Biological Control 53 (2010) 337–344

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

# *Bt* maize expressing Cry3Bb1 does not harm the spider mite, *Tetranychus urticae*, or its ladybird beetle predator, *Stethorus punctillum*

Yunhe Li, Jörg Romeis *

*Agroscope Reckenholz-Tänikon Research Station ART, Reckenholzstr. 191, 8046 Zurich, Switzerland*

ARTICLE INFO

*Article history:*
Received 21 September 2009
Accepted 3 December 2009
Available online 11 December 2009

*Keywords:*
ELISA
MON88017
Non-target effects
Environmental risk assessment

ABSTRACT

*Bt* maize varieties expressing the insecticidal protein Cry3Bb1 against larvae of the Western corn rootworm (*Diabrotica virgifera virgifera*; Coleoptera: Chrysomelidae) may harm non-target beetles due to the spectrum of activity of the protein. We have conducted studies to assess the prey-mediated effects of Cry3Bb1-expressing *Bt* maize (event MON88017) on the ladybird beetle *Stethorus punctillum* (Coleoptera: Coccinellidae). This species specifically consumes spider mites that are known to contain high amounts of *Bt* toxin when feeding on *Bt* maize. The developmental and reproduction life-history parameters did not differ for spider mites, *Tetranychus urticae* (Acari: Tetranychidae), fed *Bt* maize or non-*Bt* maize. Similarly, larval survival and development, adult survival, and adult dry weight did not differ for *S. punctillum* fed with spider mites reared on *Bt* or non-*Bt* maize for over 2 months. Female beetles that were fed *T. urticae* from *Bt* maize had a shorter pre-oviposition period, increased fecundity, and increased fertility relative to females fed spider mites from non-*Bt* maize. The reasons for these effects are unclear but may be due to unidentified differences in plant characteristics. *T. urticae* contained 56% of the Cry3Bb1 concentration in *Bt* maize leaves. Beetle larvae and adults that had consumed *Bt* maize-fed spider mites contained toxin concentrations that were six and 20 times lower, respectively, than Cry3Bb1 levels in their spider mite prey. Thus, the toxin was diluted at higher trophic levels. The results indicate that *S. punctillum* is not harmed by feeding on spider mites containing Cry3Bb1. Consequently, detrimental effects on this predator when preying in Cry3Bb1-expressing *Bt* maize fields are unlikely.



## 1. Introduction

Genetically engineered (GE) maize varieties expressing δ-endotoxins from *Bacillus thuringiensis* Berliner (*Bt*) are the most widely grown insect-resistant transgenic plants. First commercialized in 1996, *Bt* maize was grown in 17 countries on a total of 31.6 million hectares (20% of maize area worldwide) in 2008 (James, 2008). Most *Bt* maize varieties are protected against lepidopteran pests (i.e., the European corn borer and other stemborers), by expression of Lepidoptera-specific Cry1 proteins. Since 2003, several *Bt* maize varieties that express Coleoptera-active Cry proteins (i.e., Cry3Bb1, Cry34/35Ab1, mCry3A) for the control of corn rootworms, *Diabrotica* spp. (Coleoptera: Chrysomelidae), have become commercially available in the USA (Hellmich et al., 2008). Corn rootworms are the most destructive insect pests of maize in the USA (Vaughn et al., 2005; Ward et al., 2005). Among them, *Diabrotica virgifera virgifera* LeConte was accidentally introduced into Europe in the 1980s where it has been rapidly spreading (Meinke et al., 2009). The most commonly applied traditional control methods for corn rootworms are crop rotation and insecticide application (Levine and Oloumi-Sadeghi, 1991). However, these pests have developed resistance against both control strategies (Levine and Oloumi-Sadeghi, 1991; Spencer et al., 2005; Miller et al., 2009). *Diabrotica*-resistant *Bt* maize has been reported to be highly effective in controlling larvae of *Diabrotica* species and thus provides farmers with an effective alternative for managing these pests (Rice, 2004; Ward et al., 2005; Hellmich et al., 2008).

Similar to any other plant protection strategy, the adoption of *Bt* maize presents potential risks to the environment. One main concern of insect-resistant GE crops is their potential to harm non-target arthropods (Sanvido et al., 2007; Romeis et al., 2009), including natural enemies that provide the important ecological function of regulating herbivores. A reduction in natural enemies would detract from the benefits provided by a GE variety within an integrated pest management context (Kennedy, 2008; Romeis et al., 2008a). It follows that the assessment of non-target effects is an important part of the environmental risk assessment conducted before the commercialization of any novel GE variety (Garcia-Alonso et al., 2006; Romeis et al., 2008b).

In the case of *Diabrotica*-resistant GE maize particular attention should be paid to non-target species belonging to the order of Coleoptera, because they are most likely to be affected by the expressed beetle-active toxin (Raybould, 2006; Raybould et al., 2007; Romeis

* Corresponding author. Fax: +41 44 377 72 01.
*E-mail address:* joerg.romeis@art.admin.ch (J. Romeis).

1049-9644/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.biocontrol.2009.12.003

et al., 2008b, 2009). A number of previous studies have revealed no negative effects of Cry3Bb1-containing *Bt* maize pollen or purified Cry3Bb1 on *Coleomegilla maculata* (DeGeer) and *Hippodamia convergens* Guérin-Méneville (both Coleoptera: Coccinellidae) (Lundgren and Wiedenmann, 2002; Duan et al., 2002; Monsanto, 2004; Ahmad et al., 2006) and carabid beetles (Ahmad et al., 2006; Mullin et al., 2005; Duan et al., 2006). Similarly, Shirai (2006) reported no effects of *Bt* maize pollen on two non-target herbivorous beetles, *Epilachna vigintioctopunctata* (Fabricius) (Coleoptera: Coccinellidae) and *Galerucella vittaticollis* Baly (Coleoptera: Chrysomelidae).

We here present a study on the impact of Cry3Bb1-expressing *Bt* maize (event MON88017) on the ladybird beetle *Stethorus punctillum* (Weise) (Coleoptera: Coccinellidae). This species was selected because it will be exposed to the *Bt* toxin at very high levels when compared to other ladybird species. The species occurs in maize (De la Poza et al., 2005; Obrist et al., 2006a) and is a specialist predator of tetranychid mites (Acari: Tetranychidae) (Putman, 1955; Rott and Ponsonby, 2000; Biddinger et al., 2009). Earlier studies demonstrated that *Tetranychus urticae* Koch (Acari: Tetranychidae) collected from *Bt* maize (events *Bt*176 and MON88017) contained Cry proteins at levels in the same order of magnitude as the green maize leaf tissue (Obrist et al., 2006a; Meissle and Romeis, 2009a). Consequently, larvae of *S. punctillum* collected in *Bt* maize fields contained the highest toxin levels of all arthropod predators collected in those fields (Obrist et al., 2006a).

We have conducted worst-case exposure studies in which *S. punctillum* were fed *T. urticae* reared either on *Bt* maize or the corresponding non-*Bt* maize plants. To add certainty to this hazard assessment, beetles were exposed to *Bt* maize-fed spider mites for a duration exceeding the exposure duration in the field. To distinguish between potential direct effects of the toxin from indirect, prey-quality mediated effects (*sensu* Romeis et al., 2006), complementary studies determined whether the development or population growth of *T. urticae* are affected when the spider mite feeds on *Bt* maize rather than on non-transformed plants. In addition, to establish the extent to which *S. punctillum* is potentially exposed to Cry3Bb1 toxin by consumption of spider mites in *Bt* maize fields, enzyme-linked immunosorbent assays (ELISA) were used to verify and quantify the transfer of toxin from *Bt* maize to *T. urticae*, and subsequently to *S. punctillum*.

## 2. Material and methods

### 2.1. Experimental conditions

Climate chambers were used to grow plants, rear arthropods, and conduct experiments. In all cases, the conditions were 26 ± 1 °C, 60 ± 5% RH, and a 16:8 h light:dark cycle.

### 2.2. Plant material

*Bt* maize DKC5143*Bt* (event MON88017, Monsanto, St. Louis, MO, USA) and its corresponding non-transformed near isoline DKC5143 (non-*Bt* maize) as a control were used for the experiments. MON88017 plants express a synthetically modified *cry3Bb1* gene (from wild-type *Bacillus thuringiensis* ssp. *kumamotoensis* EG4691), and expression is driven by the constitutive enhanced 35S cauliflower mosaic virus promoter (P-e35s) (Monsanto, 2004). This *Bt* maize expresses Cry3Bb1 to control corn rootworms, *Diabrotica* spp. (Vaughn et al., 2005).

The two maize varieties were grown simultaneously in two separate growth chambers. Two plants were grown together in one plastic pot (12 l). 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 were watered as required.

Maize plants were replanted every 3 weeks, and old plants were removed when they reached anthesis. Plants were used for spider mite rearing after they had reached the 4–5 leaf stage.

### 2.3. Arthropod material

Two-spotted spider mites, *T. urticae,* were reared on *Bt* maize or the corresponding non-*Bt* maize plants in the growth chambers where the plants were grown. After a minimum of two generations on the respective maize plants, the spider mites were used in the experiments. A colony of the ladybird beetle, *S. punctillum*, was established from a shipment from Applied Bionomics (Victoria, BC, Canada). The beetles were kept in a transparent plastic container (50 × 30 × 30 cm). Each day, spider mites together with leaf pieces from the non-*Bt* maize plants were added to the container.

### 2.4. Effects of Bt maize on T. urticae

#### 2.4.1. Immature development

To ensure that spider mites placed on maize leaf pieces remained on those pieces, a sandwich rearing cage was developed. First, a flat layer of moisturized cotton wool was laid in a Petri dish (5.2 cm diameter, 1.3 cm height) and covered with filter paper (5 cm diameter). Then, a square section of maize leaf tissue (approximately 1.5 × 1.5 cm) was positioned in the center of the filter paper. Finally, the filter paper with the leaf tissue was covered with another filter paper containing a square hole, leaving exposed a 1 × 1 cm piece of the leaf tissue for spider mite feeding. All cages were sealed with lids containing a mesh (0.2 mm) window (0.5 cm diameter) for ventilation. Water was added each day as needed to keep the leaf tissue fresh and to keep the top filter paper wet. When the top filter paper was wet, mites could not escape from the square leaf tissue.

Two or three female spider mites collected from *Bt* or non-*Bt* maize were confined to a corresponding maize leaf tissue in the rearing cage. The leaf pieces were obtained from the 6th or 7th leaves of plants at the 8 to 10 leaf stage. The females were allowed to lay eggs for up to 12 h; then, the adults and all eggs except one were removed. A total of 60 cages were set up for each treatment. The spider mites were observed twice per day at 0800 and 1800 h. Leaf pieces were replaced every 3–4 days to ensure a constant exposure to Cry3Bb1. Previous studies have confirmed a stable toxin concentration in leave pieces from the same *Bt* maize variety for a minimum of 3 days (Meissle & Romeis, unpublished; Zurbrügg and Nentwig, 2009). The hatching rate of eggs and the developmental time and survival of each life stage were recorded until the adult stage. Individuals that died because of handling or drowning were excluded from further analyses.

#### 2.4.2. Adult survival and reproduction

Once the mites reached the adult stage, they were sexed and males were discarded. Individual females were placed in a cage together with two males to ensure mating. Males were randomly selected from the corresponding *Bt* or non-*Bt* maize rearing colony. Pieces of maize leaves were provided as described above. The cages were checked daily until the death of the female. Eggs were counted and removed. To investigate the sex ratio of the progeny, 169 and 234 eggs randomly collected from the spider mite colonies on *Bt* and non-*Bt* maize plants, respectively, were incubated, and hatched larvae were fed with the corresponding *Bt* and non-*Bt* maize leaves. Their sex was determined when they developed to adulthood. The intrinsic rate of natural increase, $r_m$ (i.e., the innate capacity to increase for an idealized population in an ideal

environment), was estimated according to the equation established by Birch (1948).

### 2.5. Prey-mediated effects of Bt maize on S. punctillum

#### 2.5.1. Immature development

Neonate larvae of *S. punctillum* were individually confined in small plastic dishes (5 cm diameter × 1.3 cm height) covered with lids containing a mesh (0.2 mm) window (0.5 cm diameter) for ventilation. A moist filter paper (5 cm diameter) was laid on the bottom of each dish, and a square piece of maize leaf tissue (approximately 3 × 3 cm) was placed upside down on the filter paper. The leaf piece was cut from a *Bt* or non-*Bt* maize plant infested with mixed stages of spider mites. Leaf pieces were replaced every 1 or 2 days to ensure that spider mites were available *ad libitum*. The experiment started with a total of 77 and 78 beetle larvae in the *Bt* and non-*Bt* treatment, respectively. The beetle larvae were observed twice per day at 0800 and 1800 h until emergence of adults. Larval survival and developmental time (duration of each instar, time to pupation, and time to adult emergence) were recorded.

#### 2.5.2. Adult survival and reproduction

Emerging adults were sexed and weighed within 12 h after eclosion and paired within 48 h. Each pair was confined to a plastic container (13 × 10.5 × 5 cm) with a mesh (0.2 mm) window (6 × 4 cm) in the lid. *Bt* or non-*Bt* maize leaf pieces infested with spider mites were provided daily as described above. In total, 26 pairs derived from the *Bt* treatment and 25 pairs from the non-*Bt* treatment were tested. Insects were checked once per day, and the following parameters were recorded: survival, pre-oviposition period, daily and total fecundity, and egg fertility. A minimum of 20 eggs were collected from each female in the period of 4 to 8 days after the first oviposition to assess fertility. The eggs were placed in a separate container, and hatched larvae were counted after 7 days. The experiment lasted for 8 weeks. After that, all adults were frozen at −80 °C for Cry3Bb1 measurement; five adults of the same sex were pooled as one sample, and a total of three samples were obtained for each sex.

### 2.6. Transfer of Cry3Bb1 toxin through the food chain

When maize plants had reached the 9-leaf stage, samples of maize leaves and spider mites were collected from *Bt* and non-*Bt* plants. Spider mites were collected in a tray kept underneath the 7th leaf of a maize plant by shaking the leaf with a stick. For leaf samples, approximately 2 g of leaf material was cut from the middle of the same leaf with a scissor. Maize leaf material and spider mites [6–10 mg fresh weight (FW)] collected from one plant were separately put into 1.5-ml microreaction tubes. A total of five leaf and spider mite samples were collected from five different maize plants per treatment. All samples were frozen at −80 °C for Cry3Bb1 measurement.

Transfer of Cry3Bb1 toxin from *Bt* maize-reared spider mites to *S. punctillum* larvae, pupae and adults was investigated. Newly hatched larvae of *S. punctillum* were placed on *Bt* maize or non-*Bt* maize (8–10 leaf stage), which had been colonized by spider mites for at least 1 week. Subsequently, larvae (3 and 6 days old for *Bt* maize but only 6 days old for non-*Bt* maize), pupae, and newly emerged adults were collected from the plants. Five *S. punctillum* of each life stage were pooled into a 1.5-ml microreaction tube as one sample, and a total of five samples per treatment were obtained for each life stage. All samples were kept at −80 °C for Cry3Bb1 measurement.

An additional experiment was conducted to test whether Cry3Bb1 toxin accumulates in adults of *S. punctillum* when continuously ingesting *Bt* maize-reared spider mites. Pupae of *S. punctillum* were collected from non-*Bt* maize and kept in a plastic container (5.2 cm diameter, 1.4 cm height) on a wet filter paper. After emergence, the adults were placed individually in plastic containers (33 × 22.5 × 5 cm) with a mesh window (12 × 20 cm). Pieces of *Bt* maize leaves covered with spider mites were provided daily. After 0, 5, 10, and 15 days, samples of females and males were separately collected and stored at −80 °C for Cry3Bb1 measurement. Five beetles were pooled as one sample, and a total of four to five samples of females and males were obtained at each sampling date.

Cry3Bb1 protein levels in maize leaves, spider mites, and beetles were measured using a double-antibody sandwich enzyme-linked-immunosorbent assay (DAS-ELISA) from Agdia (Elkhard, IN, USA). To extract Cry3Bb1 protein, phosphate-buffered saline with Tween 20 (PBST, provided in the kit) was added to the samples in 1.5-ml microreaction tubes; a 5-mm tungsten carbide bead was added, and the samples were macerated for 3 min at 30 Hz in a mixer mill MM300 (Retsch, Haan, Germany) fitted with 24 tube adapters (Quiagen, Hombrechtikon, Switzerland). After centrifugation and appropriate dilution of the supernatants, ELISA was performed according to the manufacturer's instructions. The measured OD values were calibrated using a range Cry3Bb1 standards made from purified toxin solutions provided by Monsanto (see Li et al., 2008).

### 2.7. Statistical analyses

For the spider mite bioassay, developmental time of the different *T. urticae* life stages and the lengths of the pre-oviposition and oviposition periods as affected by *Bt* and non-*Bt* maize were analyzed using Mann–Whitney *U*-tests because the data did not satisfy the assumptions for parametric analysis. Female longevity and total fecundity were analyzed by Student's *t*-test because the data were normally distributed and variances were homogenous. Frequency data were subjected to $\chi^2$-tests. Daily fecundity was analyzed using repeated-measures (RM) ANOVA. The intrinsic rate of natural increase ($r_m$) was calculated using the software $r_m$ 2.0 (Taberner et al., 1993). This software provides an estimate of the variance by means of a bootstrap resampling method. The number of replicates used for the calculation was set at 500, as recommended by Meyer et al. (1986).

In the ladybird beetle study, the frequencies of preimaginal survival, pupation, and eclosion rates were analyzed using $\chi^2$-tests. Preimaginal developmental time, mean pre-oviposition, and egg hatching rate were compared using Mann–Whitney *U*-tests because the assumptions for parametric analyses were not met. Adult weight and total fecundity were subjected to Student's *t*-tests. RM-ANOVA was conducted for daily fecundity, and pairwise Cox's *F*-test was used to compare the survival of males or females between the two treatments. The concentrations of Cry3Bb1 protein in females or males of *S. punctillum* after 5, 10, or 15 days of feeding on *Bt* maize-reared spider mites were compared using one-way ANOVA.

All analyses, except $r_m$, calculations, were conducted with STATISTICA (Version 7, StatSoft, Inc., Tulsa, OK, USA). Retrospective power analyses were conducted on non-significant ($P > 0.05$) results using PASS (Version 2005, NCCS, Kaysville, UT, USA) to avoid committing type II errors. Based on the observed control means and standard deviations and the true sample sizes, the detectable differences (percentage difference of detectable treatment means relative to control means) were calculated for $\alpha = 0.05$ and a power of 80% (Thomas, 1997). Detectable differences for means were based on *t*-tests and those for frequencies were based on $\chi^2$-tests.

## 3. Results

### 3.1. Effects of Bt maize on T. urticae

None of the developmental or reproductive parameters or the intrinsic rate of natural increase ($r_m$) differed between mites reared on *Bt* or non-*Bt* maize (Table 1). With the given means, standard deviations, and percentages of the control data, the difference detectable with a statistical power of 80% at $\alpha = 0.05$ ranged from 4% to 28% for the different parameters (Table 1). Daily fecundity of *T. urticae* was also similar on *Bt* and non-*Bt* maize (RM-ANOVA: $F_{1,34} = 0.90$, $P = 0.35$) (Fig. 1).

### 3.2. Prey-mediated effects of Bt maize on S. punctillum

None of the developmental parameters of *S. punctillum* differed for beetles fed with spider mites reared on *Bt* maize *vs.* non-*Bt* maize (Table 2). The fresh weights of emerging adult beetles (females and males) also did not differ between the two treatments. The mean pre-oviposition period of females was significantly shorter in the *Bt* treatment than in the non-*Bt* treatment (Table 2). Moreover, females had a significantly higher daily fecundity if they were fed with spider mites reared on *Bt* maize rather than on non-*Bt* maize (RM-ANOVA; $F_{1,49} = 4.30$, $P = 0.043$), with day × treatment interactions being non-significant ($F_{53,2597} = 4.30$, $P = 0.736$) (Fig. 2). Similarly, the total number of eggs laid by each female during the 56 days of the feeding experiment was higher in the *Bt* maize treatment, even though the difference was marginally not significant ($P = 0.06$) (Table 2). In addition, the egg hatching rate was significantly higher in the *Bt* treatment than in the non-*Bt* treatment (Table 2). With exception of the total fecundity, the detectable difference with a power of 80% at $\alpha = 0.05$ ranged from 4% to 21% (Table 2).

Males of *S. punctillum* had a higher survival rate than females in both *Bt* (Cox's *F*-test; $F_{12,24} = 2.07$, $P = 0.06$) and non-*Bt* ($F_{12,30} = 2.87$, $P = 0.008$) treatments (Fig. 3). Pair-wise survival analyses did not detect significant differences in the survivorship of either males ($F_{12,14} = 1.08$, $P = 0.44$) or females ($F_{24,30} = 1.62$, $P = 0.11$) between *Bt* and non-*Bt* treatments (Fig. 3).

### 3.3. Transfer of Cry3Bb1 through the food chain

Cry3Bb1 concentration (mean ± SE) in *Bt* maize leaves was 37.6 ± 4.30 μg/g fresh weight (FW) (Fig. 4). The concentration of toxin in spider mites that fed on *Bt* maize was 55.9% of the concentration in the leaves. The concentration of toxin in larvae of *S. punctillum* that were exclusively fed with *Bt* maize-reared spider mites for 3 or 6 days was 10.6% or 7.5%, respectively, of the concentration in leaves. No Cry3Bb1 was detected in pupae or in newly emerged adult beetles, i.e., adults that had not fed (Fig. 4). No Cry3Bb1 toxin was detected in any control (non-*Bt*) sample of plant or arthropod material.

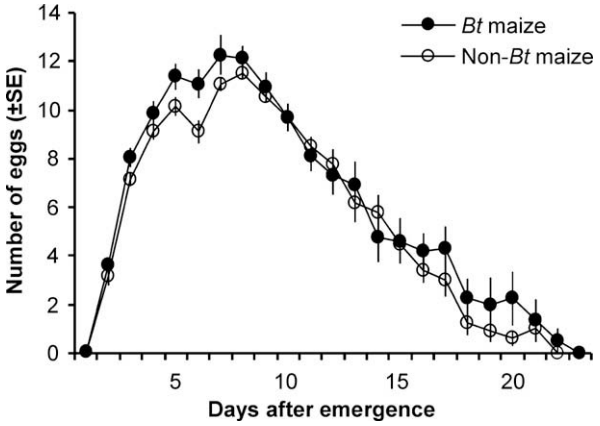


**Fig. 1.** Daily fecundity (eggs per female per day, mean ± SE) of *Tetranychus urticae* fed either Cry3Bb1-expressing *Bt* maize (MON88017) or the corresponding non-*Bt* maize. $N = 16–20$.

Cry3Bb1 concentrations were measured after adult beetles had consumed *Bt* maize-fed mites for 5, 10, or 15 days to clarify whether the toxin can accumulate in adults as a consequence of continuous ingestion. No measurable Cry3Bb1 toxin was present in the adults at the start of the experiment (day 0). In the adults that had received *Bt*-maize-fed spider mites for 5, 10, or 15 days, Cry3Bb1 concentrations ranged from 0.92 ± 0.260 to 1.06 ± 0.099 μg/g FW in females and from 0.08 ± 0.044 to 0.12 ± 0.076 μg/g FW in males. Differences among the three sampling dates were not significant for females (one-way ANOVA; $F_{2,10} = 0.11$, $P = 0.90$) or males ($F_{2,6} = 0.43$, $P = 0.67$). Similar Cry3Bb1 concentrations were detected in *S. punctillum* from the long-term feeding study in which adults were fed with *Bt*-fed spider mites for 56 days (females: 0.92 ± 0.136 μg/g FW; males: 0.17 ± 0.024 μg/g FW).

**Table 1**
Life-history parameters (means) of *Tetranychus urticae* fed Cry3Bb1-expressing *Bt* maize (MON88017) or the corresponding non-*Bt* maize.

| Parameters | *Bt* maize | | Non-*Bt* maize | | Statistics | Detectable difference (%)[a] |
|---|---|---|---|---|---|---|
| | *n* | mean | *n* | mean | | |
| Egg hatching rate (%) | 57 | 91.2 | 57 | 94.7 | $\chi^2 = 0.54$, $P = 0.46$ | 20 |
| Developmental time (days ± SE) | | | | | | |
| Egg | 52 | 4.0 ± 0.03 | 54 | 3.9 ± 0.04 | $U = 1246.0$, $P = 0.32$ | 4 |
| Larva | 51 | 1.7 ± 0.05 | 53 | 1.8 ± 0.06 | $U = 1322.0$, $P = 0.85$ | 13 |
| Protonymph | 48 | 1.8 ± 0.05 | 45 | 1.7 ± 0.06 | $U = 974.5$, $P = 0.42$ | 13 |
| Deutonymph | 40 | 1.9 ± 0.05 | 41 | 2.0 ± 0.04 | $U = 664.5$, $P = 0.14$ | 9 |
| Total immature stages | 40 | 9.2 ± 0.07 | 41 | 9.2 ± 0.08 | $U = 792.0$, $P = 0.79$ | 4 |
| Survival to adult stage (%) | 57 | 70.2 | 57 | 71.9 | $\chi^2 = 0.04$, $P = 0.84$ | 27 |
| Sex ratio (% females ± SE) | 169 | 62.8 ± 0.01 | 234 | 62.0 ± 0.007 | $\chi^2 = 0.02$, $P = 0.88$ | 15 |
| Pre-oviposition period (days ± SE) | 19 | 1.1 ± 0.05 | 25 | 1.1 ± 0.06 | $U = 206.0$, $P = 0.46$ | 24 |
| Oviposition period (days ± SE) | 16 | 14.8 ± 1.02 | 20 | 14.5 ± 0.90 | $U = 194.0$, $P = 0.91$ | 28 |
| Female longevity (days ± SE) | 16 | 17.3 ± 1.07 | 20 | 16.8 ± 1.05 | $t = 0.30$, $P = 0.77$ | 26 |
| Total fecundity (±SE) | 16 | 122.4 ± 8.38 | 20 | 111.4 ± 6.35 | $t = 1.00$, $P = 0.32$ | 26 |
| $r_m$ (95% CI) | 16 | 0.26 (0.25–0.27) | 20 | 0.25 (0.25–0.26) | | – |

[a] Retrospective power analyses were conducted to calculate the detectable difference as percentage difference of detectable treatment means relative to control means ($\alpha = 0.05$, power = 80%).

**Table 2**
Life-history parameters (means) of *Stethorus punctillum* fed *Tetranychus urticae* that were reared on Cry3Bb1-expressing *Bt* maize (MON88017) or on the corresponding non-*Bt* maize.

| Parameters | *Bt* maize | | Non-*Bt* maize | | Statistics | Detectable difference (%)[d] |
|---|---|---|---|---|---|---|
| | *n* | mean | *n* | mean | | |
| Preimaginal survival (%)[a] | 77 | 76.6 | 78 | 76.9 | $\chi^2 = 0.00$, $P = 0.99$ | 21 |
| Pupation rate (%)[b] | 74 | 93.2 | 75 | 93.3 | $\chi^2 = 0.00$, $P = 1.00$ | 16 |
| Eclosion rate (%)[c] | 69 | 85.5 | 70 | 85.7 | $\chi^2 = 0.00$, $P = 0.99$ | 21 |
| Preimaginal developmental time (days ± SE) | 59 | 10.7 ± 0.10 | 60 | 10.8 ± 0.10 | $U = 1582.5$, $P = 0.32$ | 4 |
| Female fresh weight (mg ± SE) | 32 | 0.51 ± 0.011 | 31 | 0.49 ± 0.017 | $t = 0.28$, $P = 0.78$ | 13 |
| Male fresh weight (mg ± SE) | 27 | 0.45 ± 0.014 | 29 | 0.47 ± 0.012 | $t = -0.69$, $P = 0.49$ | 10 |
| Pre-oviposition period (days ± SE) | 26 | 2.9 ± 0.21 | 25 | 3.3 ± 0.17 | $U = 212.0$, $P = 0.033$ | – |
| Total fecundity (± SE) | 26 | 383.8 ± 36.09 | 25 | 281.8 ± 39.5 | $t = 1.91$, $P = 0.06$ | 56 |
| Egg hatching rate (% ± SE) | 26 | 95.5 ± 0.60 | 24 | 94.8 ± 0.60 | $U = 250.0$, $P = 0.038$ | – |

[a] Number of pupae/number of first instars × 100.
[b] Number of pupae/number of last instars × 100.
[c] Number of emerged adults/number of pupae × 100.
[d] Retrospective power analyses were conducted for non-significant results to calculate the detectable difference as percentage difference of detectable treatment means relative to control means ($\alpha = 0.05$, power = 80%).

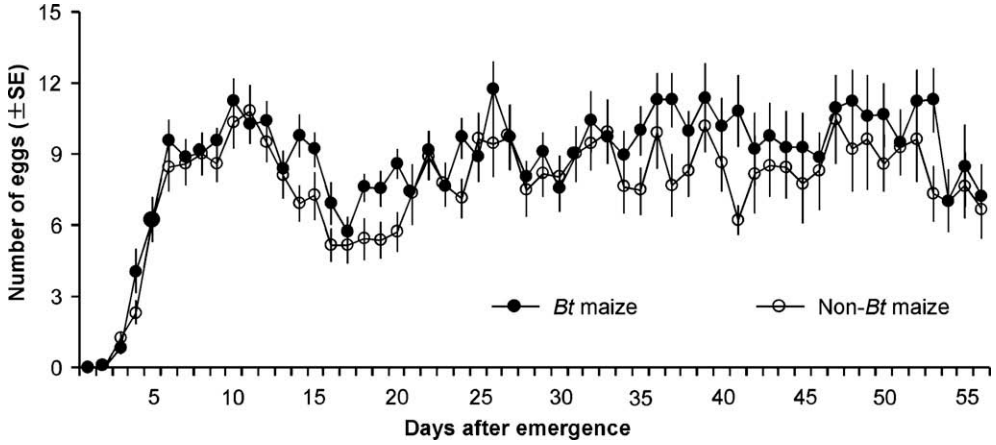


**Fig. 2.** Daily fecundity (eggs per female per day, mean ± SE) of *Stethorus punctillum* fed *Tetranychus urticae* that were reared on Cry3Bb1-expressing *Bt* maize (MON88017) or on the corresponding non-*Bt* maize for 8 weeks. *N* = 25–26.

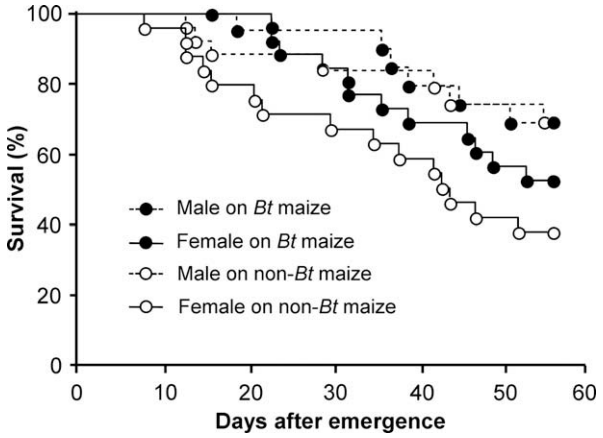


**Fig. 3.** Survival of *Stethorus punctillum* females and males fed *Tetranychus urticae* that were reared on Cry3Bb1-expressing *Bt* maize (MON88017) or on the corresponding non-*Bt* maize for 8 weeks. *N* = 25–26.

## 4. Discussion

### 4.1. Effects of Bt maize on T. urticae

Spider mites kept on *Bt* or non-*Bt* maize did not differ in any of the developmental or reproductive life-history parameters assessed. Because the study started with eggs collected from colonies that had been kept on *Bt* or non-*Bt* maize for several generations, our study also indicates that there are no long-term, multi-generational effects on this non-target herbivore. ELISA measurements revealed that spider mites feeding on *Bt* maize had ingested considerable amounts of the toxin. The data thus confirm that *T. urticae* is not affected by *Bt* maize and is not susceptible to Cry3Bb1. Consequently, indirect effects of Cry3Bb1-expressing maize (effects mediated by prey quality) on *S. punctillum* or other mite predators are not expected (Romeis et al., 2006). Similar to our results, previous studies with Cry1Ab-expressing Lepidoptera-protected maize varieties revealed no effect of the Cry protein on *T. urticae* (Dutton et al., 2002).

### 4.2. Prey-mediated effects of Bt maize on S. punctillum

No detrimental effect was found on the development and reproduction of *S. punctillum* when fed with *Bt* maize-reared spider mites *vs*. non-*Bt* maize-reared spider mites. Interestingly, however, female beetles had a shorter pre-oviposition period, an increased daily fecundity, and a higher fertility in the *Bt* treatment. The reasons for this are not clear but might be due to some unidentified differences in plant characteristics. Whether these apparently positive effects of *Bt* maize on *S. punctillum* have any relevance in the field is unclear.

*S. punctillum* larvae and adults were continuously exposed to *Bt* maize-fed prey during the whole larval development and 8 weeks

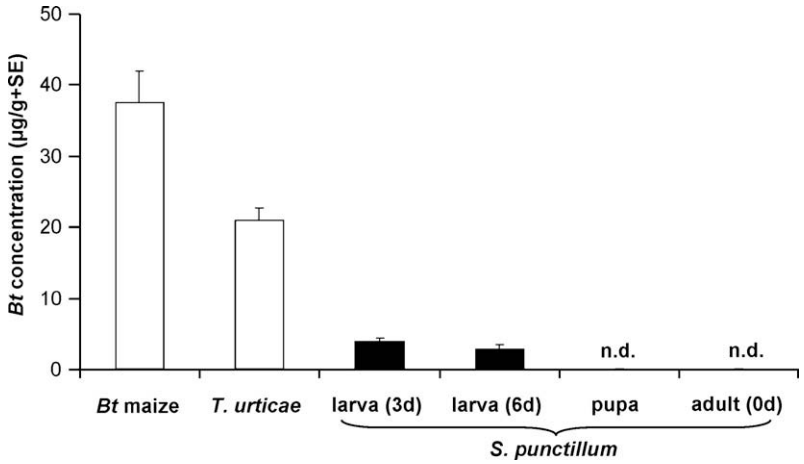


**Fig. 4.** Concentration (mean + SE) of Cry3Bb1 in *Bt* maize leaves, *Tetranychus urticae*, and different stages of *Stethorus punctillum* (based on fresh weight). *T. urticae* were collected from *Bt* maize. *S. punctillum* larvae were fed with *T. urticae* that were reared on *Bt* maize. Cry3Bb1 was analyzed in larvae after either 3 or 6 days of feeding on spider mites or in the pupal or newly emerged adult stage. $N = 3–5$; n.d., not detectable.

of the adult stage. This represents a worst-case exposure scenario because this species generally enters maize fields late in the season (after anthesis), when the mites are most abundant, and remains in these fields for only 1 month or so (Obrist et al., 2006a; Ramon Albajes, personal communication).

Spider mites fed on *Bt* maize in this study contained a high concentration of Cry3Bb1 toxin (approximately half of that in maize leaves). Sensitive insect bioassays with the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), have confirmed that the Cry3Bb1 contained in *T. urticae* has retained its full biological activity (Meissle and Romeis, 2009a). Previous studies on Cry1Ab-expressing *Bt* maize revealed similarly high Cry protein concentrations (Dutton et al., 2002) and also confirmed that the ingested protein retained its biological activity (Obrist et al., 2006b). We can therefore assume that *S. punctillum* was exposed to high levels of biologically active Cry3Bb1 protein throughout the duration of the feeding assay. The absence of any detrimental effect thus indicates a lack of toxicity of Cry3Bb1 to *S. punctillum* larvae and adults.

In agreement with our results, a number of studies have revealed no negative effect of purified Cry3Bb1 or Cry3Bb1-containing *Bt* maize pollen on ladybird beetles (Lundgren and Wiedenmann, 2002; Duan et al., 2002; Monsanto, 2004; Ahmad et al., 2006), carabids (Ahmad et al., 2006; Mullin et al., 2005; Duan et al., 2006) and non-Coleopteran arthropods (Duan et al., 2008; Li et al., 2008; Meissle and Romeis, 2009b). In addition, field studies consistently failed to detect population changes of above- or below-ground non-target arthropods in Cry3Bb1-transgenic maize fields when compared to non-transformed control maize (Al-Deeb and Wilde, 2003; Bhatti et al., 2005a,b; Bitzer et al., 2005; McManus et al., 2005; Ahmad et al., 2005, 2006; Hönemann et al., 2008). One study reported direct toxic effects of purified Cry3Bb on first instar *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae) (Schmidt et al., 2009). The results of this study were however questioned based on methodological limitations that create doubt about whether the increased mortality was caused by the ingestion of the toxin (Rauschen, in press; Ricroch et al., in press). Unfortunately, Schmidt et al. (2009) did not quantify the amount of Cry3Bb protein that was ingested by the ladybird larvae, making a comparison with our present study difficult.

### 4.3. Transfer of Cry3Bb1 through the food chain

Compared to relatively high concentrations of Cry3Bb1 in spider mite prey, the protein was substantially diluted in the next trophic level and did not accumulate in the beetles during exposure for up to 56 days. Concentrations in spider mites were six times higher than in *S. punctillum* larvae and 20 times higher than in adults. These laboratory results are in accordance with results from a recent field study in which the concentration of Cry1Ab (as total soluble protein) was more than seven times greater in *T. urticae* than in adult *S. punctillum* (Álvarez-Alfageme et al., 2008). One factor that might explain the low Cry3Bb1 concentrations measured in the predators is excretion, as previously reported for snowdrop lectin (*Galanthus nivalis* agglutinin) and larvae of two ladybird beetles (Hogervorst et al., 2006).

### 4.4. Implications for risk assessment

The non-target risk assessment for insect-resistant GE crops generally follows a tiered approach (Romeis et al., 2008b) starting with hazard (toxicity) studies in the laboratory where test species are exposed to high doses of purified toxin. More realistic and complex extended laboratory or semi-field tests are only conducted when potential hazards have been detected or when uncertainties about the risk remain. If higher tier studies are required, we believe that *S. punctillum* would be a suitable test organism, because it feeds exclusively on spider mites, which are known to contain high levels of biologically active Cry toxins when feeding on *Bt* maize.

In our present study, no adverse effect of Cry3Bb1-expressing *Bt* maize on the spider mite, *T. urticae*, or *S. punctillum* was detected in a set of laboratory studies where the test organisms were continuously exposed to high amounts of biologically active Cry3Bb1 protein. Because Cry3Bb1 expression levels of the glasshouse-grown *Bt* maize plants used in the studies were comparable to those reported from the field (Monsanto, 2004; Nguyen and Jehle, 2009), the conclusions drawn from our studies are transferrable to the field situation. Moreover, that *S. punctillum* larvae and adults were exposed to *Bt* maize-fed *T. urticae* for longer periods than would typically occur in the field increases the confidence in the relevance of the data. We therefore conclude that *S. punctillum* is unlikely to be harmed by the growing of Cry3Bb1-expressing *Bt* maize.

## Acknowledgments

We thank Michael Meissle and Fernando Álvarez-Alfageme (Agroscope ART) for discussion on the design of the experiments and comments on an earlier draft of the paper. Thanks go to Ismael Sánchez-Ramos (Departmento de Biotecnología, INIA-ALGENEX,

Madrid, Spain) for his kind help with calculation of the intrinsic rate of natural increase ($r_m$) of spider mites and to Monsanto for providing maize seeds. This research was funded by the National Centre of Competence in Research (NCCR) Plant Survival, research program of the Swiss National Science Foundation.

## References


Ahmad, A., Wilde, G.E., Zhu, K.Y., 2005. Detectability of coleopteran-specific Cry3Bb1 protein in soil and its effect on nontarget surface and below-ground arthropods. Environmental Entomology 34, 385–394.

Ahmad, A., Wilde, G.E., Whitworth, R.J., Zolnerowich, G., 2006. Effect of corn hybrids expressing the coleopteran-specific Cry3Bb1 protein for corn rootworm control on aboveground insect predators. Journal of Economic Entomology 99, 1085–1095.

Al-Deeb, M.A., Wilde, G.E., 2003. Effect of *Bt* corn expressing the Cry3Bb1 toxin for corn rootworm control on aboveground nontarget arthropods. Environmental Entomology 32, 1164–1170.

Álvarez-Alfageme, F., Ferry, N., Castañera, P., Ortego, F., Gatehouse, A.M.R., 2008. Prey mediated effects of *Bt* maize on fitness and digestive physiology of the red spider mite predator *Stethorus punctillum* Weise (Coleoptera: Coccinellidae). Transgenic Research 17, 943–954.

Bhatti, M.A., Duan, J., Head, G., Jiang, C., McKee, M.J., Nickson, T.E., Pilcher, C.L., Pilcher, C.D., 2005a. Field evaluation of the impact of corn rootworm (Coleoptera: Chrysomelidae)-protected *Bt* corn on ground-dwelling invertebrates. Environmental Entomology 34, 1325–1335.

Bhatti, M.A., Duan, J., Head, G., Jiang, C., McKee, M.J., Nickson, T.E., Pilcher, C.L., Pilcher, C.D., 2005b. Field evaluation of the impact of corn rootworm (Coleoptera: Chrysomelidae)-protected *Bt* corn on foliage-dwelling arthropods. Environmental Entomology 34, 1336–1345.

Biddinger, D.J., Weber, D.C., Hull, L.A., 2009. Coccinellidae as predators of mites: Stethorini in biological control. Biological Control 51, 268–283.

Birch, L.C., 1948. The intrinsic rate of natural increase of an insect population. Journal of Animal Ecology 17, 15–26.

Bitzer, R.J., Rice, M.E., Pilcher, C.D., Pilcher, C.L., Lam, W.F., 2005. Biodiversity and community structure of epedaphic and euedaphic springtails (Collembola) in transgenic rootworm *Bt* corn. Environmental Entomology 34, 1346–1376.

De la Poza, M., Pons, X., Farinós, G.P., López, C., Ortego, F., Eizaguirre, M., Castañera, P., Albajes, R., 2005. Impact of farm-scale *Bt* maize on abundance of predatory arthropods in Spain. Crop Protection 24, 677–684.

Duan, J.J., Head, G., McKee, M.J., Nickson, T.E., Martin, J.W., Sayegh, F.S., 2002. Evaluation of dietary effects of transgenic corn pollen expressing Cry3Bb1 protein on a non-target ladybird beetle, *Coleomegilla maculata*. Entomologia Experimentalis et Applicata 104, 271–280.

Duan, J.J., Paradise, M.S., Lundgren, J.G., Bookout, J.T., Jiang, C., Wiedenmann, R.N., 2006. Assessing nontarget impacts of *Bt* corn resistant to corn rootworms: tier-1 testing with larvae of *Poecilus chalcites* (Coleoptera: Carabidae). Environmental Entomology 35, 135–142.

Duan, J.J., Teixeira, D., Huesing, J.E., Jiang, C., 2008. Assessing the risk to nontarget organisms from *Bt* corn resistant to corn rootworm (Coleoptera: Chrysomelidae): tier-I testing with *Orius insidiosus* (Heteroptera: Anthocoridae). Environmental Entomology 37, 838–844.

Dutton, A., Klein, H., Romeis, J., Bigler, F., 2002. Uptake of *Bt*-toxin by herbivores feeding on transgenic maize and consequences for the predator *Chrysoperla carnea*. Ecological Entomology 27, 441–447.

Garcia-Alonso, M., Jacobs, E., Raybould, A., Nickson, T.E., Sowig, P., Willekens, H., van der Kouwe, P., Layton, R., Amijee, F., Fuentes, A.M., Tencalla, F., 2006. A tiered system for assessing the risk of genetically modified plants to non-target organisms. Environmental Biosafety Research 5, 57–65.

Hellmich, R.L., Albajes, R., Bergvinson, D., Prasifka, J.R., Wang, Z.Y., Weiss, M.J., 2008. The present and future role of insect-resistant GM crops in maize IPM. In: Romeis, J., Shelton, A.M., Kennedy, G.G. (Eds.), Integration of Insect-Resistant Genetically Modified Crops within IPM Programs. Springer Science + Business Media B.V., pp. 119–158.

Hogervorst, P.A.M., Ferry, N., Gatehouse, A.M.R., Wäckers, F.L., Romeis, J., 2006. Direct effects of snowdrop lectin (GNA) on larvae of three aphid predators and fate of GNA after ingestion. Journal of Insect Physiology 52, 614–624.

Hönemann, L., Zurbrügg, C., Nentwig, W., 2008. Effects of Bt-corn decomposition on the composition of the soil meso- and macrofauna. Applied Soil Ecology 40, 203–209.

James, C., 2008. Global Status of Commercialized Biotech/GM Crops: 2008. ISAAA Brief No. 39. International Service for the Acquisition of Agri-Biotech Applications, Ithaca, NY, USA.

Kennedy, G.G., 2008. Integration of insect-resistant genetically modified crops within IPM programs. In: Romeis, J., Shelton, A.M., Kennedy, G.G. (Eds.), Integration of Insect-resistant Genetically Modified Crops within IPM Programs. Springer Science + Business Media B.V., pp. 1–26.

Levine, E., Oloumi-Sadeghi, H., 1991. Management of Diabroticite rootworms in corn. Annual Review of Entomology 36, 229–255.

Li, Y., Meissle, M., Romeis, J., 2008. Consumption of *Bt* maize pollen expressing Cry1Ab or Cry3Bb1 does not harm adult green lacewings, *Chrysoperla carnea* (Neuroptera: Chrysopidae). PLoS ONE 3 (8), e2909. doi:10.1371/journal.pone.0002909.

Lundgren, J.G., Wiedenmann, R.N., 2002. Coleopteran-specific Cry3Bb toxin from transgenic corn pollen does not affect the fitness of a nontarget species, *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae). Environmental Entomology 31, 1213–1218.

McManus, B.L., Fuller, B.W., Boetel, M.A., French, B.W., Ellsbury, M.M., Head, G.P., 2005. Abundance of *Coleomegilla maculata* (Coleoptera: Coccinellidae) in corn rootworm-resistant Cry3Bb1 maize. Journal of Economic Entomology 98, 1992–1998.

Meinke, L.J., Sappington, T.W., Onstad, D.W., Guillemaud, T., Miller, N.J., Komáromi, J., Levay, N., Furlan, L., Kiss, J., Toth, F., 2009. Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) population dynamics. Agricultural and Forest Entomology 11, 29–46.

Meissle, M., Romeis, J., 2009a. Insecticidal activity of Cry3Bb1 expressed in *Bt* maize on larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). Entomologia Experimentalis et Applicata 131, 308–319.

Meissle, M., Romeis, J., 2009b. The web-building spider *Theridion impressum* (Araneae: Theridiidae) is not adversely affected by *Bt* maize resistant to corn rootworms. Plant Biotechnology Journal 7, 645–656.

Meyer, J.S., Ingersoll, C.G., McDonall, L.L., Boyce, M.S., 1986. Estimating uncertainty in population growth rates: Jackknife vs. bootstrap techniques. Ecology 67, 1156–1166.

Miller, N.J., Guillemaud, T., Giordano, R., Siegfried, B.D., Gray, M.E., Meinke, L.J., Sappington, T.W., 2009. Genes, gene flow and adaptation of *Diabrotica virgifera virgifera*. Agricultural and Forest Entomology 11, 47–60.

Monsanto, 2004. Petition for the determination of nonregulated status for MON 88017 corn. Available at: http://www.aphis.usda.gov/brs/aphisdocs/04_12501p.pdf.

Mullin, C.A., Saunders, M.C., Leslie, T.W., Biddinger, D.J., Fleischer, S.J., 2005. Toxic and behavioral effects to Carabidae of seed treatments used on Cry3Bb1- and Cry1Ab/c-protected corn. Environmental Entomology 34, 1626–1636.

Nguyen, H.T., Jehle, J.J., 2009. Expression of Cry3Bb1 in transgenic corn MON88017. Journal of Agricultural and Food Chemistry 57, 9990–9996.

Obrist, L.B., Dutton, A., Albajes, R., Bigler, F., 2006a. Exposure of arthropod predators to Cry1Ab toxin in *Bt* maize fields. Ecological Entomology 31, 143–154.

Obrist, L., Dutton, A., Romeis, J., Bigler, F., 2006b. Biological activity of Cry1Ab toxin expressed by Bt maize following ingestion by herbivorous arthropods and exposure of the predator *Chrysoperla carnea*. BioControl 51, 31–48.

Putman, W.L., 1955. Bionomics of *Stethorus punctillum* Weise (Coleoptera: Coccinellidae) in Ontario. The Canadian Entomologist 87, 9–33.

Rauschen, S., in press. A case of "pseudo science"? A study claiming effects of the Cry1Ab protein on larvae of the two-spotted ladybird is reminiscent of the case of the green lacewing. Transgenic Research 19, doi:10.1007/s11248-009-9301-0.

Raybould, A., 2006. Problem formulation and hypothesis testing for environmental risk assessment of genetically modified crops. Environmental Biosafety Research 5, 119–125.

Raybould, A., Stacey, D., Vlachos, D., Graser, G., Li, X., Joseph, R., 2007. Non-target organisms risk assessment of MIR604 maize expressing mCry3A for control of corn rootworms. Journal of Applied Entomology 131, 391–399.

Rice, M.E., 2004. Transgenic rootworm corn: Assessing potential agronomic, economic, and environmental benefits. Online. Plant Health Progress. doi:10.1094/PHP-2004-0301-01-RV, Available at: http://www.plantmanagementnetwork.org/pub/php/review/2004/rootworm/.

Ricroch, A., Bergé, J.B., Kuntz, M., in press. Is the German suspension of MON810 maize cultivation scientifically justified? Transgenic Res 19. doi:10.1007/s11248-009-9297-5.

Romeis, J., Meissle, M., Bigler, F., 2006. Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. Nature Biotechnology 24, 63–71.

Romeis, J., Van Driesche, R.G., Barratt, B.I.P., Bigler, F., 2008a. Insect-resistant transgenic crops and biological control. In: Romeis, J., Shelton, A.M., Kennedy, G.G. (Eds.), Integration of Insect-resistant Genetically Modified Crops within IPM Programs. Springer Science + Business Media B.V., pp. 87–117.

Romeis, J., Bartsch, D., Bigler, F., Candolfi, M.P., Gielkens, M.M.C., Hartley, S.E., Hellmich, R.L., Huesing, J.E., Jepson, P.C., Layton, R., Quemada, H., Raybould, A., Rose, R.I., Schiemann, J., Sears, M.K., Shelton, A.M., Sweet, J., Vaituzis, Z., Wolt, J.D., 2008b. Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. Nature Biotechnology 26, 203–208.

Romeis, J., Meissle, M., Raybould, A., Hellmich, R.L., 2009. Impact of insect-resistant transgenic crops on above-ground non-target arthropods. In: Ferry, N., Gatehouse, A.M.R. (Eds.), Environmental Impact of Genetically Modified Crops. CABI Publishing, Wallingford, pp. 165–198.

Rott, A.S., Ponsonby, D.J., 2000. Improving the control of *Tetranychus urticae* on edible glasshouse crops using a specialist coccinellid *Stethorus punctillum* Weise and a generalist mite *Amblyseius californicus* as biocontrol agents. Biocontrol Science and Technology 10, 487–498.

Sanvido, O., Romeis, J., Bigler, F., 2007. Ecological impacts of genetically modified crops: ten years of field research and commercial cultivation. Advances in Biochemical Engineering and Biotechnology 107, 235–278.

Schmidt, J.E.U., Braun, C.U., Whitehouse, L.P., Hilbeck, A., 2009. Effects of activated *Bt* transgene products (Cry1Ab, Cry3Bb) on immature stages of the ladybird *Adalia bipunctata* in laboratory ecotoxicity testing. Archives of Environmental Contamination and Toxicology 56, 221–228.

Shirai, Y., 2006. Laboratory evaluation of effects of transgenic *Bt* corn pollen on two non-target herbivorous ladybirds, *Epilachna vigintioctopunctata* (Coccinellidae) and *Galerucella vittaticollis* (Chrysomelidae). Applied Entomology Zoology 41, 607–611.

Spencer, J.L., Levine, E., Isard, S.A., Mabry, T.R., 2005. Movement, dispersal and behaviour of western corn rootworm adults in rotated maize and soybean fields. In: Vidal, S., Kuhlmann, U., Edwards, C.R. (Eds.), Western Corn Rootworm–Ecology and Management. CABI Publishing, Wallingford, pp. 121–144.

Taberner, A., Castañera, P., Silvestre, E., Dopazo, J., 1993. Estimation of the intrinsic rate of natural increase and its error by both algebraic and resampling approaches. Cabios 9, 535–540.

Thomas, L., 1997. Retrospective power analysis. Conservation Biology 11, 276–280.

Vaughn, T., Cavato, T., Brar, G., Coombe, T., DeGooyer, T., Ford, S., Groth, M., Howe, A., Johnson, S., Kolacz, K., Pilcher, C., Purcell, J., Romano, C., English, L., Pershing, J., 2005. A method of controlling corn rootworm feeding using a *Bacillus thuringiensis* protein expressed in transgenic maize. Crop Science 45, 931–938.

Ward, D.P., DeGooyer, T.A., Vaughn, T.T., Head, G.P., McKee, M.J., Astwood, J.D., Pershing, J.C., 2005. Genetically enhanced maize as a potential management option for corn rootworm: YieldGard® rootworm maize case study. In: Vidal, S., Kuhlmann, U., Edwards, C.R. (Eds.), Western Corn Rootworm–Ecology and Management. CABI Publishing, Wallingford, pp. 239–262.

Zurbrügg, C., Nentwig, W., 2009. Ingestion and excretion of two transgenic *Bt* corn varieties by slugs. Transgenic Research 18, 215–225.