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Expression of *Bacillus thuringiensis* Cry1Ac protein in cotton plants, acquisition by pests and predators: a tritrophic analysis

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Abstract

1. Studies have shown that Cry proteins of the bacterium *Bacillus thuringiensis* expressed in transgenic plants can be acquired by nontarget herbivores and predators. A series of studies under field and controlled conditions was conducted to investigate the extent to which Cry1Ac protein from Bt transgenic cotton reaches the third trophic level and to measure the amount of protein that herbivores can acquire and expose to predators.
2. Levels of Cry1Ac in Bt cotton leaves decreased over the season. Among herbivores (four species), Cry1Ac was detected in lepidopteran larvae and the amount varied between species. Among predators (seven species), Cry1Ac was detected in *Podisus maculiventris* and *Chrysoperla rufilabris*.
3. In the greenhouse, only 14% of the Cry1Ac detected in the prey (*Spodoptera exigua* larvae) was subsequently found in the predator *P. maculiventris*. Detection of Cry1Ac protein in *Orius insidiosus*, *Geocoris punctipes* and *Nabis roseipennis* was probably limited by the amount of prey consumed that had fed on Bt cotton.
4. Purified Cry1Ac was acquired by the small predatory bug *G. punctipes* but at much higher concentration than found in plants or in lepidopteran larvae.
5. Bt protein was shown to move through prey to the third trophic level. Predatory heteropterans acquired Cry1Ac from prey fed Bt cotton, but acquisition was dependent on the concentration of Cry1Ac conveyed by the prey and the amount of prey consumed. The type and availability of prey capable of acquiring the protein, coupled with the generalist feeding behaviour of the most common predators in the cotton ecosystem, probably constrain the flow of Cry1Ac through trophic levels.

Keywords Food web, nontarget effects, phytophagy, predatory bugs, transgenic plants, risk assessment.

Introduction

The environmental advantages of pest control using formulations of the bacterium *Bacillus thuringiensis* (Bt) rather than synthetic insecticides are well known (Glare & O'Callaghan, 2000). However, field cultivation of Bt transgenic plants that continuously produce Bt protein throughout the growing season is relatively new and has evoked many concerns. Bt proteins present in transgenic corn have been detected in root exudates in soil (Saxena *et al.*, 1999), in pollen drifted to areas adjacent to fields (Jesse & Obrycki, 2000), in spider mites, thrips and leafhoppers fed Bt corn (Dutton *et al.*, 2004), in honeydew produced by planthoppers fed on Bt rice (Bernal *et al.*, 2002) and in nontarget chewing herbivores (Howald *et al.*, 2003; Dutton *et al.*, 2003). The acquisition of

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Bt proteins by nontarget herbivores and by lepidopterans with low susceptibility to Bt transgenic crops indicates that Bt proteins can be transferred among trophic levels and may interfere with established food webs. However, each system is unique, being affected by the specific Bt protein, the promoter used to drive gene expression, plant species and tissues, genetic background, rainfall, soil type and soil fertility (Sachs *et al.*, 1998; Greenplate, 1999; Adamczyk & Sumerford, 2001). For example, Cry1Ab expression levels in corn are two-fold higher than Cry1Ac in cotton and the same trend occurs when both toxins are inserted in cotton plants (Perlak *et al.*, 1990; Sachs *et al.*, 1998). In addition, Cry1Ac present in Bt cotton terminal foliage can range from 19.1 μg/g dry weight in cotton cultivated in Georgia to 125.6 μg/g dry weight in cotton cultivated in Mississippi and vary between years and locations (Greenplate, 1999). Cry1Ac protein expression in Bt cotton is clearly influenced by environmental factors and these may differentially affect tritrophic associations (plant–herbivore–natural enemy).

The cotton ecosystem supports a substantial complex of arthropod pests and natural enemies. Three major groups of predatory insects (heteropterans, coleopterans and neuropterans) are recognized as important natural enemies of key and secondary pests in cotton, and these predators are capable of consuming nonpest arthropods to sustain their populations (López *et al.*, 1996). Herbivores in cotton may not be susceptible to Bt proteins but still may acquire Bt protein from the plant and convey it to higher trophic levels. Conveyance of Bt proteins in the prey/host body to predators and parasitoids has been investigated as a potential route for nontarget impact of Bt transgenic plants (Raps *et al.*, 2001; Head *et al.*, 2001; Bernal *et al.*, 2002; Dutton *et al.*, 2003; Schuler *et al.*, 1999 and 2001). The risk of Bt protein exposure to predators and parasitoids has been studied in transgenic corn under controlled conditions (Hilbeck *et al.*, 1999; Head *et al.*, 2001; Raps *et al.*, 2001; Dutton *et al.*, 2002). In the cotton ecosystem, it is possible that species moderately or not susceptible to Cry1Ac can acquire the protein from the plants and expose it to the third trophic level. Common species of lepidopterans in cotton fields only partially affected by Bt cotton (*Spodoptera* and *Pseudoplusia*) (Stewart *et al.*, 2001) could convey Cry1Ac to their predators. In addition, omnivorous predators that occasionally feed on plants may be directly exposed to Bt proteins. Although carnivory is the rule for coccinellids, chrysopids and predatory heteropterans, omnivory can occur in these species, and direct feeding on plants or their products, such as pollen and nectar, has been considered an important life history strategy (Coll & Guershon, 2002; Eubanks *et al.*, 2003).

In the present study, we investigated if the Cry1Ac protein expressed in transgenic Bt cotton plants moves from plants to herbivores and subsequently to their predators in the cotton system. Therefore, a series of experiments was conducted with three objectives: (1) to investigate the amount of Cry1Ac protein moving through trophic levels in Bt cotton fields; (2) to determine the acquisition rate of Cry1Ac by predatory heteropterans from lepidopteran prey fed-Bt cotton and from direct feeding on Bt cotton plants; and (3) to determine whether heteropteran predators (using the big-eyed bug *Geocoris punctipes*) are capable of ingesting and excreting Cry1Ac. The studies were conducted in the laboratory, greenhouse and in the field. This project covers all broad segments of trophic interactions in the cotton ecosystem, from the plant to the third trophic level, quantifying levels of Bt protein present in each trophic level under controlled and field conditions. Also, the field results cover whole crop seasons, as has been strongly recommended in risk assessment guidelines (Schuler *et al.*, 2001; Dutton *et al.*, 2003).

Materials and methods

Insects

The insects used in the laboratory and greenhouse experiments were cultured in the Biological Control Laboratory (University of Georgia, Tifton, Georgia) or were acquired from field collections. Adults of *G. punctipes* and *Orius insidiosus* were reared using corn earworm eggs [*Helicoverpa zea* (Boddie) (CEW)] as prey (obtained from the USDA-ARS-CPMRL, Tifton). To obtain enough *O. insidiosus* adults to conduct the experiments (approximately 150 adults per treatment), predators were collected from silks of non-Bt corn at the Lang Farm (University of Georgia, Tifton). Spined soldier bugs, *Podisus maculiventris* (Say), originated from a collection of females on peach trees near Plains, Georgia and were cultured in the laboratory using beet armyworm larvae, *Spodoptera exigua* (Hübner) (BAW), as prey. Damsel bugs *Nabis roseipennis* Reuter were collected from a non-Bt cotton field at the Coastal Plain Experiment Station, Tifton, Georgia. Adults of *N. roseipennis* from field collections were maintained in the laboratory in cages containing pieces of green bean and 2–5-day-old beet armyworm larvae. Field-collected predators, when used in the experiments, were held in the laboratory for 1 week to verify predator health (pathogen- and parasitoid-free) and to starve the predators to uniform hunger levels prior to the experiments.

Beet armyworm larvae were reared on a standard artificial diet for selected lepidopteran species (Burton, 1969). Moths were maintained in plastic cages with white paper towels for an oviposition substrate and were fed 10% honey/water solution. Eggs laid on the paper were collected and incubated. For the experiments, neonate larvae were caged on Bt and non-Bt cotton plants at various intervals in the greenhouse to produce larvae/prey of appropriate size for each predator species (see below).

Cry1Ac purified toxin

Activated Cry1Ac protein (65 kDa) was prepared from *Bacillus thuringiensis* ssp. *kurstaki* strain HD-73 obtained from the *Bacillus* Genetics Culture Collection (Columbus, Ohio) as previously described (Luo *et al.*, 1999). The protein used was at a concentration of 1.6 mg/mL, and stored at –25 °C until needed. The original concentration was used to prepare specified dilutions in distilled water immediately before being offered to the predators.

Cry1Ac toxin in cotton plants, prey, and predators in cotton fields

Cotton aphids, lepidopteran larvae and predators (immature or adult) were collected in three pairs of Bt and non-Bt cotton fields (5–11 ha) from different locations near Tifton, Georgia in 2004 using drop cloth samples (dislodging insects from two rows onto a 1-m long white canvas cloth laid between the cotton rows). The fields were representative of cotton production in the region and sampling focused on several of the most common predators found in cotton fields (Knutson & Ruberson, 1996).

The cotton fields [*Gossypium hirsutum* (L.)] were planted with Bt cotton (DPL 555) and non-Bt cotton (DPL 493) during the second week of May and sampled throughout the season until the fourth week of August in 2004. Leaf material was collected from six or seven randomly selected plants in each Bt cotton field. On each plant, a leaf disc was collected by snapping a centrifuge tube cap down between the main veins of the uppermost fully expanded leaf of the plant. Abundant potential prey species for predators were also assayed for toxin content. Larvae of several lepidopteran species (Noctuidae) variably susceptible to the Cry1Ac protein [soybean looper, *Pseudoplusia includens* (Walker) ($n =$ 67 larvae), southern armyworm, *Spodoptera eridania* (Cramer) ($n = 108$ larvae) and beet armyworm, *Spodoptera exigua* (Hübner) ($n = 35$ larvae)] were collected in drop cloth samples. Cotton aphids, *Aphis gossypii* (Glover) (4094 mg of sample), were brushed from infested upper leaves. Simultaneously, important predators in the cotton ecosystem were collected in drop cloth samples. Lady beetle collections focused on *Harmonia axyridis* (Pallas) ($n = 122$ larvae) because it is a common species through most of the season in the sampled cotton fields whereas other species are more sporadic. Adults of common predatory heteropterans [*G. punctipes* ($n = 231$), *O. insidiosus* ($n = \sim5000$), *N. roseipennis* ($n = 71$) and *P. maculiventris* ($n = 32$) and larval lacewings [*Chrysoperla rufilabris* (Burmeister) ($n = 116$) and *Micromus* sp. ($n = 115$)] were also collected. Immediately after collection, specimens were chilled in a cooler until return to the laboratory. In the laboratory, the material was stored in centrifuge tubes at –25 °C until protein extraction and the enzyme-linked immunosorbent assays (ELISA) were run. Plant material and *G. punctipes* were collected weekly throughout the season whereas other predators and prey were not as consistently abundant and were sampled as they occurred.

Toxin acquisition by predatory heteropterans from prey and plant

Cotton plants expressing the gene for the Bt protein Cry1Ac (variety DPL 555) and a nontransgenic variety (DPL 5415) were used in this experiment. The plants were cultivated in pots (diameter: 15 cm, height: 15 cm) filled with high porosity potting soil BM6™ (Berger Peat Moss Canada), mixed with 14-14-14 controlled-release fertilizer (Osmocote™, Scotts-Sierra Horticultural Products Company, Marysville, Ohio) and maintained under greenhouse conditions of 26 ± 4.0 °C (mean ± SD) and approximately 14 h of light. Plants were used when they were 26–32 days old, comparable in size, and had seven or eight fully expanded leaves.

Prey (neonate BAW larvae) was caged on cotton leaves using organdy fabric sleeve cages. The appropriate prey size for each predator species was achieved by offering BAW larvae of different ages to the respective predators. BAW larvae offered to *G. punctipes* and *O. insidiosus* were fed for 1 day on plants (caged for 24 h on Bt or non-Bt plants before offering to the predators). BAW larvae offered to *N. roseipennis* and *P. maculiventris* were fed on appropriate plants for 3 and 9 days, respectively. The number and weight of prey offered to the predators were obtained before confining them on the plants. The number of larvae consumed and predator weights before and after caging were used to estimate the amount of fresh material consumed by individual predators. The treatments consisted of predators caged on either: (1) Bt or non-Bt plants without prey (BAW larvae) to test for direct acquisition of toxin from the plants or (2) plants with prey to test for direct and indirect acquisition (through prey). To encourage prey and plant feeding, and to standardize predator hunger, predators were deprived of prey for 24–36 h before placement in cages. *Geocoris punctipes* ($n = 16$), *N. roseipennis* ($n = 15$) and *P. maculiventris* ($n = 10$) were singly caged with 20 BAW larvae of appropriate size, whereas *O. insidiosus* were caged in groups of 20 predators per cage, containing 60 1-day-old BAW larvae. Cages were made of 500 mL styrofoam cups, with bottoms removed, wrapped in knee-high stretch hose, and tied to the cotton leaf petioles. Only predators that were still alive 24 h after caging were assayed for Cry1Ac protein. The numbers assayed for each predator were: 12 (Bt) and 13 (non-Bt) *G. punctipes*, 12 (Bt) and ten (non-Bt) *N. roseipennis*, nine (Bt) and nine (non-Bt) *P. maculiventris* and 165 (Bt) and 148 (non-Bt) *O. insidiosus*.

We caged 16 *G. punctipes*, ten *P. maculiventris*, ten *N. roseipennis* and 120 *O. insidiosus* in the treatments with predators caged on Bt and non-Bt cotton plants deprived of prey. From this initial sampling size, again, only predators alive 24 h after caging were assayed, comprising 13 *G. punctipes* from each cotton type, ten (Bt) and nine (non-Bt) *P. maculiventris*, ten (Bt) and nine (non-Bt) *N. roseipennis*, and 102 (Bt) and 112 (non-Bt) *O. insidiosus*.

The material representing all three trophic levels of this association (cotton leaf, prey and predators) was collected at the end of the exposure period (24 h after caging predators on plants) and assayed for Cry1Ac protein. Plant material consisted of a leaf disc collected by snapping the centrifuge tube cap down between the main veins of the leaf inside the cage. Living prey (BAW larvae caged on plants), plant material and predators were collected and stored in a freezer at –25 °C until the ELISA assays were run.

Toxin ingestion by the predatory big-eyed bug *G. punctipes*

In the laboratory, a study of ingestion of various dosages of Bt protein was conducted using adult *G. punctipes* to verify the ability of this predatory heteropteran to ingest Cry1Ac protein. Male and female *G. punctipes* were starved for 48 h before beginning the experiment. Five concentrations of purified Bt Cry1Ac protein (2, 4, 8, 16 and 32 p.p.m.) were offered to predators for 1 h in a 1 μL droplet of protein-water per predator. The volume and exposure time were determined in a previous test using only distilled water to determine the volume of water ingested before substantial evaporation could alter the concentrations. Distilled water droplets were offered to the predators assigned as the control treatment. Fifteen bugs were individually placed in plastic Petri dishes (diameter: 2 cm, height: 1 cm) and allowed to acclimate for approximately 1 h. The appropriate protein–water solution was then placed in each Petri dish using a micropipettor. All predators were observed to ensure that drinking occurred for more than 1 min. Individuals that failed to drink for at least 1 min were discarded; hence, the final sample size ranged from 10–12 individuals for each concentration. After 1 h, all predators that drank were transferred to centrifuge tubes and stored at –25 °C until protein extraction and ELISA assay.

Fate of Cry1Ac protein in body and faeces of *G. punctipes*

Female and male *G. punctipes* (5–10 days old) were starved for 36–48 h to enhance thirst levels. The predators were individually placed in 2-cm diameter Petri dishes. After 0.5 h of resting in the Petri dishes, a 1μL droplet of purified Cry1Ac protein in distilled water was offered to each predator. We used 16 and 32 p.p.m. Cry1Ac protein concentrations based on the ingestion test previously conducted. The unused portion of the Cry1Ac-water dilutions was also stored at –25 °C and assayed to verify the protein levels in the dilutions. As above, only predators observed to drink from the droplet for 1 min were used in the analyses. Exposure of the droplet to predators did not run beyond 1 h to avoid significant change in droplet volume and concentration.

To investigate the fate of Cry1Ac protein ingested by *G. punctipes*, predators' bodies and their faeces were frozen at various intervals after drinking: < 1, 12, 24, 48 and 72 h after drinking. Faeces were collected during the intervals 0–12, 12–24, 24–48 and 48–72 h after drinking. Predators not immediately frozen or used to assay faeces were maintained in centrifuge tubes, with 5–6 bugs per tube. The opening of each tube was covered by screen mesh, secured by a ring inside the tube. The screen mesh permitted ventilation and held the prey (corn earworm eggs) in place. Moisture was provided to predators in the tubes using a micropipette tip containing cotton batting saturated with water. At each postingestion interval, predators were transferred to a clean centrifuge tube and stored at –25 °C until the ELISA assays. To detect and quantify Cry1Ac protein, ELISA assays were run separately for predator bodies and faeces. For protein extraction from faeces, the centrifuge tubes containing the material were washed with 100 μL of extraction buffer and the contents pooled in a single sample for each time interval. Similarly, all predators' bodies for each collection interval were pooled into a single sample for the assays and variability was derived from the OD results of multiple sub-samples of the extracted solution.

Bt toxin (Cry1Ac) analysis

Cry1Ac protein was quantified in the plant material, prey and predators for all experiments described above. All frozen material, except for *G. punctipes* faeces, was thawed, weighed, placed in a 1.5 mL centrifuge tube and mixed with phosphate-buffered saline solution and Tween 20 (1 × PBST) (Agdia® Inc., Elkhart, Indiana). Non-fat dried milk (0.4% w/v) and Tween 20 (0.5% v/v) were added to PBST to compose the final extraction buffer, which was mixed with sample material at a rate of 1 : 10 (w/v). The contents of centrifuge tubes containing faeces were individually washed with 100 μL of extraction buffer. Extraction of Cry1Ac from plant material was conducted by macerating the leaf material in buffer in a 10-mL tube. Prey and predator materials were macerated using 10-mL tubes or 1.5-mL centrifuge tubes, depending on the volume produced by the sample. The extract supernatants were transferred to clean 1.5-mL centrifuge tubes and stored at –25 °C until the ELISA assay (1–2 weeks later). On the day of protein assay, samples were thawed at room temperature and centrifuged at 5000 r.p.m. for 1 min and loaded at a rate of 100 μL per test well.

Cry1Ac levels in the samples were assayed using antibody-coated wells of PathoScreen® plates for Bt Cry1Ac/Cry1Ab ELISA in a kit using peroxidase enzyme conjugate (Agdia® Inc., Elkhart, Indiana). Standards of Cry1Ac at concentrations 0.625, 1.25, 2.5, 5, 10, 20 and 40 ng/mL (p.p.b.) were used to build a standardized optical density curve for estimating protein content of material from field collections and greenhouse experiments. For Cry1Ac detection in *G. punctipes* bodies and faeces, the standards were calibrated at concentrations of 0.312, 0.625, 1.25, 2.5, 5.0 and 10 ng/mL (p.p.b.). Absorbance measurements were taken with an EL_x808 microtiter plate reader (Bio-Tek Instruments Inc., Winooski, Vermont) reading at 450 nm. To read the results at 450 nm, 50 μL of a 3 M sulphuric acid solution was added to each well to stop further development of the reaction in the wells prior to reading. Using the optical density results generated from the standards, an assay curve was built and the concentrations of Cry1Ac protein were determined for each sample by comparing the sample reading with the optical density reading of the standard curve of pure Cry1Ac protein and correcting for the appropriate dilution and unit (μg/g fresh weight). Because there was no initial weight measurement for *G. punctipes* faeces, the results for faeces were only interpreted through optical density ($OD_{450\ nm}$) readings in the standards and sample material.

Statistical analysis

Predator body weight changes, number of BAW larvae consumed and fresh weight of prey consumed were determined for each predator species caged with BAW larvae and cotton plants in the greenhouse. The data were square-root (x + 0.5) transformed and submitted to a Student's t-test (PROC with Satterthwaite method and unequal variance; SAS Institute, 1999–2001) to compare predator weight and prey consumption when caged on Bt and non-Bt plants for each predator species. Changes in Cry1Ac detected in Bt cotton plants from the fields across sample dates and Cry1Ac levels in the bodies of *G. punctipes* as a function of Cry1Ac-water dilution concentrations were analysed using regression analysis with PROC GLM of SAS. OD readings for Cry1Ac assayed in the faeces of *G. punctipes* were submitted to two-way analysis of variance (ANOVA) (with the factors being concentration and time interval after drinking), using PROC GLM of SAS and significantly different means were separated using Tukey's high significant difference (HSD) test.

Results

Field expression of Cry1Ac protein in cotton and toxin acquisition by prey and predators

From the first week of June to the last week of August, Cry1Ac protein in upper fully expanded cotton leaves ranged from 0.20 to 0.29 μg/g fresh tissue (Table 1), with a seasonal mean of 0.24 μg (Fig. 1, commercial fields). A slight decrease in Cry1Ac level in the plants across progressive sample dates was observed ($b = -0.0045 \pm 0.001$) ($y = 0.274 - 0.0045x$, $r^2 = 0.16$, $F_{1,47} = 9.01$, $P = 0.0043$). The highest and lowest protein levels in cotton plants were detected in the second week of June and of July, respectively. Among the sampled canopy-dwelling herbivores, no Cry1Ac protein was detected in *A. gossypii*. However, all three assayed lepidopteran species exhibited detectable levels of Cry1Ac. *Spodoptera eridania* was the lepidopteran species most commonly collected on Bt cotton, except on the first sample date (Table 1). The other two species, *P. includens* and *S. exigua*, were common during the middle and later portions of the season. Cry1Ac levels detected in these species were quite variable during the season and among species. The seasonal mean for Cry1Ac was highest in *S. exigua*, followed by *S. eridania* and *P. includens* (Fig. 1, commercial fields). Almost 50, 42 and 17% of the original Cry1Ac level detected in the cotton plants were detected in *S. exigua*, *S. eridania* and *P. includens*, respectively. Among the seven representative predator species collected during the season and assayed for Bt protein, Cry1Ac was only detected in *C. rufilabris* larvae and *P. maculiventris* adults (Table 1). The presence of Cry1Ac protein was detected in two out of 7 weeks for *C. rufilabris* (late in the season) and one out of 6 weeks for *P. maculiventris* (also late in the season). The concentration of toxin observed in predators that was positive for toxin was only 8.3% (*P. maculiventris* adults) and 29% (for *C. rufilabris* larvae) of the amount found in the plants. The timing of Cry1Ac presence in predators coincided with abundant populations of *P. includens*.

Toxin acquisition by prey and predatory heteropterans in the greenhouse

Bt protein was measured in all three trophic levels in the greenhouse cage experiments. In Bt cotton plants (first trophic level), Cry1Ac was 0.18 ± 0.03 (mean ± SD of μg/g fresh weight) and decreased to 0.14 ± 0.01, 0.14 ± 0.02 and 0.12 ± 0.03 μg/g fresh weight in 10, 4 and 2-day-old BAW larvae fed Bt cotton plants (second trophic level, Fig. 1, greenhouse). Cry1Ac in the third trophic level was only detected in the predator *P. maculiventris*. The level of Cry1Ac in *P. maculiventris* was 0.02 ± 0.004 μg/g fresh body weight. The estimated fresh weight of prey (9-day-old BAW) consumed by *P. maculiventris* during the 24-h experimental period was similar whether larvae were fed Bt or non-Bt cotton plants (Table 2). However, to achieve a similar amount of food ingestion, *P. maculiventris* consumed almost twice as many BAW larvae fed on Bt cotton compared with larvae fed non-Bt cotton to compensate for the smaller size of BAW larvae fed on Bt cotton plants (Table 2). Cry1Ac protein was not detected in adult *N. roseipennis*, *G. punctipes* or *O. insidiosus* that preyed on BAW larvae fed Bt cotton. Nor was Cry1Ac detected in any of the predators fed BAW larvae that consumed non-Bt cotton. The estimated fresh prey consumption and predation rate by these three predator species preying on BAW larvae did not differ for prey fed either Bt or non-Bt cotton plants. The estimated fresh consumption of BAW by *P. maculiventris* (which had detectable Cry1Ac levels) was approximately 32, 68 and 338-fold greater than the amount of prey consumed by *N. roseipennis*, *G. punctipes*, or *O. insidiosus*, respectively (Table 2).

Toxin ingestion by *G. punctipes*

Adults of the big-eyed bug *G. punctipes* were able to ingest detectable levels of Cry1Ac protein diluted in water (Fig. 2). The lower threshold of Cry1Ac ingestion for ELISA detection was 4 p.p.m. in the tested range of 2–32 p.p.m. The levels of Cry1Ac detected in the predator body decreased linearly as a function of the concentrations from 4 to 32 p.p.m. ($F_{1,10} = 125.5$, $P < 0.0001$) at a proportion of –0.0087 ± 0.007 μg/g fresh body weight (Fig. 2). The amount of Cry1Ac toxin detected in the predator body was nearly 100-fold less than the original amount of Cry1Ac in the concentration offered to the bug.

Fate of Cry1Ac protein in body and faeces of *G. punctipes*

Cry1Ac protein exposed in 16 and 32 p.p.m. concentrations to bugs was detected in the predators' bodies and faeces in amounts proportional to the concentration offered and related to length of time passed after drinking (Fig. 3A,B). Adult *G. punctipes* had detectable Cry1Ac in their bodies up to 24 and 48 h after drinking from 16 and 32 p.p.m. concentrations of purified Cry1Ac protein, respectively. ELISA did not detect any Cry1Ac in the bodies of predators 72 h after drinking from either 16 or 36 p.p.m. concentrations. The amount of Cry1Ac measured in *G. punctipes* immediately after drinking 16 and 32 p.p.m. concentrations (< 1 h later) was approximately 46% (mean ± SD; 0.35 ± 0.003 μg/g fresh body weight) and approximately 58% (0.57 ± 0.002 μg/g fresh body weight) of the original amount available in the dilutions, respectively. The levels of Cry1Ac protein decreased linearly as the postdrinking interval increased from approximately 1–72-h (16 p.p.m.; $y = 0.035 - 0.0011x$, $r^2 = 0.95$,

Table 1 Mean ± SD Cry1Ac protein concentration (μg/g fresh weight) in uppermost fully expanded Bt cotton leaves, selected herbivores and predators collected throughout the cotton season. Tifton, Georgia, in 2004

	Sample dates											
Source	7–9 June	14–16 June	21–23 June	28–30 June	5–7 July	12–14 July	19–21 July	26–28 July	2–3 August	9–11 August	23–25 August	28–29 August
Bt cotton DPL 555	0.25 ± 0.02	0.29 ± 0.03	0.27 ± 0.06	0.27 ± 0.01	0.26 ± 0.01	0.20 ± 0.01	0.21 ± 0.02	0.27 ± 0.02	0.25 ± 0.02	0.24 ± 0.04	0.22 ± 0.04	0.22 ± 0.04
Aphis gossypii	– [a]	–	ND[b]	ND	ND	ND	–	–	–	–	–	–
Spodoptera eridania	–	0.17 ± 0.01	0.15 ± 0.01	0.06 ± 0.008	0.11 ± 0.02	0.12 ± 0.02	0.09 ± 0.01	0.06 ± 0.02	0.12 ± 0.05	0.08 ± 0.05	0.07 ± 0.05	0.10 ± 0.008
Pseudoplusia includens	–	–	–	–	0.03 ± 0.004	0.02 ± 0.001	0.03 ± 0.004	0.02 ± 0.003	0.05 ± 0.02	0.09 ± 0.004	0.02 ± 0.004	0.04 ± 0.003
Spodoptera exigua	–	–	–	–	0.13 ± 0.02	0.09 ± 0.02	0.08 ± 0.007	0.13 ± 0.02	0.14 ± 0.02	0.07 ± 0.007	0.21 ± 0.02	–
Chrysoperla rufilabris	–	–	–	ND	ND	ND	ND	ND	0.12 ± 0.01	–	0.012 ± 0.01	–
Hemerobiid larvae	–	–	–	ND	ND	ND	ND	ND	–	ND	ND	ND
Geocoris punctipes	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Orius insidiosus	–	–	–	–	–	–	ND	ND	ND	ND	–	–
Nabis roseipennis	–	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND
Podisus maculiventris	–	–	–	ND	–	–	ND	ND	ND	0.02 ± 0.005	ND	–
Harmonia axyridis	–	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	–

[a] Signifies absence in the field or collected individuals did not compose a minimum of 10 mg sample to permit testing.

[b] Not detected (ND) indicates specimens tested for which no Cry1Ac protein was detected at a standard detection limit of 0.5 p.p.b.

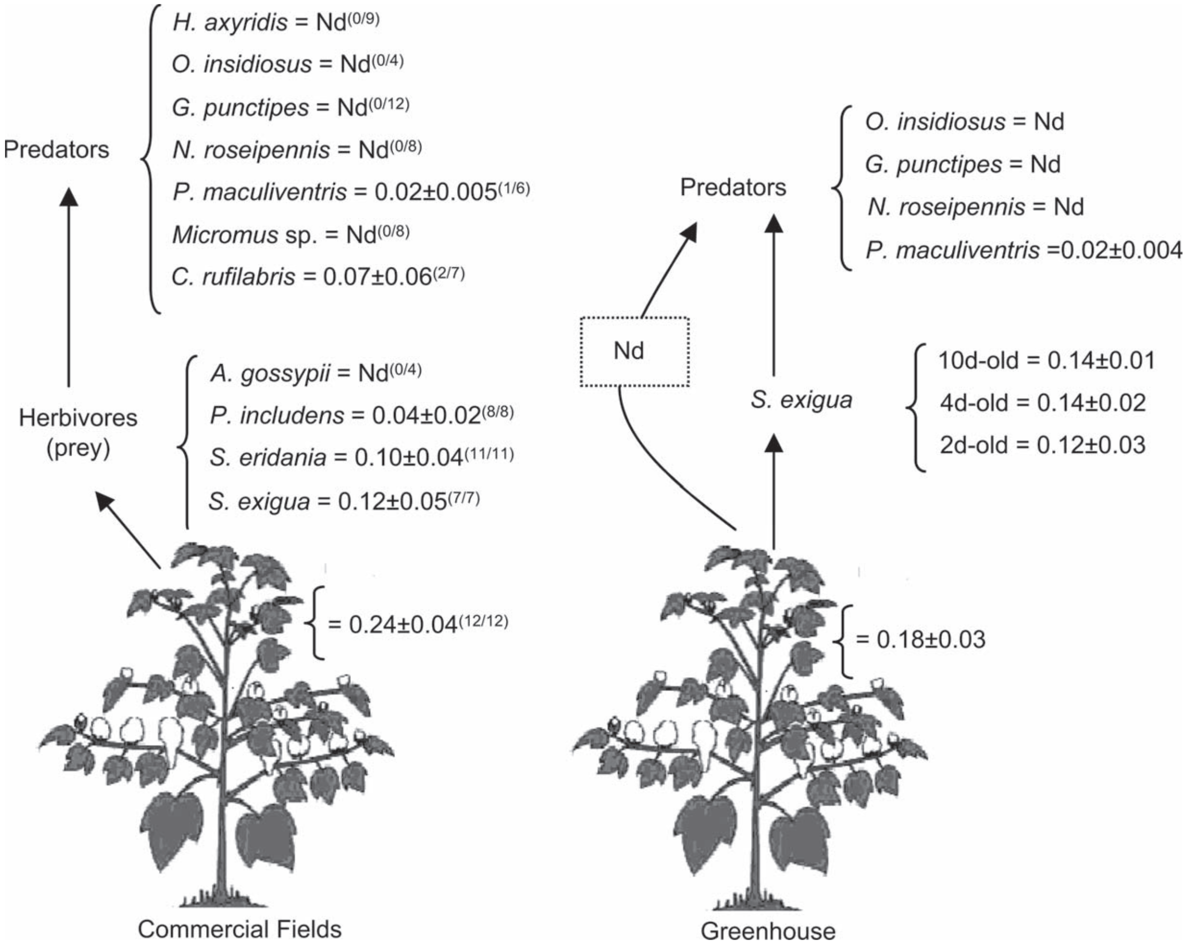

Figure 1 Levels (μg/g^{-}fresh weight) of *Bacillus thuringiensis* Cry1Ac protein in Bt cotton plants (DPL 555), herbivores and predators (representing the three trophic levels in the cotton ecosystem) in commercial fields and under greenhouse conditions. Seasonal mean is presented for material collected from cotton fields throughout the growing season; Nd, not detected; numerators on superscript values for the field results represent the number of sample dates tested positive to Cry1Ac on which the respective organisms were found and sampled in the field out of the 12 sample weeks.

$F = 133.10$, $P < 0.0001$; 32 p.p.m.; $y = 0.055 - 0.00037x$, $r^2 = 0.87$, $F = 67.88$, $P < 0.0001$; Fig. 3A). Although no Cry1Ac was detected in predators' bodies 72 h after drinking from either concentration offered, the Cry1Ac levels in bugs fed 16 p.p.m. concentration declined approximately threefold as rapidly as levels in bugs that drank from the 32 p.p.m. concentration ($b_{16ppm} \div b_{32ppm}$).

OD readings from ELISA assays (Fig. 3B) indicated detectable amounts of Cry1Ac in faeces of *G. punctipes* during the four intervals after drinking from both concentrations tested (16 and 32 p.p.m). Two-way ANOVA indicated that concentration had an effect on the concentration of Cry1Ac detected in big-eyed bug faeces (average OD across time intervals was 0.42 ± 0.10 and 1.58 ± 0.48 for 16 and 32 p.p.m., respectively; $F_{1,16} = 565.13$, $P < 0.0001$) as did time interval after drinking; with significant interactions between concentration and time intervals ($F_{3,16} = 163.42$, $P < 0.0001$). Faeces collected during the 1–12-h postdrinking interval produced the lowest concentration of Cry1Ac for bugs drinking the 32 p.p.m. concentration, but did not differ significantly from amounts observed in the 24–48 and 48–72-h intervals for bugs drinking 16 p.p.m. The peak of Cry1Ac excretion in the big-eyed bug faeces was observed during the 12–24-h interval for both concentrations.

Discussion

The amount of Cry1Ac protein detected in cotton plants in the field decreased slightly across the season (Table 2), but toxin amounts were sufficient to move up through trophic levels in the ecosystem. Many factors contribute to Cry1Ac expression in transgenic Bt cotton. Detailed studies of environmental and plant factors affecting Cry1Ac expression in the field were conducted by Greenplate (1999) and Adamczyk & Sumerford (2001). Their results indicated that Bt cotton grown in Georgia expressed the lowest rate of Cry1Ac in terminal foliage relative to cotton cultivated in six other Southeastern U.S. States. Adamczyk & Sumerford (2001) found a decrease of Cry1Ac expression in 13 Bt cotton varieties

Table 2 Body weight changes, number of *Spodoptera exigua* larvae (BAW) killed, and estimated fresh weight of BAW consumed by individual predators caged on Bt or non-Bt cotton plants under greenhouse conditions (mean ± SD: 27.1 ± 4 °C and approximately 14 h of light)

Predator	Cotton	Weight gain (mg)[a]	BAW killed (no.)[b]	Prey consumed (mg)[c]
Podisus maculiventris	*Bt*	24.9 ± 3.49	10.1 ± 1.28	101.3 ± 14.96
	Non-Bt	27.2 ± 5.51	5.7 ± 0.49	104.3 ± 12.03
		$t = -0.17$, $P = 0.8660$	$t = 3.28$, $P = 0.0053$	$t = -0.61$, $P = 0.5522$
Nabis roseipennis	Bt	1.39 ± 0.21	6.1 ± 1.41	3.18 ± 0.70
	Non-Bt	1.28 ± 0.36	5.9 ± 0.81	2.65 ± 0.65
		$t = 0.96$, $P = 0.3530$	$t = -0.19$, $P = 0.8485$	$t = 0.56$, $P = 0.5853$
Geocoris punctipes	Bt	0.83 ± 0.14	8.0 ± 0.81	1.49 ± 0.16
	Non-Bt	0.64 ± 0.29	6.7 ± 0.71	1.84 ± 0.18
		$t = 0.83$, $P = 0.4321$	$t = 1.02$, $P = 0.3213$	$t = -1.62$, $P = 0.1155$
Orius insidiosus	Bt	0.0417 ± 0.011	3.4 ± 0.37	0.30 ± 0.03
	Non-Bt	0.0200 ± 0.015	2.9 ± 0.31	0.38 ± 0.04
		$t = 1.27$, P = 0.2954	$t = 0.94$, P = 0.3601	$t = -1.57$, $P = 0.1356$

[a] Difference between individual predator weight before and after caging with prey on respective cotton types.

[b] Average number of *Spodoptera exigua* larvae killed by individual predators. BAW larvae were offered to predators at different ages (*P. maculiventris* = 9 days; *N. roseipennis* = 3 days; *G. punctipes* and *O. insidiosus* = 1 day).

[c] Fresh weight of prey material consumed per individual predator, considering the number of prey killed and predator weight change during the exposure period.

across the season in Mississippi ranging from approximately 1.5–0.5 p.p.m. (μg/g or μg/ml) per fresh weight of tissue. Considering that the toxin amounts in Mississippi Bt cotton should be around 6.57-fold greater than those detected in Georgia Bt cotton (Greenplate, 1999), the Cry1Ac amounts in our sampled plants across the season fit the expected values. The lower Cry1Ac expression in the Georgia cotton does not compromise its value for pest management because the lethal concentration for neonate larvae of the major target lepidopterans is much lower than that expressed (Perlak *et al.*, 2001).

Among the major prey items available in the cotton canopy, aphids failed to acquire toxin, whereas the lepidopteran larvae did. No aphid species have been reported to acquire Cry protein by feeding on plants. Our findings with *A. gossypii* collected in Bt cotton fields agree with those reported for *Rhopalosiphum padi* L. and *Rhopalosiphum maidis* (Fitch) (Raps *et al.*, 2001; Dutton *et al.*, 2002; Head *et al.*, 2001). Among prey that can acquire toxin, the amount of Cry protein conveyed to the third trophic level appears to be dependent on herbivore species. Larvae of the lepidopterans *S. exigua*, *S. eridania* and *P. includens* can expose their predators to approximately 50, 42 and 17%, respectively, of the original Cry1Ac concentrations in Bt cotton plants (Fig. 1). Similarly, almost 21% of original Cry1Ab expressed in Bt corn (event N4640Bt) was detected in *S. littoralis* larvae, whereas 73% was detected in the spider mite, *Tetranychus urticae* (Koch) (Dutton *et al.*, 2002). Studying four herbivores fed Bt corn, Dutton *et al.* (2003) found no Cry1Ab in the aphid *R. padi*, and the highest amount of Cry1Ab was detected in the spider mite, *T. urticae* (5.56 μg/g fresh weight), followed by the thrips *Frankliniella tenuicornis* (Uzel) (0.91 μg), and the leafhopper *Zyginidia scutellaris* (Herrich-Schaefer) (0.20 μg). Larvae of the sawfly *Athalia rapae* (L.) exhibited 18% of Cry1Ac that was present in the plants when fed rape expressing the Bt protein Cry1Ac, and almost the same amount was detected in their faeces but feeding on Bt rape had no effect on the fecundity and fertility of the sawfly (Howald *et al.*, 2003). In addition, Head *et al.* (2001) reported a large decrease in Cry1Ab in lepidopteran larvae fed Bt corn (MON 810) compared with the original levels of Cry1Ab in transgenic Bt plants. Cry1Ab in the European corn borer, *Ostrinia nubilalis* (Hübner), *H. zea* and *Agrotis ipsilon* (Hufnagel) larvae fed Bt corn was 0.07, 0.15 and 0.17 p.p.m. (μg/g or μg/ml), respectively, of the fresh weight of *O. nubilalis*, *H. zea* and *A. ipsilon*. We observed a similar range of variation in Cry1Ac levels among the three lepidopteran species fed Bt cotton in our field collections (0.04–0.12 μg/g fresh weight (Fig. 1).

Cry1Ac was detected in larvae of the predator *C. rufilabris* late in the season when aphids, a common prey for lacewings

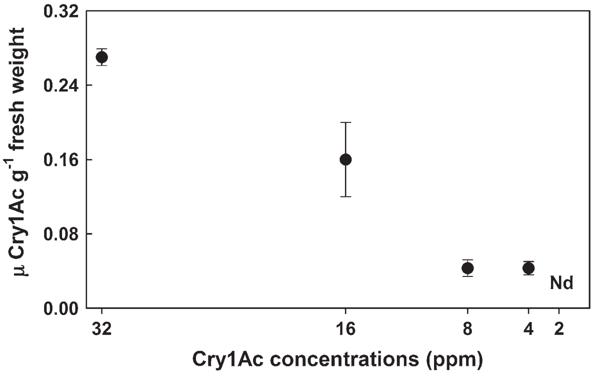

Figure 2 Amount of Cry1Ac protein detected in bodies of *Geocoris punctipes* after drinking Cry1Ac protein–water (concentrations from 2 to 32 p.p.m). Nd, not detected at 0.5 p.p.b. of standard detection limit.

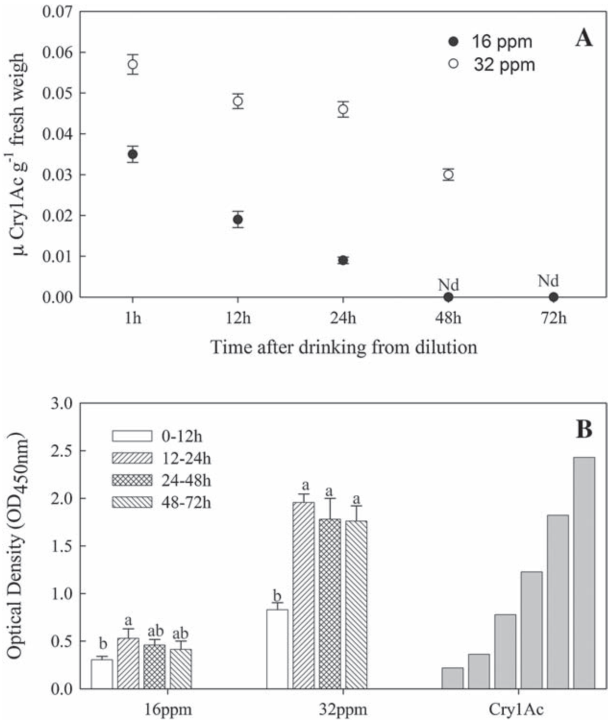

Figure 3 (A) Mean ± SD Cry1Ac protein concentrations in the bodies of *Geocoris punctipes* in five intervals after drinking from purified Cry1Ac-water dilutions of 16 and 32 p.p.m. (Nd, not detected). (B) Optical density (OD) readings of enzyme-linked immunosorbent assays for detection of Cry1Ac protein in *G. punctipes* faeces at different intervals after drinking from 1 μL of purified Cry1Ac-water dilution (16 and 32 p.p.m), corrected for distilled water drinking bugs. Grey bars from left to right represent OD readings for standards consisting of purified Cry1Ac (0.312, 0.625, 1.25, 2.5, 5.0 and 10 ng/mL). Bars under same letter do not differ among time intervals within the same concentration at 0.05 significance levels (Tukey's HSD test).

and one that does not acquire Bt protein, were scarce and Cry1Ac-containing lepidopteran larvae were abundant. Therefore, late in the season, predation on lepidopteran larvae could be enhanced because of reduced numbers of alternative prey. Nordlund & Morrison (1990) found that *C. rufilabris* preferred *Heliothis virescens* (F.) larvae to cotton aphids. The late-season availability of lepidopteran larvae, a preferred prey of *P. maculiventris*, is probably the source of Cry1Ac detected in this predator in the field because these predators do not acquire Cry1Ac directly by plant feeding (Fig. 1, greenhouse). Lepidopteran larvae partially susceptible to Cry1Ac usually become much more abundant in the middle or late in the season, replacing the once-abundant aphids, which are free of Cry1Ac. Therefore, there is an important seasonal element to dynamics of prey and predators in the cotton ecosystem (in this case, between Bt cotton, lepidopteran larvae eating Bt cotton plants, *C. rufilabris* larvae and *P. maculiventris* adults) that is highly relevant to assessing the possible risks of exposure to Cry proteins. These findings reinforce the recommendations made by Schuler *et al.* (2001) and Dutton *et al.* (2003) suggesting that field experiments should consider more than one generation of the organisms during the crop season to ascertain risks.

Despite detecting Cry1Ac in *C. rufilabris* larvae and in *P. maculiventris* in the field, it is not apparent that these predators are adversely affected by the protein. Seasonal means per 40-drop cloth samples for three successive cotton growing seasons covering multiple generations of both predators were similar in Bt and non-Bt cotton fields, including in 2004 when *C. rufilabris* and *P. maculiventris* were positive for Cry1Ac (Torres & Ruberson, 2005). The Cry1Ac ingested by these predators may be handled similarly to other undigested/unused material from the diet and excreted, as observed in the laboratory experiment with *G. punctipes* (Fig. 2B). Similarly, direct ingestion of Bt Cry1Ab by the lacewing *Chysoperla carnea* (Stephens) from sucrose solution did not affect development and survival of this predator (Romeis *et al.*, 2004). Several other examples are summarized by Lövei & Arpaia (2005).

Supporting our field results, we found that predatory heteropterans (a group of predators well known for their plant feeding behaviour) failed to acquire Cry protein from direct feeding on Bt cotton plants in the greenhouse (Fig. 1, greenhouse). Unlike aphids, feeding by predatory heteropterans is not confined to phloem, where Cry-protein would not be expected to occur (Raps *et al.*, 2001). Plant feeding by predatory heteropterans is believed to occur by insertion of stylets randomly into plant tissue and removal of liquid contents and materials liquefied by the action of salivary enzymes, such as amylase and proteinases that are found in salivary glands of *O. insidiosus*, *P. maculiventris* and *G. punctipes* (Stamopoulos *et al.*, 1993; Cohen, 1996; Zeng & Cohen, 2000). Therefore, Cry1Ac protein could be picked up as a component of digested cell debris and other material. However, in our trials, if it was acquired at all, the toxin level was not enough to be detected via ELISA assays. Armer *et al.* (2000) also reported no ingestion of Cry3A from direct feeding on Bt potato plants by *Orius tristicolor* (White), *Nabis* sp. and *Lygus hesperus* Knight, although the last species is a phytozoophagous species that is recognized as a pest in some crops. It would be reasonable to expect Cry3A to be detected at least in *L. hesperus* fed Bt potato plants because the salivary glands of this bug produce pectinase (Strong & Krutitwagen, 1968), which is responsible for digesting plant cell walls.

The wide size range of heteropteran predators used in the greenhouse cage experiments was predetermined to broadly assess the exposure risk of this important predator group to Cry1Ac in Bt transgenic cotton. The sizes of predators caged on Bt cotton deprived of prey in the present study [*P. maculiventris* (84.4 ± 18.5), *N. roseipennis* (7.8 ± 4.1), *G. punctipes* (4.6 ± 0.8) and *O. insidiosus* (0.27 ± 0.05)] covered a large portion of predatory heteropterans not only common in cotton fields but also in other crop ecosystems. In addition, the species studied exhibit feeding behaviour and enzymatic profiles representative of extra-oral and gut digestion found among predatory taxa in the Pentatomomorpha (*Podisus* and *Geocoris*) and Cimicomorpha (*Nabis* and *Orius*). We would expect larger predators to consume more prey, and thereby acquire more toxin and increase exposure risk. Because the estimated amount of BAW larval fresh weight consumed played a role in Cry1Ac acquisition and detection, it would be expected that, if Cry1Ac were to be detected in any of the heteropteran predators tested, it would be found in *P. maculiventris*, one of the largest predatory heteropterans found in cotton. This proved to be the case and was verified in the field samples.

In the laboratory, *G. punctipes* was able to ingest Cry1Ac protein in its purified form at high doses relative to that expressed in the cotton plants. Similarly, the lacewing *C. carnea* consumed detectable amounts of Cry1Ab toxin directly from a sucrose diet (Romeis *et al.*, 2004) and the lacewing *C. rufilabris* acquired toxin in the field in the present study, most probably from prey that fed on Bt cotton in the field (Table 2). The ability to ingest Cry1Ac at detectable levels in a purified form directly from water dilutions provides opportunities to directly test toxicity of Cry proteins to these predators by ingestion. Direct toxicity has been used as a basic laboratory screening of selectivity (Sims, 1995, 1997; Romeis *et al.*, 2004). For example, Romeis *et al.* (2004) offered Cry1Ab in a sucrose diet to second-instar larvae of *C. carnea* and demonstrated no direct effect of purified Cry1Ab on the development of lacewing larvae, although Cry1Ab was detected in larvae fed Cry1Ab-sucrose diet. A study by Romeis *et al.* (2004) demonstrated the lack of toxicity of Cry1Ab for *C. carnea* larvae, in contrast to previous results indicating negative effects (Hilbeck *et al.*, 1998). Consumption of detectable amounts of Cry1Ac by *G. punctipes* will allow much higher doses of Cry-proteins to be tested than usually expressed in transformed plants, permitting generation of direct and quick information on the safety of the protein.

The amounts of Cry1Ac in the body of *G. punctipes* decreased approximately 100-fold from the original concentrations provided to them in the droplets (e.g. $32/0.27 = 118.5$) (Fig. 3). This dilution effect may explain the negative results obtained when this predator consumed prey fed Bt cotton in the greenhouse, as well as the negative results from field samples. Based on the results obtained in the present study, and assuming no loss of Cry1Ac during prey consumption, the predator would have to consume a minimum of 24 mg of BAW to acquire sufficient Cry1Ac to be detectable at the lower detection limit for the ELISA (0.5 p.p.b.). The 12 *G. punctipes* assayed consumed a total of 17.88 mg of prey (individual predators consumed 1.49 mg fresh weight of BAW larvae; (Table 2). Only *P. maculiventris*, which individually consumed 101.4 mg fresh weight of BAW, consumed sufficient prey material to acquire detectable levels of Cry1Ac (Table 2).

Insect faeces appear to accumulate ingested Cry protein. *Geocoris punctipes* faeces, as is the case for other predatory heteropterans, consist of semiliquid excreta, which can lose water quickly, increasing the concentration of undigested material in the faeces. Overall, insect excretion is slower than ingestion and undigested contents are more concentrated than was the original food (Chapman, 1998). This may explain why levels of Cry protein detected in the faeces were higher than levels in the original food. Similarly, Cry1Ab in faeces of the lepidopteran *Spodoptera littoralis* (Boisduval) fed-Bt corn for 24 h was ten-fold greater than in the larvae (Raps *et al.*, 2001). Cry proteins also were detected in the faeces of *A. rapae* larvae fed Bt rape (Howald *et al.*, 2003) and in honeydew produced by brown planthopper, *Nilaparvata lugens* (Stål) fed-Bt rice (Bernal *et al.*, 2002). However, the high level of Cry1Ac in *G. punctipes* faeces does not exclude the possibility that some of the protein was broken down during ingestion or digestion and discarded or used in an altered form in the bug. There is no published information regarding the fate of Cry proteins in heteropterans. Considering the short time for digestion in heteropterans (liquid feeders), it is possible that Cry1Ac ingested by *G. punctipes* may be restricted to the digestive tract and eliminated in the faeces. However, Cry1Ac traces could remain in the digestive system at levels not detectable by ELISA (0.5 p.p.b.) but be concentrated sufficiently in the faeces to produce detectable levels.

To date, studies of ingestion of Cry proteins by predators through diets, prey fed-Bt plants or Bt plant products have yielded no evidence of adverse effects of Bt proteins on predators. These studies have been conducted using green lacewings, *C. carnea*, that were fed Bt sucrose diet (Sims, 1995, 1997; Romeis *et al.*, 2004), pollen of Bt corn (Pilcher *et al.*, 1997) and aphids fed Bt corn (Lozzia *et al.*, 1998; Meier & Hilbeck, 2001); with lady beetles *Coleomegilla maculata* Timberlake and *Hippodamia convergens* Guerin-Meneville fed pollen of Bt plants, prey fed Bt plants, or prey fed diet containing Cry proteins (Sims, 1995, 1997; Pilcher *et al.*, 1997); and with predatory bugs, *O. insidiosus*, *Orius majusculus* (Reuter) and *Cyrtorhinus lividipennis* Reuter that consumed prey fed on Bt plants (Zwahlen *et al.*, 2000; Pilcher *et al.*, 1997; Bernal *et al.*, 2002). Therefore, the ability of herbivorous prey to acquire Bt proteins from host plants and the resulting exposure to predators appears to have no adverse effects on predator populations. The presence of Cry protein in lepidopteran larvae does not necessarily imply negative impacts on the third trophic level in cotton fields. Most of the common predatory arthropods in cotton are generalists and can feed on herbivores free of Bt protein, which could ameliorate or limit adverse effects, if they exist. This is supported by the demonstration that negative effects on green lacewing larvae attributed to Bt proteins (Hilbeck *et al.*, 1999) were later shown to be due to suboptimal prey quality rather than Cry1Ab protein (Dutton *et al.*, 2002). Further, direct toxicity of Cry proteins to predators has not been reported (Sims, 1995, 1997; Romeis *et al.*, 2004).

Attempts to determine the safety of Bt transgenic plants for nontarget organisms, particularly natural enemies, have often been conducted in laboratories or have focused on just one trophic level. Although there is some agreement on identification of potential negative interactive effects, it is not yet possible to predict the impact of Bt proteins in the field with certainty because most conclusions rely heavily on artificial conditions. A hierarchy of studies from the laboratory to the field, and from small- to large-scale studies, is important for making appropriate assessments (Schuler *et al.*, 1999, 2001; Dutton *et al.*, 2003), but the conditions in standard farm investigations can make such studies difficult because they are susceptible to many sources of uncontrolled variation. Nevertheless, by using stepwise tri-trophic experiments extending from the laboratory to the field, the results of the present study provide a number of insights into the movement of Cry1Ac toxin in the cotton ecosystem. In the laboratory and greenhouse, predatory heteropterans were found to be capable of ingesting purified Cry1Ac protein in concentrations above 4 p.p.m. and, in some cases, acquired toxin from prey fed on Bt cotton. However, predator exposure to toxin can be dependent on prey species because respective lepidopteran species collected in the field contained different levels of Bt protein (Table 1 and Fig. 1, commercial fields). In addition, greenhouse experiments demonstrated that Cry1Ac acquisition from prey fed Bt plants was dependent on the amount of prey consumed (Table 2 and Fig. 1, greenhouse) and that important predatory heteropterans were unable to acquire detectable amounts of Cry1Ac directly by feeding on plants. ELISA results established that the predators *C. rufilabris* and *P. maculiventris* were able to pick up Cry1Ac conveyed by prey fed Bt cotton in the field but only during periods when large numbers of lepidopteran larvae were present and there was no evidence for adverse population effects on either species (Torres & Ruberson, 2005). In conclusion, despite continuous expression of Cry1Ac by Bt cotton plants, the degree to which toxin reaches the third trophic level in cotton fields appears to be related to the community structure and dynamics of lepidopteran larvae and their predators, coupled with availability of alternative prey free of Cry1Ac-protein.

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