

## RESEARCH ARTICLE

# Spider mite infestations reduce *Bacillus thuringiensis* toxin concentration in corn leaves and predators avoid spider mites that have fed on *Bacillus thuringiensis* corn

S.M. Prager<sup>1,2\*</sup>, X. Martini<sup>1,3\*</sup>, H. Guvvala<sup>4</sup>, C. Nansen<sup>1,5</sup> & J. Lundgren<sup>6</sup>

1 Department of Entomology, Texas AgriLife Research, Lubbock, TX, USA

2 Department of Entomology, University of California, Riverside, CA, USA

3 Entomology and Nematology Department, Citrus Research and Education Center, University of Florida, Lake Alfred, FL, USA

4 Department of Biological Sciences, Texas Tech University, Lubbock, TX, USA

5 School of Animal Biology, The UWA Institute of Agriculture, The University of Western Australia, Perth, WA, Australia

6 USDA-ARS, North Central Agricultural Research Laboratory, Brookings, SD, USA

## Keywords

Cry1Ab; Cry3Bb1; predation; *Phytoseiulus persimilis*; *Tetranychus cinnabarinus*.

## Correspondence

Sean M. Prager, Department of Entomology, University of California, Riverside, 900 University Ave., Riverside, CA, USA. Email: sprager@ucr.edu

\*These authors contributed equally to this study.

Received: 8 July 2013; revised version accepted: 3 February 2014; published online: 17 March 2014.

doi:10.1111/aab.12120

## Abstract

Perceived benefits of insecticidal transgenic crops include reduced usage of broad-based insecticides, and therefore lower risk to non-target organisms. Numerous studies have documented low or no direct toxicity of *Bacillus thuringiensis* (*Bt*)-derived toxins against non-target organisms, but there has been less research on (a) effects of secondary pest infestations on *Bt* expressing in crops and (b) behavioural responses by predators feeding on host arthropods from *Bt* crops – both topics are investigated in this study. We quantified predation by the obligate spider mite predator *Phytoseiulus persimilis* of carmine spider mites (*Tetranychus cinnabarinus*), reared on *Bt* or non-*Bt* corn (*Zea mays*). Both no-choice and two-choice studies were conducted. In addition, we quantified toxin levels in corn leaves with/without spider mite infestation. Under no-choice conditions, *P. persimilis* consumed non-*Bt* spider mites at a faster rate than *Bt* spider mites. Under two-choice conditions, *P. persimilis* spent more time in the vicinity of non-*Bt* spider mites than near *Bt* spider mites. Corn infested with spider mites exhibited lower toxin levels than non-infested plants. These results suggest potentially complex interactions among non-target herbivores, their natural enemies and *Bt* crops.

## Introduction

Transgenic crops containing genes derived from the soil bacterium, *Bacillus thuringiensis* Berliner (*Bt*), have been commercially available since 1996. These genes produce toxic proteins that specifically target coleopteran or lepidopteran pests (Schnepf *et al.*, 1998). However, concerns persist over how *Bt* crops may affect food webs within agroecosystems (Lövei *et al.*, 2009; Hilbeck *et al.*, 2014). The current approach to assessing non-target risks of transgenic insecticidal crops focuses primarily on direct toxicological effects. In the USA, where *Bt* crops are widely used, characterising non-target effects and assessing ecological compatibility of *Bt* crops and biodiversity have followed a tiered approach (USDA, 2007).

This approach is based on USDA standards developed for pesticide and chemical risk assessment and initially involves laboratory assays to assess the toxicity of a high dose of purified insecticidal protein against a small suite of non-target species that are indicative of functional or taxonomic guilds. If toxicity is observed, affected taxa are further examined under more realistic scenarios within the laboratory or under semi-field conditions. Finally, larger-scale field trials that examine the effects of *Bt* crops against arthropod abundance and community structure are conducted to validate observations conducted in the laboratory (Tencalla *et al.*, 2009). In Europe, the cultivation of genetically modified *Bt* crops is limited because of significant public opposition and concerns about potential adverse effects on health and environment (Myhr, 2010).

For a genetically modified organism to be regulatory approved in Europe, an environmental risk assessment is required (Hilbeck *et al.*, 2011). This environmental risk assessment has to evaluate both direct and indirect risks to human health and environment (The Council of the European Union, 2001).

However, despite these requirements, there is a relative paucity of studies investigating indirect effects of *Bt* toxins on the foraging behaviour and prey preference of natural enemies (Lundgren *et al.*, 2009; but see Desneux *et al.*, 2010; Liu *et al.*, 2011), and the effects that transgenic crops have on the behaviour of secondary (non-target) pests. This is despite the fact that secondary pests, such as spider mites on corn (*Zea mays* L.), can be important pests that may undermine the benefits of *Bt* crops (Men *et al.*, 2004; Zhao *et al.*, 2011). It also discounts the evidence that *Bt* toxins may affect the behaviour of non-target organisms and natural enemies. Critically, some of these risk assessments served as support for a ban on the MON810 corn line in Germany and in five other EU countries (Bøhn *et al.*, 2012; Hilbeck *et al.*, 2012).

Numerous studies have examined the toxicological effects of *Bt* toxins offered as either purified proteins or *Bt* plant tissue on various life history parameters of dozens of putative non-target organisms. However, despite this extensive literature, the effects of *Bt* crops on non-target organisms remain a controversial topic. The majority of the laboratory and field studies conclude that *Bt* toxins have no detectable toxicity to non-target insects (Sanvido *et al.*, 2007; Romeis *et al.*, 2008; Naranjo, 2009; Duan *et al.*, 2010; Peterson *et al.*, 2011; Alcantara, 2012). Yet, there are noticeable examples reporting that *Bt* crops were found to affect non-target arthropods. As an example, honeybees (*Apis mellifera* L.) exposed to Cry1Ab had disrupted learning performances (Ramirez-Romero *et al.*, 2008). It has also been demonstrated that *Bt* crops may adversely affect natural enemies. Studies on the lacewing, *Chrysoperla carnea* Stephens, showed the avoidance of prey items reared on *Bt*-expressing corn versus non-*Bt* isogenic controls (Meier & Hilbeck, 2001). Finally, the predatory mite *Phytoseiulus persimilis* Athias-Henriot prefers two-spotted spider mites (*Tetranychus urticae* Koch) reared on eggplant (*Solanum melongena* L.) that does not express Cry1Ab to conspecific spider mites reared on the same plant isolate that did not contain *Bt* genes (Zemkova Rovenska *et al.*, 2005). This is significant because two-spotted spider mites contained *Bt* toxins from *Bt* corn at levels above those found in the host plant (Obrist *et al.*, 2006b) and because studies measuring life history parameters of two-spotted spider mites showed that Cry1Ab toxin (Lozzia *et al.*, 2000; Dutton *et al.*, 2002) or Cry3Bb1 (Li & Romeis, 2010) contents were not associated with any fitness costs

for the spider mites (Hilbeck *et al.*, 1998a; Lövei *et al.*, 2009; Schmidt *et al.*, 2009; Stephens *et al.*, 2012).

Expression of transgenes in crops is influenced by a wide range of abiotic factors, including temperature (Zhou *et al.*, 2009), salinity (Luo *et al.*, 2008), vernalisation (Burgio *et al.*, 2011) and light intensity (Dong & Li, 2007). To our knowledge, few studies, have investigated whether herbivory affects *Bt* toxin concentrations in transgenic plants, although any alterations in *Bt* contents could have important implications for the efficacy and long-term sustainability of this technology Olsen *et al.* (2005).

The overall goal of this study was to examine interactions between spider mites, their predators and *Bt* corn. Under both no-choice and two-choice conditions, we quantified the predation rate by predatory mites (*P. persimilis*) of carmine spider mites (*Tetranychus cinnabarinus* Boudreaux), reared on corn containing genes for *Bt* toxins (*Bt*-mites) or not containing those genes (non-*Bt* mites). Spider mites may be considered important secondary pests on corn, and they can potentially undermine the economic benefits of growing *Bt* crops (Men *et al.*, 2004; Zhao *et al.*, 2011). In addition, we quantified toxin levels in corn leaves with/without spider mite infestation. The results showed that *P. persimilis* consumed *Bt*-mites at a lower rate than non-*Bt* mites, preferred non-*Bt* mites when given a choice, and that herbivory by this secondary pest of corn adversely affected the quantity of *Bt* toxin contained within by host plants. These results could have implications for how pest management and resistance management are approached in transgenic crops.

## Materials and methods

### Plant and mite cultures

Experiments were conducted using spider mites reared on one of two commercial corn varieties with similar genetic backgrounds (i.e. same parent cultivars), both of which are widely planted in West Texas. The *Bt* hybrid (207-03VT3, Monsanto, St. Louis, MO, USA) is a YieldguardVT triple-stacked hybrid that expresses two *Bt* toxins, Cry3Bb1 and Cry1Ab, along with glyphosate tolerance. The conventional (non-*Bt*) hybrid (207-01, Monsanto) contains similar base genetics to the *Bt* hybrid. The agronomic characteristics and susceptibility to diseases are the same between the two varieties (<http://www.channel.com>, accessed on 23 June 2013). Consequently, the only known and documented difference between the two varieties is the presence of *Bt*-derived genes in 207-03VT3 that are absent in 207-01. Both varieties have a maturity of 107 days, are glyphosate tolerant and were factory treated with the 500 rate (0.500 mg of a.i. per kernel) of the insecticide

Clothianidin (Poncho, Bayer CropScience, Research Triangle Park, NC, USA). Clothianidin has been shown not to affect the survival of spider mites (Cloyd & Bethke, 2011). The use of two commercially available hybrids with similar but not identical genetics is more reflective of a real-world scenario in which an organism is likely to encounter sister varieties rather than true isolines. Corn plants were grown in 3.8-L plastic pots with Metromix 900 Professional Growing Mix (Sun Gro Horticulture, Vancouver, BC, Canada) under greenhouse conditions at the Texas AgriLife Research and Extension Center (Lubbock, TX, USA). Corn plants were watered *ad libitum* and fertilised weekly with 238 ppm/N Peter's Professional 20:20:20 (N-P-K) growing media (J.R. Peters Inc, Allentown, PA, USA). To avoid accidental infestations of corn plants by spider mites, corn plants were grown in spider mite proof cages ( $96 \times 26$  mesh  $\text{cm}^{-2}$ ; Bug Dorm, MegaView Science, Taichung, Taiwan). A colony of carmine spider mites was maintained on non-*Bt* corn plants (DeKalb, IL, USA). Even though toxicology studies of *Bt* corn on carmine spider mites are lacking, field data indicated that *Bt* cotton does not control this pest (Cui & Xia, 2000). At the seven-leaf stage, about 4 weeks after emergence, eight plants each of the two varieties were infested with approximately 100 spider mites from the main colony. This process was repeated until all leaves of infested plants contained 100–200 spider mites each (approximately three times per plant). This level of infestation was high enough to maintain populations but did not create visible plant damage. Female spider mites were collected 7–21 days after the final addition of spider mites. Consequently, all but the most recently added spider mites would have been on the Monsanto corn for a generation, as longevity of these spider mites is approximately 3 weeks (Northcraft & Watson, 1987).

In this study, we used the predatory mite *P. persimilis* as a representative natural enemy. *P. persimilis* is an obligate predator of, and is widely used for biocontrol of, several species of spider mites. Commercially sourced *P. persimilis* (Syngenta Bioline, Oxnard, CA, USA) were used to start a laboratory colony that was reared in mite proof tents (Bug Dorm, MegaView Science) containing two to four each of tomato (*Solanum lycopersicum* L.) and bean (*Phaseolus vulgaris* L.) plants infested with carmine spider mites. The use of plant species other than corn ensured that predators were naïve to corn prior to use in bioassays. Cages were maintained at  $25 \pm 1^\circ\text{C}$  and  $60 \pm 5\%$  humidity. To maintain high humidity, a 1-L beaker containing distilled water was placed in each cage. In addition, bean plants were occasionally sprayed with water using a household misting bottle. To standardise the feeding level, and because a starved predator is likely to show less preference, we exposed *P. persimilis* to spider

mites from the rearing culture (spider mites from bean or tomato) in 10 cm diameter Petri dishes for 24 h prior to use in no-choice and choice experiments.

### No-choice experiment

To examine predator response to spider mites reared on either *Bt*- or non-*Bt*-expressing plants (*Bt*- and non-*Bt* mites, respectively), we conducted no-choice feeding experiments with individual *P. persimilis*. This experiment was conducted in arenas consisting of a 35-mm circular piece of P8 Fisherbrand<sup>®</sup> filter paper (Fisher Scientific, Pittsburgh, PA, USA) with a sticky and repelling (The Tanglefoot Company, Grand Rapids, MI, USA) border. Arenas contained one of four food sources (three spider mites in each arena): frozen ( $15$  min at  $-80 \pm 2^\circ\text{C}$ ) *Bt* spider mites, frozen non-*Bt* spider mites, live non-*Bt* spider mites, live *Bt* spider mites. Spider mites were frozen to prevent movement, and frozen mite treatments were included to test that freezing did not influence preference. A preliminary experiment indicated that a time of 310 min was enough for *P. persimilis* to consume two live spider mites, but rarely three were consumed in this time. Consequently, a period of 310 min was set as a maximum duration for the experiment. The consumption of spider mites was assessed after 10 min and every 30 min thereafter until 310 min after the onset of the experiment. A total of 10 replications were conducted for each food source.

### Two-choice experiment

*Phytoseiulus persimilis* movement was examined using an automated video-tracking system consisting of software (Ethovision; Noldus Information Technology, Leesburg, VA, USA) and a CCD video camera (Panasonic wv-cp484, Panasonic Corporation of North America, Newark, NJ, USA). The experiments were conducted in arenas consisting of 70-mm filter paper circles with a Tanglefoot border. Arenas were positioned 40 cm below the camera lens. Two tabletop lamps with soft cold 13 W white bulbs were placed 18 cm from the arenas to produce maximum contrast and uniform light for the tracking of *P. persimilis*. Using the Ethovision software, arenas were divided into two virtual zones provisioned with different prey items. As in the feeding experiment, freezing spider mites allowed us to restrict the prey items to a particular location, and to exclude confounding effects, such as defensive behaviour and webbing. As a control, we conducted a preliminary experiment in which one zone of the arena contained three frozen non-*Bt* spider mites in a row and the alternate zone contained three pencil marks similar in size to a spider mite. In a second experiment, three frozen *Bt* spider mites were placed in one zone of

the arena, and three frozen non-*Bt* spider mites were placed in the other. To avoid positional bias, treatments were randomly assigned to either side. Experiments were conducted for either 15 min (control) or 20 min (*Bt*/non-*Bt*), and total time spent by individual *P. persimilis* in each zone was recorded. A total of 42 and 35 replications were conducted for each of the two experiments.

### Enzyme linked immunosorbent assay

The concentration of *Bt* toxins was quantified in both *Bt* and non-*Bt* corn plants used for spider mite rearing using double antibody sandwich enzyme linked immunosorbent assays (ELISA) performed with a commercially available kit (Catalogue # PSM11200, Agdia, Elkhart, IN, USA). Fresh corn tissue was diluted 1:10 (weight: volume) in phosphate buffered saline (PBS) Tween-20 (hereafter PBST), and tissue was ground for 45 s with a plastic pestle. Samples (100 µL) were incubated for 1 h on the pre-coated plates; subsequently, the samples were ejected and washed 3× with PBST. The conjugate enzyme (100 µL) was then incubated for 1 h on the plates, ejected and the wells washed again were washed 3× with PBST. To detect Cry1Ab, 50 µL of *para*-nitrophenylphosphate (pNPP) substrate was added to each well, incubated at room temperature for 30 min and absorbance was read at 405 nm on a microplate reader (Plate Chameleon™, Hidex, Turku, Finland). The pNPP was ejected, and each well was washed thrice with PBST. To detect Cry3Bb1, 50 µL of TMB substrate solution was added to each well, incubated for 20 min, and absorbances were read at 590 nm. A series of sample-free control (PBS only) wells ( $n = 3$ ) were included on each plate; a sample was considered positive if its absorbance (or optical density) exceeded the mean negative absorbance + 3 SD of the negative control series (Sutula et al., 1986). Absorbance values below this threshold were adjusted to zero for subsequent analyses. Thresholds were plate-specific, but the cross-plate mean (SEM) absorbance value was  $0.30 \pm 0.01$ . For each treatment combination, corn tissue samples were collected randomly from middle tier leaves of 10 different plants at the six- to nine-leaf stage. A total of 20 individual plants were sampled (five for each infestation by *Bt* combination), for a total of 20 samples per treatment combination. In addition to plants, ELISA was also performed on the individual spider mites and *P. persimilis* used in experiments. However, ELISA did not prove sensitive enough to be accurate, and consequently those results were excluded.

### Statistical analysis

Statistical analyses were performed using the R statistical package version 2.11.1. Results from no-choice

experiments were tested using Cox's proportional hazards model (Cox, 1972; Crawley, 2007) for the time until the first and the second prey were consumed. In each instance, the model contained fixed factors for frozen versus alive, *Bt* or non-*Bt* and the interaction term. The interaction was not significant in any instance and was subsequently removed from the model. While no *P. persimilis* died of natural causes, some were caught in the Tanglefoot and these were censored as were those that did not consume any spider mites within the duration of the experiment. Results from two-choice experiments were analysed with a non-parametric Wilcoxon sign ranked sum test on differences. *Bt* toxin levels in corn plants were analysed separately for each toxin using a general linear model with the following treatment factors: spider mite presence, *Bt* or non-*Bt* hybrid, an interaction term and the date when samples were collected. The date term was included to account for abiotic factors that may influence *Bt* levels among planting groups. Date was not significant in either analyses and was subsequently removed from the models.

## Results

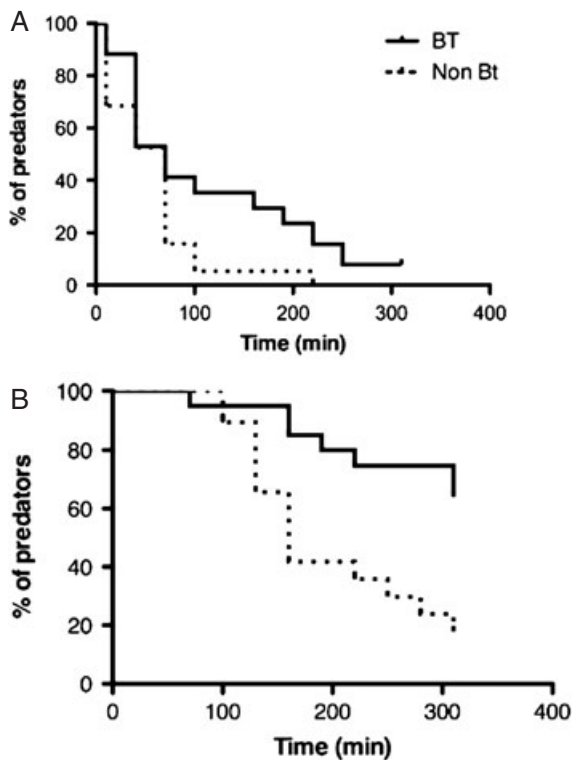
### No-choice experiment

After 310 min, three *P. persimilis* mites did not consume any spider mites, and six were caught in the Tanglefoot and then considered as censored values. The condition (frozen or live) of spider mites did not influence the predation rate of either non-*Bt* or *Bt* spider mites (Table 1), which confirmed that *a priori* freezing of spider mites did not affect their acceptability as a food source for *P. persimilis*. *P. persimilis* consumed both sources (frozen and alive) of non-*Bt* spider mites at a faster rate than *Bt* spider mites (Fig. 1; Table 1). The first non-*Bt* spider mite was always consumed during the 310-min period, but in some instances with *Bt* spider mites, no items were consumed. In both conditions, *P. persimilis* occasionally failed to feed on a second spider mite, but this was more common when presented with *Bt* spider mites. Similarly, *P. persimilis* individuals that consumed a *Bt* spider mite frequently failed to consume a second mite or took longer to do so (Fig. 1).

**Table 1** Cox analysis of prey consumption;  $n = 10$  for all treatments

	Covariate	$\beta$	SE ( $\beta$ )	(exp) $\beta$	z	P-value
First prey item	<i>Bt</i> or non- <i>Bt</i>	0.804	0.3598	2.234	2.23	<0.026
	Frozen or alive	0.049	0.3413	1.050	0.14	0.887
Second prey item	<i>Bt</i> or non- <i>Bt</i>	1.402	0.4754	4.064	2.95	<0.003
	Frozen or alive	-0.210	0.4427	0.811	-0.47	0.636

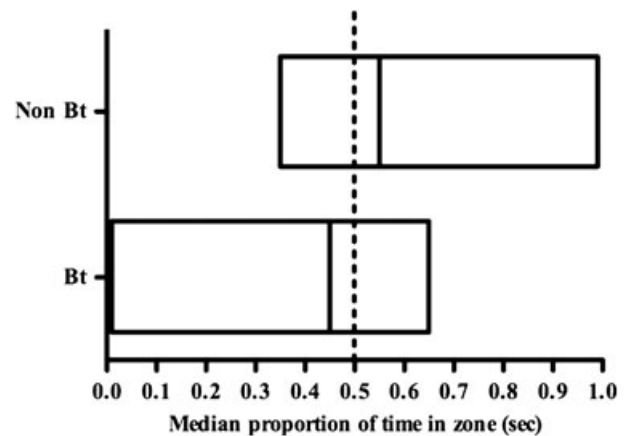
*Bt*, *Bacillus thuringiensis*; SE, standard error;  $\beta$ , estimated regression coefficients.



**Figure 1** The percent of *Phytoseiulus persimilis* that failed to consume prey over time (A) initial prey item and (B) subsequent prey item. Solid lines indicate when prey items were spider mites reared on *Bacillus thuringiensis* (*Bt*) corn; dashed lines indicate that spider mites were reared on non-*Bt* corn. There were 10 replications for each treatment; because preferences of frozen and live spider mites did statistically differ, data were pooled. During the experiment, six *P. persimilis* were caught in the Tanglefoot and added as censored data.

### Two-choice experiment

*Phytoseiulus persimilis* spent significantly more time in the zone containing frozen spider mites ( $411.5 \pm 147.2$  s) than in the zone with pencil marks ( $264.0 \pm 154.1$  s) (Wilcoxon signed ranks test:  $W = 350$ ,  $P < 0.01$ ,  $n = 21$ ). This indicated that the *P. persimilis* could distinguish the frozen spider mites from other objects and supported the use of frozen spider mites in the experiment. When offered a choice between frozen non-*Bt* and *Bt* spider mites, *P. persimilis* mites spent significantly more time in the zone containing non-*Bt* spider mites (Wilcoxon signed ranks test:  $W = 133$ ,  $P < 0.03$ ,  $n = 35$ ) (Fig. 2). Specifically, the majority of time was spent in the half of arenas containing non-*Bt* spider mites. Moreover, *P. persimilis* mites never spend an entire observation period with *Bt* spider mites, whereas they will spend the full period on the side of the arena with non-*Bt* spider mites (Fig. 2).



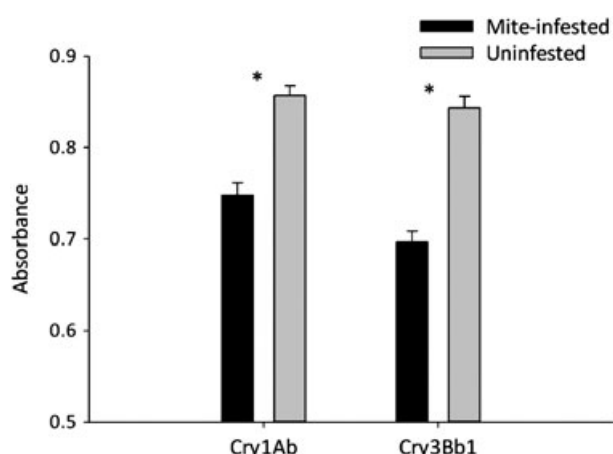
**Figure 2** Median proportion of time spent on the side of the arena containing frozen spider mites reared on *Bt*-corn or reared on non-*Bt* corn. Boxes indicate maximum and minimum, solid line indicates median and the dashed vertical line indicates 50%.

### Enzyme linked immunosorbent assays

Cry toxin quantity was significantly affected by whether the plant was *Bt* or non-*Bt* and whether it was infested by spider mites or not. For both Cry3Bb1 (*Bt* versus non-*Bt*:  $F_{1,75} = 7114$ ,  $P < 0.0001$ ; mite versus no mite:  $F_{1,75} = 66.42$ ,  $P < 0.0001$ ; interaction:  $F_{1,75} = 66.77$ ,  $P < 0.0001$ ) and Cry1Ab (*Bt* versus non-*Bt*:  $F_{1,71} = 8776$ ,  $P < 0.0001$ ; mite versus no mite:  $F_{1,71} = 28.58$ ,  $P < 0.0001$ ; interaction:  $F_{1,71} = 36.75$ ,  $P < 0.0001$ ), mite infestation significantly reduced Cry toxin in the leaf tissue on the *Bt* plants (Fig. 3). As expected, there was no effect of spider mite infestation on Cry toxin levels in non-*Bt* control plants; these plants did not contain Cry toxins. However, spider mite infestation was associated with reduced toxin levels in spider mite-infested plants (Fig. 3), with average absorbances on the non-*Bt* plants  $< 0.001$  for Cry1Ab, and  $< 0.01$  for Cry3Bb1.

### Discussion

The results presented here constitute the first evaluation of behavioural responses of an obligate spider mite predator to spider mites reared on pyramid stacked *Bt* corn. These results inform our current understanding of how *Bt* crops may affect food webs in agricultural systems, and we identified a particular interaction that merits additional research from the perspective of non-target risk assessments for *Bt* crops. Specifically, we found that in a no-choice experiment, *P. persimilis* individuals consumed non-*Bt* spider mites at a faster rate than spider mites reared on *Bt* corn. We also found that when given a choice, *P. persimilis* individuals spend more time in the vicinity of non-*Bt* spider mites than *Bt* spider mites.



**Figure 3** Cry toxin contents [represented as absorbance from the enzyme linked immunosorbent assays (ELISA) assays] of genetically modified corn plants with or without herbivorous mite infestations. Asterisks indicate significant differences between treatments within a Cry toxin ( $P = 0.0001$ ).

Our behavioural results compliment those of Zemkova Rovenska *et al.* (2005) who found that *P. persimilis* consumed significantly fewer spider mites that had fed on eggplant expressing the Cry3Bb protein than spider mites that had been reared on non-*Bt* eggplants. Interestingly, the authors also found that the spider mites preferred to settle on the leaves of *Bt* eggplant leaves rather than on non-*Bt* eggplant leaves, suggesting that spider mites may use *Bt* eggplant as a refuge. Conversely, Esteves Filho *et al.* (2010) found no preference when the predatory mite *Phytoseiulus macropilis* Banks was exposed to spider mites reared on cotton plants expressing Cry1Ac or spider mites reared on non-*Bt* cotton plants. Lacewings [*C. carnea*] showed a significant preference for Egyptian cotton leafworms [*Spodoptera littoralis* (Boisduval)] fed non-*Bt*-corn versus conspecifics fed transgenic corn expressing Cry1Ab toxin (Meier & Hilbeck, 2001). However, no preference was observed when lacewings had a choice between plum aphids (*Rhopalosiphum padi* L.) fed *Bt* and non-*Bt* corn (Meier & Hilbeck, 2001). Finally, a coccinellid beetle (*Harmonia axyridis* Pallas) did not show any preferences between *Spodoptera* larvae fed *Bt* and non-*Bt* corn (Dutra *et al.*, 2012). Consequently, it appears that avoidance by predators of prey items fed on *Bt* host plants is more system specific than a universal behaviour.

Ingestion and accumulation of Cry toxin by spider mites is a possible explanation for the observed preference of *P. persimilis* for non-*Bt* spider mites. Indeed, previous studies have demonstrated the presence of Cry toxins in two-spotted spider mites (*T. urticae*) exposed to *Bt*-corn (Obrist *et al.*, 2006a,b). In contrast, Obrist *et al.* (2006a,b)

found negligible toxin presence in aphids (*Metopolophium dirhodum* Walker), thrips (*Frankliniella tenuicornis* Uzel) and leafhoppers (*Zyginidia* spp.) and suggest that factors such as corn cultivar and phenology (stage relative to flowering) may influence Cry toxin accumulation.

As noted previously, life history parameters of two-spotted spider mites are not influenced by the ingestion of *Bt* toxins (Dutton *et al.*, 2002; Obrist *et al.*, 2006c; Li & Romeis, 2010). Similarly, when reared on spider mites known to contain Cry1Ab, the predatory mite *Neoseiulus cucumeris* did not differ in any life history trait from controls reared on a non-*Bt* near isoline (Obrist *et al.*, 2006c). This would suggest that the behavioural patterns we observed are probably not associated with a fitness consequence to the predators. *P. persimilis* has complex and well-studied foraging behaviour that includes the ability to learn (Rahmani *et al.*, 2009) and to distinguish among volatiles under various scenarios (i.e. Takabayashi & Dicke, 1992; Gols *et al.*, 1999; Krips *et al.*, 1999; Dicke *et al.*, 2003). It is likely that these same mechanisms are involved with the behaviours observed in this study. The specific cues and factors involved with these behaviours will require further investigation. Such follow up could include exposure to pure proteins; however, studies have shown differing responses between microbial *Bt* sprays and toxins acquired indirectly from plants (Hilbeck *et al.*, 1998a,b; Dutton *et al.*, 2003).

These results may help answer questions about the susceptibility of *Bt* corn to infestation by spider mites. Several studies performed in China demonstrated resurgences of spider mites in *Bt*-cotton (Yang *et al.*, 2005; Yu *et al.*, 2011; Zhao *et al.*, 2011). This indicates that spider mites may take advantage of *Bt* crops. A decrease in predation rate by *P. persimilis* due to behavioural avoidance is one possible mechanistic explanation of these findings. Although *Bt* crops also receive less insecticide applications, which in turn contributes to keeping populations of natural enemies higher, and this might be expected to offset the decreased predation due to behavioural effects. How these two factors compensate for each other needs to be studied in more detail.

Our controlled greenhouse studies indicated that <2 weeks of feeding by relatively moderate numbers of spider mites reduced the amounts of *Bt* toxins found in corn leaves. This is a clear indication that herbivory by spider mites results in a reduction in *Bt*-toxin levels in leaf tissue. To our knowledge, this is the first report of such a trend. In fact, to the contrary, Olsen *et al.* (2005) showed that damage by chewing or sucking insects do not induce changes in the expression of Cry1Ac in cotton. Given the novelty of this finding, what mechanisms are involved is unclear. The reduction may derive from a plant response that turns off *Bt*-protein production,

degradation of proteins within the plant, changes in the levels of photosynthesis or loss of chlorophyll. Regardless of mechanisms involved, this is an important finding that warrants further research. In particular, studies are required that specifically address the implications for the effectiveness of resistance management plans for Bt crops.

This research provides clear evidence for two potential ways in which a secondary pest could potentially undermine one of the key benefits of Bt corn: its ability to reduce the need for insecticide applications. First, consuming Bt corn may give spider mites a refuge from their predator community, thereby necessitating insecticidal inputs to manage secondary pest outbreaks. This aspect is a particular concern with the introduction of blended bags of Bt and non-Bt crop seeds (compared to physically separated non-Bt crop refuges in specific blocks), as it means that Bt and non-Bt crop plants are mixed and therefore enable easy movement by spider mites between adjacent plants. In addition, if spider mites alter the levels of Cry toxin in Bt corn, the effectiveness of this technology in controlling target herbivore pests may be partially compromised, potentially leading to resistance in pest populations. Additional research is needed to explore whether either of these scenarios occurs under realistic conditions.

In summary, the results presented here strongly underscore the importance of comprehensive assessments of the impact of Bt toxins on food webs in agricultural food webs. Toxicity studies are important and necessary. However, it is also clear that factors such as behaviour, plant physiological effects and the differences among Cry toxins must also be considered when considering the ecological risks posed by transgenic crops. We offer additional support to the idea that Cry toxins will influence predator behaviour, but also demonstrate that these effects may be specific to certain toxins, rather than a response to all Bt events. In addition, we have demonstrated that there may be critically important (and so far neglected) feedback mechanisms between herbivory and Bt toxin expression in crop plants. We suggest that future assessments of Bt toxins need to go beyond toxicity and consider the numerous potential indirect ecological impacts of these crops.

### Acknowledgements

We thank Patrick Porter for advice on experimental design and Kathy Vaughn for colony maintenance. Mention of any proprietary products does not constitute endorsement by the USDA.

### References

Alcantara E.P. (2012) Postcommercialization monitoring of the long-term impact of Bt corn on non-target arthropod communities in commercial farms and adjacent riparian

areas in the Philippines. *Environmental Entomology*, **41**, 1268–1276.

Bøhn T., Primicerio R., Traavik T. (2012) The German ban on GM maize MON810: scientifically justified or unjustified? *Environmental Sciences Europe*, **24**, 1–7.

Burgio G., Dinelli G., Marotti I., Zurla M., Bosi S., Lanzoni A. (2011) Bt-toxin uptake by the non-target herbivore, *Myzus persicae* (Hemiptera: Aphididae), feeding on transgenic oilseed rape in laboratory conditions. *Bulletin of Entomological Research*, **101**, 241–247.

Cloyd R.A., Bethke J.A. (2011) Impact of neonicotinoid insecticides on natural enemies in greenhouse and interiorscape environments. *Pest Management Science*, **67**, 3–9.

Cox D.R. (1972) Regression models and life-tables (with discussion). *Journal of the Royal Statistical Society, Series B*, **34**, 187–220.

Crawley M.J. (2007) *The R Book*. West Sussex, UK: Wiley.

Cui J.J., Xia J.Y. (2000) Effects of Bt (*Bacillus thuringiensis*) transgenic cotton on the dynamics of pest population and their enemies. *Acta Phytophyca Sinica*, **27**, 141–145.

Desneux N., Ramirez-Romero R., Bokonon-Ganta A., Bernal J. (2010) Attraction of the parasitoid *Cotesia marginiventris* to host (*Spodoptera frugiperda*) frass is affected by transgenic maize. *Ecotoxicology*, **19**, 1183–1192.

Dicke M., De Boer J.G., Höfte M., Rocha-Granados M.C. (2003) Mixed blends of herbivore-induced plant volatiles and foraging success of carnivorous arthropods. *Oikos*, **101**, 38–48.

Dong H., Li W. (2007) Variability of endotoxin expression in Bt transgenic cotton. *Journal of Agronomy and Crop Science*, **193**, 21–29.

Duan J.J., Lundgren J.G., Naranjo S., Marvier M. (2010) Extrapolating non-target risk of Bt crops from laboratory to field. *Biology Letters*, **6**, 74–77.

Dutra C.C., Koch R.L., Burkness E.C., Meissle M., Romeis J., Hutchison W.D., Fernandes M.G. (2012) *Harmonia axyridis* (Coleoptera: Coccinellidae) exhibits no preference between Bt and non-Bt maize fed *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *PLoS One*, **7**, e44867.

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.

Dutton A., Romeis J., Bigler F. (2003) Assessing the risks of insect resistant transgenic plants on entomophagous arthropods Bt-maize expressing Cry1Ab as a case study. *BioControl*, **48**, 611–636.

Esteves Filho A.B., de Oliveira J.V., Torres J.B., Gondim M.G. Jr. (2010) Compared biology and behaviour of *Tetranychus urticae* Koch (Acari: Tetranychidae) and *Phytoseiulus macropilis* (Banks) (Acari: Phytoseiidae) on Bollgard and non-transgenic isoline cotton. *Neotropical Entomology*, **39**, 338–344.

- Gols R., Posthumus M.A., Dicke M. (1999) Jasmonic acid induces the production of gerbera volatiles that attract the biological control agent *Phytoseiulus persimilis*. *Entomologia Experimentalis et Applicata*, **93**, 77–86.
- Hilbeck A., Baumgartner M., Fried P.M., Bigler F. (1998a) Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environmental Entomology*, **27**, 480–487.
- Hilbeck A., Moar W.J., Pusztai-Carey M., Filippini A., Bigler F. (1998b) Toxicity of *Bacillus thuringiensis* Cry1Ab toxin to the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environmental Entomology*, **27**, 1255–1263.
- Hilbeck A., Meier M., Römbke J., Jänsch S., Teichmann H., Tappeser B. (2011) Environmental risk assessment of genetically modified plants – concepts and controversies. *Environmental Sciences Europe*, **23**, 1–12.
- Hilbeck A., McMillan J.M., Meier M., Humbel A., Schläpfer-Miller J., Trtikova M. (2012) A controversy re-visited: Is the coccinellid *Adalia bipunctata* adversely affected by Bt toxins? *Environmental Sciences Europe*, **24**, 1–12.
- Hilbeck A., Weiss G., Oehen B., Römbke J., Jänsch S., Teichmann H., Lang A., Otto M., Tappeser B. (2014) Ranking matrices as operational tools for the environmental risk assessment of genetically modified crops on non-target organisms. *Ecological Indicators*, **36**, 367–381.
- Krips O.E., Willems P.E.L., Gols R., Posthumus M.A., Dicke M. (1999) The response of *Phytoseiulus persimilis* to spider mite-induced volatiles from Gerbera: influence of starvation and experience. *Journal of Chemical Ecology*, **25**, 2623–2641.
- Li Y., Romeis J. (2010) Bt maize expressing Cry3Bb1 does not harm the spider mite, *Tetranychus urticae*, or its ladybird beetle predator, *Stethorus punctillum*. *Biological Control*, **53**, 337–344.
- Liu X., Chen M., Onstad D., Roush R., Shelton A. (2011) Effect of Bt broccoli and resistant genotype of *Plutella xylostella* (Lepidoptera: Plutellidae) on development and host acceptance of the parasitoid *Diadegma insulare* (Hymenoptera: Ichneumonidae). *Transgenic Research*, **20**, 887–897.
- Lövei G.L., Andow D.A., Arpaia S. (2009) Transgenic insecticidal crops and natural enemies: a detailed review of laboratory studies. *Environmental Entomology*, **38**, 293–306.
- Lozzia G.C., Rigamonti I.E., Manachini B., Rocchetti R. (2000) Laboratory studies of the effects of transgenic corn on the spider mite *Tetranychus urticae* Koch. *Bolletino di Zoologia Agraria e di Bachicoltura*, **32**, 35–47.
- Lundgren J.G., Gassmann A.J., Bernal J., Duan J.J., Ruberson J. (2009) Ecological compatibility of GM crops and biological control. *Crop Protection*, **28**, 1017–1030.
- Luo Z., Dong H., Li W., Ming Z., Zhu Y. (2008) Individual and combined effects of salinity and waterlogging on *Cry1Ac* expression and insecticidal efficacy of Bt cotton. *Crop Protection*, **27**, 1485–1490.
- Meier M.S., Hilbeck A. (2001) Influence of transgenic *Bacillus thuringiensis* corn-fed prey on prey preference of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Basic and Applied Ecology*, **2**, 35–44.
- Men X., Ge F., Edwards C., Yardim E. (2004) Influence of pesticide applications on pest and predatory arthropods associated with transgenic Bt cotton and nontransgenic cotton plants. *Phytoparasitica*, **32**, 246–254.
- Myhr A.I. (2010) A precautionary approach to genetically modified organisms: challenges and implications for policy and science. *Journal of Agricultural and Environmental Ethics*, **23**, 501–525.
- Naranjo S.E. (2009) Impacts of Bt crops on non-target invertebrates and insecticide use patterns. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, **4**, 011.
- Northcraft P.D., Watson T.E. (1987) Developmental biology of *Tetranychus cinnabarinus* (Boisduval) under three temperature regimes. *Southwestern Entomologist*, **12**, 45–50.
- 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.B., 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.
- Obrist L.B., Klein H., Dutton A., Bigler F. (2006c) Assessing the effects of BT maize on the predatory mite *Neoseiulus cucumeris*. *Experimental and Applied Acarology*, **38**, 125–139.
- Olsen K.M., Daly J.C., Finnegan E.J., Mahon R.J. (2005) Changes in Cry1Ac Bt transgenic cotton in response to two environmental factors: temperature and insect damage. *Journal of Economic Entomology*, **98**, 1382–1390.
- Peterson J.A., Lundgren J.G., Harwood J.D. (2011) Interactions of transgenic *Bacillus thuringiensis* insecticidal crops with spiders (Araneae). *Journal of Arachnology*, **39**, 1–21.
- Rahmani H., Hoffmann D., Walzer A., Schausberger P. (2009) Adaptive learning in the foraging behavior of the predatory mite *Phytoseiulus persimilis*. *Behavioral Ecology*, **20**, 946–950.
- Ramirez-Romero R., Desneux N., Decourtye A., Chaffiol A., Pham-Delegue M.H. (2008) Does Cry1Ab protein affect learning performances of the honey bee *Apis mellifera* L. (Hymenoptera, Apidae)? *Ecotoxicology and Environmental Safety*, **70**, 327–333.
- 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. (2008) Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. *Nature Biotechnology*, **26**, 203–208.
- Sanvido O., Romeis J., Bigler F. (2007) Ecological impacts of genetically modified crops: ten years of field research



- and commercial cultivation. In *Green Gene Technology*, pp. 235–278. Eds A. Fiechter and C. Sautter. Berlin, Germany: Springer.
- Schmidt J.E., 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.
- Schnepf E., Crickmore N., Van Rie J., Lereclus D., Baum J., Feitelson J., Zeigler D.R., Dean D.H. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews*, **62**, 775–806.
- Stephens E.J., Losey J.E., Allee L.L., DiTommaso A., Bodner C., Breyre A. (2012) The impact of Cry3Bb Bt-maize on two guilds of beneficial beetles. *Agriculture, Ecosystems & Environment*, **156**, 72–81.
- Sutula C.L., Gillett J.M., Morrissey S.M., Ramsdell D.C. (1986) Interpreting ELISA data and establishing the positive–negative threshold. *Plant Disease*, **70**, 722–726.
- Takabayashi J., Dicke M. (1992) Response of predatory mites with different rearing histories to volatiles of uninfested plants. *Entomologia Experimentalis et Applicata*, **64**, 187–193.
- Tencalla F., Nickson T., Garcia-Alonso M. (2009) Environmental risk assessment. In *Environmental Impact of Genetically Modified Crops*, pp. 61–73. Eds N. Ferry and A. Gatehouse. Wallingford, UK: CAB International.
- The Council of the European Union (2001) Directive 2001/18/EC of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EC, 17.4. *Official Journal of the European Communities*, **L106**, 1–38.
- USDA. (2007) White paper on tier-based testing for the effects of proteinaceous insecticidal plant-incorporated protectants on non-target invertebrates for regulatory risk assessments. Ed. R.I. Rose. USDA. URL [http://www.aphis.usda.gov/brs/pdf/NTO\\_White\\_Paper\\_1.pdf](http://www.aphis.usda.gov/brs/pdf/NTO_White_Paper_1.pdf) [accessed on 29 November 2013].
- Yang P., Iles M., Yan S., Jolliffe F. (2005) Farmers' knowledge, perceptions and practices in transgenic Bt cotton in small producer systems in Northern China. *Crop Protection*, **24**, 229–239.
- Yu H., Li Y., Wu K. (2011) Risk assessment and ecological effects of transgenic *Bacillus thuringiensis* crops on non-target organisms. *Journal of Integrative Plant Biology*, **53**, 520–538.
- Zemkova Rovenska G., Zemek R., Schmidt J.E., Hilbeck A. (2005) Altered host plant preference of *Tetranychus urticae* and prey preference of its predator *Phytoseiulus persimilis* (Acari : Tetranychidae, Phytoseiidae) on transgenic Cry3Bb-eggplants. *Biological Control*, **33**, 293–300.
- Zhao J., Ho P., Azadi H. (2011) Benefits of Bt cotton counterbalanced by secondary pests? Perceptions of ecological change in China. *Environmental Monitoring and Assessment*, **173**, 985–994.
- Zhou G-S., Zhou F-C., Xie Y-M., Feng C-N., Yang Y-Z. (2009) Effects of temperature stress on Bt insecticidal protein expression in Bt transgenic cotton leaves and death rate of cotton bollworm. *Cotton Science*, **21**, 302–306.