

ORIGINAL CONTRIBUTION

Bacillus thuringiensis* plants expressing Cry1Ac, Cry2Ab and Cry1F are not toxic to the assassin bug, *Zelus renardiiH.-H. Su^{1,2}, J.-C. Tian^{1,3}, S. E. Naranjo⁴, J. Romeis⁵, R. L. Hellmich⁶ & A. M. Shelton¹

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Abstract

Cotton- and maize-producing insecticidal crystal (Cry) proteins from the bacterium, *Bacillus thuringiensis* (Bt), have been commercialized since 1996. Bt plants are subjected to environmental risk assessments for non-target organisms, including natural enemies that suppress pest populations. Here, we used Cry1F-resistant *Spodoptera frugiperda* (J.E. Smith) and Cry1Ac and Cry2Ab-resistant *Trichoplusia ni* (Hübner) as prey for the assassin bug, *Zelus renardii* (Kolenati), a common predator in maize and cotton fields. In tritrophic studies, we assessed several fitness parameters of *Z. renardii* when it fed on resistant *S. frugiperda* that had fed on Bt maize expressing Cry1F or on resistant *T. ni* that had fed on Bt cotton expressing Cry1Ac and Cry2Ab. Survival, nymphal duration, adult weight, adult longevity and female fecundity of *Z. renardii* were not different when they were fed resistant-prey larvae (*S. frugiperda* or *T. ni*) reared on either a Bt crop or respective non-Bt crops. ELISA tests demonstrated that the Cry proteins were present in the plant at the highest levels, at lower levels in the prey and at the lowest levels in the predator. While *Z. renardii* was exposed to Cry1F and Cry1Ac and Cry2Ab when it fed on hosts that consumed Bt-transgenic plants, the proteins did not affect important fitness parameters in this common and important predator.

Introduction

Genetically engineered insect-resistant crops, producing insecticidal crystal (Cry) proteins from the bacterium *Bacillus thuringiensis* (Bt), have revolutionized insect control (Shelton et al. 2002) and become a major tool for integrated pest management (IPM) programmes (Romeis et al. 2008). The commercial production of Bt crops is considered another form of host plant resistance, which is a cornerstone of IPM (Kennedy 2008). In 2013, these crops were grown on nearly 75 million ha worldwide (James 2013). The majority of Bt crops have been designed for control of

Lepidoptera by expressing Cry1Ab, Cry1Ac, Cry1F, Cry2Ab and Vip3A proteins.

Another cornerstone of IPM is biological control (Naranjo et al. 2008) and numerous studies have been conducted to assess whether Bt crops disrupt the biological function of important natural enemies (reviewed by Romeis et al. 2006; Naranjo 2009). The consumption of prey that have fed on Bt crops and ingested Cry proteins represents a critical route of exposure to arthropod natural enemies and is referred to as a tritrophic exposure pathway. When a prey or host species feeds on a Bt crop and is susceptible to the plant-produced Cry

protein, it typically suffers deleterious effects. Furthermore, when a predator feeds on these compromised prey, it may also suffer negative effects on various life-history traits. An approach to eliminate these potential prey/host-quality-mediated effects when assessing the direct effects of the Bt crop on the predator is to utilize prey or hosts that have evolved resistance to Cry proteins (Chen et al. 2008; Lawo et al. 2010; Li et al. 2011; Tian et al. 2012, 2013, 2014). Thus, the natural enemy can then be exposed to realistic levels of Cry proteins but not simultaneously suffer from any associated prey- or host-quality-mediated effects.

Zelus renardii (Kolenati) (Hemipteran: Reduviidae) is a generalist natural enemy that is broadly distributed in the New World (Ables 1978; Weirauch et al. 2012). It injects lethal venom (Cohen 1993) when it inserts its proboscis in prey and then consumes the host's contents. Adults and nymphs feed on caterpillars and many insect species in multiple crops, including cotton and maize and can be an important biological control agent (Lingren et al. 1968; Ables 1978; Cortez and Trujillo 1994; Cisneros and Rosenheim 1998). This predator species has received little attention relative to risk assessment in Bt crops (Ponsard et al. 2002). However, because Bt cotton and Bt maize are grown in regions where *Z. renardii* occurs, it is important to determine if it may be harmed by feeding on prey that have consumed tissue from Bt crops.

Here, we used Cry1F-resistant *Spodoptera frugiperda* (J.E. Smith) and Cry1Ac/Cry2Ab-resistant *Trichoplusia ni* (Hübner) populations as prey for *Z. renardii*. These resistant caterpillars were allowed to feed on Cry1F maize and Cry1Ac/Cry2Ab cotton, or their respective non-Bt near-isolines, and then fed to *Z. renardii*. Nymphal survival and development time, adult longevity, mass, fecundity and egg-hatching rates of *Z. renardii* were evaluated. Additionally, the amount of each Cry protein was determined in the leaf tissue, the host insect and the predator.

Materials and Methods

Plants

Seeds of Bt maize (Mycogen 2A517, Herculex[®] I, event TC1507), producing Cry1F protein, and the corresponding non-transformed near-isoline (Mycogen 2A496) were obtained from Dow AgroSciences (Indianapolis, IN) and grown in the greenhouses at Cornell University's New York State Agricultural Experiment Station in Geneva, NY. The two maize varieties were

grown in Ray Leach Cone-tainer Cells (diam. 3.8 cm; depth 21 cm; vol. 164 ml) (Stuewe & Sons, Tangent, OR) with Cornell Mix potting soil (Boodley and Shelldrake 1977) and 500 ml Power-Gro liquid fertilizer (Wilson Laboratories Inc., Dundas, ON, Canada) was applied weekly. When maize plants reached the tasselling stage but before pollen was shed, leaves were fed to *S. frugiperda* larvae. All maize plants were grown in the same greenhouse at $21 \pm 2^\circ\text{C}$ under a 16 : 8 L : D regime.

Seeds of Bt cotton (BollGard II[®], event 15895), which has genes coding for Cry1Ac and Cry2Ab, and the corresponding non-transformed near-isoline Stoneville 474, were obtained from Monsanto Company (St. Louis, MO). The two cotton varieties were grown in 6-l plastic pots with Cornell Mix potting soil. Approximately, 6 g Osmocote[®] Plus release fertilizer (Scotts, Marysville, OH) was placed in each pot and 500 ml Power-Gro liquid fertilizer was applied weekly. When cotton reached the seedling stage, cotton leaves were fed to *T. ni*. All cotton plants were grown in the same greenhouse at $27 \pm 2^\circ\text{C}$ under a 16 : 8 L : D regime.

Insects

A Cry1F-resistant strain of *S. frugiperda* was obtained from Dow AgroSciences in 2010 and reared on artificial diet (General Purpose Lepidoptera #F9772; Bio-Serv Inc., Frenchtown, NJ) in our Cornell University laboratory. This strain developed resistance to Cry1F maize in Puerto Rico (Storer et al. 2010) and is able to survive on Cry1F maize (Tian et al. 2012, 2014).

A Cry1Ac/Cry2Ab-resistant *T. ni* strain (GLEN-BGII) was originally collected from commercial greenhouses in British Columbia and was further selected on Cry1Ac/Cry2Ab cotton foliage. Previous studies have shown that Cry1Ac/Cry2Ab-resistant *T. ni* larvae can survive well on Cry1Ac/Cry2Ab cotton plants (Li et al. 2011; Tian et al. 2013, 2014).

Zelus renardii were collected in cotton fields near the USDA-ARS, Arid-Land Agricultural Research Center, Maricopa, AZ during 2012. *Z. renardii* were subsequently reared in our laboratory by feeding them eggs and larvae of *Plutella xylostella* (Lepidoptera: Plutellidae), which were reared on artificial diet (Shelton et al. 1991). Newly hatched (<12 h old) *Z. renardii* nymphs were used in bioassays.

All insect strains were maintained in a climatic chamber at $27 \pm 1^\circ\text{C}$, $50 \pm 10\%$ RH and 16 : 8 L : D regime. All experiments were conducted under these same conditions.

Expression of Cry proteins in Bt cotton and Bt maize

Three leaf samples (20 mg per sample) were collected from Bt plants and non-Bt plants at different plant growth stages (see tables 1 and 2). The second new leaf of cotton and maize was sampled at each stage, weighed and kept at -20°C until Cry protein levels were measured within 1 month.

Tritrophic bioassay with *Z. renardii*

To evaluate the effect of Cry1F on *Z. renardii* growth and development, 1st instar *Z. renardii* were individually kept in 59-ml plastic transparent cups and supplied with Cry1F maize-fed or non-Bt maize-fed *S. frugiperda* larvae. As the body size of *Z. renardii* increased, the 59-ml cups were replaced by 237-ml cups. Second to 3rd instar *S. frugiperda* were provided *ad libitum* to *Z. renardii* nymphs. *S. frugiperda* larvae were changed daily, and *Z. renardii* nymphs were checked daily at the same time. The survival and developmental duration of each nymphal stage was recorded. Sixty *Z. renardii* neonates were used for each treatment.

The sex of newly emerged *Z. renardii* adults was determined and they were weighed. Fifteen mating pairs of newly emerged adults from each treatment were randomly selected and used to assess fecundity. Each pair was kept in a 946-ml plastic box and fed Cry1F maize-fed or non-Bt maize-fed *S. frugiperda* larvae for 40 days. Eggs were removed and counted daily. All egg masses from each treatment were put into individual 59-ml plastic cups to monitor egg-hatching rates. One pair of *Z. renardii* from Cry1F maize treatment and four pairs of *Z. renardii* from non-Bt maize treatment were excluded from statistical analysis as one or both *Z. renardii* were killed during handling.

For assessing the effect of Cry1Ac and Cry2Ab on *Z. renardii*, bioassays were performed as described

above but using 2nd–3rd instar of the Cry1Ac/Cry2Ab-resistant strain of *T. ni* and Cry1Ac/Cry2Ab cotton and non-transformed cotton plants. Thirty *Z. renardii* neonates were used in the bioassay for each treatment, and 10 mated pairs of adults were used for assessing fecundity and fertility. Two pairs of *Z. renardii* from Cry1Ac/Cry2Ab cotton treatment and one pair of *Z. renardii* from non-Bt cotton treatment were excluded from statistical analysis as one or both *Z. renardii* were killed during handling.

Cry protein residue in insects

Another 60 1st instar *Z. renardii* for each treatment were reared as described for the tritrophic bioassay. When they reached 4th and 5th instars, nine *Z. renardii* (three *Z. renardii* per replication) were collected for each development stage in each treatment. *S. frugiperda* fed with tasselling stage Cry1F maize or non-Bt maize were collected as 2nd and 3rd instars. *T. ni* fed with seedling stage Cry1Ac/Cry2Ab cotton or non-Bt cotton were also collected as 2nd and 3rd instars. For each prey sample, three replications (10 prey per replication) were used. Cry1F, Cry1Ac and Cry2Ab titres in insect samples were determined by ELISA.

ELISA measurement

The concentration of Cry1F in maize leaves and insects, and those of Cry1Ac and Cry2Ab in cotton leaves and insects, were measured by ELISA using Cry1F detection kits from Agdia (Elkhart, IN) and Cry1Ac and Cry2Ab detection kits from EnviroLogix (Portland, ME). Kits were identified as: QualiPlate™ Kit for Cry1Ab/Cry1Ac – AP 003 CRBS, QuantiPlate™ Kit for Cry2A – AP 005 and Bt-Cry1F ELISA Kit (Quantitative) PSP 11700. Prior to analysis, all insects were washed with PBST buffer four times to remove any Cry protein from the surface. Leaf samples were

Table 1 Cry1F concentration ($\mu\text{g/g}$ fresh weight) in Cry1F maize leaves, *Spodoptera frugiperda* larvae and *Zelus renardii* nymphs

	Maize	<i>S. frugiperda</i> larvae		<i>Z. renardii</i> nymphs	
Trifoliolate stage	5.23 \pm 0.67 a	2nd instar	0.41 \pm 0.02 a	4th instar	0.06 \pm 0.01 a
Before jointing stage	4.59 \pm 0.18 a	3rd instar	0.23 \pm 0.04 b	5th instar	0.08 \pm 0.01 a
Tasselling stage	2.64 \pm 0.05 b				
Blossom stage	2.45 \pm 0.17 b				
	F = 23.47; d.f. = 9; P < 0.01		t = 4.14; d.f. = 4; P = 0.01		t = 1.41; d.f. = 4; P = 0.23

Means (\pm SE) followed by different letters in the same column are significantly different (One-way ANOVA or Student's *t*-test, P < 0.05).

S. frugiperda were fed with tasselling stage Cry1F maize leaves before being sampled.

Z. renardii were fed with 2nd–3rd instar Cry1F maize-fed *S. frugiperda* before being sampled.

Table 2 Cry1Ac and Cry2Ab concentrations ($\mu\text{g/g}$ fresh weight) in Cry1Ac/Cry2Ab cotton leaves, *Trichoplusia ni* larvae and *Zelus renardii* nymphs

	Cotton	<i>T. ni</i> larvae	<i>Z. renardii</i> nymphs
Cry1Ac			
Seedling stage	2.08 \pm 0.01 a	2nd instar	0.22 \pm 0.05 a
Bloom stage	1.63 \pm 0.03 a	3rd instar	0.21 \pm 0.04 a
Boll opening stage	0.58 \pm 0.29 b		
F = 22.22; d.f. = 8; P < 0.01		t = 0.07; d.f. = 4; P = 0.95	t = 5.01; d.f. = 4; P = 0.02
Cry2Ab			
Seedling stage	28.58 \pm 0.92 a	2nd instar	0.48 \pm 0.10 a
Bloom stage	10.34 \pm 3.27 b	3rd instar	0.37 \pm 0.21 a
Boll opening stage	5.29 \pm 0.41 b		
F = 38.52; d.f. = 8; P < 0.01		t = 0.02; d.f. = 4; P = 0.99	t = 4.78; d.f. = 4; P = 0.04

Means (\pm SE) followed by different letters in the same column of Cry1Ac or Cry2Ab are significantly different (One-way ANOVA or Student's *t*-test, $P < 0.05$).

T. ni were fed with seedling stage Cry1Ac/Cry2Ab cotton leaves before being sampled.

Z. renardii were fed with 2nd–3rd instar Cry1Ac/Cry2Ab cotton -fed *T. ni* before being sampled.

diluted at a rate of 1 : 20 (mg sample: μl PBST buffer) and fully ground by mortar and pestle. Insect samples were diluted at a rate of at least 1 : 10 (mg sample: μl PBST buffer) in 1.5 ml-centrifuge tubes, and ground by hand using a plastic pestle. ELISA was performed according to the manufacturer's instructions.

Statistical analyses

Data on Cry proteins in plant leaves were analysed using one-way analysis of variance (ANOVA) and the Tukey's HSD test; data on Cry protein residues in insects were analysed using Student's *t*-test. Data on survival and life-table parameters of *Z. renardii* were analysed using the Wilcoxon test and Student's *t*-test, respectively. All statistical calculations were performed with SAS version 9.1 package (SAS Institute 2001). To avoid committing type II errors, that is failing to reject a false null hypothesis, retrospective power analyses were conducted on non-significant results ($P > 0.05$) using PASS 12 (Hintze 2013). 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%. Depending on the statistical method that was applied, detectable differences for means were calculated based on Student's *t*-tests or chi-square tests.

Results

Cry proteins in Bt maize, *S. frugiperda* and *Z. renardii*

Leaves of the Cry1F maize variety contained Cry1F at levels ranging from 2.45 to 5.23 $\mu\text{g/g}$ fresh weight

(FW) (table 1). These amounts are similar to Cry1F maize Event DAS-06275-8 that contained 10.7 to 23.8 $\mu\text{g/g}$ dry weight of maize leaves (which was \sim 5 times higher than fresh weight) (USEPA 2005). The highest concentration of Cry1F was recorded at the trifoliate stage and decreased as the plant grew. Cry1F residues in *S. frugiperda* ranged from 0.23 to 0.41 $\mu\text{g/g}$ FW (table 1) and significantly decreased from 2nd instar to 3rd instar. The Cry1F concentrations in *Z. renardii* nymphs were \sim 3- to 7-fold lower compared to those in *S. frugiperda* larvae, ranging from 0.06 to 0.08 $\mu\text{g/g}$ FW (table 1). In this case, the Cry1F concentration did not change significantly as *Z. renardii* grew. As expected, no Cry1F was detected in any samples from non-Bt maize and non-Bt maize-fed insects.

Cry proteins in Bt cotton, *T. ni* and *Z. renardii*

The Bt cotton variety contained Cry1Ac at levels ranging from 0.58 to 2.08 $\mu\text{g/g}$ FW of cotton leaves and Cry2Ab from 5.29 to 28.58 $\mu\text{g/g}$ FW of cotton leaves (table 2). These amounts are similar to Bollgard II cotton that contained 6.50 to 32.5 $\mu\text{g/g}$ FW of cotton leaves (USEPA 2003). Cry1Ac/Cry2Ab cotton leaves contained the highest concentration of Cry proteins at the seedling stage and significantly decreased as the plants developed. The average Cry1Ac residue in Bt cotton-fed *T. ni* ranged from 0.21 to 0.22 $\mu\text{g/g}$ FW, and 0.37 to 0.48 $\mu\text{g/g}$ FW for Cry2Ab (table 2). The Cry1Ac concentrations in *Z. renardii* nymphs were at least 1.7-fold lower than those of *T. ni* larvae, ranging from 0.01 to 0.13 $\mu\text{g/g}$ FW. For Cry2Ab, the concentration was at least 2.7-fold lower than those of *T. ni* larvae, ranging from 0.03 to 0.13 $\mu\text{g/g}$ FW (table 2). In both cases for *Z. renardii* nymphs, Cry protein concentrations were significantly higher in 4th instars

compared to 5th instars. As expected, no Cry protein was detected in any samples from non-Bt cotton and non-Bt cotton-fed insects.

Tritrophic bioassay with *Z. renardii*

Except for a significant difference in the developmental duration of the 4th stadia, there were no significant differences in any other developmental stages, survival and longevity of *Z. renardii* when fed *S. frugiperda* reared on Bt or non-Bt maize (table 3). The 0.5 days slower growth in 4th instars fed prey that had fed on Bt maize did not affect the total development time to adulthood. There were no significant differences in fresh weight of newly emerged female and male adults between the Bt maize and the control treatment. Fecundity and egg hatch rate were also not significantly different between the two treatments when one pair from the Bt treatment and four pairs from non-Bt treatment were excluded from analysis because of *Z. renardii* killed by handling or females with infertile eggs. Retrospective power analyses revealed detectable effect sizes between 9% and 55% depending on the parameter measured. The most sensitive parameters with detectable effect sizes below 30% were nymphal survival and development time, adult longevity and adult fresh weight.

The 4th stadia of *Z. renardii* fed with Bt cotton-fed *T. ni* grew significantly faster (0.5 days) than those fed with non-Bt cotton-fed *T. ni*; but there was no significant difference in total development time or any other life-table parameters (table 4). Two pairs from

the Bt treatment and one pair from non-Bt treatment were excluded from fecundity and egg-hatching rate analysis for *Z. renardii* because they were killed in handling or females had infertile eggs. Retrospective power analyses revealed detectable effect sizes between 8% and 67% depending on the parameter measured. The most sensitive parameters with detectable effect sizes below 30% were nymphal development time and adult fresh weight.

Discussion

Our study revealed no biologically significant toxic effects of Cry1F producing maize and Cry1Ac/Cry2Ab producing cotton on the predator *Z. renardii*. There were no significant differences in survival rate, adult longevity, fresh weight of newly moulted males or females, total fecundity and fertility between *Z. renardii* that consumed *S. frugiperda* fed Cry1F maize and a non-Bt near-isoline. Although duration of the 4th stadia of *Z. renardii* was slightly longer (by ca. 8%) when they fed on prey exposed to Bt maize, there was no significant difference in duration of the entire nymphal stage between the two groups. Similarly, there were no significant differences in any of the recorded life-table parameters between *Z. renardii* fed with *T. ni* reared on Bollgard II and those fed with *T. ni* reared on non-Bt near-isoline cotton leaves. Although duration of the 4th stadia of *Z. renardii* fed with *T. ni* reared on Bollgard II was slightly shorter (by ca. 9%), there were no significant differences in duration of the entire nymphal stage between the two groups.

Table 3 Tritrophic effects on life-table parameters of *Zelus renardii* when fed with Cry1F-resistant *Spodoptera frugiperda* larvae that were reared on Cry1F maize leaves or non-Bt near-isoline maize leaves

Parameters	Cry1F maize	Non-Bt near-isoline	Statistics	Detectable difference (%)
Survival (%)	60.0	60.0	$\chi^2 = 0.05$; d.f. = 1; P = 0.82	27
Development time (days)				
1st stadia	8.0 ± 0.2 (50)	8.2 ± 0.2 (51)	$t = 0.38$; d.f. = 99; P = 0.70	9
2nd stadia	6.1 ± 0.2 (37)	6.1 ± 0.3 (36)	$t = 0.16$; d.f. = 98; P = 0.87	20
3rd stadia	5.7 ± 0.2 (36)	5.7 ± 0.2 (36)	$t = 0.12$; d.f. = 70; P = 0.91	14
4th stadia	6.8 ± 0.2 (36)	6.3 ± 0.2 (36)	$t = 2.04$; d.f. = 70; P = 0.04	13
5th stadia	9.6 ± 0.2 (36)	9.4 ± 0.2 (36)	$t = 0.92$; d.f. = 70; P = 0.36	9
Nymphs to adult (days)	36.4 ± 0.6 (36)	34.3 ± 0.8 (36)	$t = 1.93$; d.f. = 70; P = 0.06	9
Adult longevity (days)	29.1 ± 2.4 (27)	34.5 ± 1.7 (26)	$t = 0.57$; d.f. = 51; P = 0.57	20
Male fresh weight (mg)	17.4 ± 0.86 (13)	17.0 ± 0.48 (14)	$t = 0.58$; d.f. = 25; P = 0.57	12
Female fresh weight (mg)	22.5 ± 0.71 (18)	23.6 ± 0.55 (22)	$t = 1.21$; d.f. = 38; P = 0.24	10
Total fecundity (eggs/female)	276.7 ± 19.8 (14)	256.5 ± 32.3 (11)	$t = 0.59$; d.f. = 23; P = 0.56	49
Egg-hatching rate (%)	79.9 ± 4.8 (14)	76.9 ± 5.0 (11)	$t = 0.43$; d.f. = 23; P = 0.67	55

Data are means ± SE. Number of replications is given in parenthesis. The experiment started with 60 nymphs in each treatment. Treatment means were compared using Wilcoxon test or Student's *t*-test. The detectable difference was calculated for $\alpha = 0.05$ and a power of 80%.

Table 4 Tritrophic effects on life-table parameters of *Zelus renardii* when fed with Cry1Ac/Cry2Ab-resistant *Trichoplusia ni* larvae that were reared on Cry1Ac/Cry2Ab cotton leaves or non-Bt near-isoline cotton leaves

Parameters	Cry1Ac/Cry2Ab cotton	Non-Bt near-isoline	Statistics	Detectable difference (%)
Survival (%)	66.7	76.7	$\chi^2 = 0.29$; d.f. = 1; P = 0.59	39
Development time (days)				
1st stadia	6.3 ± 0.4 (25)	6.2 ± 0.4 (24)	$t = 0.14$; d.f. = 47; P = 0.89	26
2nd stadia	5.8 ± 0.2 (24)	6.2 ± 0.3 (24)	$t = 1.33$; d.f. = 46; P = 0.19	19
3rd stadia	5.0 ± 0.3 (23)	5.1 ± 0.2 (24)	$t = 0.54$; d.f. = 45; P = 0.59	16
4th stadia	5.3 ± 0.2 (22)	5.8 ± 0.2 (23)	$t = 2.27$; d.f. = 45; P = 0.03	14
5th stadia	8.5 ± 0.1 (20)	8.5 ± 0.2 (23)	$t = 0.08$; d.f. = 41; P = 0.94	9
Larva to adult stage (days)	30.6 ± 0.5 (20)	32.1 ± 0.6 (23)	$t = 1.77$; d.f. = 41; P = 0.09	8
Adult longevity (days)	27.2 ± 1.1 (16)	26.9 ± 3.1 (12)	$t = 0.08$; d.f. = 26; P = 0.93	44
Male fresh weight (mg)	20.9 ± 0.6 (10)	20.0 ± 0.5 (15)	$t = 0.87$; d.f. = 23; P = 0.39	12
Female fresh weight (mg)	24.6 ± 1.7 (11)	25.3 ± 1.0 (9)	$t = 0.69$; d.f. = 17; P = 0.50	16
Total fecundity (eggs/female)	181.3 ± 17.8 (8)	244.1 ± 33.9 (9)	$t = 1.58$; d.f. = 17; P = 0.13	61
Egg-hatching rate (%)	88.3 ± 2.6 (8)	85.9 ± 4.4 (9)	$t = 0.34$; d.f. = 15; P = 0.74	67

Data are means ± SE. Number of replications is given in parenthesis. The experiment started with 30 nymphs in each treatment. Treatment means were compared using Wilcoxon test or Student's *t*-test. The detectable difference was calculated for $\alpha = 0.05$ and a power of 80%.

The reason for the differences in the duration of the 4th stadium of *Z. renardii* in the Bt and non-Bt treatment, one longer and the other shorter, are unclear, but such minor effects have been reported from other laboratory studies (Li and Romeis 2010). Given that the observed differences were less than the observation interval of the experiment and that overall nymphal develop was unaffected, these results suggest no casual relationship of the differences and they would have little biological significance. For most of the parameters recorded, retrospective power analyses revealed detectable effect sizes below 30%. We thus consider the tests sufficiently sensitive. For comparison, detectable effect size of 30% or 50% is suggested for laboratory toxicity studies with insecticidal proteins by the European Food Safety Authority (EFSA 2010) or the US Environmental Protection Agency (Rose 2007), respectively. Parameters that were generally less sensitive included *Z. renardii* fecundity and egg-hatching rate.

Previous studies had reported that when *S. frugiperda* were fed Cry1F maize and *T. ni* were fed Cry1Ac/Cry2Ab cotton, the Bt protein residues in the Lepidoptera larvae were still biologically active (Tian et al. 2013). Even though nymphs and adults were constantly exposed to bioactive Cry proteins in our tritrophic feeding experiments, levels of these proteins in the predators were only a small fraction of those detected in plant tissue. Cry1F protein levels in Bt maize decreased with the growth and development of the plant, which is consistent with our previous results (Tian et al. 2012). Maize leaves at the tassell-

ing stage expressed 2.64 $\mu\text{g/g}$ FW of Cry1F protein while that of *S. frugiperda* feeding on these leaves had a Cry1F concentration of 0.41 $\mu\text{g/g}$, and 5th instar *Z. renardii* feeding on these prey contained an average of 0.08 $\mu\text{g/g}$ FW. Our ELISA results thus show that the Cry1F concentration in the prey and the predator was only ca. 16% and 3%, respectively, of the Cry1F concentration in the plant tissue. In the case of Bollgard II cotton plants, both Cry1Ac and Cry2Ab protein concentrations in leaves gradually decreased during development. Cotton leaves used in our study contained 2.08 $\mu\text{g/g}$ FW Cry1Ac protein and 28.58 $\mu\text{g/g}$ FW Cry2Ab in the seedling stage. Again, Cry protein concentrations were significantly lower in the *T. ni* larvae and *Z. renardii* nymphs. In the case of Cry1Ac, concentrations detected in the lepidopteran larvae and in the 4th instar predator were only ca. 11% and 6%, respectively, of that found in the plant. In the case of Cry2Ab, concentrations in the lepidopteran larvae (2nd instar) and the 4th instar predator were ca. 1.5% and 0.5%, respectively, of those in the plant leaves. A similar dilution of Cry proteins across three trophic levels (Bt crop, herbivorous pest and natural enemy) has been reported in other studies. For example, Tian et al. (2012) found that only 10–20% of the Cry1F protein in *S. frugiperda* larvae fed with Cry1F maize was detected in *Coleomegilla maculata* (Coleoptera: Coccinellidae). Li and Romeis (2010) reported that 56% of the Cry3Bb1 protein in Bt maize leaves was detected in *Tetranychus urticae* (Trombidiformes: Tetranychidae) that were fed Bt maize (event MON88017); and Cry3Bb1 protein levels in larvae

and adults of the spider mite predator *Stethorus punctillum* (Coleoptera: Coccinellidae) were 6- and 20-fold lower, respectively, than that in their prey. Likewise, Li et al. (2011) found that Cry1Ac was sixfold lower and Cry2Ab 21-fold lower in *C. maculata* than its prey *T. ni* fed with Cry1Ac and Cry2Ab-expressing cotton. When the spider *Phylloneta impressa* (Araneae: Theridiidae) was fed with a single *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) or *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) reared on Cry3Bb1 maize, about 90% of the Cry protein in *P. impressa* had already been excreted or degraded 5 days after feeding (Meissle and Romeis 2012). These results suggest that, instead of bioaccumulation and biomagnification of Cry proteins, it is common for smaller amounts of Cry protein to be found at each step in the food chain.

Using Bt-resistant *S. frugiperda* and *T. ni* populations is a useful technique for eliminating any potential effect of prey quality in a tritrophic study. Our results demonstrate that neither Cry1F in Bt maize nor Cry1Ac and Cry2Ab in Bt cotton accumulate in the assassin bug, *Z. renardii*. Furthermore, these proteins do not negatively affect important fitness parameters of *Z. renardii*, including its survival rate, nymphal duration, adult weight and longevity and female fecundity and fertility at the concentrations tested. These results suggest that Bt plants expressing Cry1Ac, Cry2Ab and Cry1F are not directly toxic to *Z. renardii* and would not likely compromise its function as a biological control agent. However, there may be a question of indirect effects.

As we used resistant hosts, we were not able to determine whether a compromised (=susceptible) host would have had an effect on the development or reproduction of *Z. renardii* when it fed on such a host. Indirect effects are difficult to measure and have often and have been confused with direct effects (Romeis et al. 2013). In the field, indirect effects could result as a consequence of any pest management tactic that has a direct effect on the prey or host. This includes insecticide effects, other host plant resistance factors (including defensive reactions triggered by herbivory) and parasitism, all of which could cause harm to the prey or host or induce sublethal effects that would alter their quality as food for a predator or parasitoid (Tian et al. 2014). However, direct toxic effects would have a much greater impact on a natural enemy and our studies clearly demonstrate that Bt plants expressing Cry1Ac, Cry2Ab and Cry1F do not have any direct toxicity to *Z. renardii*.

Recently, it was demonstrated that the predator *C. maculata* helps delay the evolution of resistance to

Bt crops in a target pest species (Liu et al. 2014). Similar contributions might be made by other important predators, such as *Z. renardii*, that are unaffected by the common Cry proteins used to control Lepidoptera in maize and cotton.

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