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Uptake of Bt endotoxins by nontarget herbivores and higher order arthropod predators: molecular evidence from a transgenic corn agroecosystem

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Abstract

The planting of transgenic crops expressing *Bacillus thuringiensis* endotoxins is widespread throughout the world; the prolific increase in their application exposes nontarget organisms to toxins designed to control pests. To date, studies have focused upon the effects of Bt endotoxins on specific herbivores and detritivores, without consideration of their persistence within arthropod food webs. Here, we report the first quantitative field evaluation of levels of Bt endotoxin within nontarget herbivores and the uptake by higher order arthropods. Antibody-based assays indicated significant quantities of detectable Cry1Ab endotoxin within nontarget herbivores which feed on transgenic corn (including the corn flea beetle, *Chaetocnema pulicaria*, Japanese beetle, *Popillia japonica* and southern corn rootworm, *Diabrotica undecimpunctata howardi*). Furthermore, arthropod predators (Coccinellidae, Araneae, and Nabidae) collected from these agroecosystems also contained significant quantities of Cry1Ab endotoxin indicating its movement into higher trophic levels. This uptake by predators is likely to have occurred by direct feeding on plant material (in predators which are facultatively phytophagous) or the consumption of arthropod prey which contained these proteins. These data indicate that long-term exposure to insecticidal toxins occurs in the field. These levels of exposure should therefore be considered during future risk assessments of transgenic crops to nontarget herbivores and arthropod predators.

Keywords: *Bacillus thuringiensis*, ELISA, endotoxin transfer, generalist predators, nontarget effects, risk assessment

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Introduction

The planting of transgenic crops in the USA and other parts of the world has increased dramatically since the commercialization of *Bacillus thuringiensis* (Bt) corn in the mid-1990s (Cannon 2000; Pray *et al*. 2002; Shelton *et al*. 2002). Bioengineered crops can confer significant advantages to natural enemies over conventional broad-spectrum insecticides (Gould 1998; Hoy *et al*. 1998; Way & Van Emden 2000) and provide effective levels of control of target pests. However, concern still exists with regard to their long-term and potentially negative effects upon nontarget components of the food chain (O'Callaghan *et al*. 2005) and their role in integrated pest management is poorly understood (Way & Van Emden 2000; Wolfenbarger & Phifer 2000; Hilbeck 2001; Obrycki *et al*. 2001, 2004; Groot & Dicke 2002; Lövei & Arpaia 2005). Although some studies report that Bt toxins negatively impact (through increased mortality, longer development time and reduced weight gain) the fitness of nontarget herbivores and predators (e.g. Hilbeck *et al*. 1998; Losey *et al*. 1999; Jesse & Obrycki 2000; Zwahlen *et al*. 2003a), other studies suggest that such exposure has little or no consequence for such species (e.g. Pilcher *et al*. 1997; Al-Deeb *et al*. 2001; Lundgren & Wiedenmann 2002; Anderson *et al*. 2004). However, no study has, to date, examined how transgenic toxins move through nontarget food webs in the field. This is especially important given their slow degradation rate in the field (Zwahlen *et al*. 2003b), thus exposing many organisms in the decomposer food chain to Bt endotoxins for extended periods of time.

Monoclonal antibodies (Symondson *et al*. 2000; Harwood *et al*. 2004, 2005; Winder *et al*. 2005) and DNA approaches (Sheppard *et al*. 2004; Harper *et al*. 2005) have been highly

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successful in measuring trophic interactions between predator and prey populations in the field. Crucially, these methods of gut-content analysis allow the feeding behaviour of predators to be evaluated after predation events have occurred naturally (Sunderland 1988; Harwood & Obrycki 2005). However, the application of such techniques to quantify transgenic insecticidal concentrations in arthropod populations has been limited to relatively few laboratory studies (Raps *et al*. 2001; Dutton *et al*. 2002; Wandeler *et al*. 2002; Howald *et al*. 2003; Zwahlen *et al*. 2003b). Using an EnviroLogix Quantiplate enzyme-linked immunosorbent assay (ELISA) kit for the detection of Cry1Ab endotoxin, we tested the hypothesis that nontarget herbivores and natural enemies occurring in transgenic corn fields would contain significant quantities of Bt endotoxin. Dutton *et al*. (2002) was able to demonstrate, using a quantitative ELISA, that Cry1Ab endotoxins [which have been developed for the control of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae)] were readily transferred between Bt maize, *Zea mays* L. (N4640Bt, Syngenta, Stein, Switzerland), and *Tetranychus urticae* (Koch) (Acari: Tetranychidae), *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and, to a lesser extent, *Rhopalosiphum padi* (L.) (Homoptera: Aphididae). Evidence for this flow of transgenic material in the laboratory illustrates the potential for movement of these compounds through food webs in the field. Despite this likely movement of endotoxins, there have been no documented field studies recording its presence in nontarget invertebrates.

In this study, we quantify the concentration of Bt endotoxins (Cry1Ab) present within nontarget herbivores and their natural enemies (spiders and predatory insects) in a corn agroecosystem using an antibody-based assay. Previous field studies have documented the relative abundance of nontarget species in Bt vs. non-Bt fields (Jasinski *et al*. 2003; Obrycki *et al*. 2004; Sisterson *et al*. 2004) but have not tested for the presence of Bt endotoxins in these species. We specifically tested the hypothesis that higher order natural enemies and nontarget herbivores would contain significant quantities of insecticidal toxins. It was further predicted that those predators which supplement their food intake by feeding on plant material (e.g. many heteropterans) (Alomar & Wiedenmann 1996) would contain elevated concentrations of Cry1Ab endotoxins in their bodies. This could provide the basis for future risk assessment of the interactions between nontarget arthropods and transgenic crops in the field, indicating natural exposure levels of different trophic levels to Bt endotoxins.

Methods

Sample collection

Arthropods were collected on 11 dates between early June and August 2004 from a field of transgenic corn (Bt hybrid N79-L3, Bt-11 event, Syngenta Seeds, Golden Valley, MN) planted at the University of Kentucky Spindletop Research Station, Lexington, KY, USA (Universal Trans-Mercator Grid References: 4224676 Northing, 689850 Easting, Zone 16). All samples were collected individually by aspirator, transferred into 1.5 mL Eppendorf tubes or 7 mL Sterilin Bijou containers (Dynalab, Rochester, NY, USA) (depending upon size) and frozen at –20 °C immediately after collection in a portable freezer.

In addition to the collection of arthropods from transgenic corn, 21 representative arthropods from seven orders (Araneae, Coleoptera, Heteroptera, Homoptera, Diptera, Neuroptera, Mollusca) (10 replicates of each species) were collected from a nontransgenic field (alfalfa, *Medicago sativa* L.) at Spindletop Research Station, Lexington, KY, USA. These arthropods were collected to ensure specificity of the assay to Cry1Ab endotoxins given that unexpected 'false-positive' results can be obtained from antibody-based assays (Harwood & Obrycki 2005). If any arthropod collected from a nontransgenic crop and maintained on nontransgenic food elicited a positive absorbance in the assay, the presence of target Cry1Ab endotoxins could not be assured. This cross-reactivity testing is a requirement before such assays can be applied to study trophic connectance in the field. These species were maintained in the laboratory on water and nontransgenic food (alfalfa, non-Bt corn isoline, Collembola, Diptera and/or Aphididae) prior to screening for the presence of Cry1Ab endotoxins. One additional arthropod control was included to evaluate potential error caused by contamination with pollen falling from the crop onto the samples in the field. Ten replicates of a common linyphiid spider, *Bathyphantes pallida* (Banks) (Araneae: Linyphiidae), were collected from alfalfa fields as above. Following a starvation period and one-week nontransgenic diet in the laboratory (consisting of Collembola and *Drosophila melanogaster* Meigen), female spiders were lightly dusted with pollen (*c*. 20 pollen grains/predator) from transgenic corn (N79-L3, Bt-11 event) and screened for the presence of Cry1Ab endotoxins. Pollen was collected from the field by bagging tassels during anthesis and used immediately in cross-reactivity tests.

Sample preparation and screening protocol

Invertebrates (n = 642 from Bt-corn fields and 21 species (10 replicates/species) from a non-Bt field (alfalfa) for cross-reactivity tests) were screened using the EnviroLogix Quantiplate Kit (EnviroLogix Inc.) for the detection of Cry1Ab residues. This kit is based on a quantitative sandwich ELISA and will be referred to as an EnviroLogix Quantiplate ELISA. The protocol was designed to optimize the detection of Bt endotoxins whilst minimizing the potential for false positive reactivity. Therefore, extensive characterization against nontarget negative control samples

was performed prior to the screening of field-collected arthropods (see Results).

All arthropods (except Coccinellidae) were weighed, whole bodies homogenized and diluted × 500 (mg/μL) in EnviroLogix 1× Extraction/Dilution buffer. Two samples were analysed for all coccinellids. These predators were allowed to thaw at room temperature and the foreguts extracted by carefully teasing apart between the thorax and abdomen. This allowed the separation of foregut from the remaining body. Both the foregut and the body were weighed (separately), homogenized and diluted × 500 (mg/μL) in EnviroLogix Extraction buffer as mentioned earlier. The solution was mixed for 1 min on a vortex mixer and centrifuged at 5000 *g* for 5 min to remove solid particulate matter. The supernatant was transferred to a sterile 7 mL Sterilin Bijou container prior to screening without further dilution by EnviroLogix Quantiplate ELISA.

To each EnviroLogix Quantiplate ELISA plate, 100 μL of EnviroLogix Negative Control and three calibrators (containing 0.5 ng Cry1Ab per g fresh weight, 2.5 ng Cry1Ab per g fresh weight and 5.0 ng Cry1Ab per g fresh weight) were added to representative wells on the plate. To the remaining wells, 100 μL of each invertebrate sample was added, the contents carefully mixed within their wells by rotating in a rapid circular motion, all wells were covered with Parafilm and allowed to incubate at room temperature for 15 min. A further 100 μL of EnviroLogix Cry1Ab-Enzyme Conjugate (horseradish-peroxidase) was added to each well after incubation and thoroughly mixed as described above. The wells were covered with Parafilm and allowed to incubate at room temperature for 1 h. After this further incubation, the contents of all wells were ejected and the plates washed three times with 10 mM phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 (Sigma-Aldrich). One hundred microlitre of EnviroLogix Substrate was added to all wells, the contents mixed as above, all wells were covered with a Parafilm sheet and allowed to incubate for 30 min at room temperature. After this time period elapsed, 100 μL of 1.0 N hydrochloric acid stop solution was added to all wells and spectrophotometric measurement recorded at 450 nm using a Thermo Labsystems Multiskan Plus Spectrophotometer (Fisher Scientific L.L.C.).

Calculation of Cry1Ab endotoxin concentration

Absorbance readings of EnviroLogix Negative Controls were subtracted from all wells and a linear curve fitted to the calibrators (0.5 ng/g, 2.5 ng/g, 5.0 ng/g Cry1Ab endotoxin concentrations). The OD_{450} of invertebrates, if positive, was entered into the regression to extrapolate the concentration of Cry1Ab endotoxin, multiplied by the dilution factor (500) and divided by 1000 to convert values into μg/g concentration of Cry1Ab endotoxin present within each sample. Only positive samples were incorporated into this regression. Samples eliciting an absorbance less than the lowest calibrator (0.5 ng Cry1Ab per g fresh weight) were considered as containing no Cry1Ab endotoxin.

Results

Cross-reactivity screening

The screening of arthropods from non-Bt fields (alfalfa) for potential false-positive cross-reactivity with the assay at optimized concentrations (1 : 500 mg/μL) indicated no detectable quantities for any of the 21 species screened (Table 1) vs. the EnviroLogix Quantiplate ELISA for Cry1Ab

Table 1 Arthropods screened for false-positive cross-reactivity vs. the EnviroLogix Quantiplate ELISA for Cry1Ab endotoxins (n = 10 for each species/family). All samples contained no significant quantities of recognizable proteins above the lowest calibrator (0.5 ng Cry1Ab per g fresh weight) and were statistically similar to the OD_{450} for the EnviroLogix Negative Control on each plate. All samples were adults unless stated

Order	Species/family
Araneae	*Bathyphantes pallida* (Banks), *Erigone autumnalis* Emerton, *Meioneta unimaculata* (Banks), *Tetragnatha laboriosa* Hentz, *Pardosa distincta* (Blackwall)
Coleoptera	*Coleomegilla maculata** (De Geer), *Harmonia axyridis** (Pallas), *Cycloneda munda** (Say), *Diabrotica undecimpunctata howardi* Barber, *Popillia japonica* (Newman), *Hypera postica* Gyllenhal, *Photinus pyralis* (L.), *Chaetocnema pulicaria* Melsh.
Heteroptera	*Orius insidiosus* Say, *Nabis roseipennis* Reuter (nymph), *Sehirus cinctus* (Palisot)
Homoptera	*Empoasca fabae* (Harris), *Acyrthosiphum pisum* (Harris)
Diptera	Mycetophilidae
Neuroptera	*Chrysoperla carnea* Stephens
Mollusca	*Deroceras reticulatum* Müller
Contaminated Araneae†	*Bathyphantes pallida* (Banks)

*Bodies and foreguts of coccinellid specimens were screened separately
†Contaminated spiders were lightly dusted with pollen (*c*. 20 pollen grains/predator) from Bt corn to determine potential 'false-positive' reactivity from pollen-contaminated specimens.

Table 2 Presence of Cry1Ab endotoxins within nontarget insect herbivores collected from Bt corn fields. All samples screened were adults

Species [Order: Family]	*n*	% positive*	Mean concentration of Cry1Ab endotoxin in positive samples (μg/g)	Ratio† (sample/negative control)	*t*-value‡	*P*
Chaetocnema pulicaria Melsheimer [Coleoptera: Chrysomelidae]	71	87.3	2.43 ± 0.13	10.24 ± 0.64	20.37	< 0.001
Nodonota tristis Olivier [Coleoptera: Chrysomelidae]	51	2.0	2.09	1.20 ± 0.17	1.29	0.203
Diabrotica undecimpunctata howardi (Barber) [Coleoptera: Chrysomelidae]	11	21.3	0.78 ± 0.50	2.07 ± 0.63	2.30	0.044
Popillia japonica (Newman) [Coleoptera: Scarabeidae]	51	23.5	0.43 ± 0.08	1.92 ± 0.14	8.40	< 0.001
Sphenophorus callosus (Olivier) [Coleoptera: Curculionidae]	18	0	—	1.02 ± 0.06	0.01	0.994
Sphenophorus maidis Chittenden [Coleoptera: Curculionidae]	11	0	—	0.98 ± 0.01	2.09	0.063
Scaphoideus sp. [Hemiptera: Cicadellidae]	46	2.2	2.59	0.96 ± 0.01	0.18	0.859

*Positive is defined as invertebrates which screen more positive than the lowest calibrator on each ELISA plate (0.5 ng Cry1Ab per g fresh weight). Above this threshold indicates the presence of Cry1Ab endotoxin; † A ratio > 1 indicates OD_{450} of predators was higher than the OD_{450} of the EnviroLogix negative control; ‡ Statistics presented for the comparison of OD_{450} values for samples vs. OD_{450} of EnviroLogix negative controls.

Table 3 Field-collected arthropods (adults) from Bt-corn fields screened vs. the EnviroLogix Quantiplate ELISA for Cry1Ab-endotoxin. All replicates did not elicit a positive reaction above the EnviroLogix negative control threshold. Numbers in parentheses indicate number of individuals tested

Order	Species/family
Diptera	Mycetophilidae (12), Agromyzidae (12), Muscidae (11), Phoridae (7), Cecidomyiidae (7), Syrphidae (6), Chironomidae (3), Lonchopteridae (2), Drosophilidae (1), Tachinidae (1), Sphaeroceridae (1)
Hymenoptera	Ichneumonidae (6), Chalcidae (2), Braconidae (2), Proctotrupidae (1), *Apis mellifera* L. (1)
Coleoptera	*Gastroidea polygoni* L. (6), *Carpophilus lugubris* (Murray) (3), *Stenolophus lecontei* Chaudoir (3), *Mordella marginata* Melsheimer (2), *Leptinotarsa decimlineata* (Say) (2), *Chauliognathus pennsylvanicus* (De Geer) (1), *Carpophilus dimidiatus* (F) (1), *Clivinia impressifrons* Le Conte (1), *Oulema melanopus* L. (1), Aleocharinae (1), Cantharidae (1), Chrysomelidae (1)
Homoptera	*Empoasca fabae* (Harris) (2), Cercopidae (2)
Orthoptera	*Melanoplus femurrubrum* (De Geer) (2)
Heteroptera	*Sehirus cinctus* (Palisot) (7), Cydnidae (1)
Collembola	Entomobryidae (1)
Lepidoptera	Unidentified adult Lepidoptera (4)
Dictyoptera	Mantidae (1)

endotoxin. An ELISA was also performed on laboratory food (Collembola, *Drosophila melanogaster*, alfalfa plants (leaves, stems, roots), non-Bt corn isoline plants (leaves, stems, roots) and *Acyrthosiphon pisum* (Harris) (to ensure no cross-reactivity with food items). All results were nonsignificant, with absorbance levels indicating no significant quantities Bt-endotoxins in the food.

In addition to individual species tested for cross-reactivity, light surface-level dusting of female *Bathyphantes pallida* with pollen from Bt-corn fields elicited no reactivity when screened against the assay.

Cry1Ab endotoxin in nontarget herbivores

Examination of nontarget herbivores collected from transgenic corn fields indicated detectable quantities of Cry1Ab proteins in five species (Table 2), although arthropods from nine orders screened negative (Table 3).

The majority (87.3%) of corn flea beetles, *Chaetocnema pulicaria* Melsheimer, screened positive for Cry1Ab proteins. The average recorded concentration of Bt endotoxin within *C. pulicaria* was 2.43 ± 0.13 μg Cry1Ab per g fresh weight (n = 71). Almost 25% of Japanese beetles, *Popillia japonica*

Table 4 Presence of Cry1Ab endotoxins within nontarget arthropod predators collected from Bt-corn fields. All samples were adults unless stated

Species [Order: Family]	*n*	% positive*	Mean concentration of Cry1Ab endotoxin in positive samples (μg/g)	Ratio† (sample/ negative control)	*t*-value‡	*P*
Araneae§	91	7.7	0.48 ± 0.09	1.23 ± 0.08	3.13	0.002
Coleomegilla maculata (De Geer) [Coleoptera: Coccinellidae]	69	5.8	0.88 ± 0.20	1.33 ± 0.12	9.59	< 0.001
Harmonia axyridis (Pallas) [Coleoptera: Coccinellidae]	25	4.0	0.66	1.12 ± 0.12	1.02	0.317
Cycloneda munda (Say) [Coleoptera: Coccinellidae]	6	33.3	0.42 ± 0.14	1.75 ± 0.48	1.56	0.179
Coccinella septempunctata L. [Coleoptera: Coccinellidae]	2	50.0	0.51	2.17 ± 1.15	–	–
Coccinella septempunctata larvae [Coleoptera: Coccinellidae]	1	0	—	1.11	—	—
Coleomegilla maculata larvae [Coleoptera: Coccinellidae]	2	0	—	1.16 ± 0.29	—	—
Photinus pyralis (L.) [Coleoptera: Lampyridae]	33	0	—	0.95 ± 0.01	5.25	< 0.001¶
Nabis roseipennis Reuter (nymph) [Heteroptera: Nabidae]	30	76.7	1.85 ± 0.21	7.26 ± 0.89	9.59	< 0.001
Orius insidiosus (Say) [Heteroptera: Anthocoridae]	2	100.0	2.53 ± 1.54	12.35 ± 6.49	—	—
Chrysoperla carnea (Stephens) [Neuroptera: Chrysopidae]	2	0	—	1.28 ± 0.32	—	—

*Positive is defined as invertebrates which screen more positive than the lowest calibrator (0.5 ng Cry1Ab per g fresh weight). Above this threshold indicates the presence of Cry1Ab-endotoxin.
†A ratio > 1 indicates OD_{450} of predators was higher than the OD_{450} of the EnviroLogix negative control.
‡Statistics presented for the comparison of OD_{450} values for samples vs. OD_{450} of EnviroLogix negative controls.
§Spiders consisted of 17 different species belonging to the Linyphiidae (n = 54), Tetragnathidae (n = 25), Lycosidae (n = 3), Theridiidae (n = 3) and Thomisidae (n = 6).
¶OD_{450} of *Photinus pyralis* was significantly lower than the EnviroLogix negative control, therefore the significant difference does not indicate the presence of Cry1Ab endotoxins.

(Newman), also contained concentrations of Cry1Ab endotoxin above the 0.5 ng Cry1Ab per g fresh weight calibrator, but the concentration was significantly lower than within corn flea beetles which screened positive (Mann–Whitney U = 2689.5, P < 0.001). The only other nontarget herbivore which contained a large proportion of individuals with Bt endotoxins above this threshold was the southern corn rootworm, *Diabrotica undecimpunctata howardi* (Barber) (Table 2). Despite large numbers of *Nodonota tristis* Olivier (n = 51) and *Scaphoideus* sp. (n = 46) being collected from transgenic corn, only a single representative from each of these groups tested positive for Cry1Ab endotoxins (*N. tristis* = 2.09 μg Cry1Ab per g fresh weight; *Scaphoideus* = 2.59 μg Cry1Ab per g fresh weight), and mean Cry1Ab concentrations in all samples were not significantly different to EnviroLogix negative controls (Table 2).

Cry1Ab endotoxin in natural enemies

The screening of predators from the field indicated that natural enemies from three orders (Araneae, Coleoptera and Heteroptera) contained Cry1Ab endotoxins above the threshold of 0.5 ng Cry1Ab per g fresh weight (Table 4). Out of 91 spiders collected from Bt corn, 7.7% contained concentrations of Cry1Ab endotoxins above this threshold. The spiders collected from Bt corn fields were dominated by the Linyphiidae [*Bathyphantes pallida* (Banks), *Meioneta unimaculata* (Banks), *Erigone autumnalis* Emerton, *Tennesseellum formicum* (Emerton), *Grammonota inornata* Emerton and *Linyphia pusilla* Sundevall] but also contained representatives from the Tetragnathidae [*Tetragnatha versicolor* Walckenaer, *Tetragnatha labouriosa* Hentz, *Mimognatha foxi* (McCook) and *Pachygnatha tristriata* C.L. Koch], Theridiidae

[*Euryopis funebris* (Hentz)], Thomisidae [*Xysticus discursans* Keyserling and *Misumena calycina* (L.)], and Lycosidae [*Pardosa milvina* (Hentz) and *Pardosa distincta* (Blackwall)].

The majority of *Nabis roseipennis* Reuter contained Cry1Ab endotoxins (Table 4). The mean concentration of these proteins in the 76.7% testing positive (1.85 ± 0.21 μg Cry1Ab per g fresh weight) was significantly higher than in spiders ($U = 421.0$, $P = 0.002$) but lower than in corn flea beetles ($U = 783.0$, $P = 0.042$). Although very few individuals were sampled ($n = 2$), the only other heteropteran collected from transgenic corn fields, *Orius insidiosus* (Say), tested positive on both occasions for Cry1Ab endotoxins.

Large numbers of predatory coccinellids were also collected, with 5.8% of adult *Coleomegilla maculata* (De Geer) foreguts containing a protein concentration eliciting an absorbance above the 0.5 ng Cry1Ab per g fresh weight calibrator. The mean OD_{450} of these predators was significantly higher than EnviroLogix negative controls (Table 4). In addition to *C. maculata*, data were gathered to suggest the uptake of Cry1Ab endotoxins in other coccinellids [*Harmonia axyridis* (Pallas), *Cycloneda munda* (Say) and *Coccinella septempunctata* (L.)] (Table 4). Only foreguts of these coccinellids screened positive; the bodies of all species did not contain any recognizable quantities of Cry1Ab endotoxins when assayed vs. the EnviroLogix Quantiplate ELISA.

Discussion

Quantifying the concentration of Cry1Ab endotoxins present in nontarget arthropods in the field is essential for determining natural exposure levels within complex food webs. These experiments present the first field data measuring the concentration of Bt endotoxin in nontarget herbivores and higher order arthropod predators. Our hypothesis that detectable quantities of toxins would be found in nontarget species in the field was therefore supported.

There was clear evidence that feeding upon Bt corn by nontarget herbivores occurred in the field and Cry1Ab-endotoxins flowed from plant material into the first trophic level of the nontarget food web. The incidence of these proteins within plant-feeding arthropods could have significant implications for their predators. If feeding events upon Cry1Ab-endotoxin-containing prey occur over extended periods of time, significant reductions in fitness could result, given that continued exposure to transgenic material induces weight loss in some organisms even though short-term exposure does not (Zwahlen *et al*. 2003a). To date, evidence from laboratory trials tends to suggest predator communities are rarely affected by exposure to transgenic material (e.g. Pilcher *et al*. 1997; Armer *et al*. 2000; Al-Deeb *et al*. 2001; Lundgren *et al*. 2004), although further research is required to identify those trophic connections most likely to facilitate the transfer of endotoxins along the food chain.

Predators from three orders (Araneae, Coleoptera and Heteroptera) contained concentrations of Cry1Ab endotoxins above the lowest calibrator (Table 4). Spiders are particularly important natural enemies of many herbivorous pests of agroecosystems throughout the world (Sunderland 1999; Nyffeler & Sunderland 2003), often impacting pest population dynamics by feeding early in the season before their populations increase exponentially (Harwood *et al*. 2004). In total, 7.7% of spiders (dominated by the Linyphiidae) contained significant quantities of Cry1Ab endotoxins. Given the sensitivity of these predators to insecticidal toxins (e.g. Dinter & Poehling 1995; Pekar 2002) and their value in biological control (Sunderland 1999; Nyffeler & Sunderland 2003), exposure to transgenic endotoxins could, in the long term, affect their fecundity and role in integrated pest management (if exposure levels are sufficiently high to reduce reproductive output). Clearly, further research is required to reveal the magnitude and consequence of toxins flowing through the food chain, either by feeding on Bt pollen (Smith & Mommsen 1984; Vogelei & Greissl 1989), which may be of low quality to spiders (Sunderland *et al*. 1996), or the consumption of more nutritious prey which contain Bt endotoxins.

The levels of Cry1Ab endotoxins recorded in heteropteran predators supported the hypothesis that a significant flow of endotoxins would occur to these natural enemies due to plant feeding. The nymphal stages of *Nabis roseipennis* feed upon plant material (Alomar & Wiedenmann 1996), thus taking up toxins directly from Bt corn, much like herbivorous pests. Adult nabids are an important component of the natural enemy assemblage impacting pest populations in the field (Sunderland *et al*. 1997; Cardinale *et al*. 2003; Snyder & Ives 2003), but plant feeding is an important resource during their development. The high incidence of Cry1Ab endotoxins within these nymphs and continued exposure should therefore be considered during the risk assessment of transgenic crops, although no effect was reported for nabid exposure to Bt toxins in cotton (Ponsard *et al*. 2002). It is also likely that nabids were, in part, responsible for the flow of Cry1Ab endotoxins to predator communities as these nymphs are readily consumed by some spiders (Howell & Pienkowski 1971), which are themselves eaten by many other predators (Sunderland *et al*. 1997; Harwood *et al*. 2001). Similar to nabid nymphs, *Orius insidiosus* is an important predator of many pests (Corey *et al*. 1998; Sansone & Smith 2001), but adults supplement their diet by feeding on plant material. Very few *Orius* were collected ($n = 2$), but both contained very high quantities of Cry1Ab endotoxins (Table 4). Despite high levels of transgenic endotoxins in *O. insidiosus* in the field, previous laboratory research investigating the impact of Bt crops on *Orius* sp. (e.g. Pilcher *et al*. 1997; Armer *et al*. 2000; Zwahlen *et al*. 2000; Al-Deeb *et al*. 2001), indicated little or no effect of transgenic crops to fitness parameters.

Significant numbers of coccinellids also contained detectable quantities of Cry1Ab endotoxins in their foreguts. These important generalist predators are known to feed on pollen (Lundgren *et al*. 2004, 2005) and the uptake of endotoxins could be through plant feeding and/or the consumption of herbivores which contained Cry1Ab endotoxins. The exposure levels documented here may not be sufficient to reduce fitness, but further research is required (particularly with species sensitive to insecticidal toxins) to reveal the consequence of extended exposure to Cry1Ab (and other) endotoxins within their guts and possible routes of transfer of endotoxins into coccinellid foreguts. Long-term evaluation of nontarget populations is important given that some species experience significant weight loss after extended exposure to transgenic material (Zwahlen *et al*. 2003a) even though short-term exposure of the same organisms tends to indicate limited fitness consequences (Saxena & Stotzky 2001). If persistent feeding on food containing Bt endotoxin is evident in the field, further risk assessments are required, targeted towards species whose exposure is a secondary consequence. Scavenging can also lead to the transfer of detectable proteins (Calder *et al*. 2005), thus predators which feed on dead or decaying prey (which themselves may contain Bt endotoxins) may be at an increased risk of negative transgenic effects.

This research has provided the first clear evidence for the uptake of Cry1Ab endotoxins from transgenic corn by nontarget herbivores and several arthropod predators in the field. It is unclear if these endotoxins are flowing to primary, secondary or tertiary predators via the herbivore and/or detritivore food chain or whether their presence is a result of direct consumption of plant material. However, the significance and ecological consequences for such toxin transfer needs further investigation during the development and risk assessment of existing and future transgenic varieties. Particular focus is required into investigating the consequence of long-term uptake of insecticidal endotoxins by nontarget organisms in the field.

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