tween-20. The extracts were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Bio-Rad Criterion protein separation system (Hercules, CA). After electrophoresis, separated proteins were transferred to a 0.2-μm nitrocellulose membrane at 300 mA for 50 min and blocked overnight using 10% (wt:vol) nonfat dry milk (NFDM) in 10 mM Tris-base, 150 mM NaCl, and 0.05% (vol:vol) Tween-20 (TBST). The membrane was probed for the presence of the Cry3Bb1 protein using a 1:2,500 dilution of purified goat anti-Cry3Bb1 antibodies in TBST with 1% (wt:vol) NFDM for 1 h. Unbound antibodies were removed with three TBST washes (5 min each). Bound antibodies were probed with a 1:5,000 dilution of rabbit anti-goat IgG antibodies conjugated to horseradish peroxidase (Sigma, St. Louis, MO) in TBST with 1% (wt:vol) NFDM for 1 h. Unbound secondary antibodies were rinsed away as previously described. The membrane was incubated in an enhanced chemiluminescence substrate (Amersham, Piscataway, NJ) and exposed to Kodak X-OMAT AR film at varying lengths of time to record an image of the immunoreactive bands. The Western blot films were scanned using a Bio-Rad model GS-800 calibrated imaging densitometer and the immunoreactive bands were reduced using Quantity One Software (Version 4.4.0; Bio-Rad). The Cry3Bb1 protein concentration in the carabid diet samples was extrapolated using densitometric analysis from a Cry3Bb1 protein standard curve run on each blot.

In addition, separate sensitive insect bioassays of *L. decemlineata* larvae indicated that Cry3Bb1 protein incorporated into the artificial diet remained biologically active for a minimum of 5 d under environmental conditions similar to those used in this study (J.J.D. and M. S. Paradise, unpublished data). Thus, the frequent diet changes (every 48–72 h) in this study ensured minimal degradation of Cry3Bb1 protein in the diet during testing.

Data Analysis. For analysis of the percent survival and developmental stage of larvae at different observation times, the following log linear model was used:

$$\log\ (m_{ij}) = \mu + \lambda_i^{Rep} + \lambda_j^{Treat} \qquad [1]$$

where m_{ij} is the expected number of survivors or the instar under a given treatment in a given replicate, μ is the overall mean of all treatments and replicates, λ_i^{Rep} is the replicate effect, and λ_j^{Treat} is the treatment effect.

The analysis of the percent survival and developmental stage with the above model was conducted separately for each of the four observation times using the SAS procedure PROC FREQ (SAS Institute 1999–2001).

To compare the larval weights among treatments, data were first transformed with the logarithm function and then analyzed with the following linear mixed model:

$$y_{ijkl} = \mu + \beta_i + \tau_j + \lambda_k + (\tau \cdot \lambda)_{jk} + \delta_{ijk} + \epsilon_{ijkl} \qquad [2]$$

where y_{ijkl} is the logarithm of the observed larval weight, μ is the overall mean of treatments and replicates, β_i is the replicate effect, τ_j is the treatment effect, λ_k is the effect of the observation day, $(\tau \cdot \lambda)_{jk}$ is the interaction between treatment and observation day, δ_{ijk} is the experimental residual variation, and ϵ_{ijkl} is the sampling variation.

SAS procedure PROC MIXED was used for the analysis with the above model; the observation day was treated as the repeated measurement in the analysis. This was because the same group of individuals was observed at two different (14 and 28) days for each treatment and each replicate. The ESTIMATE option, with the above model without the effect of observation day and the interaction, was used to compare different treatments on each observation day.

In addition to the above statistical analyses for treatment effects, a power analysis was performed to estimate the minimum differences in percent survival and larval weight that can be statistically detected for each observation day at an 80% level of power and a preset type I error rate of 0.05. The SAS procedure

Table 2. Mean percent survival ± SE of *P. chalcites* larvae exposed to diets treated with test, control, and reference substances

Dietary treatment	Number of replicates[a]	Day 7[b]	Day 14	Day 21	Day 28
Cry3Bb1 protein	6	98.3 ± 1.7a	93.6 ± 4.9a	92.2 ± 5.2a	89.2 ± 5.5a
Buffer control(assay control)	6	98.3 ± 1.7a	93.6 ± 4.9a	93.6 ± 4.9a	93.6 ± 4.9a
Assay control (diet only)	6	98.6 ± 1.4a	96.8 ± 2.1a	95.4 ± 2.1a	94.0 ± 2.9a
Positive control (arsenate)	6	86.1 ± 5.3b	77.2 ± 7.7b	69.7 ± 6.3b	63.6 ± 7.8b

[a] Ten to 12 larvae were tested for each replicate of the treatment.
[b] Means followed by the same letter in a column are not significantly different at the 0.05 error level according to likelihood ratio χ^2 tests.

POWER was used for this power analysis (SAS Institute 1999–2001).

Analysis of variance (ANOVA) was used to determine if there were any significant differences in the estimated Cry3Bb1 concentrations among different diet samples collected from the top, middle, and bottom parts of the test diet prepared at the beginning of the study (day 0). A two-sample *t*-test (SAS procedure PROC GLM) was used to evaluate if there was any significant difference in the estimated Cry3Bb1 concentration between diet samples prepared at day 0 and those stored at −20°C for 28 d.

Results

Dietary Assay. There were no significant differences in the percent survival of *P. chalcites* larvae among Cry3Bb1 treatment, buffer control, and assay control groups at any of the four observation days (days 7, 14, 21, and 28). The mean percentages of survival of *P. chalcites* larvae at 7 and 28 d were ≈98 and 89% for the Cry3Bb1 treatment, 98 and 94% for the buffer control, 99 and 94% for the assay control (diet-only), and 86 and 64% for the positive control (arsenate) treatment (Table 2). χ^2 tests detected significant differences in the mean percent survival at each of the 4 observation d between the positive arsenate control group and CryBb1 treatment, buffer control, or assay control groups.

There were no significant differences in the mean biomass of the larvae surviving 14 and 28 d among the Cry3Bb1, buffer control, and assay control groups; the mean biomass of the larvae ranged from 26.0 to 27.4 mg after 14 d and from 39.2 to 40.1 mg after 28 d (Table 3). However, mean biomass of the larvae surviving in the positive control group after 14 and 28 d was significantly lower than for the Cry3Bb1 treatment, buffer, or assay control groups (Table 3).

The developmental stages of *P. chalcites* after 7, 14, 21, and 28 d of dietary exposure are presented in Fig. 1 for each of the dietary treatments. For each of the observation days, *P. chalcites* larvae exposed to the Cry3Bb1 treatment, buffer control, and assay control were developmentally more advanced (second or higher instar) than those in the potassium arsenate treatment. At the termination of the assay (day 28), ≈42, 48, and 52% of the larvae in the Cry3Bb1 treatment, buffer control, and assay control groups, respectively, had pupated or eclosed to adults, whereas no larvae in the arsenate treatment advanced beyond the larval stage (Fig. 1).

Likelihood ratio χ^2 tests detected a significant difference in development of *P. chalcites* larvae exposed to Cry3Bb1, buffer control, and assay control at day 14 of the dietary exposure. No significant differences were detected among these three treatments at days 7, 21, and 28. Pairwise comparisons of the treatment effect at day 14 indicated that there was no significant difference in larval development between the Cry3Bb1 and buffer control groups, whereas the assay control differed significantly from the Cry3Bb1 and buffer control treatments.

The power analysis (Table 4) indicated that, at levels of 80% power and 5% type I error rate, the study design with 66 individuals of *P. chalcites* larvae for each treatment would have been able to detect a minimum ≈14 (at day 7) to ≈19% (at days 14, 21, and 28) reduction in survival rate of the test larvae relative to the buffer control group. At levels of 80% power and 5% type I error rate, the study design with 60 insects surviving at each treatment would have been able to detect a minimum ≈21% reduction or ≈27% increase in biomass measured at day 14 of the dietary exposure and an ≈18% reduction or ≈22% increase in biomass at 28 d.

Cry3Bb1 Protein Analysis. Mean levels of Cry3Bb1 in the diet samples taken from different positions within the diet containers (i.e., day 0 samples) ranged from 832 to 958 μg/g of diet (Table 5). The differences of the Cry3Bb1 concentrations in all the test diet

Table 3. Mean biomass of *P. chalcites* larvae exposed to diets treated with test, control, and reference substances

Dietary treatment	Day 14		Day 28	
	No. insects weighed	Mean ± SE (mg)[a]	No. insects weighed	Mean ± SE (mg)[a]
Cry3Bb1 protein	61	26.9 ± 1.2a	58	40.1 ± 1.6a
Buffer control (assay control)	62	26.0 ± 0.9a	61	39.9 ± 1.1a
Assay control (diet only)	63	27.4 ± 0.8a	61	39.2 ± 1.3a
Positive control (arsenate)	51	5.9 ± 0.4b	42	12.7 ± 0.7b

[a] Means followed by the same letter in a column are not significantly different at the 0.05 error level according to mixed linear model with repeated measures for the two different times of observation.

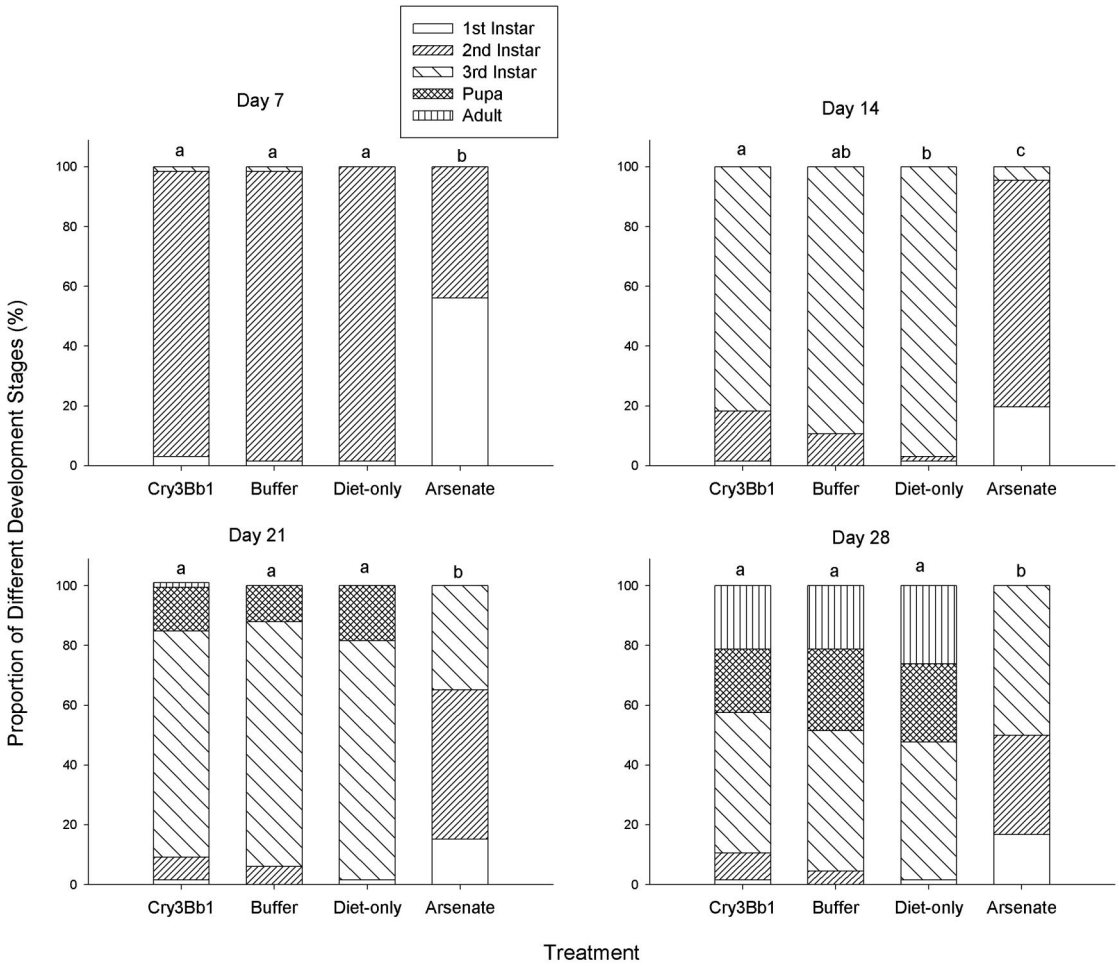

Fig. 1. Developmental stages of *P. chalcites* reached at 7, 14, 21, and 28 d after larvae were exposed continuously to different diet treatments. Bars followed by the same letter at each observation day within each figure are not significantly different at the 0.05 error level according to likelihood ratio χ^2 tests. A total of 60–66 larvae were tested for each of the diet treatments.

samples are within 10.5% of the target concentration (930 μg/g of diet). Thus, the presumed concentration of 930 μg Cry3Bb1 protein per gram of diet in the study was considered to be accurate. Statistical analyses of the data showed significant differences in levels of Cry3Bb1 in diet samples collected from different container positions ($P = 0.0202$) and no significant differences at different storage times ($P = 0.2249$). Although there was a statistically significant difference in levels of the Cry3Bb1 concentration among the top, middle, and bottom of the diet preparation, the mean Cry3Bb1 concentration in all the test diet samples differed within ≈11% of the target concentration. This range of differences in Cry3Bb1 protein concentration

Table 4. Power analyses on detectable differences in percent of survival and mean biomass of *P. chalcites* larvae between negative (buffer) control and the other treatments with 80% power and 5% type I error rate

Endpoints	Observation time (day)	Number of insects observed for each treatment (n)	Observed means (O_m) from negative (buffer) control	Treatment means (T_m) to be detected with 80% power	Percent differences of detectable treatment means relative to control means[a]
Survival (%)	7	66	98.3	<85.0	<13.5%
	14	66	93.6	<76.0	<18.8%
	21	66	93.6	<76.0	<18.8%
	28	66	93.6	<76.0	<18.8%
Biomass (mg)	14	60	25.1	<19.8 or >31.8	<21.1% or >26.8%
	28	60	39.1	<32.0 or >47.9	<18.2% or >22.3%

[a] Calculated as $[(|T_m - O_m|)/O_m] \times 100\%$.

Table 5. Levels of Cry3Bb1 proteins estimated with Western blot analysis in diet samples used in *P. chalcites* feeding assays

Dietary treatment	Storage duration (d) (−20°C)	Sampling location	Number of diet samples analyzed	Estimated mean concn of Cry3Bb1 (μg/g of diet) by Western blot analysis[a]	Percent difference relative to nominal concentration (930 μg/g)[b]
Cry3Bb1 protein	0	Top	6	889	−4.4%
	0	Middle	6	832	−10.5%
	0	Bottom	6	958	+3.0%
	28	Middle	6	1007	+8.3%
Buffer control	0	Middle	4	<LOQ	NA
	0	Middle	2	0.250	NA

[a] Limit of quantification (LOQ) = 0.0625 ng.
[b] Calculated as [(estimated mean concentration − nominal concentration)/nominal concentration] × 100%.
NA, not applicable.

among different test diet samples is generally acceptable for these kinds of regulatory toxicity studies. Western blot analyses showed that two of the six buffer control diet samples analyzed were contaminated with small amounts of Cry3Bb1 protein (mean concentration from the two samples = 0.25 μg/g diet, or <0.03% of the target test substance concentration).

Discussion

Results from this study show that the Cry3Bb1 protein at 10 times the maximum concentration expressed in MON 863 corn tissues (930 μg/g of diet) had no apparent adverse effects on the survival, growth, and development of larvae of *P. chalcites*, and thus is expected to pose no risk to this nontarget beneficial insect. The robustness of these findings is supported by the extended period (28 d) of continuous exposure, the high dietary Cry3Bb1 concentration that remained at the original level (930 μg/g diet) throughout the assay, and the sensitivity of the assay design as shown by the statistical power analyses.

Tier-I toxicity testing has been used in regulatory risk assessment for analyzing the potential hazard of insecticidal products under worst-case scenarios. Data generated through tier-I testing often help scientists decide whether higher-tier field studies are warranted for specific natural enemy communities. Under the current EPA regulatory risk framework for PIPs (e.g., Cry proteins produced in *Bt* plants), if no adverse effects are detected under worst-case conditions of the tier-I toxicity tests, minimal risks are assumed, and no further data are required. Because testing all taxa in an exposed ecosystem or environment is impossible, appropriate surrogate species are selected and tested in tier-I laboratory studies to serve as indicators of risk to other nontarget arthropods. Because of its high abundance and role in biological control in the Midwestern corn belt, *P. chalcites* may be an appropriate surrogate species for predatory guilds of Carabidae that have similar ecological and biological characteristics for assessing potential risk of genetically modified corn expressing coleopteran-active PIPs (such as Cry3Bb1 protein).

Diet-incorporated assays are an important early step in assessing the risk of PIPs to nontarget organisms, but they are only one aspect of the risk-analysis framework. The dietary-exposure assay reported in this study did not involve study into exposure of *P. chalcites* through its prey feeding on the transgenic plants or artificial diet containing Cry3Bb1 proteins. However, recent studies have shown that the magnitude of secondary exposure of plant-produced *Bt* Cry proteins through prey-mediated trophic interactions is much less than the primary (direct) exposure (e.g., Head et al. 2001, Raps et al. 2001, Dutton et al. 2002, Brandt et al. 2004). This is largely because the maximum amount of plant-produced *Bt* Cry proteins that an insect herbivore (prey) can carry is limited by the total volume of its alimentary canal, the rate of intake of the *Bt* plant tissues, and its susceptibility to the insecticidal proteins. Studies by Head et al. (2001) and Raps et al. (2001) also suggest that some sap-feeding insect herbivores (e.g., aphids) may not ingest plant-produced *Bt* Cry proteins if those proteins are not present in phloem tissue. In nature, larvae of *P. chalcites* likely do not feed on plant tissues, but instead prey on insect herbivores that may have fed on the plant tissues containing the insecticidal protein. In the direct, dietary-exposure assay reported here, the concentration of Cry3Bb1 in the test diet was 10 times greater than the maximum level of the Cry3Bb1 protein expressed in MON863 corn plant tissue, and thus ensures a highly conservative margin of safety in concluding there are no adverse effects on *P. chalcites* larvae even when a reasonable level of season long exposure is assumed.

Dutton et al. (2002) suggested that direct toxicity from *Bt* crops through prey to predators is minimal and that the adverse effects of *Bt* Cry proteins on certain predators previously reported in the literature (e.g., Hilbeck et al. 1998a, b, 1999) were likely the result of reductions in prey quality (e.g., Romeis et al. 2004). Although reductions in prey quality may have important implications for the fitness of specialist predators that rely only on *Bt*-crop impacted prey, this type of prey-mediated trophic effect on generalist predators such as *P. chalcites* is likely to be minimal. Recent field studies revealed no significant differences in populations of *P. chalcites* in MON 863 versus nontransgenic fields (Bhatti et al. 2005, J.J.D., unpublished data). Taken together, these data support the conclusion that MON 863 corn will not adversely affect populations of the nontarget species *P. chalcites*.

One component of this study that is seldom included in published records of diet-incorporated toxicity assays is substantiation that the toxin is stable in the test diet and administered at the same level throughout the entire experiment. Proteinases that are present in artificial diets that contain animal or plant tissues may degrade the Cry proteins over time. In this study, Western blot analyses showed that the Cry3Bb1 protein was intact in all the diet samples used in the study and that the levels were fairly consistent in all test diet samples throughout the experiment.

Marvier (2001) criticized some nontarget organism studies designed with small sample sizes ($n = 4$ or less), pointing out that inadequate sample sizes could hinder statistical detection of small treatment differences when the amount of variability is large among replicates. In this study, the statistical power analysis (Table 4) indicates that, at levels of 80% power and 5% type I error rate, the study design with 66 or 60 *P. chalcites* larvae per treatment is able to detect a 14–19% reduction in survival and an 18–21% reduction or 22–27% increase in larval biomass, respectively. Although there are no stringent rules about how much power is sufficient for a study of this nature, power of 80% used in this study is generally recognized to be adequate for detecting the specified magnitude of treatment effect (i.e., 20% reduction in insect survival from an insecticide treatment relative to control) (Cohen 1988, Murphy and Myors 1998, Candolfi et al. 2000). The ability of this study design to detect the small differences in survival and larval biomass further indicates that the effect of MON 863 corn expressing the Cry3Bb1 protein on *P. chalcites*, if any, would be minimal, and practically undetectable.

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