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Exposure of arthropod predators to Cry1Ab toxin in Bt maize fields

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Abstract. 1. To assess the risks of an insect-resistant transgenic plant for non-target arthropods, it is important to investigate the exposure of non-target species to the transgene product. Exposure of predators in the field depends on the toxin levels in food sources, their feeding ecology and that of their prey.

2. To verify the transmission of Cry1Ab toxin through the food chain, and thus exposure of predators in the field, samples from different plant tissues, herbivores, and predators in Bt maize fields in Spain (Event 176) were collected at different periods over the season and the toxin content was measured using ELISA. Complementary laboratory studies were performed with the omnivorous predator *Orius majusculus* to assess the toxin uptake and persistence after feeding on variable Bt-containing food sources.

3. Field results revealed that toxin content in some herbivores was negligible (aphids, thrips, leafhoppers) compared with those in spider mites. The latter herbivore only occurred after pollen shed and contained three times greater toxin levels than Bt maize leaves.

4. Data confirmed that the Bt toxin can be transferred to predators, that is to say to *Orius* spp., *Chrysoperla* spp., and *Stethorus* sp. This only applied when Bt maize pollen or spider mites were available. The passage of Bt toxin to *O. majusculus* via these two food sources was also confirmed in the laboratory. Contrastingly, some predators in the field (hemerobiids, *Nabis* sp., *Hippodamia* sp., *Demetrias* sp.) contained no or negligible toxin levels even when pollen or spider mites were present.

5. Besides essential information for exposure assessment of numerous arthropod predators, this study provides an insight into the feeding ecology of different arthropods in the maize system.

Key words. ELISA, food chain, insect-resistant transgenic plants, non-target arthropods, *Orius majusculus*, risk assessment.

Introduction

To assess potential risks that insect-resistant transgenic plants pose to non-target arthropods, several factors have to be taken into account. Besides the investigation of the toxicity of an insecticidal protein expressed by a transgenic plant, it is important to assess the extent to which non-target organisms are exposed to the toxin (U.S. EPA, 1998; Cowgill & Atkinson, 2003; Dutton *et al*., 2003). Exposure of predators to the insecticidal protein may be indirect when preying on herbivores containing the toxin, or direct when feeding on plant parts containing the toxin (Groot & Dicke, 2002). The latter route of exposure applies for omnivorous predators (e.g. many heteropteran species) as they can feed on pollen and/or other plant tissue (Alomar & Wiedenmann, 1996) as well as on arthropod herbivores. To assess the exposure of arthropods to an insecticidal protein,

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the level of toxin contained in their food source has to be determined (Dutton *et al*., 2002, 2003). Laboratory studies with herbivores feeding on Cry1Ab expressing Bt maize revealed large differences in the quantity of ingested toxin among species (Head *et al*., 2001; Raps *et al*., 2001; Obrist *et al*., 2005). Using enzyme-linked immuno-sorbent assays (ELISA), Dutton *et al*. (2002) measured Cry1Ab toxin concentrations in *Tetranychus urticae* Koch (Acarina: Tetranychidae) that were similar to those found in Bt maize leaves. Lower levels were found in *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) larvae and no or only trace amounts of toxin were detected in aphids [*Rhopalosiphum padi* (L.); Homoptera: Aphididae] reared on Bt maize. Accordingly, toxin levels measured in first-instar *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) were shown to correlate with the amount of toxin in the Bt maize-fed herbivores they fed upon (Obrist *et al*., 2006).

To date, many field studies have been performed with Bt maize, which primarily assessed its impact on the abundance of numerous arthropod species (for review see Dutton *et al*., 2003; O'Callaghan *et al*., 2005, Romeis *et al*., 2006). However, little is known about the extent to which beneficial arthropods are effectively exposed to Bt toxin in the field. Exposure under field conditions is influenced by numerous factors such as variable expression rates in different plant parts over the growing season and the feeding ecology, behaviour, and mobility of both herbivores and predators. Moreover, various environmental factors such as wind or rain may influence exposure in the field and complicate a quantitative estimation based on laboratory studies.

In Europe, transgenic Bt maize expressing Cry1Ab toxin is predominantly grown in Spain where it has been commercialised since 1998. Increasing areas are covered with Bt maize, which is resistant to the Mediterranean corn borer [*Sesamia nonagrioides* (Lefèbvre); Lepidoptera: Noctuidae] and the European corn borer [*Ostrinia nubilalis* (Hübner); Lepidoptera: Crambidae], two severe pest species in Mediterranean countries. Until 2002, Compa (CB® Event 176 Syngenta, Basle, Switzerland) was the only Bt maize variety that was commercially grown in Spain (Brookes, 2002). This transgenic cultivar is known to express significant levels of Cry1Ab toxin in leaves and pollen, but not in roots, pith, or kernels (Koziel *et al*., 1993; Fearing *et al*., 1997).

A number of arthropod predator species are regularly present in Spanish maize fields (Iraola *et al*., 1997; Asin & Pons, 1999; Albajes *et al*., 2003; de la Poza *et al*., 2005) among which *Orius majusculus* (Reuter) (Heteroptera: Anthocoridae) is one of the dominant species. *Orius* spp. are known to be omnivorous and generalist predators that feed on various arthropods such as thrips, spider mites, leafhoppers, aphids, and lepidopteran eggs or young larvae (McMurtry *et al*., 1970; Coll & Bottrell, 1991; Corey *et al*., 1998). Pollen can be an important alternative food source for them in maize fields (Dicke & Jarvis, 1962; Corey *et al*., 1998; Musser & Shelton, 2003a) and some *Orius* spp. can develop exclusively on pollen (Pilcher *et al*., 1997; Vacante *et al*., 1997). The role of green plant tissue feeding for *Orius* spp. is not entirely understood but it is assumed that it is at least used for water provisioning (Armer *et al*., 1998). The impact of Bt maize on *Orius* spp. was investigated in several studies, all of which detected no detrimental effects on this predator group (Pilcher *et al*., 1997; Zwahlen *et al*., 2000; Bourguet *et al*., 2002; Jasinski *et al*., 2003; Musser & Shelton, 2003b; Pons *et al*., 2004; de la Poza *et al*., 2005). Due to their complex feeding ecology, the degree of their exposure to Bt toxin in the field remains unclear. *Orius* spp. and other predatory species usually found in maize are also recorded in other crops, like alfalfa, that is often grown in rotation with maize in irrigated Mediterranean areas. Toxin acquired by arthropods in Bt maize may thus be transferred to alfalfa in neighbouring fields.

The present study was conducted to assess the exposure of relevant arthropod predators to Cry1Ab toxin in a transgenic maize ecosystem in Spain by considering the pathways through which the toxin may reach predators. In addition, preliminary studies were conducted to evaluate the possibility of toxin transfer to neighbouring fields by predator dispersal. Samples of three successive trophic levels (maize plants, arthropod herbivores, and predators) were collected in Bt maize fields at different periods in the growing season and Cry1Ab toxin content was determined using ELISA. The toxin content in predators collected from adjacent alfalfa fields was also determined. Complementary laboratory studies were performed with the omnivorous predator *O. majusculus* to verify the toxin uptake when fed with different Bt-containing food sources and to assess the persistence of the toxin in adults.

Materials and methods

Laboratory experiments

Plants. Commercial cultivars of transgenic Bt maize (Zea mays L.) (Event 176, Compa CB®, Syngenta) (designated as Bt^+) expressing a gene encoding a truncated version of the Cry1Ab protein derived from Bacillus thuringiensis Berliner ssp. kurstaki HD-1 and the corresponding non-transformed near-isogenic variety (Dracma®, Syngenta) (Bt^-) as a control were used for experiments. In this transgenic maize variety the Cry1Ab expression is driven by the constitutive PEPC promoter as well as a pollen-specific promoter. Plants used for experiments were grown individually in plastic pots (3 litre volume) in the greenhouse at 24 ± 4 °C, 70 ± 10% RH and used when at vegetative stages V10 to V12 (6–9 weeks) according to Ritchie et al. (1993). All plants were fertilised (10% N : 10% P_2O_5 : 8% K_2O) every 2 weeks with 0.5 litre of a 0.2% aqueous solution. Additional fertiliser (15–20 g of 27.5% N) was added after 3–4 weeks. Plants used for pollen collection were grown in plastic pots (10 litre volume) under the same environmental conditions. Pollen was collected by placing individual

inflorescences into air-permeable cellophane bags (20.5 × 40 cm) for 3–4 days upon emergence. Pollen was subsequently sieved and dried for 24 h at ambient conditions before it was transferred into Eppendorf tubes and stored at −24 °C until use.

Arthropods. Spider mites (T. urticae, originating from a permanent rearing at the Agroscope FAL Reckenholz) were kept in separate colonies on either Bt^- or Bt^+ maize plants (older than 2 months) in the greenhouse (24 ± 4 °C, 70 ± 10% RH). Spider mites were collected by shaking infested leaves over a tray.

Colonies of O. majusculus (provided by Andermatt Biocontrol, Grossdietwil, Switzerland) were kept in a climatic chamber (25 ± 1 °C, 75 ± 10% RH, L:D 16:8 h), fed with Ephestia kuehniella Zeller (Lepidoptera: Pyralidae) eggs and provided with water. Potato tubers (Solanum tuberosum L.) and green bean pods (Phaseolus vulgaris L.) were supplied as a substrate for oviposition. For experiments, either fifth instar or adult O. majusculus were used; old fourth or fifth instars were separated from the colony, and kept individually in Plexiglas cages (2.7 × 10 mm, diameter × height) each containing 10 µl of water. Cages were kept in a climatic chamber (see above) for a total of 48 h. After the first 24 h, water in the cages was removed and cages were kept in the climatic chamber for another 24 h. Only individuals that had moulted during the 2 days of food deprivation (and thus have become fifth-instar nymphs or adults respectively) were used for experiments.

ELISA analyses. To determine the levels of Cry1Ab protein, ELISA was performed using kits from EnviroLogix Inc. (Portland, Maine). Cry1Ab standards at concentrations 0, 0.5, 2.5, and 5 p.p.b. were used as calibrators. Spectrophotometric measurements were conducted with a microtitre plate reader (Tecan, Spectrafluor PLUS for samples from the laboratory and Titertek Multiskan® PLUS MKII for samples from the field) at 450 nm. The toxin was defined as not detectable if the measured optical density value was lower than the intercept of the Y-axis of the standard regression line. The quantification limit was at approximately 0.03 µg g^{-1} sample material (dry weight) when analysed at the maximal concentration of 15 mg ml^{-1} (sample material per millilitres extraction buffer). Sample extracts were diluted for analyses if necessary when too high toxin levels led to measurements beyond the quantifiable range.

Nymphs and adults of Orius majusculus feeding on different food sources containing Bt toxin. Experiments were conducted to assess Bt toxin content in different stages (adults or nymphs) of O. majusculus when provided with different food sources containing Cry1Ab toxin (spider mites, pollen, and leaf) for different periods of time.

Spider mites (T. urticae) collected from the Bt^+ maize colony were distributed into cages (2.7 × 1 cm, diameter × height). Thirty to 40 O. majusculus adults and 30–40 nymphs were transferred into individual cages each containing a minimum of 15 spider mites (adults or deutonymphs). After feeding for 3 h, the predators were transferred into Eppendorf tubes and frozen at −24 °C.

Pollen (approximately 5–10 mg) was placed into 30–40 cages (2.7 × 1 cm, diameter × height) and 6 µl of water was added. The cages were closed and incubated for at least 1 h in the climatic chamber in order to swell the pollen with water and make it accessible for O. majusculus. Before placing the insects individually into the cages, all water was removed. Two additional treatments were made with pollen, both lasting for 24 h. In one of the two treatments, 30–40 individuals were transferred into cages containing pollen as described above but provided with water (6 µl). In the other treatment, 30–40 individuals were transferred into a cellophane bag (20 × 40 cm) which was fixed on a pollen shedding tassel of a Bt^+ maize plant.

Maize leaves (sixth or seventh leaf) abscised from Bt^+ maize plants were used as a substrate for 90–120 O. majusculus nymphs. These were kept for 3 h in clip cages (1 × 1 cm, diameter × height) fixed on the leaves. One third of the nymphs was additionally supplied with water-soaked cotton balls (0.3 cm diameter), another third with E. kuehniella eggs ad libitum and the last third without additional food or water.

For each Bt^+ treatment described above, a minimum of four samples (each consisting of 30–40 individuals) was collected for analyses with ELISA. Furthermore, a sample of each food source (Bt^+ maize-fed spider mites, Bt^+ maize pollen, and Bt^+ maize) was taken together with each predator sample to analyse the toxin concentration with ELISA. In addition, a control, which consisted of two samples of O. majusculus nymphs and adults fed with the corresponding Bt^- food source, was performed. To verify if toxin uptake is related to food ingestion, the weight gain of O. majusculus was determined by measuring the weights of at least 40 individuals (a minimum of 10 from each sample) per treatment (except that in which O. majusculus were kept together on tassels) before and after feeding using a microbalance (Mettler Toledo, MX5 Greifensee, Switzerland, division = 1 µg; tolerance = 2 µg). As the food uptake in some treatments was very restricted and weight gain sometimes even negative, the weight loss within 3 h of a food- and water-deprived control group was measured.

Persistence of Bt toxin in adult *Orius majusculus*. Food-deprived adults (150–200 individuals) were allowed to feed on T. urticae originating from the Bt^+ colony for 3 h as described above. Thereafter, 30 randomly chosen individuals were frozen at −24 °C whereas the rest was distributed in five boxes (13 × 10.5 × 5 cm) containing water and leaves infested with spider mites originating from the Bt^- colony. After 24 h, one third of the O. majusculus was collected and new spider mites were supplied to the remaining individuals. The same was repeated after 48 h and the remaining individuals were collected after 96 h. This experiment was repeated three times in order to have three ELISA replicates.

Field experiments

Commercial fields of Bt^+ and Bt^- maize (Event 176) belonging to a farm located 35 km to the West of Lleida

(Catalunya, Spain) were used to take samples of plant material and arthropods during the growing season 2004. The coordinates of the fields were estimated using global positioning system (eMap, Garmin, Olathe, Kansas). Georeferenced Universal Mercator are utm-31n, $X = 294\,015$–$294\,715$; $Y = 4\,628\,243$–$4\,628\,985$. The crop was managed according to common growing practices in the region but without insecticide application. Field size was variable, ranging from 0.4 to 0.7 ha. Samples were collected in three Bt^+ and one Bt^- maize (control) fields. The distance between the experimental Bt^+ fields was at least 200 m with wheat or other maize fields in between. The Bt^- field was next to one of the Bt^+ fields, but as fields were arranged in terraces, this field was at least 1.5 m higher than the bordering Bt^+ field.

An alfalfa field located next to one of the Bt^+ fields was used to collect additional predator samples. The two fields were separated by a weedy strip of approximately 1 m width. The alfalfa had not been treated with pesticides for at least 1 year.

Toxin content in Bt maize. Samples were collected at three developmental stages: (I) before pollen shed (end of June to beginning of July); (II) during pollen shed (mid to end of July) and (III) after pollen shed (mid to end of August).

In period I, samples were taken from the sixth (during vegetative stages V7–V10 according to Ritchie *et al*., 1993), 11th (during V14–V15), and 19th leaf (during tasselling before pollen was shed). Leaf samples were excised from the distal third of the leaves. Samples of developing, unfolded leaves from the whorl, leaf sheaths (from the 11th leaf) and stem (from the lowest fourth of the plant) were taken during vegetative stages V14–V15. During pollen shed (period II), samples of pollen and the stem within the tassels (designated as tassel axis) were taken. After pollen shed (period III), additional leaf samples were collected from the 15th and 19th leaf and from silk. One sample of each plant part was taken in each of the three Bt^+ fields. A plant sample consisted of a pool of material from four plants chosen randomly. Three samples of pollen of mixed age were also collected from leaves and axils after the period of pollen shed. All samples were collected in Eppendorf tubes and put directly on ice in the field. In the laboratory, the samples were immediately frozen at −24 °C. After freezing, appropriate quantities of each sample (5–60 mg fresh weight; depending on the water content and toxin concentration) were taken and weighed on a semimicrobalance (HA-202M, A & D Company Ltd, Oxford, U.K., division = 10 μg ± 20 μg). Subsequently, the samples were lyophilised, and re-weighed to determine their fresh/dry weight ratios. The dried samples were stored at −24 °C until ELISA was performed.

Toxin content in arthropods from Bt maize. During three periods (I–III) two samples of different arthropod groups were collected in each of the three Bt^+ maize fields (Table 1). The two samples per field were taken from different areas both located in the centre of the field. The size of these areas (i.e. the number of plants that needed to be inspected to collect sufficient arthropods) depended on the arthropod density.

Thrips were sampled during the first and second period. In the first period (I), maize plants were cut and dissected on site and all stages of *Frankliniella* spp. (Thysanoptera: Thripidae) were collected together in a vial (4.7 × 4 cm, diameter × height). A rough estimation, which was done by dissecting 20 plants and counting all visible thrips, showed that pre-imaginal stages were approximately twice as abundant as adults. The predominant thrips species was *Frankliniella tenuicornis* (Uzel). Samplings during the second period were made by shaking tassels (inflorescences) into a white plastic tray, from where thrips were immediately collected. The predominant species in inflorescences was identified as *Frankliniella occidentalis* (Pergande).

Spider mites (exclusively *T. urticae*) were only found after pollen shed. For collections, infested leaves were cut and transferred into a plastic bag, which was kept in a cooling box for the transport to the laboratory. Under binocular microscope, all stages of spider mites were collected directly from the underside of the leaves using a camel-hair brush.

All other arthropod groups were collected using a sweep net and/or an aspirator. The collected arthropods were immediately put on ice in order to reduce the metabolism and the excretion of the toxin. Once in the laboratory, arthropods were frozen at −24 °C. Later, samples were sorted out, and, when needed, pollen and other impurities were removed under the binocular microscope. Further processing of the samples was identical to that of plant samples. The initial amount of sample material that was collected in the field ranged from 10 to 60 mg fresh weight. If individuals were big (e.g. adult chrysopids, chrysomelids, nabids), a minimum of four individuals per sample was collected. In Table 1 concentrations at which samples were extracted and dilutions made for the ELISA are provided.

Estimation of prey abundance. During each sampling period (I–III), the abundance of herbivores was estimated by checking a minimum of 25 randomly chosen plants per plot. The abundance of herbivores was estimated according to the following abundance class system: 0 = absent (no individual detected); 1 = present (at least one individual); 2 = few (aphids: one or two small colonies; spider mites: one or two colonies covering restricted leaf areas); 3 = medium (aphids: at least three colonies on different sites of the plant; spider mites: major part of at least two leaves infested); 4 = high (aphids: four or more colonies; spider mites: at least four entire leaves infested and some visible damage); 5 = very high (aphids: heavy infestation covering large areas of the plant; spider mites: at least six leaves infested and clearly visible damage). The assessment of leafhoppers (cicadellids) was carried out in a similar way but was mainly based on the amount of damage (decoloured strips) on the leaves, as this insect is very mobile and moves away when approaching a plant. Thrips were counted individually during the first sampling period by dissecting the whole plants. Thrips were also

Table 1. Arthropods collected before (I), during (II), and after (III) pollen shed in Bt maize fields and analysed using ELISA. Concentrations at which samples were extracted (milligrams of lyophilised sample material per millilitre extraction buffer) are provided together with the dilutions (in parentheses) made for ELISA measurements.

Arthropods	Genus or species	Stage[a]	I	II	III
Heteroptera					
Anthocoridae	*Orius* spp.	N	15	15 (4×)	15 (4×)
		A	15	15 (4×)	15 (4×)
Nabidae	*Nabis provencalis* Remane	N	–	–	15
		A	15	15	15
Miridae	*Trigonotylus* spp.	Mix	15[b] (16×)	15 (16×)	–
Homoptera					
Cicadellidae	*Zyginidia* spp.	Mix	15	–	–
Aphididae	*Metopolophium dirhodum* (Walker)	Mix	15	–	–
Thysanoptera					
Thripidae	*Frankliniella* spp.	Mix	15	15 (4×)	–
Neuroptera					
Chrysopidae	*Chrysoperla* spp.	L	15	15	15 (4×)
		A	–	15 (4×)	15
Hemerobiidae	Not identified	A	15	15	–
Coleoptera					
Carabidae	*Demetrias atricapillus* L.	A	15	15	15
Staphylinidae	*Tachyporus* sp.	A	15	–	–
Coccinellidae	*Hippodamia variegata* (Goeze)	A	–	15	–
	Scymnus spp.	A	–	15	15
	Stethorus punctillum Weise	L	–	–	4 (4×)
		A	–	–	8 (16×)
Chrysomelidae	*Oulema melanopus* (L.)	A	15 (25×)	15	15
Acarina					
Tetranychidae	*Tetranychus urticae* Koch	Mix	–	–	2 (16×)

[a]A, adults; L, larvae; N, nymphs; Mix, mixture of all available stages.
[b]Samples were only taken from two fields.

observed to be present during the following sampling periods (II + III) but their abundance was not estimated.

Toxin content in predators collected in alfalfa field adjacent to Bt maize. During pollen shed, samples of selected adult predators were taken from the alfalfa field to verify if the toxin is carried out of the Bt maize field by predators. Three samples of adult *Orius* spp., *Nabis provencalis*, *Chrysoperla* spp., and *Hippodamia variegata* were taken at two distances (1–3 m and 4–6 m from the Bt^+ field) from different spots by means of a sweep net.

Statistical analyses

All statistical analyses were computed using STATISTICA (Version 6, Statsoft Inc., Tulsa, Oklahoma).

Laboratory experiments. One-way and/or main effects ANOVA were performed to determine the influence of Treatment and Stage. The data for weight changes of *O. majusculus* were transformed by $\log_e(x + 100)$, the data for toxin uptake by $\log_e(x + 1)$ prior to analyses.

Field experiments. The average toxin concentration from two samples per plot was used to calculate the mean concentration in samples of the three Bt^+ plots. One-way ANOVA was performed to determine the influence of a single categorical factor (Sampling period) and main effects ANOVA of two categorical factors (Sampling period and Stage). Data sets were transformed by $\log_e(x + 1)$ prior to analyses.

Results

Laboratory experiments

Toxin content and persistence in *Orius majusculus*. Weight change of O. majusculus depended on the food sources they were offered (one-way ANOVA, nymphs: $F_{5,269} = 38.0$, $P < 0.0001$; adults: $F_{2,176} = 64.0$, $P < 0.0001$) (Fig. 1). When offered pollen or spider mites, nymphs of O. majusculus gained significantly more weight than adults (mean ± SE, nymphs: 57.4 ± 3.8 μg, adults: 25.4 ± 3.5 μg; main effects ANOVA, $F_{1,330} = 56.1$; $P < 0.0001$). The toxin concentrations measured in nymphs and adults were also significantly different depending on the food sources (one-way ANOVA, nymphs: $F_{6,24} = 39.3$, $P < 0.0001$; adults: $F_{3,13} = 4.37$, $P = 0.025$) (Fig. 2). Toxin concentrations in nymphs were significantly higher than those in adults after feeding on Bt-containing spider mites and Bt^+ pollen (mean ± SE, nymphs:

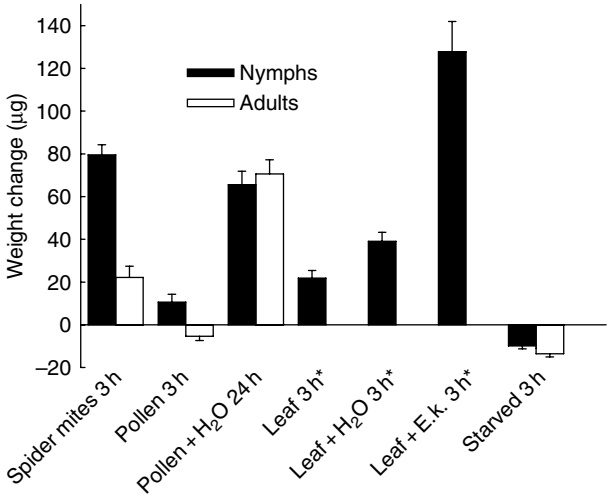

Fig. 1. Mean (+ SE) weight change of *Orius majusculus* nymphs and adults after starvation or feeding on various food sources for 3 or 24 h ($n = 40$–80). H_2O, water; E.k., *Ephestia kuehniella* eggs. *Only nymphs were tested.

0.75 ± 0.11 µg g^{-1}, adults: 0.51 ± 0.10 µg g^{-1}; main effects ANOVA, $F_{1,31} = 7.56$, $P = 0.01$). Levels measured in nymphs that were exposed to Bt^+ maize leaves for 3 h were mostly below detection level independent of the presence of food (E. kuehniella eggs) or water. No toxin was detected in any of the O. majusculus samples fed on control food sources (Bt^-). Bt toxin contained in adult O. majusculus after feeding on Bt^+ maize-fed spider mites decreased rapidly upon transfer to Bt^- maize-fed spider mites. One day (24 h) after the transfer, the toxin was no longer detectable.

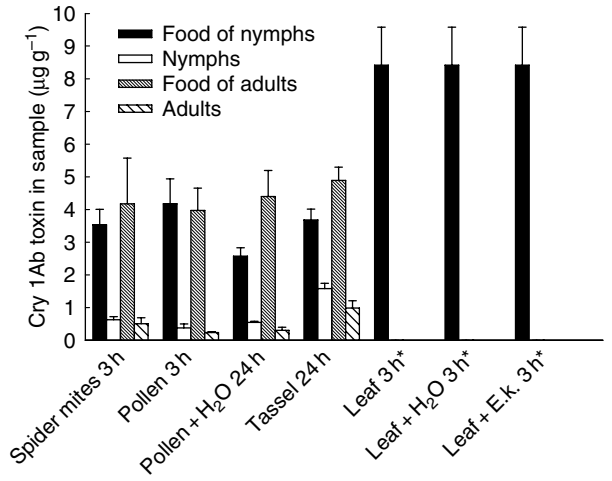

Fig. 2. Mean (+ SE) Cry1Ab toxin concentration (µg g^{-1} dry weight) measured in different food sources and in nymph and adult *Orius majusculus* fed with the respective food ($n = 4$–6). H_2O, water; E.k., *Ephestia kuehniella* eggs. *Only nymphs were tested.

Field experiments

Toxin content in Bt maize. Different plant tissues were shown to contain different toxin levels (one-way ANOVA, $F_{10,22} = 90.3$, $P = 0.0001$) (Fig. 3). Relatively high levels of toxin were measured in leaves [> 4 µg g^{-1} dry weight (DW)] and in fresh pollen (approximately 3 µg g^{-1} DW). Concentrations in old pollen collected from leaves and axils after pollen shed remained comparatively high. Low levels were measured in tissues originating from inner parts of the plant (e.g. whorl, stem, sheath). Toxin concentration in silk was below the quantification limit. The toxin concentrations in leaves collected during the first period (6th, 11th or 19th leaf) was shown to be variable when taking the dry weight as a reference (one-way ANOVA, $F_{2,6} = 16.3$, $P = 0.004$). However, this difference was not detected when toxin concentrations relating to the fresh weight were taken for statistical analyses (one-way ANOVA, $F_{2,6} = 0.46$, $P = 0.65$).

Toxin content in arthropods from Bt maize. Toxin levels in different arthropod groups collected during three different periods in the season (I–III) are presented in Fig. 4. During the first sampling period, concentrations of Cry1Ab toxin in most of the arthropod groups (including some herbivore groups) were low or not detectable. Only mirids (Trigonotylus spp.) and adult chrysomelids (Oulema melanopus) contained substantial levels of toxin. In the second sampling period, when pollen was available, the toxin was additionally detected in several other arthropod groups including Orius spp., adults of Chrysoperla spp., and Frankliniella spp. In contrast, at that time, the toxin

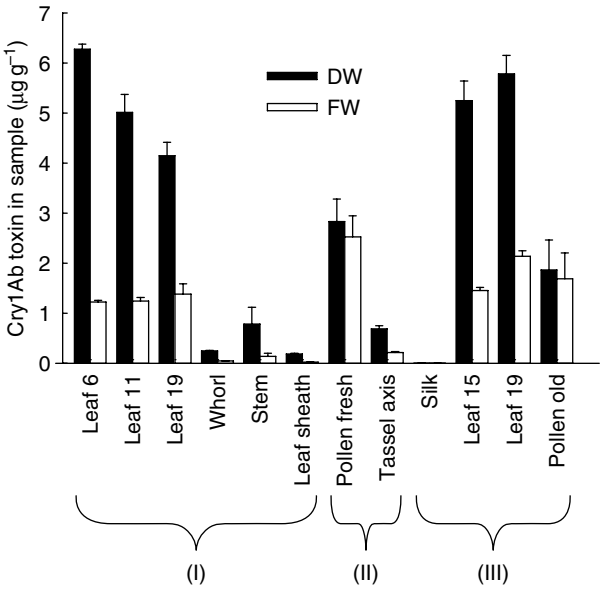

Fig. 3. Mean (+ SE) Cry1Ab toxin concentrations [µg g^{-1} dry weight (DW) or fresh weight (FW)] measured in different plant parts of Bt maize from the field. Samples were taken before (I), during (II), or after pollen shed (III) ($n = 3$).

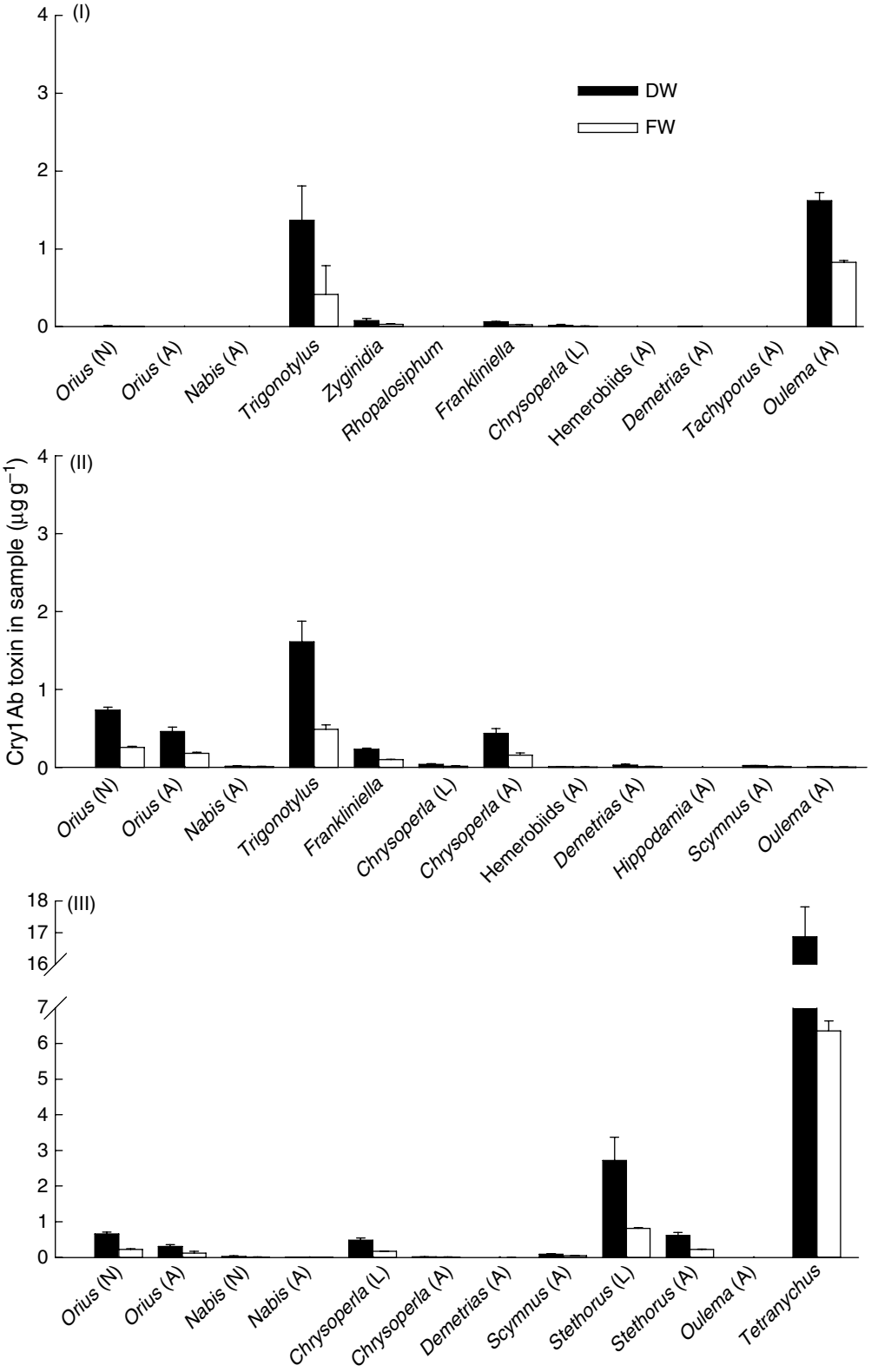

Fig. 4. Mean (+ SE) Cry1Ab toxin concentrations [µg g^{-1} dry weight (DW) or fresh weight (FW)] measured in different arthropod groups that were collected from Bt maize fields before (I), during (II), and after (III) pollen shed. Taxonomic details can be seen in Table 1. A, adults; L, larvae; N, nymphs. $n = 3$ except for *Trigonotylus* where $n = 2$ during period I).

levels were below the quantification limit in adult chrysomelids. During the third sampling period, very high toxin levels were found in spider mites (T. urticae). These concentrations were almost three times higher than those measured in leaves. Nymphs of the spider mite predator Stethorus punctillum contained the highest toxin levels of all predators. Further, similar levels of the toxin were found in adults of S. punctillum, Orius spp., and larvae of Chrysoperla spp. In all but one control (Bt^-) arthropod groups no toxin was detected. The exception were spider mites, in which traces of toxin (below quantification level) were detected. Traces in the control group of spider mites have already been detected in previous studies and are considered to be caused by cross-reactions with other proteins (Dutton et al., 2002; Obrist et al., 2006).

Statistical analyses over the whole sampling season showed that toxin levels in Orius spp. depended on sampling period, being higher in the second and third sampling periods than in the first one (main effects ANOVA, $F_{2,14} = 74.1$, $P < 0.0001$), and stage, as higher concentrations where found in nymphs than in adults ($F_{1,14} = 17.4$, $P = 0.001$). A higher toxin concentration in larvae than in adults was also found for S. punctillum ($F_{1,4} = 15.6$, $P = 0.017$). In other predators like N. provencalis the toxin concentration was very low, independent of the sampling period (one-way ANOVA, $F_{2,6} = 1.68$, $P = 0.26$) and in Demetrias atricapillus the toxin was never detected at all.

Estimation of prey abundance. Mean number of adult thrips per plant during the first sampling period was 16.5 (± 0.75 SE, n = 95). Other prey groups are presented as medians (± quartile) of the estimated abundance group during the sampling periods (I–III) (Fig. 5). Aphids were predominantly found in the beginning of the season. Leafhoppers (cicadellids) were present during all periods and spider mites only occurred after pollen shed.

Toxin content in predators collected in alfalfa field adjacent to Bt maize. Most predators collected in the adjacent alfalfa field contained negligible levels of Cry1Ab toxin (Fig. 6). Only adults of Chrysoperla spp. contained significant Bt toxin levels, in particular, when collected close to the adjacent Bt maize field (1–3 m).

Discussion

Risk assessment studies of an insect-resistant transgenic crop require previous knowledge of the agro-ecosystem. Arthropod species to be tested for adverse effects should be selected according to their ecological and economic importance in the crop and the likelihood of exposure to the insecticidal protein (Dutton *et al.*, 2003). Exposure of predators depends on their feeding habits as well as on the feeding ecology of the prey (e.g. herbivores). The toxin ingestion by herbivores depends on the expression levels and pattern in plants. Thus, as a first step, the toxin expression in different plant tissues was verified. As expected from previous studies (Koziel *et al.*, 1993; Fearing *et al.*, 1997), the toxin was mainly present in leaves and pollen.

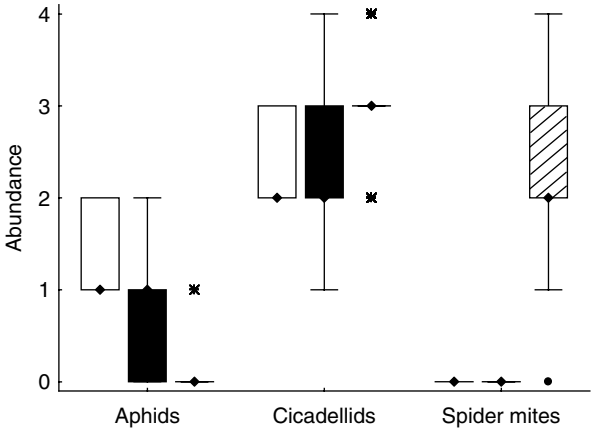

Fig. 5. Abundance of herbivores in Bt maize fields before (white bars), during (black bars), and after (hatched bars) pollen shed, which was estimated by means of a classification system ranging from one (no individual) to five (very high infestation). Black rhombus, median; box, quartile; whiskers, non-outlier range; circles, outlier; asterisk, extreme ($n = 75$–95).

In order to track the toxin through the trophic levels, the Bt toxin content in the most abundant herbivores found in Bt maize fields was determined. No or negligible amounts of toxin were detected in aphids and leafhoppers. These findings are in agreement with previous laboratory studies (Head *et al.*, 2001; Raps *et al.*, 2001; Dutton *et al.*, 2002, 2004). Thrips collected from Bt^+ maize fields did not contain significant toxin levels either, even when collected directly from inflorescences during pollen shed. This was not expected, as in a previous laboratory study, the toxin concentrations in larvae of *F. tenuicornis* reared on Bt maize plants (Bt11, Syngenta) were up to 50 times higher than those found in the field before pollen shed (Obrist *et al.*, 2005). The discrepancy with the present study could

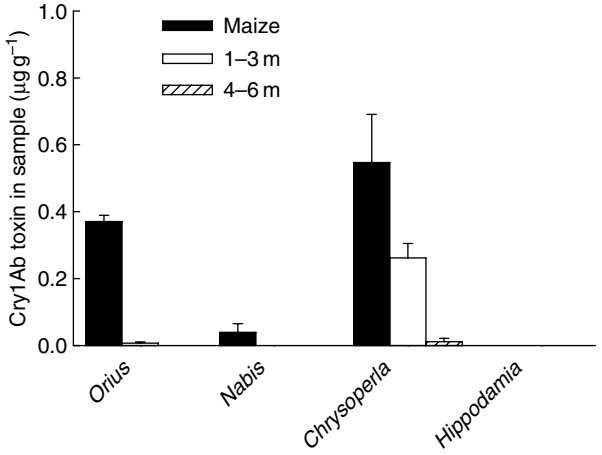

Fig. 6. Mean (+ SE) Cry1Ab toxin concentrations (µg g^{-1} dry weight) measured in predatory arthropods collected in Bt^+ maize fields and two distances (1–3 m and 4–6 m) in the bordering alfalfa fields during maize pollen shed ($n = 3$).

be due to the different cultivars used in the two studies (Event 176 vs. Bt11) as they have different expression levels and spatial patterns (see Dutton *et al*., 2003). This assumption is supported by the fact that before pollen shed most thrips were found in leaf sheaths and whorl, two parts in which very low toxin levels were measured in Event 176. In contrast, spider mites collected in the field contained toxin concentrations that were almost three times higher than those measured in plants and four times higher than those in spider mites used in our laboratory study. Fluctuations in the toxin content in spider mites have been observed in previous laboratory studies (Dutton *et al*., 2002; Obrist *et al*., 2006). It is possible that variable proportions of different stages in the population (including diapausing individuals) accounts for this inconsistency. Among all herbivores tested to date, spider mites contain by far the highest toxin levels. Moreover, the toxin ingested by them has been shown to remain in an active form (Obrist *et al*., 2006). Thus, these herbivores bear a high likelihood of transferring the toxin to their predators. Another interesting herbivore regarding the toxin content is the chrysomelid beetle *O. melanopus*. At the beginning of the sampling season, considerable toxin concentrations were measured in adults. However, during the following sampling periods, no toxin was detected. This finding can be explained by the biology of *O. melanopus*. Adults of the F_1 were shown to feed on maize from late May to late June and subsequently stop feeding and become less active (Grant & Patrick, 1993).

Exposure of predators does not only depend on the presence of toxin in herbivores but also on its own feeding habits. *Orius* spp. are important predators in maize, and thus our studies aimed at describing their routes of exposure to Bt toxin in more detail by performing complementary laboratory experiments. These have shown that *O. majusculus* ingests Bt toxin when feeding on pollen or spider mites in a 'no-choice' situation, but that toxin persistence in adults is shorter than 24 h. Because a previous study reported that the toxin concentration in *C. carnea* was related to the toxin content in the respective herbivore and also corresponded to the amount of food that was taken up by the predator (Obrist *et al*., 2006), it could have been expected that the toxin uptake by *O. majusculus* was also related to the weight gain. Such a relation was not found when *O. majusculus* was fed with pollen or Bt maize leaves. Tested individuals contained detectable toxin levels after feeding on pollen for 3 h (0.38 μg g^{-1} for nymphs; 0.24 μg g^{-1} for adults) even though their weight gain was extremely restricted. The present findings therefore suggest that the toxin is rather concentrated in the content of the pollen grain leading to proportionally high toxin uptake by *O. majusculus* when sucking out Bt maize pollen. In contrast, no toxin was detected in *O. majusculus* nymphs that were fed with Bt maize leaves, although plant feeding was confirmed by the weight gain of insects kept on maize leaves. This finding stands in agreement with a study by Armer *et al*. (2000) who could not detect Cry3A toxin in *Orius insidiosus* after feeding on Bt potato leaves. Armer *et al*. (1998) have shown in a previous study that the same predator ingests xylem sap. Given that the overall protein concentration in xylem is low and that most of these proteins are specific to the xylem (Biles & Abeles, 1991; Buhtz *et al*., 2004), it can be assumed that Bt toxin content is negligible in xylem. The fact that the toxin could not be detected in *Orius* spp. after feeding on Bt maize leaves suggests that Cry1Ab toxin ingestion from Bt maize leaves is not important when compared with uptake via prey or pollen.

In the field, the toxin concentrations measured in *Orius* spp. is related to those measured in the food sources available during the three sampling periods. Before pollen shed, these predators mainly prey on thrips and occasionally on aphids, leafhoppers, lepidopteran eggs, or other predatory arthropods (Dicke & Jarvis, 1962; Coll & Bottrell, 1991; Corey *et al*., 1998; L. B. Obrist, pers. obs.). As none of these herbivores contained significant toxin levels, and the laboratory experiments indicated that toxin uptake by *O. majusculus* through plant feeding is negligible (except from pollen), the exposure of the predator to the toxin before pollen shed can be estimated to be minor. However, during pollen shed, the toxin in *Orius* spp. was detectable. These results leave no doubt that pollen was responsible for the toxin found in *Orius* spp., in particular, as our laboratory experiments have shown that *O. majusculus* can take up considerable amounts of toxin from pollen. In the last sampling period, levels of toxin measured in *Orius* spp. were similar to those found during pollen shed. Although *Orius* spp. were often observed feeding on spider mites (L. B. Obrist, pers. obs.), it cannot be ruled out that they also fed on old pollen. Further investigations would be needed to determine to what extent decomposing pollen can be used as a food source by *Orius* spp. or other arthropods.

The different toxin concentrations found in other heteropteran groups suggest that their feeding habits must differ from those of *Orius* spp. Mirids (*Trigonotylus* spp.) contained toxin during the first sampling period, and when pollen was available their toxin content exceeded that of all other groups collected during this period. Species of this family are known to be omnivorous and even pests (DeBach & Rosen, 1991; Naranjo & Gibson, 1996). Apparently, the mirids collected for this study substantially feed on maize leaves and possibly on pollen. In contrast, the toxin content in nabids could hardly be detected during either of the sampling periods. For nabids, some plant feeding has been reported (Ridgway & Jones, 1968), but generally they are predacious, presumably preying on small invertebrates including leafhoppers, aphids, and various other arthropods (Lattin, 1989). Although we cannot exclude the possibility that they ingested the toxin at any time, the exposure of nabids to Bt toxin in maize in Spain can be estimated to be negligible. A similar situation was found for the carabid beetle, *D. atricapillus*, an efficient predator that is known to climb plants to feed on soft-bodied prey items such as aphids and insect eggs (Sunderland & Vickerman, 1980; Luff, 2002; Sunderland, 2002).

Chrysopids are of particular interest with regard to the risk assessment of Bt maize, because previous laboratory studies revealed inconsistent effects of Cry1Ab on *C. carnea* (Hilbeck *et al*., 1998; Dutton *et al*., 2002; Romeis *et al*., 2004). However, the exposure of this predator to the toxin under field conditions has not yet been assessed. Negligible toxin levels were found in *Chrysoperla* spp. larvae when collected before and during pollen shed. Apparently, larvae did not or only insignificantly feed on pollen in our fields, even though pollen feeding was reported in no-choice laboratory studies (Pilcher *et al*., 1997; J. Romeis, pers. comm.). In contrast, relatively high toxin levels in larvae were measured after pollen shed, possibly as a consequence of consuming spider mites. The toxin content measured in adult *Chrysoperla* spp. differed from that in larvae. Adults of this genus are not predacious and feed primarily on pollen and carbohydrates (McEwen *et al*., 2001). Accordingly, highest toxin levels in adults were detected during pollen shed, whereas negligible levels were detected after pollen shed. In contrast to the chrysopids, adults of the related family of hemerobiids did not contain any Bt toxin. This family is generally predacious with adults having similar feeding habits as larvae (Stelzl, 1991; McEwen *et al*., 2001). The total lack of Bt toxin in the hemerobiid samples collected during pollen shed indicates that they probably did not feed on pollen in the maize fields, even though pollen feeding for some species of this family has been reported (Stelzl, 1991). It can also be assumed that pollen feeding for adults of the coccinellid *H. variegata* is of minor importance, given that Bt toxin was not detected during pollen shed. This is an interesting finding as several coccinellid species are known to feed on pollen (Smith, 1965; Hodek & Honěk, 1996; Lundgren & Wiedenmann, 2002, 2004). Pollen can represent a substantial food source for *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae) an important predator in North America and thus, pollen feeding is addressed in many risk assessment studies of transgenic Bt maize (Pilcher *et al*., 1997; Duan *et al*., 2002; Lundgren & Wiedenmann, 2002, 2004). *Stethorus punctillum*, another coccinellid that was analysed for the presence of Bt toxin, is a specialist predator feeding exclusively on spider mites (McMurtry *et al*., 1970; Hodek & Honěk, 1996). As expected from the high toxin concentrations in spider mites, this predator contained very high toxin concentrations. Exposure of *S. punctillum* to Bt toxin in the field, in particular of the larvae, was therefore highest of all predators that were analysed.

A consistent result of this study is that Bt toxin concentrations in pre-imaginal stages (nymphs and larvae) of predators were higher than in adults, when both stages fed on the same prey items. For *O. majusculus* the data for weight uptake substantiate these results by showing that nymphs ingested significantly more food than adults. The finding that such nymphs or larvae are generally exposed to higher toxin concentrations than adults should be taken into account in risk assessment studies.

In spite of the Cry1Ab toxin levels found in adult *Orius* spp. when collected in Bt maize fields, the toxin was hardly detectable in individuals collected in the bordering alfalfa field. This could be due to the short persistence of the toxin in adults as indicated by the laboratory experiments, or to low movement between the two crops. Bt toxin was still detectable in adult *Chrysoperla* spp. when collected in the alfalfa field closest (1–3 m) to the Bt maize field. The low concentrations measured in adults collected farther away from the Bt maize field (4–6 m) indicate that the concentrations rapidly decrease with distance. It is possible that *Chrysoperla* spp. adults ingested the toxin because they were feeding on pollen in Bt maize fields or that Bt maize pollen was carried by wind into the margin of the alfalfa field, where it was taken up by them. The second assumption, however, seems less likely when considering the fact that the toxin was hardly detected in *Orius* spp. collected in the alfalfa field margin. This pollen-feeding predator would probably also contain the toxin if Bt maize pollen was available in this zone.

The field studies presented here show that the level of exposure of arthropod predators to Cry1Ab in Bt maize fields depends on the time in the growing season and reveal the most likely food sources transmitting the Bt toxin to higher trophic levels. These data, together with the information gained about feeding behaviour of different arthropods, provide the information needed for a science-based exposure assessment of relevant predators in Bt maize.

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