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# Laboratory toxicity studies demonstrate no adverse effects of Cry1Ab and Cry3Bb1 to larvae of *Adalia bipunctata* (Coleoptera: Coccinellidae): the importance of study design

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**Abstract** Scientific studies are frequently used to support policy decisions related to transgenic crops. Schmidt et al., Arch Environ Contam Toxicol 56:221–228 (2009) recently reported that Cry1Ab and Cry3Bb were toxic to larvae of *Adalia bipunctata* in direct feeding studies. This study was quoted, among others, to justify the ban of *Bt* maize (MON 810) in Germany. The study has subsequently been criticized because of methodological shortcomings that make it questionable whether the observed effects were due to direct toxicity of the two Cry proteins. We therefore conducted tritrophic studies assessing whether an effect of the two proteins on *A. bipunctata* could be detected under more realistic routes of exposure. Spider mites that had fed on *Bt* maize (events MON810 and MON88017) were used as carriers to expose young *A. bipunctata* larvae to high doses of biologically active Cry1Ab and Cry3Bb1. Ingestion of the two Cry proteins by *A. bipunctata* did not affect larval mortality, weight, or development time. These results were confirmed in a subsequent experiment in which *A. bipunctata* were directly fed with a sucrose solution containing dissolved purified proteins at concentrations approximately 10 times higher than measured in *Bt* maize-fed spider mites. Hence, our study does not provide any evidence that larvae of *A. bipunctata* are sensitive to Cry1Ab and Cry3Bb1 or that *Bt* maize expressing these proteins would adversely affect this predator. The results suggest that the apparent harmful effects of Cry1Ab and Cry3Bb1 reported by Schmidt et al., Arch Environ Contam Toxicol 56:221–228 (2009) were artifacts of poor study design and procedures. It is thus important that decision-makers evaluate the quality of individual scientific studies and do not view all as equally rigorous and relevant.

**Keywords** Environmental risk assessment · MON810 · MON88017 · Non-target effects · Study design

## Introduction

In 2009, genetically engineered (GE) maize expressing insecticidal Cry proteins derived from the bacterium *Bacillus thuringiensis* (*Bt*) was grown on more than 35 million hectares worldwide (James 2009). Most of the *Bt* maize varieties express either Cry1Ab for the control of stemborers, such as the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (events MON810 and Bt11), or Cry3Bb1 for the control of corn rootworms (*Diabrotica* spp.; Coleoptera: Chrysomelidae) (events MON863 and MON88017) (Hellmich et al. 2008; CERA 2010).

Before their commercial release, GE crops must undergo an environmental risk assessment to ensure

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that they do not cause unacceptable detrimental effects to the environment. In the case of insecticidal GE crops, one focus of the risk assessment is the potential adverse impact on non-target arthropods. The regulatory non-target risk assessment should follow a tiered approach, where the assessment increases in complexity and realism based on the knowledge gained during previous tests (Garcia-Alonso et al. 2006; Rose 2007; Romeis et al. 2008). Initial toxicity studies are conducted under controlled conditions in the laboratory and adopt certain quality criteria to ensure that the study can be reconstructed and that the results can be reasonably interpreted and repeated (Romeis et al. 2008). These criteria include confirmation of the validity of the testing procedure (e.g., a low control mortality in the untreated test organism) as well as information on the test compound (e.g., concentration tested and biological activity of the insecticidal protein).

One focus of the research on non-target arthropods has been on beneficial species such as those contributing to the biological control of herbivores, including predatory ladybird beetles (Coleoptera: Coccinellidae). For the two most common Cry proteins expressed in *Bt* maize varieties, i.e., Cry1Ab and Cry3Bb1, laboratory studies for regulatory purposes have indicated no detrimental effects on the ladybird beetles *Coleomegilla maculata* (DeGeer) and *Hippodamia convergens* Guérin-Méneville exposed to protein concentrations several times higher than those expected in the field (US EPA 2001, 2003; Duan et al. 2002). In addition, a number of peer-reviewed laboratory studies in which ladybird beetles were fed Cry1Ab- or Cry3Bb1-expressing maize material (pollen) or *Bt* maize-fed herbivores have revealed no negative effects on different life-history parameters of *C. maculata* (Pilcher et al. 1997; Lundgren and Wiedenmann 2002; Ahmad et al. 2006) or *Stethorus punctillum* (Weise) (Álvarez-Alfageme et al. 2008; Li and Romeis 2010). However, one study reported inconclusive results for Cry1Ab effects on larvae of *Cheilomenes sexmaculatus* (Linnaeus) (Dhillon and Sharma 2009). The authors detected direct toxic effects in some bioassays, but no effect in others and no convincing explanation was presented for the observed differences. That ladybird beetle populations are not adversely affected by *Bt* maize has been confirmed in numerous field studies, both with maize varieties expressing Cry1Ab (e.g., Musser and Shelton 2003; Daly and Buntin 2005; de la Poza et al. 2005; Pilcher et al. 2005; Rose and Dively 2007) and with maize varieties expressing Cry3Bb1 (e.g., Bhatti et al. 2005; Ahmad et al. 2006; Rauschen et al. 2010). Overall, the large body of published literature provides no indication that the currently grown *Bt* maize varieties cause direct adverse effects on arthropods that are not closely taxonomically related to the target pest (Romeis et al. 2006; Wolfenbarger et al. 2008; Meissle and Romeis 2008; Naranjo 2009; Ricroch et al. 2010; Duan et al. 2010).

A recent study by Schmidt et al. (2009), however, raised concern in the scientific and regulatory communities because it reported toxicity of *Escherichia coli*-produced recombinant Cry1Ab and Cry3Bb to first-instar *Adalia bipunctata* (Linnaeus), a two-spotted ladybird beetle species that predominantly feeds on aphids but that also consumes other soft-bodied arthropods and pollen (Hodek and Honěk 1996). The study was, among others, subsequently cited by the German authorities as new scientific evidence for potential environmental harm to justify the suspension of *Bt* maize (event MON810) cultivation in 2009 (BVL 2009). The results of the study have been questioned because of methodological shortcomings that undermine the study's inferences and that also prevent the reconstruction of the study (Meissle and Romeis 2008; Rauschen 2010; Ricroch et al. 2010). Schmidt et al. (2009) exposed larvae of *A. bipunctata* to the Cry proteins deposited on the outside of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs. Given that young ladybird larvae do not consume entire prey items but instead puncture the prey and suck out the contents (Banks 1957; Hagen 1962; Hodek and Honěk 1996), it is questionable whether the test insects actually ingested the insecticidal proteins. Furthermore, the control mortality in the first larval instar was very high (21%), indicating the unsuitability of the bioassay set-up.

We have thus conducted a set of experiments to determine whether *A. bipunctata* larvae are adversely affected by Cry1Ab and Cry3Bb1 at levels above those to which the predator larvae would be exposed to in a *Bt* maize field. In an initial experiment, we investigated whether young *A. bipunctata* larvae consume whole *E. kuehniella* eggs and ingest test compounds deposited on the outside of the egg shell. We then performed tritrophic studies to test whether early instar *A. bipunctata* are sensitive to *Bt*

maize-expressed Cry1Ab and Cry3Bb1 (events MON810 and MON88017, respectively) when delivered through prey herbivores. The spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), was selected as the carrier because among arthropod prey of *A. bipunctata*, this species is known to contain the highest concentrations of Cry proteins when fed on *Bt* maize (Dutton et al. 2002; Obrist et al. 2006a; Meissle and Romeis 2009a; Li and Romeis 2010). In a final experiment, *A. bipunctata* larvae were directly fed with purified Cry proteins at a concentration that was approximately 10 times higher than that detected in the *Bt* maize-fed spider mites.

## Materials and methods

### Plant material

Two transgenic maize varieties, DKc3421*Bt* (event MON810) and DKc5143*Bt* (event MON88017), both from Monsanto Company (St. Louis, MO, USA), and their corresponding non-transformed near isolines, DKc3421 and DKc5143, were used for the experiments. DKc3421*Bt* plants express a truncated, synthetic version of the *cry1Ab* gene from *B. thuringiensis* ssp. *kurstaki* HD-1, targeting Lepidoptera, while DKc5143*Bt* plants express a synthetically modified *cry3Bb1* gene from wild-type *B. thuringiensis* ssp. *kumamotoensis* EG4691, targeting Coleoptera (CERA 2010). *Bt* expression levels in the plants used for the experiments were measured using double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) from Agdia (Elkhard Indiana, USA) (see below for details).

The four maize varieties were grown simultaneously under the same environmental conditions of 25 ± 1°C, 70 ± 5% RH, and a 16-h photoperiod. Different growth chambers were used for transgenic and non-transgenic maize plants to prevent spider mites from moving between treatments. Three seeds were planted in one plastic pot (12 l) filled with humus-rich soil (Ökohum-Staudenerde, Obi-Ter, Märwil, Switzerland). Plants were fertilized weekly with 400–800 ml of a 0.2% aqueous solution of Vegesan standard (80 g N, 70 g $P_2O_5$, and 80 g $K_2O$ per liter, Hauert HBG Dünger AG, Grossaffoltern, Switzerland) and watered as required.

Maize plants were used for the spider mite rearing once they had reached the 4–5 leaf stage and were removed when they reached anthesis.

### Arthropod material

Mixed stages of two arthropod species and eggs of a third species were used as prey for *A. bipunctata*. The two-spotted spider mite, *T. urticae,* was reared on *Bt* maize or the corresponding control plants in the growth chambers where the plants were raised. A colony of the pea aphid, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae), was kept as a continuous culture on broad bean plants (*Vicia faba* L.) in the glasshouse at 22 ± 3°C, 70 ± 5% RH, and a 16-h photoperiod. UV-irradiated eggs of the lepidopteran *E. kuehniella* were supplied by Biotop (Valbonne, France) and stored at 4°C. Mixed stages of *A. pisum* and eggs of *E. kuehniella*, both of which are high quality food for *A. bipunctata* larvae (Blackman 1967; De Clercq et al. 2005), were used to determine the food quality of mixed stages of *T. urticae* for *A. bipunctata* larvae.

Eggs of *A. bipunctata* were purchased from Andermatt Biocontrol (Grossdietwil, Switzerland). Upon arrival, egg masses were placed in Petri dishes (9 cm diameter) and kept at 25 ± 1°C, 70 ± 5% RH, and a 16-h photoperiod until larvae emerged. Once larvae had eaten their egg shell and started searching for food (≈12 h old), they were individually transferred to the experimental arenas and offered a single egg of *E. kuehniella* to enhance larval survival. After about 8 h, larvae were switched to their respective prey treatments.

### Insecticidal compounds

Stock solutions of purified Cry1Ab in 25 mM carbonate/bicarbonate buffer (pH 10.6) and Cry3Bb1 in 10 mM sodium carbonate/bicarbonate buffer (pH 10.0) were provided by Monsanto Company. The Cry1Ab protein used for the bioassays was the purified trypsin-resistant core of Cry1Ab from recombinant *B. thuringiensis* strain SIC1837. The Cry3Bb1 protein used was the purified Cry3Bb1.11098 (Q349R) produced by recombinant *E. coli* containing the pMON72735 expression plasmid. Both proteins were purified using SDS–Page/Densitometry. The Cry3Bb1 protein is equivalent to protein expressed in

*Bt* maize (events MON863 and MON88017) in terms of biochemical or toxical characteristics (US EPA 2007). The purity-corrected Cry1Ab and Cry3Bb1 protein concentration was 2.1 and 6.3 mg $ml^{-1}$, respectively. Bioactivity of both Cry proteins was confirmed by Monsanto Company in sensitive insect bioassays (unpublished information, provided with the protein certificate of analysis). Cry1Ab was tested on larvae of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) and a 50% effect ($EC_{50}$) on larval weight was reported for 0.012 µg trypsin resistant core. Cry3Bb1 was tested on larvae of *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) and a concentration causing 50% mortality ($LC_{50}$) of 0.4 µg protein was reported. In addition, the bioactivity of the Cry3Bb1 protein was confirmed in a *L. decemlineata* bioassay in our laboratory (Meissle and Romeis 2009a).

Lyophilized snowdrop lectin (*Galanthus nivalis agglutinin*, GNA) was obtained from Els van Damme (Ghent University, Belgium). A detailed description of the isolation from snowdrop bulbs is given by Van Damme et al. (1987).

The inorganic toxin potassium arsenate was purchased from Sigma–Aldrich (Buchs, Switzerland).

## Bioassay set-up

Experimental arenas consisted of small plastic Petri dishes (5 cm diameter, 1.3 cm height) covered with lids containing a mesh window (0.5 cm diameter with 0.2 mm openings) for ventilation. All experiments were conducted in a climate chamber at 25 ± 1°C, 70 ± 5% RH, and a 16-h photoperiod.

## Consumption of *E. kuehniella* eggs by *A. bipunctata*

Visual observations were conducted to determine how young *A. bipunctata* larvae feed on *E. kuehniella* eggs. First and second instars of *A. bipunctata* were offered undamaged eggs and were observed with a stereomicroscope. Furthermore, the number of *E. kuehniella* eggs consumed during the first two instars was determined. Neonate *A. bipunctata* were fed daily ad libitum with undamaged *E. kuehniella* eggs, and the depleted egg shells were counted daily until *A. bipunctata* reached the third instar. Before eggs were provided to the predator, they were examined with a stereomicroscope, and damaged eggs were removed. The assay was performed using eight larvae.

## Assessment of prey quality

A bioassay was conducted to investigate whether *A. bipunctata* larvae are able to develop by feeding exclusively on *T. urticae*. Neonates of *A. bipunctata* were fed ad libitum with one of the following food sources: *E. kuehniella* eggs or mixed stages (immatures and adults) of *A. pisum* or *T. urticae*. Mixed stages of *A. pisum* were brushed from bean leaves and added to the test arenas. For *T. urticae*, an approximately 9-$cm^2$ maize (Dkc5143) leaf disc infested with spider mites was placed upside-down in each Petri dish. All food sources were replaced daily. The experiment was performed with 30 *A. bipunctata* larvae per treatment. Assessment of variables is described in the next section.

## Tritrophic feeding study

This bioassay assessed the prey-mediated effects of *Bt* protein-expressing maize on *A. bipunctata*. Neonates were fed ad libitum with *T. urticae* reared on *Bt*-transgenic plants or on the respective non-transformed near isolines for several generations as described above. The bioassay was conducted in three subsequent runs with 15 replications each, resulting in a total of 45 *A. bipunctata* larvae per treatment. For every run, the *A. bipunctata* larvae were obtained from a different shipment and were fed with *T. urticae* reared on different plants.

In both the prey-quality and tritrophic feeding assays, *A. bipunctata* larvae were observed twice daily, in the morning and in the evening, until they reached the third instar. Then, larvae were frozen at −20°C, dried at 50°C for 24 h, and subsequently weighed on a microbalance (Mettler Toledo MX5, division $d = 1$ µg; tolerance ± 2 µg). Larval development time (L1–L3), dry weight of third-instars, and mortality were measured. We focused on the early larval instars for three main reasons: (1) early instars are generally regarded as being more sensitive to *Bt* Cry proteins than older instars or adults (Glare and O'Callaghan 2000), (2) *T. urticae* are not an optimum food for *A. bipunctata* larvae, and extended feeding on *T. urticae* would have caused unacceptably high

control mortality levels, and (3) Schmidt et al. (2009) only reported Cry protein effects on the first instar in their bioassays.

### Verification of the transfer of Cry proteins through the food chain

This experiment was conducted to verify that *A. bipunctata* larvae were exposed to Cry protein when feeding on *Bt* maize-fed spider mites and to quantify the protein levels. Samples of maize leaves and *T. urticae* were collected from *Bt* plants. Spider mites were collected in a tray kept below a maize leaf by shaking the leaf with a stick. Maize leaf material and *T. urticae* obtained from the 7th leaf from one plant were separately put into 1.5-ml micro-reaction tubes. For each of the three runs, two samples of leaves and *T. urticae* (7–10 mg fresh weight) were collected from different maize plants, resulting in a total of six samples. Leaf material and *T. urticae* were similarly obtained from control plants.

Neonate *A. bipunctata* were fed *Bt* maize-reared *T. urticae* for either 2 or 5 days, equivalent to first and second larval instars, respectively. As a control, larvae were fed *T. urticae* raised on non-transgenic maize plants. Five *A. bipunctata* were pooled into a 1.5-ml micro-reaction tube as one sample; for each of the three runs, two samples of both larval instars were analyzed.

All plant and insect samples were frozen and stored at −20°C for less than 11 weeks for Cry1Ab or Cry3Bb1 measurements. *Bt* protein levels in maize leaves, *T. urticae*, and *A. bipunctata* were measured using DAS-ELISA from Agdia following the protocol described in detail in Meissle and Romeis (2009b). Standard curves were made using solutions of the purified Cry1Ab or Cry3Bb1 proteins that were provided by Monsanto Company and for which purity was known. Protein concentrations in μg $g^{-1}$ fresh weight (FW) were calculated from the standard curves using regression analysis. For the clear separation of positive readings from controls, the limit of detection (LOD) of the test was determined based on the standard deviation of the OD values of buffer-only controls multiplied by three (ICH 2005). Subsequently, the detection limit of each sample was calculated from the dilution, sample weight and amount of added buffer. Measurements of all *Bt* samples revealed ODs above the respective LOD.

### Direct feeding study

A direct feeding study was conducted to evaluate the effect of purified Cry1Ab and Cry3Bb1 proteins on the pre-imaginal development of *A. bipunctata* and the weight of the emerging adult beetles. The bioassay aimed to cover some of the limitations of the tritrophic experiment that used *T. urticae* as a carrier for the Cry proteins, namely (1) *T. urticae* is a suboptimum food for *A. bipunctata*, (2) the tritrophic experiment had to be restricted to the first two intars of *A. bipunctata,* and (3) we could not include a positive control to show that the experimental set-up was able to detect adverse effects on the life-history parameters that were measured.

*Bt* protein concentrations were approximately 10 times higher than those measured in spider mites that had fed on *Bt* maize. Snowdrop lectin (GNA) and potassium arsenate were used as positive control treatments. That GNA can have a deleterious effect on larvae of *A. bipunctata* at high concentrations has been previously demonstrated (Hogervorst et al. 2006), and potassium arsenate is an inorganic compound that is highly toxic to insects and that is often used as a positive control in toxicological studies including those with ladybird beetles (Duan et al. 2002, 2006, 2008). The aim of the positive controls was to show that the ladybird larvae actually ingested the sucrose solution containing the test compounds and that the experimental set-up was able to detect adverse effects on the measured life-history parameters (Rose 2007).

The experiment was initiated with neonate *A. bipunctata*. Each larva received two droplets of a 2 M sucrose solution that was prepared with deionized water (L1 and L2: 0.5 μl, L3 and L4: 1 μl) or a sucrose solution containing 45 μg $ml^{-1}$ Cry1Ab, 200 μg $ml^{-1}$ Cry3Bb1, 10,000 μg $ml^{-1}$ GNA, or 300 μg $ml^{-1}$ potassium arsenate on the first day of each instar. After 24 h, larvae were transferred to clean Petri dishes and subsequently fed ad libitum with *E. kuehniella* eggs to continue development. After emergence, adults were frozen at −20°C and later dried at 50°C for 24 h. Subsequently, the dried adults were weighed on the microbalance and their sex was determined by dissection. Larval and pupal development time, adult dry weight, and mortality were recorded. The experiment was conducted with 34–41 *A. bipunctata* larvae per treatment.

### Statistical analysis

In the assay in which *A. bipunctata* was fed with three different prey species, larval development time (L1–L3) and L3 dry weight were analysed using non-parametric statistics because data were not normally distributed (Kolmogorov–Smirnov test). Three pairwise comparisons were conducted using the Mann–Whitney *U* test, adjusted for ties, and significance levels were adjusted using the sequential Bonferroni procedure (Holm 1979). In the experiment that determined the impact of *Bt*-expressing maize on *A. bipunctata*, data for larval development time (L1–L3) and L3 dry weight were compared between the *Bt* maize and the respective non-*Bt* maize treatments using Student's *t*-test.

In the direct feeding assay, larval and pupal development time and adult dry weight were analyzed between the control treatment and the respective insecticidal compounds in pairwise comparisons using Student's *t*-test. Significance levels were adjusted using the sequential Bonferroni procedure.

In all assays, mortality data were compared using Chi-square tests. Again, significance levels were adjusted using the sequential Bonferroni procedure to correct for multiple pairwise comparisons.

For all tests, the overall $\alpha$-level was set at 5%. Statistical analyses were conducted using the software package Statistica (Version 7.1, StatSoft Inc., Tulsa, OK, USA).

Statistical power analyses were performed before conducting the tritrophic feeding study using PASS (Version 2005, NCCS, Kaysville, UT, USA). Data (mean and standard deviation) recorded for the *A. bipunctata* larvae fed with *T. urticae* in the prey-quality bioassay were used for the calculations. With 45 replications, a power of 80%, and $\alpha = 0.05$, the detectable differences between control and *Bt* treatment for larval development time (L1–L3), L3 dry weight, and larval mortality were 12, 8, and 29%, respectively. Calculations were based on two-sided *t*-tests (larval development time and weight) or Chi-square tests (mortality). The sensitivity of the experiment was judged to be sufficient because 80% power at $\alpha = 0.05$ to detect a 50% effect is often stated as acceptable for risk assessment research (Rose 2007; EFSA 2009). Power analyses were not conducted for the direct feeding bioassay because the positive control treatments that were included provide some indication about the size of the detectable effect in that bioassay.

## Results

Visual observations revealed that, when preying on *E. kuehniella* eggs, both first and second instars of *A. bipunctata* sucked out their contents until they were completely depleted. No larva was observed consuming whole eggs or even parts of the egg shell. During the first instar, a single *A. bipunctata* larva consumed the contents of 41.0 ± 3.26 (mean ± SE) *E. kuehniella* eggs. The average egg consumption of a second instar was 56.3 ± 6.26. In no case did the larvae consume the egg shell. Thus, young *A. bipunctata* larvae cannot be dosed with test compounds deposited on the outside of *E. kuehniella* eggs.

A comparison among different prey items revealed that mixed stages of *A. pisum* and *E. kuehniella* eggs were of high and equal quality as prey for *A. bipunctata* resulting in similar larval development time, weight, and mortality (Table 1). Although *A. bipunctata* larvae were also able to reach the third instar when exclusively fed mixed stages of *T. urticae*, development time (L1–L3) was doubled, L3 larval weight was halved, and mortality was increased >5-fold (but not significantly) when *A. bipunctata* consumed *T. urticae* instead of *A. pisum* or *E. kuehniella* eggs. The results revealed that spider mites are suitable carriers to expose the first two larval instars of *A. bipunctata* to *Bt* maize-expressed Cry proteins.

When *A. bipunctata* larvae were fed exclusively with *T. urticae* that had been reared on *Bt* maize expressing Cry1Ab or Cry3Bb1, none of the recorded life-history parameters, i.e., development time (L1–L3), weight, and mortality, was significantly affected when compared to larvae that were fed *T. urticae* collected from the respective non-transformed control plants (Table 2).

That the *A. bipunctata* larvae had ingested Cry protein when feeding on *Bt* maize-fed *T. urticae* was confirmed in subsequent ELISA analyses (Table 3). While Cry1Ab and Cry3Bb1 were detected in *Bt* maize leaves, *T. urticae* reared on transgenic plants, and *A. bipunctata* fed with spider mites collected from *Bt* plants, a drastic reduction (>90%) of the *Bt* concentration through the food chain occurred for

**Table 1** Development time, weight, and mortality of larvae (L1–L3) of *Adalia bipunctata* fed with *Ephestia kuehniella* eggs, mixed stages of *Acyrthosiphum pisum*, or mixed stages of *Tetranychus urticae* ($N = 30$)

| Parameters | Prey | | |
|---|---|---|---|
| | *E. kuehniella* eggs | *A. pisum* | *T. urticae* |
| Development time (days ± SE) | | | |
| L1 | 2.0 ± 0.02 | 2.0 ± 0.03 | 2.8 ± 0.16 |
| L2 | 1.2 ± 0.04 | 1.1 ± 0.04 | 3.4 ± 0.16 |
| L1–L3 | 3.2 ± 0.04 a | 3.1 ± 0.03 a | 6.2 ± 0.24 b |
| L3 dry weight (mg ± SE) | 0.56 ± 0.021 a | 0.52 ± 0.038 a | 0.22 ± 0.006 b |
| Mortality (%) | | | |
| L1 | 3.3 | 0.0 | 13.3 |
| L2 | 0.0 | 0.0 | 3.8 |
| L1–L3 | 3.3 a | 0.0 a | 16.7 a |

Values followed by different letters in the same row represent significant differences ($P < 0.05$; Mann–Whitney $U$ test for development time and dry weight, and Chi-square test for mortality). The sequential Bonferroni procedure was applied to account for three pairwise comparisons

**Table 2** Development time, weight, and mortality of larvae (L1–L3) of *Adalia bipunctata* fed with mixed stages of *Tetranychus urticae* reared on *Bt* maize expressing either Cry1Ab (DKc3421*Bt*) or Cry3Bb1 (DKc5143*Bt*) or reared on the corresponding non-transformed varieties (DKc3421 and DKc5143, respectively) ($N = 45$)

| Parameters | DKc3421 | | DKc5143 | |
|---|---|---|---|---|
| | *Bt* (Cry1Ab) | Non-*Bt* | *Bt* (Cry3Bb1) | Non-*Bt* |
| Development time (days ± SE) | | | | |
| L1 | 3.2 ± 0.16 | 3.0 ± 0.16 | 2.9 ± 0.18 | 3.0 ± 0.13 |
| L2 | 3.6 ± 0.13 | 3.5 ± 0.14 | 3.3 ± 0.16 | 3.4 ± 0.12 |
| L1–L3 | 6.8 ± 0.14 | 6.5 ± 0.25 | 6.1 ± 0.22 | 6.4 ± 0.29 |
| L3 dry weight (mg ± SE) | 0.22 ± 0.007 | 0.23 ± 0.009 | 0.21 ± 0.006 | 0.22 ± 0.005 |
| Mortality (%) | | | | |
| L1 | 13.3 | 15.6 | 11.1 | 13.3 |
| L2 | 5.1 | 5.3 | 5.0 | 5.1 |
| L1–L3 | 17.8 | 20.0 | 15.6 | 17.8 |

Pairwise comparisons were conducted between the *Bt*-transgenic plants and their corresponding non-transformed near isoline (Student's $t$-test for L1–L3 development time and L3 dry weight, and Chi-square test for mortality). None of the pairwise comparisons was significant ($0.29 \leq P \leq 0.79$)

both proteins (Table 3). Neither Cry1Ab nor Cry3Bb1 protein was detected in any control sample of plant or insect material.

When purified Cry1Ab and Cry3Bb1 dissolved in a sucrose solution were fed directly to *A. bipunctata* larvae, no effect on any of the measured life-history parameters were detected (Table 4). In contrast, both positive controls (GNA and potassium arsenate) caused adverse effects on the *A. bipunctata* larvae, indicating the sensitivity of the bioassay set-up and the exposure of the larvae to the test compounds. Ingestion of potassium arsenate significantly increased the mortality of *A. bipunctata* larvae and pupae and prolonged the development of surviving larvae (Table 4). GNA also significantly prolonged larval development by almost 10% and caused a significant reduction (by 23%) in the dry weight of emerging male beetles.

**Table 3** Mean concentration of Cry1Ab and Cry3Bb1 in *Bt* maize leaves (DKc3421*Bt* or DKc5143*Bt*, respectively), in mixed stages of *Tetranychus urticae*, and in *Adalia bipunctata* larvae

| Tissue sampled | Concentrations of Cry protein (μg $g^{-1}$ FW ± SE) | |
|---|---|---|
| | Cry1Ab | Cry3Bb1 |
| *Bt* maize | 8.9 ± 0.92 (100) | 34.5 ± 2.28 (100) |
| *T. urticae* | 4.7 ± 0.53 (52.1) | 19.9 ± 1.26 (57.8) |
| *A. bipunctata* | | |
| L1 | 0.7 ± 0.06 (7.7) | 2.4 ± 0.08 (7.0) |
| L2 | 0.5 ± 0.05 (5.7) | 1.6 ± 0.09 (4.8) |

*T. urticae* were collected from the two kinds of *Bt* maize and *A. bipunctata* were fed with *Bt* maize-reared *T. urticae*. Values in parentheses represent the percentage of Cry protein relative to the concentration measured in the leaves ($N = 6$)

## Discussion

The current study provides no indication that larvae of *A. bipunctata* are adversely affected after ingestion of Cry1Ab or Cry3Bb1. A tritrophic bioassay in which first and second instars of *A. bipunctata* were fed Bt maize-fed spider mites provided no evidence that young larvae are sensitive to *Bt* maize-expressed Cry1Ab or Cry3Bb1. That the *A. bipunctata* larvae were exposed to plant-expressed Cry proteins in our tritrophic experiments was confirmed by ELISA. Compared to ladybird beetle larvae collected in Cry1Ab- or Cry3Bb1-expressing *Bt* maize fields (Harwood et al. 2005, 2007; Obrist et al. 2006b; Meissle and Romeis 2009b), the larvae in our bioassay contained considerably more Cry protein. For example, the first and second instars of *A. bipunctata* in the current study contained 164–329 times more Cry3Bb1 protein than the larvae of *Coccinella septempunctata* (Pallas) and *Propylea quatuordecimpunctata* (L.) collected in a field of the same Cry3Bb1-expressing *Bt* maize variety used in the present study (see supporting information, Table S1, in Meissle and Romeis 2009b). Our tritrophic experiments thus provided very conservative, worst-case exposure conditions. Because the Cry1Ab and Cry3Bb1 leaf-expression levels of the climate chamber-grown *Bt* maize plants used in our study were comparable to those reported from the field (Monsanto 2002, 2004; Nguyen and Jehle 2007, 2009), the conclusions drawn are applicable to the field situation.

Spider mites are an alternative prey for ladybird beetles including *A. bipunctata*, even though mites cannot support complete larval development (Robinson 1951). Our study showed that *T. urticae* is of lower nutritional quality than the aphid *A. pisum* or *E. kuehniella* eggs, both of which are known to be

**Table 4** Impact of Cry1Ab, Cry3Bb1, GNA, and potassium arsenate on larval development time, larval mortality, and adult dry weight of *Adalia bipunctata*

| Food solution | Larval mortality (%) | Larval development (days ± SE) | Pupal mortality (%) | Pupal development (days ± SE) | Adult dry weight (μg ± SE) | |
|---|---|---|---|---|---|---|
| | | | | | Female | Male |
| Sucrose | 15.0 | 11.9 ± 0.20 | 0 | 4.2 ± 0.09 | 3.4 ± 0.21 | 3.1 ± 0.15 |
| Sucrose + Cry1Ab | 20.6 | 11.7 ± 0.14 | 3.7 | 4.2 ± 0.09 | 3.4 ± 0.12 | 2.8 ± 0.10 |
| Sucrose + Cry3Bb1 | 17.1 | 11.8 ± 0.17 | 0 | 4.2 ± 0.06 | 3.2 ± 0.14 | 2.9 ± 0.10 |
| Sucrose + GNA | 26.3 | 12.9 ± 0.22* | 0 | 4.1 ± 0.13 | 3.2 ± 0.09 | 2.4 ± 0.10* |
| Sucrose + potassium arsenate | 90.0* | 16.7 ± 0.75* | 50.0* | 4.2 ± 0.25 | 1.9 ± 0.10 | –[a] |

The experiment was started with 34–41 larvae per treatment. Larvae were fed a 2 M sucrose solution containing either one of four different insecticidal compounds or a pure sucrose solution (negative control) on the first day of each larval stage. Subsequently, larvae were fed ad libitum exclusively with *Ephestia kuehniella* eggs to support their development. Test compounds were dissolved in the sucrose solution at the following concentrations: Cry1Ab (45 μg $ml^{-1}$), Cry3Bb1 (200 μg $ml^{-1}$), GNA (10,000 μg $ml^{-1}$), potassium arsenate (300 μg $ml^{-1}$)

Means that differed significantly from the control (sucrose) are marked with an asterisk ($P < 0.05$; pairwise comparisons between the control and the insecticidal solutions using Student's *t*-test for development time and dry weight, and Chi-square test for mortality). The sequential Bonferroni procedure was applied to account for four pairwise comparisons

[a] No male emerged from the sucrose + potassium arsenate treatment

high quality food sources for *A. bipunctata* larvae (Blackman 1967; De Clercq et al. 2005). Nevertheless, feeding exclusively on *T. urticae* allowed *A. bipunctata* larvae to reach the third instar with mortality levels remaining below 20%. To keep mortality low, however, we had to feed neonate *A. bipunctata* a single egg of *E. kuehniella* before the start of the experiment. Preliminary observations had revealed that the provision of a single egg increased the ability of the neonates to capture spider mites and significantly reduced mortality (Álvarez-Alfageme et al., unpublished data). Dixon (1959) and Hurst and Majerus (1993) have shown that the chance of capturing prey increases for neonate ladybirds once the first prey has been eaten.

*Bt* maize-fed *T. urticae* are suitable carriers to expose *A. bipunctata* larvae to plant-expressed Cry proteins for three main reasons. First, the spider mites contain high concentrations of Cry protein when fed *Bt* maize. In our study, *T. urticae* contained more than 50% of the Cry protein concentration found in green leaf tissue. Similar or even higher Cry protein levels have been reported in previous studies with different *Bt* maize events (Dutton et al. 2002; Obrist et al. 2006a; Meissle and Romeis 2009a; Li and Romeis 2010). Second, *T. urticae* is not itself affected when feeding on Cry1Ab- or Cry3Bb1-expressing *Bt* maize (Dutton et al. 2002; Li and Romeis 2010), and this excludes potential indirect effects on the predator that may be caused by changes in prey quality (see Romeis et al. 2006 and Naranjo 2009 for a discussion of such prey-quality mediated effects). Third, sensitive insect bioassays have confirmed that Cry1Ab and Cry3Bb1 remain biologically active after ingestion by *T. urticae* when feeding on *Bt* maize (Obrist et al. 2006a; Meissle and Romeis 2009a). Spider mites might thus be used as a carrier of plant-expressed insecticidal proteins to expose other predators that are known to feed occasionally on this herbivore. This has already been documented for larvae of the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) (Dutton et al. 2002; Obrist et al. 2006a).

Even though the tritrophic feeding experiment provided no evidence that *Bt* maize-expressed Cry1Ab or Cry3Bb1 detrimentally affect *A. bipunctata* larvae, a confirmatory experiment was conducted using purified Cry proteins at a concentration that was approximately 10 times higher than measured in the *Bt* maize-fed *T. urticae*. Larvae were dosed with the test compounds dissolved in a sucrose solution only during the first day of each larval instar. They subsequently received ad libitum *E. kuehniella* eggs as food because the larvae would not be able to develop on sucrose solution alone. A similar set-up has been used to conduct direct toxicity tests with larvae of *C. carnea* (Lawo and Romeis 2008). The results confirmed our previous findings that ingestion of either of the two Cry proteins does not harm *A. bipunctata* larvae. In contrast to the tritrophic experiment, the direct feeding experiment involved all larval instars and the pupal stage (in terms of development time and mortality) and the adults (in terms of weight at emergence) of *A. bipunctata*. Two positive control treatments that were tested in parallel confirmed that the predator larvae had ingested the test compounds and that the bioassay was sensitive enough to detect adverse effects on larval mortality, development time and adult weight. The GNA treatment revealed that even a 10% prolongation of the larval development time and a 23% reduction in adult weight were detectable.

The results obtained in our experiments contradict those of Schmidt et al. (2009), who reported direct toxic effects of purified Cry1Ab and Cry3Bb1 proteins to first-instar *A. bipunctata*. One possibility for this contradiction is differences in the protein exposure levels. While we confirmed and quantified the ingestion of Cry proteins by *A. bipunctata* larvae in our experiments, the dose that the larvae ingested in Schmidt et al. (2009) was not measured and remains unclear. Schmidt et al. (2009) fed the ladybird larvae with eggs of *E. kuehniella* that had been sprayed with a water solution containing the dissolved Cry proteins. While the protein concentration of the solution was indicated, the authors did not provide information on the amount of protein solution sprayed on the *E. kuehniella* eggs, the residual Cry protein concentration on the treated eggs, or the amount of treated eggs consumed by the *A. bipunctata* larvae. It is thus impossible to calculate the dose with which larvae were treated in Schmidt et al. (2009). Another confusing factor about the Schmidt et al. (2009) study is that young coccinellid larvae are known to bite into their prey (aphids and insect eggs) and suck out the contents (Banks 1957; Hagen 1962; Hodek and Honěk 1996). Our visual observations demonstrated that this is also the case for first and

second instars of *A. bipunctata* when provided eggs of *E. kuehniella*. Consequently, uptake of test compounds deposited on the outside of the *E. kuehniella* egg shell was probably very low in Schmidt et al. (2009), especially for first instar *A. bipunctata,* for which the detrimental effects of Cry proteins were observed. Therefore, the possibility that factors other than direct toxicity of the two Cry proteins were responsible for the reported effects is likely.

In addition to these shortcomings, additional weaknesses in Schmidt et al. (2009) have been pointed out in three other publications (Meissle and Romeis 2008; Rauschen 2010; Ricroch et al. 2010). First, the authors did not observe a relationship between the Cry protein concentration and the measured mortality. Second, the only effect seen was mortality; no sublethal effects for surviving *A. bipunctata* were reported on larval development time or *A. bipunctata* body weight. Third, the larvae suffered an unusually high and variable control mortality. In the three experiments, first-instar *A. bipuncata* suffered control mortalities ranging from 7.5 to 20.8%. This is unexpectedly high given the fact that *E. kuehniella* eggs are generally regarded as high quality food for *A. bipunctata* (De Clercq et al. 2005) and points to methodological problems in the bioassay. Our results support this: first-instar *A. bipunctata* suffered a mortality of only 3% when fed exclusively with *E. kuehniella* eggs.

Our study is in accordance with a range of laboratory studies that have reported a lack of direct toxic effects of Cry1Ab and Cry3Bb1 on different species of ladybird beetles (Pilcher et al. 1997; US EPA 2001, 2003; Duan et al. 2002; Lundgren and Wiedenmann 2002; Ahmad et al. 2006; Álvarez-Alfageme et al. 2008; Li and Romeis 2010). A recent direct feeding study with trypsin-activated Cry1Ab has also revealed no toxic effects on *A. bipunctata* larvae (Porcar et al. 2010). In addition, exposure to the Cry proteins in the field is generally low for ladybird beetles that use aphids as their major food source given that aphids ingest no or only trace amounts of Cry proteins when feeding on *Bt* maize (Raps et al. 2001; Head et al. 2001; Dutton et al. 2002; Lundgren and Wiedenmann 2005; Meissle and Romeis 2009b; Romeis and Meissle 2010). Under field conditions, significant exposure only seems to occur for species such as *S. punctillum* that specifically feed on spider mites (Obrist et al. 2006b) or for species such as *C. maculata* that consume large amounts of maize pollen (Lundgren et al. 2005). The lack of toxicity together with generally low exposure levels indicates a negligible risk of the current *Bt* maize varieties to ladybird beetles, and this has been confirmed in various field studies (e.g., Musser and Shelton 2003; Bhatti et al. 2005; Daly and Buntin 2005; de la Poza et al. 2005; Pilcher et al. 2005; Ahmad et al. 2006; Rose and Dively 2007; Rauschen et al. 2010).

## Importance of study design

Laboratory toxicity studies are important for assessing potential non-target effects of insecticidal GE crops (Garcia-Alonso et al. 2006; Rose 2007; Romeis et al. 2008; Duan et al. 2010). Such studies must be properly designed and executed to maximize the probability that compounds with adverse effects are detected. The confidence in the conclusions drawn from those laboratory studies thus depends on the study's ability to detect such adverse effects, if present.

Using spider mites as a carrier of Cry proteins, we exposed first and second instars of *A. bipunctata* to unrealistically high levels of biologically active, plant-expressed Cry1Ab and Cry3Bb1. The larvae were not adversely affected by the two Cry proteins. This was confirmed in a second bioassay where *A. bipunctata* larvae were fed with purified Cry proteins dissolved in a sucrose solution at a concentration approximately 10 times higher than in the *Bt* maize-fed spider mites. Our confidence in the conclusion that Cry1Ab and Cry3Bb1 at plant-expression levels do not adversely affect larvae of *A. bipunctata* is high because (1) the *A. bipunctata* larvae were exposed to and ingested concentrations of the test compounds that were several times higher than the predicted exposures in the field; (2) the ingested Cry proteins were biologically active; (3) the negative control mortality in both the tri-trophic experiment and in the direct feeding study were acceptable, showing the suitability of the bioassay set-up; and (4) statistical power analyses or positive control treatments confirmed the sensitivity of our test systems for detecting adverse effects of the test compounds on the measured *A. bipunctata* life-history parameters.

Our results show that the harmful effects of the two Cry proteins reported in Schmidt et al. (2009) were likely false positives, i.e., artifacts of poor study

design and procedures. This shows the importance of following minimum quality standards in conducting laboratory non-target studies for assessing the environmental risk of GE crops. It is thus important that decision-makers evaluate the quality of individual scientific studies and do not view the conclusions of all studies as equally trustworthy, a fact that has for example been explicitly stated by the Australian Office of the Gene Technology Regulator (OGTR 2009). Consequently, not all peer-reviewed scientific studies are equally relevant for environmental risk assessment and decision-making.

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