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Effects of exposure to the toxin Cry1Ab through Bt maize fed-prey on the performance and digestive physiology of the predatory rove beetle *Atheta coriaria*

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ABSTRACT

In this study we assess the prey-mediated effects of *Bacillus thuringiensis* (Bt) Cry1Ab-expressed in maize on the performance and digestive physiology of the rove beetle *Atheta coriaria* (Coleoptera: Staphylinidae), using *Tetranychus urticae* (Acari: Tetranychidae) as prey in tritrophic bioassays. The toxin was detected in both *T. urticae* and *A. coriaria* adults and larvae, with concentrations of Cry1Ab decreasing through the trophic chain. In *A. coriaria* adults, the toxin decayed following an exponential curve, not being detectable in their bodies 24 h after the exposure to Bt-fed mites. When the performance of *A. coriaria* reared on (i) Bt maize infested with *T. urticae*, (ii) non-Bt maize infested with *T. urticae*, and (iii) rearing food were compared, no differences were found in any of the parameters analyzed (duration of immature stages, sex ratio, survivorship, fecundity and egg fertility). Proteolytic activities of *A. coriaria* adults fed with mites raised on Bt maize did not show differences with those reared on non-Bt fed-prey, indicating that the nutritional quality of the prey was not affected by exposure to the toxin. This work represents the first study dealing with prey-mediated effects of Bt maize on larvae and adults of a rove beetle. The use of this cosmopolitan rove beetle as an indicator species to assess potential effects of genetically modified crops on non-target arthropods is feasible.

1. Introduction

Genetically modified (GM) crops expressing insecticidal Cry toxins from *Bacillus thuringiensis* Berliner (Bt) represent one of the major biotechnological tools used to control insect pests all over the world (James, 2009). Particularly, Bt maize varieties expressing Cry1Ab provide an effective control of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), a major lepidopteran pest in Europe and North America; and *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae), a pest restricted to the Mediterranean area. Other varieties of Bt maize commercialized are those expressing the Cry1Ac or Cry1F proteins, aiming to control lepidopteran pests and Cry34Ab1/Cry35Ab1 or Cry3Bb1 proteins to control *Diabrotica* spp. (Coleoptera: Chrysomelidae) (Bravo and Soberón, 2008). Currently, GM maize expressing Cry1Ab toxin is the only Bt crop authorized and cultivated in the European Union, with Spain being the EU member state with highest adoption rate of Bt maize in agriculture, since it was first introduced in 1998. Non-target organisms present in maize agroecosystems can also be exposed to Bt toxin in several ways; i.e., feeding directly on plant parts (aerial or subterranean), being transferred through the food chain, or by contact with root exudates (Saxena et al., 2002; Andow et al., 2006; Icoz and Stotzky, 2008). Thus, prior to commercialization GM crops must go through a process of evaluation for assessing the potential impact on non-target organisms. However, it is not possible to perform risk assessment tests for all species present in an agroecosystem; therefore, appropriate representative indicator species should be used as surrogates (Garcia-Alonso et al., 2006).

Generally, indicator species selection is done according to their economic/ecological relevance and their potential exposure to the toxin (Dutton et al., 2003; Romeis et al., 2008). To date, many studies have investigated the potential effects of Bt toxin on non-target arthropods (Romeis et al., 2004; Lövei et al., 2009). Some of the species proposed or assumed as surrogates for certain guilds are: *Folsomia candida* (Willem) (Collembola: Isotomidae) for decomposers (Clark and Coats, 2006); *Apis mellifera* L. (Hymenoptera: Apidae) for pollinators (Duan et al., 2008); *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) for parasitoids (Vojtech et al., 2005); *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) for generalist polyphagous predators (Romeis et al., 2004); *Orius* spp. Wolff (Hemiptera: Anthocoridae) for predators and pollen feeders (Obrist et al., 2006); and *Poecilus cupreus* L. (Coleoptera: Carabidae) for ground-dwelling predators (Meissle et al., 2005). However, no or very few published laboratory studies exist on other groups commonly present in maize fields, such as spiders (Araneae) and rove beetles (Coleoptera: Staphylinidae) (Ludy and Lang, 2006; Porcar et al., 2010).

Staphylinids are among the most common and important ground-dwelling generalist predator fauna in semi-natural and

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farming systems and they are considered suitable and sensitive bioindicators (Bohac, 1999; Pohl et al., 2007). Nevertheless, there is a gap concerning possible effects of Bt toxin on this group of insects under controlled conditions, possibly because of the difficulty associated with rearing rove beetles in the laboratory and because of their high species richness (Bhatti et al., 2005; Lövei and Arpaia, 2005; Farinós et al., 2008). Evaluations of the effects of Bt toxins on staphylinids have been performed as part of broader field studies, aimed to determine the effects of Bt plants on the arthropod community. Generally, these studies show variable responses that do not attain any reliable conclusion on the effects of Bt maize against rove beetles (Jasinski et al., 2003; Dively, 2005; Higgins et al., 2009; Balog et al., 2010). Particularly, field studies performed in Spain, as a part of the post-market environmental monitoring program for Bt maize expressing Cry1Ab toxin showed that staphylinids were the only group of ground-dwelling predators that exhibited a significant decrease in abundance in Bt plots, although differences varied depending on the year and location (de la Poza et al., 2005). Further studies under more controlled conditions are needed to elucidate the results obtained from field surveys.

Atheta coriaria Kraatz (Staphylinidae: Aleocharinae) is the only species of rove beetle commercially available on the market (Cloyd et al., 2009), and it belongs to a subfamily commonly found in farming systems (Farinós et al., 2008; Balog et al., 2010). This cosmopolitan species, adventive on many parts of the world, is a soil-dwelling predator that shows an excellent potential for biological control of soil-dwelling pests. It is commonly used as a biological control agent for certain greenhouse pests such as fungus gnats (Diptera: Sciaridae) (Carney et al., 2002), but both larvae and adults may also feed on other arthropods, such as shore flies, mites and thrips (Helyer et al., 2003). *A. coriaria* could be a suitable species in risk assessment of GM crops to be used as indicator generalist predator, since it is easily identified and can be readily maintained under laboratory conditions, and studies using larvae and adults have given consistent results. A recent study has evaluated the effects of Bt toxins incorporated in an artificial rearing diet on *A. coriaria* adults (Porcar et al., 2010), but there is no literature with this species regarding the effects of exposure to the toxin through the food chain.

The main aim of this paper is to evaluate possible prey-mediated effects of Cry1Ab-expressing Bt maize on the performance and digestive physiology of *A. coriaria*, using *Tetranychus urticae* Koch (Acari: Tetranychidae) as prey in tritrophic bioassays. Hence we performed experiments to: (1) determine the level of exposure and decay rate of Cry1Ab toxin in *A. coriaria* when ingested through prey; (2) assess prey-mediated effects of Bt maize on the performance of *A. coriaria* larvae and adults; and (3) investigate if there were effects on the digestive physiology of *A. coriaria* when they fed on a Bt toxin containing prey.

2. Materials and methods

2.1. Arthropod colonies

A colony of *A. coriaria* was established from adults and larvae purchased from Syngenta Bioline Ltd. (Staphyline C®, Almeria, Spain). Rearing was based on the method developed by Carney et al. (2002) as described below. Adults and larvae were reared in plastic boxes (23.5 × 23.5 × 5.5 cm) containing the rearing substrate: a mix of peat (substrate Compo Sana Universal®, Compo Agricultura SL, Barcelona, Spain), coconut fiber and vermiculite (at a 4:2:1 volume ratio). The moisture level was achieved by adding 200 ml of water weekly. Rearing diet was a mixture of dog food (Brekkies Excel Tender & Delicious®, Affinity Petcare SA, Barcelona, Spain) and oatmeal (Kllön®, Peter Kllön KGaA, Elmshorn, Germany) (at a 4:1 weight ratio) ground to a thin powder. Once a week, 2 g of this mixture was added and mixed thoroughly into each culture. This environment permitted the development and maintenance of other organisms like nematodes and sciarid larvae. Since *A. coriaria* is a generalist predator, adults and larvae feed on these organisms as well as rearing diet. The colony was maintained in a climatic chamber at 20 ± 0.3 °C, 80 ± 5% RH and L:D 16:8 h photoperiod. Sex of adults was determined according to Klimaszewski et al. (2007).

Tetranychus urticae came from a stable laboratory colony, established from laboratory specimens provided in 2006 by Dr. Vicente Marco (Universidad de La Rioja, Spain). They were reared on maize (*Zea mays* L.) plants at 25 ± 0.3 °C, 70 ± 5% RH and L:D 16:8 h photoperiod. Eggs, immatures and adults were used to feed rove beetles *ad libitum*.

2.2. Plant material

Feeding trials were performed with transgenic maize (cv. DKC 6575 YG, Event MON810) expressing a version of the Cry1Ab gene from *B. thuringiensis* (hereafter called Bt maize) or an unmodified maize hybrid (Tiétar) (hereafter called non-Bt maize). Plants were grown in plastic pots (20 cm diameter) using Compo Sana® Universal as a substrate and maintained in a growth chamber at 25 ± 0.3 °C, 70 ± 5% RH and L:D 16:8 h photoperiod. Plants were used when they reached the five-leaf stage.

2.3. Fate of the Cry1Ab protein through the trophic chain

Newly emerged larvae and adults of 0- to 3-day-old of *A. coriaria* were individually placed in a plastic (38 mm diameter × 19 height) test arena containing maize leaf pieces of either Bt maize or non-Bt maize infested with enough number of *T. urticae* to feed specimens *ad libitum*. A group of first instar larvae (L_1) were frozen after feeding on *T. urticae* for 2 days. The rest were allowed to feed on mites for 2 or 3 more days to obtain second instar larvae (L_2). Finally, third instar larvae (L_3) were obtained after 6 to 8 days preying on *T. urticae*. Adult rove beetles were fed *ad libitum* on *T. urticae* for 4 days and initial and final weights were recorded. All the experiments were conducted at 20 ± 0.3 °C, 80 ± 5% RH and L:D 16:8 h photoperiod. At the end of the assay, *A. coriaria* adults were transferred into 1.5 ml Eppendorf tubes and frozen at −20 °C until Cry1Ab determination by ELISA. To assure that the prey–predator system proposed was reliable and to rule out the possibility that *A. coriaria* acquired the toxin directly from the plant, three control treatments without *T. urticae* were assayed, consisting of larvae and adults of *A. coriaria* maintained on moistened filter paper, non-Bt maize or Bt maize, in the same conditions as explained above.

Levels of the Cry1Ab toxin in samples of *T. urticae* raised either on Bt or non-Bt maize and larvae and adults of *A. coriaria* fed on them were screened by sandwich ELISA using the Abraxis Bt Cry1Ab/Cry1Ac Microtiter Plate Kit (Abraxis L.L.C., Warminster, PA, USA). Treatment, quantities of material and buffer solutions (Abraxis L.L.C. Extraction/Dilution Buffer) used in the different treatments are summarized on Table 1. To rule out the presence of Cry toxin on the rearing food random samples were analyzed. Cry1Ab levels in Bt and non-Bt plants were also quantified. Leaf samples were taken at the same phenological stage and at the same time leaves were harvested to be used in the experiments. All samples were centrifuged for 5 min at 12,000*g* and 100 ml of each sample was introduced into the ELISA plate following the manufacturer's instructions. Cry1Ab standards at concentrations 0, 0.25, 0.5, 1.0, 1.5, 2.0 and 4.0 ng/ml were used as calibrators. Spectrophotometric measurements were conducted with a micro-

Table 1
Summary of treatments and subjects screened by sandwich ELISA to measure Cry1Ab toxin levels.

Treatment	Samples analyzed (n)	No of specimens per sample	Weight (mg ± SE)	Buffer volume (ml)
1. Fate of the Cry1Ab protein through the trophic chain				
T. urticae raised on Bt plants	8	70	1.1 ± 0.08	0.3 (1:2 diluted)
T. urticae raised on non-Bt plants	3	70	1.0 ± 0.09	0.3
A. coriaria L_1 fed on *T. urticae* raised on Bt maize	5	10	0.6 ± 0.07	0.3
A. coriaria L_1 fed on *T. urticae* raised on non-Bt maize	2	10	0.6 ± 0.02	0.3
A. coriaria L_2 fed on *T. urticae* raised on Bt maize	5	9	0.9 ± 0.03	0.3
A. coriaria L_2 fed on *T. urticae* raised on non-Bt maize	2	9	0.9 ± 0.03	0.3
A. coriaria L_3 fed on *T. urticae* raised on Bt maize	5	5	1.0 ± 0.12	0.3
A. coriaria L_3 fed on *T. urticae* raised on non-Bt maize	2	5	1.0 ± 0.04	0.3
A. coriaria adults fed on *T. urticae* raised on Bt maize	5	25	11.3 ± 0.28	0.5 (1:2 diluted)
A. coriaria adults fed on *T. urticae* raised on non-Bt maize	5	25	12.4 ± 0.20	0.5
A. coriaria adults maintained on Bt maize	2	10	5.5 ± 0.20	0.3
Rearing food	5	–	55.2 ± 5.08	0.5
Bt plants	10	–	4.8 ± 0.10	0.3 (1:20 diluted)
Non-Bt plants	5	–	5.4 ± 0.10	0.3
2. Detection time of the Cry1Ab protein in A. coriaria adults				
A. coriaria adults fed on *T. urticae* raised on Bt maize at 0 h	4	10	4.7 ± 0.30	0.5
A. coriaria adults fed on *T. urticae* raised on Bt maize at 4 h	4	10	5.5 ± 0.02	0.5
A. coriaria adults fed on *T. urticae* raised on Bt maize at 8 h	4	10	5.5 ± 0.04	0.5
A. coriaria adults fed on *T. urticae* raised on Bt maize at 12 h	4	10	5.4 ± 0.05	0.5
A. coriaria adults fed on *T. urticae* raised on Bt maize at 24 h	4	10	4.8 ± 0.01	0.5
A. coriaria adults fed on *T. urticae* raised on Bt maize at 48 h	4	10	5.5 ± 0.01	0.5

T. urticae = *Tetranychus urticae*.
A. coriaria = *Atheta coriaria*.

titer plate reader at 450 nm (VersaMax™ Microplate Reader, Molecular Devices Inc., Sunnyvale, CA, USA).

2.4. Detection time of the Cry1Ab protein in A. coriaria adults

A bioassay at different post-exposure times was carried out to measure the detection time of the Cry1Ab protein in *A. coriaria* adults after ingestion of prey fed with Bt maize. Newly emerged adults of 0 to 3 days old were individually placed in the test arena containing maize leaf pieces of Bt maize infested with *T. urticae*. Adults were fed for 4 days. At the end they were individually transferred to a new plastic container containing one piece of filter paper moistened with 200 μl of water and 50 mg of rearing food. At different time intervals (0, 4, 8, 12, 24 and 48 h) adults were weighed and immediately frozen at −20 °C. The experiment was replicated four times with groups of 10 adults at each time interval, and it was conducted at 20 ± 0.3 °C, 80 ± 5% RH and L:D 16:8 h photoperiod. Levels of Bt toxin in *A. coriaria* were determined by ELISA following the same protocol as above. Quantities of material and buffer volumes used are summarized on Table 1.

2.5. Prey-mediated effects of Bt maize on predator performance

2.5.1. Effects due to larval exposure to Cry1Ab

Eggs from *A. coriaria* were randomly collected from the laboratory colony. After emergence, larvae were individually placed in the test arena containing one piece of filter paper moistened with 200 μl of water and assigned to one of the following treatments: (1) pieces of Bt maize infested with *T. urticae*; (2) pieces of non-Bt maize infested with *T. urticae*; and (3) rearing food (50 mg). Leaves were replaced every 3 days to ensure an *ad libitum* feeding whereas rearing food was added weekly. The experiment was daily recorded to assess development time, mortality of immature stages, emergence of adults and sex ratio. The experiment was conducted three times with 30 eggs per treatment. Effects upon survivorship, fecundity and egg fertility on adults emerged from each treatment were evaluated as follows. One female and 2 males randomly selected from each treatment were placed in the test arena containing rearing substrate (500 mg), rearing food (50 mg) and humidity (0.5 ml of water). Weight, total length and elytra length were measured in each adult specimen at the beginning of the assay. To ensure an optimum level of nutrients, one frozen third instar larva of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) was added weekly. Adults were examined daily for 14 weeks and mortality, preoviposition period, oviposition period, and numbers of eggs laid were recorded. Ten replicates per treatment were performed. For assessment of fertility, eggs from each treatment were collected daily and individually placed in the test arena containing a piece of filter paper moistened with 200 μl of water. Egg hatching was checked daily.

2.5.2. Effects due to adult exposure to Cry1Ab

Egg fertility was also measured in adults given *T. urticae* fed on Bt or non-Bt maize. Ten males and 10 females of 0–3 days old were placed together in a Petri dish (89 × 23 mm) with the base covered with filter paper and 2.0 g of sterile rearing substrate. The dish was moistened weekly with 3 ml of water to ensure an appropriate level of humidity. Maize leaf pieces from either Bt maize or non-Bt maize infested with *T. urticae* were added. Adults were fed *ad libitum* with *T. urticae* during the bioassay. A third treatment with rearing food (500 mg) was performed. Eggs from each dish were collected weekly until 30 eggs were accumulated. They were individually placed in the test arena containing a piece of filter paper moisted with 200 μl of water and egg hatching was checked daily. Three replicates per treatment were performed.

2.6. Proteolytic activities in A. coriaria adults exposed to Bt toxin

For the characterization of *A. coriaria* proteolytic enzymes, abdomens of *A. coriaria* from the laboratory colony were dissected, homogenized in 0.15 M NaCl (10 abdomens/600 μl ClNa), centrifuged at 12,000*g* for 5 min, and the supernatants pooled and stored frozen (−20 °C) until needed. All protease activities were performed at 30 °C, assays were carried out in triplicate and blanks were used to account for spontaneous breakdown of substrates. A series of overlapping buffers were used to generate a pH gradient from 2 to 11: 0.1 M citric acid–NaOH (pH 2.0–5.0), 0.1 M citrate (pH 3.0–5.0), 0.1 M Tris–HCl (pH 6.5–9.0), 0.1 M phosphate (pH

6.0–8.0) and 0.1 M glycine–NaOH (pH 9.0–11.0). All buffers contained 0.15 M NaCl and 5 mM $MgCl_2$. All substrates and protease inhibitors and activators were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Trypsin-like activity was measured using 1 mM BApNa (Nα-benzoyl-DL-arginine-*p*-nitroanilide) and incubating for 1.45 h, chymotrypsin-like activity using 0.25 mM SA_2PPpNa (*N*-succinyl-(alanine)$_2$-proline-phenylalanine-*p*-nitroanilide) and incubating for 1 h; elastase-like activity using 0.5 mM SA_3pNa (*N*-succinyl-(alanine)$_3$-*p*-nitroanilide) and incubating for 24 h; carboxypeptidase A-like activity with 1 mM HPA (hippuryl-phenylalanine) and incubating for 2 h; carboxypeptidase B-like activity with 1 mM HA (hippuryl-L-arginine) and incubating for 2.5 h; and leucine aminopeptidase-like activity with 1 mM LpNa (L-leucine-*p*-nitroanilide) with an incubation time of 45 min, as described by Ortego et al. (1996). Cathepsin D-like activity was measured with 0.2% hemoglobin solution and incubating for 24 h; and cathepsin B-like activity with 50 mM ZAA_2MNA (*N*-carbobenzoxy-alanine-arginine-arginine 4-methoxy-β-naphthyl amide) and incubating for 4 h, as described by Novillo et al. (1997). Total protein was determined according to the method of Bradford (1976) using bovine serum albumin as the standard. The proteolytic activities were assayed in the presence of the following specific protease inhibitors: the serine protease inhibitor SBBI (Soybean Bowman-Birk inhibitor); the cysteine protease inhibitor E-64 (L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane); the aspartic protease inhibitor pepstatin-A; the serine protease inhibitors STI (soybean trypsin inhibitor), TEI (Turkey Egg White inhibitor) and LBI (lima bean protease inhibitor); and the heavy metal ion $CuCl_2$, inhibitor of aminopeptidases. The cysteine protease activators L-cysteine and dithiothreitol (DTT) were also tested. Protease inhibitors and activators were incubated at 30 °C with the extract for 15 min, prior to adding the substrate. All compounds were added in 20 µl of 0.15 M NaCl, except pepstatin-A, which was added in 4 µl of DMSO. Spectrophotometric measurements were made using a VersaMax™ Microplate Reader and a Hitachi U-2000 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan).

To investigate the potential effects on proteolytic activities of *A. coriaria* when ingesting a prey containing Cry1Ab toxin, adults from the laboratory colony were fed individually *ad libitum* during 4 days on *T. urticae* raised on Bt or non-Bt maize and then frozen at −20 °C. A total of 40 adults for each treatment were used. Abdomens were dissected, homogenized in 0.15 M NaCl (2 abdomens/140 µl), centrifuged at 12,000*g* for 5 min, and the supernatants were frozen. Enzymatic assays were carried out as described above.

2.7. Data analysis

Data were tested for normal distribution using the Kolmogorov–Smirnof test. If normality was not achieved, data were transformed using logarithmic transformation (when variables were continuous) or square root transformation (when variables were counts) to normalize distributions and to stabilize the variance before statistical analysis. Measurements of the standardized skewness and kurtosis were used to determine the intensity of departure from normal distribution. Those data that were unable to improve distribution with transformations and showed major departures from normality were analyzed using non-parametric tests. Toxin concentration in Bt maize, *T. urticae* and *A. coriaria* were compared by Student's *t*-test or one way-ANOVA, followed by Tukey's multiple comparisons tests. Differences in adult weight and fertility were analyzed by Kruskal–Wallis test, followed by Dunn's multiple comparisons tests. Larvae and pupae development times, adult body measurements, preoviposition period and total fecundity were compared between treatments by one way-ANOVA, followed by Tukey's multiple comparisons tests. Weekly fecundity was analyzed using repeated-measurements (RM) one way-ANOVA. The Kaplan–Meier survival analysis was applied to compare survival of the tested specimens on the different diets and the survival distributions were compared by the Mantel log-rank test. Specific activities of extracts of *A. coriaria* adults fed on *T. urticae,* raised on non-Bt maize or Bt maize, were compared by analysis of the covariance using as covariate protein content, whereas total protein was compared by Student's *t*-test (Ortego et al., 1999). The significance level of $P < 0.05$ was considered for all tests.

3. Results

3.1. Fate of the Cry1Ab protein through the trophic chain

Atheta coriaria adults and larvae fed upon *T. urticae* during the treatment period, whereas no feeding activity was observed on maize leaves. Indeed, no larvae survived for more than 4 days in those treatments where *T. urticae* was not present (Bt maize, non-Bt maize and water), while all larvae survived in treatments where *T. urticae* was present as a food source (data not shown). Weight gains in *A. coriaria* adults presented significant differences among treatments after 4 days (K-$W = 176.9$; df = 4; $P = 0.00$) (Fig. 1). Adults fed with *T. urticae* raised on non-Bt maize and on Bt maize, presented an average weight increase of 1.9% and 7.3%, respectively. On the contrary, starved *A. coriaria* adults (Bt maize, non-Bt maize and water) showed a significant reduction in weight after 4 days.

Mean concentrations of 5.49 (Bt maize leaves), 1.28 (*T. urticae* raised on Bt maize) and 0.21 (*A. coriaria* adults fed on mites raised on Bt maize) µg Cry1Ab per gram of fresh weight were detected (Fig. 2A). Thus, Cry1Ab protein level was about 4-fold lower in *T. urticae* than in Bt plants and toxin level in *A. coriaria* adults diminished about six folds compared with mites. Mean concentration of Cry1Ab was different for *A. coriaria* larvae exposed to the toxin depending on the instar analyzed; L_1, L_2 and L_3 presented a mean concentration of 0.16, 0.42 and 0.72 µg Cry1Ab per g of fresh weight, respectively (Fig. 2B). When toxin levels were compared between the two bioassays performed (Fig. 2A and B), no differences were found for Bt maize ($t = 0.18$; $P = 0.85$) and for *T. urticae* raised on Bt maize ($t = 0.91$; $P = 0.37$). Thus, levels of the toxin in adults and larvae could be compared, presenting significant differences ($F_{3,21} = 33.09$ $P = 0.00$). Cry1Ab levels in adults and L_1 presented no differences between them but were significantly lower than values displayed by L_2 and L_3. In addition, L_3 larvae showed significantly higher toxin values than L_2. Bt toxin was not detected in rearing food, non-Bt maize leaves, *T. urticae* raised on non-Bt maize, *A. coriaria* larvae and adults fed with *T. urticae* raised on

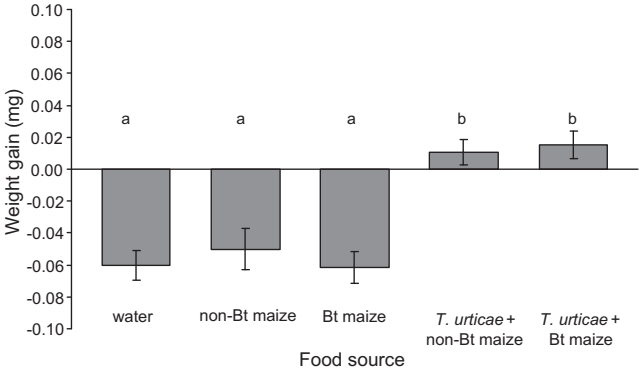

Fig. 1. *Atheta coriaria* adult mean weight gain after 4 days of treatment. Error bars represent ±SE. $N = 100$ for treatments without mites and $N = 125$ for treatments with mites. Different letters above bars indicate significant differences (Kruskal–Wallis test followed by Dunn's multiple comparison test, $P < 0.05$).

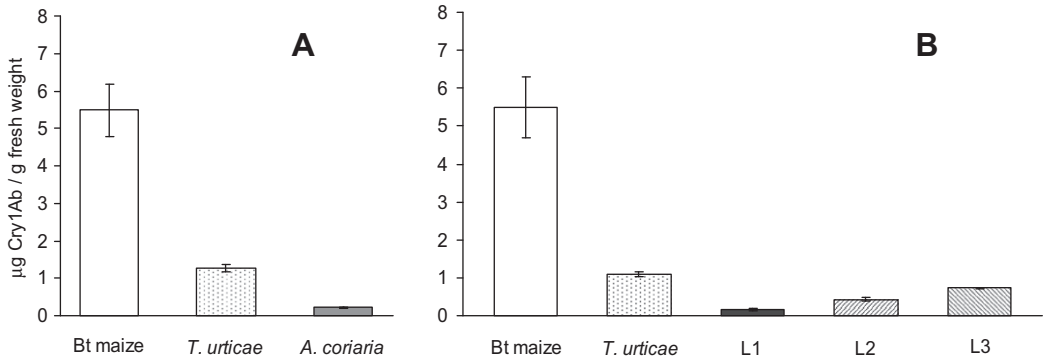

Fig. 2. Mean Cry1Ab toxin level in (A) Bt maize ($N = 10$), *Tetranychus urticae* fed with Bt maize ($N = 8$), and *Atheta coriaria* adults fed on *T. urticae* raised on Bt maize ($N = 5$); and (B) Bt maize ($N = 10$), *T. urticae* fed with Bt maize ($N = 8$), and *A. coriaria* larvae (L1–L3) fed on *T. urticae* raised on Bt maize ($N = 5$ for each larval instar). Error bars represent ±SE.

non-Bt maize and *A. coriaria* adults maintained on Bt maize (data not shown).

3.2. Detection time of the Cry1Ab protein in A. coriaria

Results herein demonstrate that Bt toxin can be detected at different post-exposure times, and that Cry1Ab toxin within *A. coriaria* adults decay at an exponential rate over time (Fig. 3). There was a rapid decline in Cry1Ab concentration during the first 3–4 h after exposure to the toxin, presenting a half-life (t ½) of 3.3 h. The maximum time that Cry1Ab could be detected in the body of *A. coriaria* adults was 12 h after the exposition to the toxin.

3.3. Prey-mediated effects of Bt maize on predator performance

3.3.1. Effects due to larval exposure to Cry1Ab

Rove beetles were able to complete their development from egg to adult when they fed on any of the three diets evaluated in this bioassay. The development time of each larval instar differed among diets (L_1: $F_{2,216} = 8.01$, $P = 0.00$; L_2: $F_{2,216} = 17.48$, $P = 0.00$; L_3: $F_{2,216} = 4.45$, $P = 0.01$). Nevertheless, when the total larval development time was analyzed (L_1–L_3), no significant differences were found ($F_{2,216} = 0.10$, $P = 0.90$) (Table 2). The duration of the pupal stage was significantly different among diets ($F_{2,216} = 14.03$, $P = 0.00$); the longest corresponding to those rove beetles fed on rearing food. Total immature development time (L_1-adult) of *A. coriaria* bred on rearing food presented a significant increase (24.2 days) with respect to those reared on *T. urticae* raised on Bt or non-Bt maize (23.1 and 23.6 days, respectively) ($F_{2,216} = 5.30$, $P = 0.00$). Different diets did not affect the probability to reach the adult stage (Log-Rank test, $X^2 = 0.12$, $P = 0.94$), being the percentage of survival to adults about 80% in all cases. The number of males was slightly higher than that of females in the three treatments; nevertheless there were no significant differences in sex ratio among them ($F_{2,6} = 0.49$, $P = 0.63$) (Table 2).

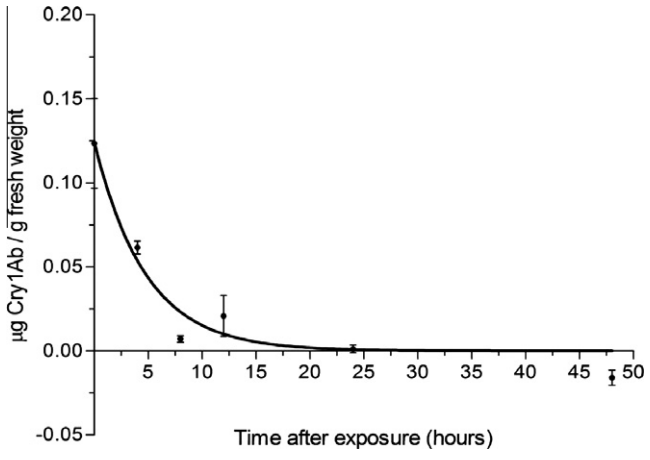

Fig. 3. Mean Cry1Ab toxin level at different post-exposure times in *Atheta coriaria* adults fed during 4 days on *Tetranychus urticae* raised on Bt maize. Decay rate is determined by regression of concentration *vs.* time. Decay rate followed an exponential rate over time: $Y(t) = -124.6\,e^{-0.210t}$ where: $Y0$ is the initial concentration of Cry1Ab (µg/g fresh weight); t ½ (half-life) = 3.3 h; df = 22; $r^2 = 0.78$. Data are means of four measurements for each time period. Error bars represent ±SE.

Females and males emerged from larvae fed with different diets were randomly selected to measure possible effects upon survivorship, fecundity and egg fertility. They did not show significant differences in weight ($F_{2,27} = 0.77$, $P = 0.47$ for females; $F_{2,57} = 1.41$, $P = 0.25$ for males), body length ($F_{2,27} = 1.64$, $P = 0.21$ for females; $F_{2,57} = 0.14$, $P = 0.87$ for males) and elytra length ($F_{2,27} = 2.29$, $P = 0.12$ for females; $F_{2,57} = 1.68$, $P = 0.19$ for males). This is important, since differences among treatments inherent to individual specimens can be discarded. No differences in survivorship were detected among the three treatments for females (Log-Rank test; $X^2 = 0.42$, $P = 0.80$) and males (Log-Rank test; $X^2 = 0.71$, $P = 0.96$), and about 20–30% adults of both sexes were still alive at the end of the 14 weeks of the bioassay (Fig. 4). The preovipositon period was similar (7–9 days) in the different treatments ($F_{2,27} = 1.25$, $P = 0.30$). Average total number of eggs laid by female ranged between 170 and 180, presenting no differences among treatments ($F_{2,27} = 0.02$, $P = 0.97$) (Table 2). Similarly, no differences were displayed when fecundity was analyzed using weekly fecundity data by RM ANOVA ($F_{2,13} = 0.00$, $P = 0.99$). Egg hatching ranged from 90% to 93% in the three treatments and there were not significant differences among them (K-$W = 1.40$, df = 2; $P = 0.49$).

3.3.2. Effects due to adult exposure to Cry1Ab

The percentage of fertile eggs laid by *A. coriaria* adults actively eating *T. urticae* raised on non-Bt maize (92.2 ± 1.1), *T. urticae* raised on Bt maize (93.3 ± 1.1) or rearing food (95.5 ± 1.9) did not differ statistically (K-$W = 2.61$, df = 2, $P = 0.27$).

3.4. Atheta coriaria proteolytic enzymes

Proteolytic activities in *A. coriaria* abdomen extracts were characterized using specific substrates and inhibitors (Table 3). The trypsin and chymotrypsin substrates, BApNa and SA_2PPpNa, were specifically inhibited by SBBI and both presented the maximal hydrolysis at pH 10.0. These activities were corroborated using other specific inhibitors like STI, TEI and LBI at a concentration of

Table 2
Atheta coriaria performance when larvae (L1–L3) were fed on rearing food, *Tetranychus urticae* raised on non-Bt maize and *T. urticae* fed on Bt maize.

	Parameter measured[a]	Diet		
		Rearing food	*T. urticae* + non-Bt maize	*T. urticae* + Bt maize
Pre-imaginal development time (days)	L_1	2.2 ± 0.0a	2.3 ± 0.0a	2.6 ± 0.0b
	L_2	2.7 ± 0.0a	3.3 ± 0.0b	2.6 ± 0.0a
	L_3	6.1 ± 0.1b	5.5 ± 0.1a	5.9 ± 0.1ab
	Larval ($L_1 + L_2 + L_3$)	11.0 ± 0.1a	11.0 ± 0.1a	11.1 ± 0.2a
	Pupae	13.2 ± 0.1a	12.6 ± 0.1b	12.0 ± 0.1 c
	L_1-adult	24.2 ± 0.1b	23.6 ± 0.2a	23.1 ± 0.2a
% Survival to adults[b]		81.1 ± 4.0a	80.0 ± 1.9a	80.0 ± 1.9a
Sex ratio (males/females)		1.1 ± 0.1a	1.3 ± 0.1a	1.3 ± 0.0a
Adult body measurements				
Female	Weight (mg)	0.5 ± 0.0a	0.5 ± 0.0a	0.5 ± 0.0a
	Body length (mm)	3.4 ± 0.0a	3.4 ± 0.0a	3.6 ± 0.0a
	Elytra length (mm)	0.5 ± 0.0a	0.5 ± 0.0a	0.5 ± 0.0a
Male	Weight (mg)	0.5 ± 0.0a	0.5 ± 0.0a	0.4 ± 0.0a
	Body length (mm)	3.4 ± 0.0a	3.4 ± 0.0a	3.2 ± 0.1a
	Elytra length (mm)	0.5 ± 0.0a	0.5 ± 0.0a	0.5 ± 0.0a
Reproductive measurements	Preoviposition period (days)	8.7 ± 1.2a	7.2 ± 0.7a	7.7 ± 0.9a
	Total fecundity (No. eggs)	170.0 ± 33.4a	179.7 ± 31.8a	172.3 ± 34.3a
	Fertility (%)	91.9 ± 2.6a	93.1 ± 1.1a	90.3 ± 1.8a

Data are means ± S.E.
[a] Means followed by the same letter within a row are not significantly different.
[b] Kaplan–Meier survival analysis followed by Log-Rank test ($X^2 = 0.12$, $P = 0.94$).

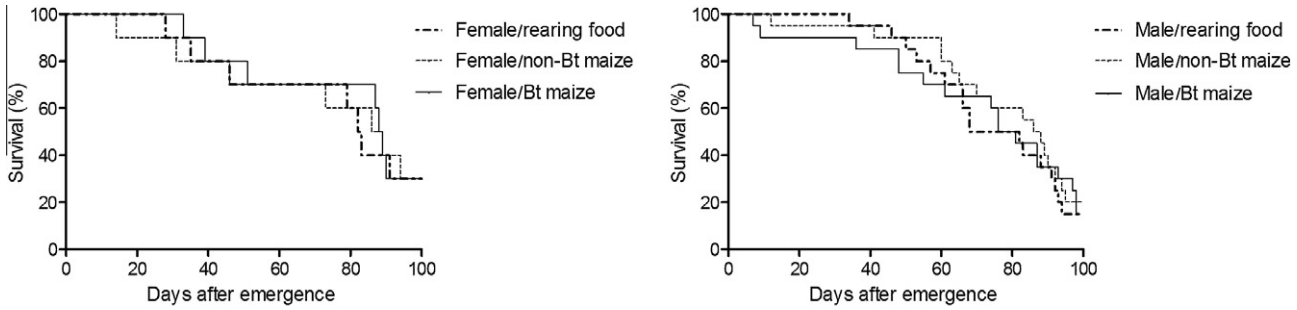

Fig. 4. Survival probability of *Atheta coriaria* females ($N = 10$) and males ($N = 20$) fed during their larval stage with *Tetranychus urticae* reared on non-Bt maize, Bt maize and rearing food. Adults were examined daily for 14 weeks.

Table 3
Proteolytic activity of *Atheta coriaria* abdomens extracts against general and specific substrates and effects of proteases inhibitors and activators.

Substrate	Optimum pH	Specific activity[a]	% Relative activity[b]					
			SBBI (10μM)	E-64 (10μM)	CuCl2 (1mM)	Pepstatin-A (10 μM)	L-cysteine (5mM)	DTT (5mM)
BApNa	10.0	77 ± 1	3 ± 0	ne	ne	ne	ne	ne
SA_2PPpNa	10.0	182 ± 4	21 ± 2	ne	ne	ne	ne	ne
SA_3pNa	8.0	1.6 ± 0.0	ne	ne	ne	ne	ne	ne
ZAA_2MNA	7.5	6.0 ± 0.7	71 ± 7	ne	38 ± 5	ne	63 ± 4	ne
Hemoglobin	6.0	22 ± 2	54 ± 4	68 ± 10	ne	144 ± 5	124 ± 9	ne
LpNa	7.0	115 ± 3	ne	ne	18 ± 0	ne	52 ± 1	ne
HPA	7.0	1650 ± 31	ne	152 ± 14	44 ± 3	ne	–	43 ± 5
HA	7.5	1230 ± 131	ne	ne	35 ± 1	ne	–	ne

Substrates: BApNa, (Nα-benzoyl-DL-arginine *p*-nitroanilide); HA, (hippuryl-L-arginine); HPA, (hippuryl-phenylalanine); LpNa, (L-leucine-*p*-nitroanilide); SA3pNa, (*N*-succinyl-(alanine)3-*p*-nitroanilide); SA2PPpNa, (*N*-succinyl-(alanine)2-proline-phenylalanin-*p*-nitroanilide); ZAA2MNA, (*N*-carbobenzoxy-alanine-arginine-arginine 4-methoxy-β-naphthyl amide).
[a] Specific activities as nanomoles of substrate hydrolyzed/(min mg protein), except for proteolytic activity against hemoglobin as mU ΔAbs 280 nm/(min mg protein). Values are means± SE of triplicate measurements.
[b] Values are means± SE of triplicate measurements from a pool of abdomens extracts treated with an inhibitor or activator *vs*. their corresponding control without them. No effect (ne) was considered for activities between 80%and 120%. Dash (–) means that the inhibitor is not compatible with bioassay.

100 μM, here values did not exceed the 11% of relative activity (data not shown). A peak of maximum activity for the hydrolysis of the elastase substrate, SA_3pNa, was observed at pH 8.0, but none of the serine protease inhibitors tested (SBBI, STI, TEI and LBI) showed inhibitory activity on this substrate. Hydrolysis of ZAA_2M-NA, a substrate for cysteine proteases, was optimum at pH 7.5, but this activity was not inhibited by E-64 or activated by either L-cysteine or DTT, whereas some inhibition was found with SBBI and $CuCl_2$. The hydrolysis of hemoglobin reached a maximum at pH 6.0 and was inhibited by SBBI and E-64 and slightly activated by pepstatin-A and L-cysteine. The hydrolysis of the leucine amino-peptidase substrate, LpNa, presented its maximum activity at pH

7.0, and its hydrolysis was inhibited by the divalent heavy metal ion, $CuCl_2$ and cysteine. The optimum pH for the hydrolysis of HPA and HA, substrates of carboxypeptidase A and carboxypeptidase B-like activities, were 7.0 and 7.5, respectively. The hydrolysis of both substrates was specifically inhibited by $CuCl_2$, although HPA hydrolysis was activated by E-64 and inhibited by DTT.

The proteolytic activity of *A. coriaria* adults fed on *T. urticae* raised on Bt and non-Bt maize was compared (Table 4). Total protein content was similar in rove beetles fed with *T. urticae* raised on non-Bt maize (10.4 ± 0.8 μg protein/insect) and on Bt maize (9.2 ± 0.5 μg protein/insect) ($t = 1.44$, $P = 0.15$). Proteolytic activities of *A. coriaria* adults fed with mites raised on Bt maize did not show differences with those reared on non-Bt fed-prey.

4. Discussion

To the best of our knowledge, this is the first study dealing with prey-mediated effects of Bt maize on larvae and adults of a staphylinid species, a group commonly found in maize fields but that has been scarcely considered in laboratory studies focused on non-target insects. The tritrophic bioassay presented in our paper allowed the exposure of *A. coriaria* larvae and adults to the Cry1Ab toxin in a "worst-case scenario". *Tetranychus urticae* was used as a vehicle for the toxin, since it has been proved that the toxin conserves its biological activity intact after being ingested by this mite (Obrist et al., 2006).

Results obtained in the laboratory by means of tritrophic bioassay give us an idea about the fate of the toxin along food webs in nature. Our results demonstrate that *A. coriaria* larvae and adults were exposed to the toxin when they fed on *T. urticae* raised on Bt maize under laboratory conditions. The passage of Cry1Ab from Bt maize to higher trophic levels has been reported for other coleopteran predators, such as the ground beetle *P. cupreus* (Meissle et al., 2005; Álvarez-Alfageme et al., 2009) and the ladybird *Stethorus punctillum* Weise (Coleoptera: Coccinellidae) (Álvarez-Alfageme et al., 2008). Moreover, Bt toxins may also reach natural enemies when they feed on plant tissues (Obrist et al., 2006). In our tritrophic experiments, we ruled out the possibility that this species acquires the toxin directly from the plant, since no feeding activity was observed on maize leaves and Cry1Ab toxin was not detected in *A. coriaria* adults maintained exclusively on Bt maize. We also proved that toxin titer declined through the trophic chain, although at a different rate in larvae and adults. Depletion movement of the toxin was: Bt maize → *T. urticae* (4-fold lower) → *A. coriaria* (6-fold lower for adults and L_1; 3-fold lower for L_2 and 2-fold lower for L_3). According to our results, the acquisition of the toxin was higher in second and third larval instars, representing in *A. coriaria* the main vehicle to transfer slight amounts of the toxin to other trophic levels. Obrist et al. (2006) showed in a previous study that nymphs of the omnivorous predator *Orius majusculus* (Reuter) (Heteroptera: Anthocoridae) contained higher levels of toxin after feeding on Bt-fed spider mites and Bt pollen than adults. At the same time, when these authors measured the toxin content of different predators collected in Bt maize fields, they found that concentrations were higher in pre-imaginal stages (nymphs and larvae) than in adults. Nevertheless, the toxin is not always detected in tritrophic bioassays with soil-dwelling predators. Thus, no Cry1Ab toxin was found in *Scarites subterraneus* Fabricius (Coleoptera: Carabidae) fed on *Deroceras leave* (Müller) (Mollusca: Agriolimacidae) reared on Bt maize, despite the uptake of Cry1Ab toxin by the slug (Harwood et al., 2006). When specimens of *S. subterraneus* collected from Bt maize fields were analyzed, the toxin could not be detected in this species either (Peterson et al., 2009). This points out the importance of choosing the right species for GM crops post-market monitoring risk assessment.

When the concentration of Cry1Ab was measured in *A. coriaria* adults after they were exposed to Bt fed-prey, toxin levels decreased rapidly following an exponential curve (t ½ was reached after 3.3 h) and did not accumulate in the bodies (the toxin could not be detected 24 and 48 h after exposure). Experiments with other predators conducted to measure the detectable quantities of Cry toxins following consumption of Bt maize fed-prey only considered specific time estimations and did not measure the decay rate of Cry toxins through time. Thus, when larvae of the predator *C. carnea* were fed on *Plutella xylostella* L. (Lepidoptera: Plutellidae) containing Cry1Ac, the toxin could not be detected 24 h after exposure to the toxin (Wei et al., 2008). In the same species, when larvae fed on a sucrose solution containing trypsinized Cry1Ab, the toxin was not detected after 6 days (Romeis et al., 2004). Since defecation takes place only after imaginal molting in this species, both authors cautiously concluded that toxins could have been digested by the larvae. Our study indicates that *A. coriaria* adults rapidly eliminate the plant-produced toxin, although other approximations would be necessary to estimate if the toxin was excreted or metabolized.

Bioassays carried out to determine the Bt fed-prey-mediated effects upon *A. coriaria* performance indicated that Cry1Ab toxin has no negative effects on the biological parameters measured. No alterations on development time, sex ratio and larval survival were detected when larvae ingested the toxin through Bt-fed *T. urticae*. Likewise, no detrimental effects on body measurements, adult survival, preoviposition period, fecundity and fertility were observed in *A. coriaria* adults emerged from larvae exposed to Cry1Ab toxin, and in the egg fertility of *A. coriaria* adults actively eating Bt-fed mites. Our results agree with those reported in a study carried out with *A. coriaria* adults that had acquired the toxin through an artificial diet (Porcar et al., 2010). These authors did not find effects on mortality after feeding adults of *A. coriaria* on a diet treated with Cry1Ab or Cry3Aa toxins for 15 days. Nevertheless, *A. coriaria* are mainly predatory insects; hence toxin exposure mediated by prey represents a more realistic and likely scenario by which rove beetles would enter in contact with Bt toxin in nature. Interestingly, values of total fecundity and adult life-span obtained in our study were much higher than those previously reported for this species (Carney et al., 2002; Porcar et al., 2010). Average total fecundity ranged between 170 and 180 eggs per female, and the maximum number of eggs laid by a female was 331 in the treatment of *A. coriaria* larvae fed on *T. urticae* raised on Bt maize. The fecundity was not age-dependent, with females laying a rather constant number of eggs during their whole life-span in the three treatments (data not shown). These results contrast with a mean of 15.6 progeny per female reported by Carney et al. (2002). Values of adult life-span (about 50% of females survived more than 80 days and about 50% of males more than 70 days) were also higher than previously reported for *A. coriaria* reared in the laboratory (adult longevity did nor exceed 21 days) (Carney et al., 2002; Porcar et al., 2010). This

Table 4
Proteolytic activity on adults exposed to Cry1Ab toxin.

Substrate	% Specific activity[a]	
	Non-Bt maize	Bt maize
BApNa	43 ± 4a	50 ± 6a
SA_2PPpNa	62 ± 7a	71 ± 8a
LpNa	49 ± 2a	40 ± 3a
HPA	1132 ± 174a	1163 ± 172a

Means followed by the same letter within rows are not significantly different.
Mean ± SE for each treatment of measurement from abdomens extract of *Atheta coriaria* fed on *Tetranychus urticae* raised on non-Bt ($N = 20$) and Bt maize ($N = 20$).
[a] Specific activities and substrate as in Table 4.

information is relevant because it provides evidence that the conditions used in our trial are optimal for performing bioassays with *A. coriaria* adults over extended periods of time. Furthermore, the biological parameters analyzed were not affected when *A. coriaria* larvae fed on mites compared to those fed on a rearing diet (a mixture of dog food and oatmeal), indicating that *T. urticae* can be used as a high-quality prey in this type of tritrophic bioassay.

Most studies performed with predatory beetles have found no adverse effects due to the acquisition of Cry toxins through the trophic chain. Larvae of the ground beetle *P. cupreus* were not adversely affected when fed on *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) larvae raised on Bt maize expressing the Cry1Ab toxin (Álvarez-Alfageme et al., 2009). The ladybird *S. punctillum* was not impaired by feeding on spider mites reared on Cry1Ab- or Cry3Bb1-expressing Bt maize (Álvarez-Alfageme et al., 2008; Li and Romeis, 2010). Similarly, bioassays with larvae of *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae) that consumed Cry1Ab or Cry3Bb toxin through Bt pollen did not disclose acute detrimental effects, showing no differences in parameters such as duration of pre-imaginal stages and adult survivorship (Pilcher et al., 1997; Lundgren and Wiedenmann, 2002). Nevertheless, it has been reported that indirect effects due to a reduction in the nutritional quality of the prey may occur, as in the case of *P. cupreus* fed with *S. littoralis* larvae raised on Bt maize that experienced higher mortality than when fed with larvae raised on conventional maize (Meissle et al., 2005).

The analysis of the proteolytic activities involved in digestion could be utilized to elucidate potential alterations in tritrophic bioassays due to a reduced quality of Bt maize fed-prey (Álvarez-Alfageme et al., 2009). Hydrolysis of specific substrates, maximal activity under different pH level, and sensitivity to protease inhibitors reveals that proteolytic activity of *A. coriaria* adults rely on trypsin-, chymotrypsin-, leucine aminopeptidase-, and carboxypeptidase A-, and B-like proteases for digestion. The substrates SA_3pNa, ZAA_2MNA and hemoglobin were also hydrolyzed, but they presented unusual pH profiles and no inhibition was observed when specific protease inhibitors were tested, thus the presence of elastase-, cysteine- and aspartyl-like proteases as part of *A. coriaria* digestive enzymes can be discharged. The proteolytic characterization of *A. coriaria* adults represents the first report on digestive enzymes in rove beetles. In our present study, none of the proteolytic activities of *A. coriaria* adults fed on *T. urticae* raised on Bt or non-Bt maize were significantly different. These data suggest that the quality of the prey was not affected when reared on Bt maize plants expressing the Cry1Ab toxin. Prey with a proper nutritional quality is necessary in tritrophic bioassays, because low nutritional prey may mask real susceptibility of insects to Bt toxin.

Currently there is a trend toward which the evaluation of potential negative effects of GM crops on non-target organisms should take into account two key features; (1) the use of representative surrogate/indicator species and (2) the information acquired during tritrophic bioassays. In the case of rove beetles, native species are sometimes difficult to identify and to rear under laboratory conditions. The species *A. coriaria* belongs to the subfamily Aleocharinae, which represents about 30% of the total number of rove beetles species (Klimaszewski et al., 2007) and is very frequent in farming systems. In addition, it is easy to handle in the laboratory and it is commercially available. Arrangement of bioassays should focus on some key information in order to obtain reliable data. One crucial aspect in risk assessment of Bt plants is length of exposure, and the end point of the study so long-term exposure bioassays that include different developmental stages (larvae-pupa-adult) are more likely to give reliable results (Dively et al., 2004; Harwood et al., 2005). Moreover, generally young larvae are more sensitive than mature larvae or adults (James et al., 1999). Other key factors are the determination of possible adverse effects from a realistic level of exposure and effects on natural enemies' metabolism (Raybould et al., 2007; Wei et al., 2008). In this study we have established that *A. coriaria* are exposed to the toxin Cry1Ab when they feed on Bt maize raised mites, since the toxin was detected in both larvae and adults, but no adverse effects were noticed on its performance or digestive physiology. Based on our findings, we believe that *A. coriaria* can be considered as a reliable and useful model species to be used in laboratory studies for GM crops' risk evaluation.

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