Using Resistant Prey Demonstrates That Bt Plants Producing Cry1Ac, Cry2Ab, and Cry1F Have No Negative Effects on *Geocoris punctipes* and *Orius insidiosus*

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ABSTRACT *Geocoris punctipes* (Say) and *Orius insidiosus* (Say) are generalist predators found in a wide range of crops, including cotton (*Gossypium hirsutum* L.) and maize (*Zea mays* L.), where they provide important biological control services by feeding on an array of pests, including eggs and small larvae of caterpillars. A high percentage of cotton and maize in the United States and several other countries are transgenic cultivars that produce one or more of the insecticidal Cry proteins of *Bacillus thuringiensis* Berliner (Bt). Here we quantify effects of three Cry proteins on the life history of these predators over two generations when they are exposed to these Cry proteins indirectly through their prey. To eliminate the confounding prey quality effects that can be introduced by Bt-susceptible prey, we used Cry1Ac/Cry2Ab-resistant *Trichoplusia ni* (Hübner) and Cry1 F-resistant *Spodoptera frugiperda* (J.E. Smith) in a series of tri-trophic studies. Survival, development, adult mass, fecundity, and fertility were similar when predators consumed larvae feeding on Cry1Ac/Cry2Ab cotton or Cry1 F maize compared with prey feeding on isogenic or near-isogenic cotton or maize. Repeated exposure of the same initial cohort over a second generation also resulted in no differences in life-history traits when feeding on non-Bt- or Bt-fed prey. Enzyme-linked immunosorbent assay showed that predators were exposed to Bt Cry proteins from their prey and that these proteins became increasingly diluted as they moved up the food chain. Results show a clear lack of effect of three common and widespread Cry proteins on these two important predator species. The use of resistant insects to eliminate prey quality effects provides a robust and meaningful assessment of exposure and hazard.

KEY WORDS transgenic Bt crop, *Trichoplusia ni*, *Spodoptera frugiperda*, biological control service, prey quality

The adoption of insect-resistant transgenic crops producing the insecticidal proteins of *Bacillus thuringiensis* Berliner (Bt) continues to grow rapidly on a global scale. Cotton (*Gossypium hirsutum* L.) and maize (*Zea mays* L.), the two Bt crops currently under commercial production, were grown on nearly 70 million hectares in 27 countries in 2012 (James 2012). In the United States, Bt cotton and Bt maize represent ≈77 and 67% of total crop production, respectively (USDA-NASS 2012). These genetically engineered crops have been associated with large increases in yield and substantial reductions in insecticide use for key Lepidoptera throughout most adopting countries (Fernandez-Cornejo and Caswell 2006, Wu et al. 2008, Brookes and Barfoot 2012, Kathage and Qaim 2012). A large body of literature also has shown that the Bt proteins produced in these crops and others are selective against lepidopteran and coleopteran pests, while having little to no effect on a wide range of nontarget arthropods (Romeis et al. 2006, Marvier et al. 2007, Wolfenbarger et al. 2008, Naranjo 2009). Nonetheless, concerns about effects on nontarget organisms persist (Lövei et al. 2009).

Generalist arthropod predators represent a very important component of the biological control services in agriculture (Symondson et al. 2002). Many species are common residents in a number of agronomic and horticultural crops, where they feed on key pests and

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effectively suppress secondary and other minor pests. *Geocoris punctipes* (Say) (big-eyed bug, Hemiptera: Geocoridae) and *Orius insidiosus* (Say) (insidious flower bug, Hemiptera: Anthocoridae) are among the most abundant and cosmopolitan species of generalist predators in the United States and are frequently found in cotton (Whitcomb and Bell 1964, Ehler 1977, Wilson and Gutierrez 1980, Head et al. 2005, Naranjo 2005, Torres and Ruberson 2005) and maize (Dicke and Jarvis 1962, Coll and Bottrell 1991, Dively 2005, Pilcher et al. 2005). Both predators feed on a wide range of small-sized prey, including eggs and small caterpillars, several of which are the targets of Bt cotton and Bt maize (Whitcomb and Bell 1964, Orphanides et al. 1971, Crocker and Whitcomb 1980, Corey et al. 1998, Naranjo and Hagler 1998, Torres and Ruberson 2006, Bickerton and Hamilton 2012). These predators also function as omnivores and feed directly on plant sap, nectar, and pollen (Naranjo and Gibson 1996, Coll 1998). Thus, *G. punctipes* and *O. insidiosus* are potentially exposed to Bt proteins in plants through several pathways in nature.

The consumption of prey that have fed on the Bt crop and ingested Bt proteins represents a critical route of exposure to arthropod natural enemies. This so-called tri-trophic exposure pathway has been examined in a number of different arthropod predators and parasitoids (reviewed by Romeis et al. 2006, Naranjo 2009). When a prey or host species that is susceptible to Bt proteins feeds on a Bt crop or Bt-containing diet, it typically suffers deleterious effects including mortality, but also sublethal effects such as reduced growth and vigor, if the insect is only partially susceptible (Dutton et al. 2005, Ramirez-Romero et al. 2007, Sanders et al. 2007, Torres and Ruberson 2008). In turn, predators or parasitoids offered such prey or hosts sometimes suffer negative effects on various life-history traits. These negative effects can confound an assessment of the direct effects of the Bt protein on that natural enemy (Romeis et al. 2006, Naranjo 2009, Shelton et al. 2009, Romeis et al. 2013). An approach to eliminate these potential prey/host quality-mediated effects is to use prey or hosts that are either not susceptible to the Bt proteins under investigation (Zwahlen et al. 2000, Bernal et al. 2002, Dutton et al. 2002, Bai et al. 2006, Álvarez-Alfageme et al. 2011) or use prey or hosts that have evolved resistance to these Bt proteins (Ferry et al. 2006; Chen et al. 2008; Lawo et al. 2010; Li et al. 2011; Tian et al. 2012, 2013). In either approach, the natural enemy can then be exposed to realistic levels of Bt proteins but not simultaneously suffer from any associated prey or host quality-mediated effects.

While the effects of Bt cotton and maize have been examined for *G. punctipes* and *O. insidiosus* as part of larger community studies in the field (Bhatti et al. 2005, Dively 2005, Head et al. 2005, Naranjo 2005, Pilcher et al. 2005, Torres and Ruberson 2005), there have been relatively few studies examining the effects of Bt proteins on these species under controlled conditions in the laboratory (Pilcher et al. 1997, Armer et al. 2000, Al-Deeb et al. 2001, Ponsard et al. 2002, Torres and Ruberson 2006, Duan et al. 2008) and even fewer that have examined realistic exposure to Bt proteins through their prey (Al-Deeb et al. 2001, Ponsard et al. 2002, Torres and Ruberson 2006).

The objectives of this study were to quantitatively assess the effects of three Bt Cry proteins commonly found in currently grown Bt cotton and Bt maize cultivars on *G. punctipes* and *O. insidiosus* via tri-trophic exposure routes. To eliminate potential confounding effects of prey quality, we took advantage of cultures of two lepidopteran prey that have evolved resistance to certain Cry proteins either in the field, Cry1 F-resistant *Spodoptera frugiperda* (J.E. Smith), or through greenhouse or laboratory selection, Cry1Ac/Cry2Ab-resistant *Trichoplusia ni* (Hübner) (both Lepidoptera: Noctuidae). We quantified nymphal survival and development, as well as adult mass, survival, fecundity, and fertility, over two consecutive generations of exposure.

Materials and Methods

Plants. Seeds of Bt cotton (Event 15895), which carries genes coding for Cry1Ac and Cry2Ab, and the corresponding nontransformed near-isoline Stoneville 474, were obtained from the Monsanto Company (St. Louis, MO). The two cotton varieties were grown in 6-liter plastic pots with Cornell Mix potting soil (Boodley and Sheldrake 1977) in greenhouses at Cornell's New York State Agricultural Experiment Station (NYSAES). Approximately 6 g of Osmocote Plus patterned release fertilizer (Scotts, Marysville, OH) was placed in each pot and 500 ml of Power-Gro liquid fertilizer (Wilson Laboratories Inc., Dundas, ON, Canada) was applied weekly. When cotton reached the 12-leaf stage, cotton leaves were used to feed *T. ni*. All cotton plants were grown in the same greenhouse at 27 ± 2°C under a photoperiod of 16:8 (L:D) h.

Seeds of Bt maize (Mycogen 2A517), producing Cry1 F protein, and the corresponding nontransformed near-isoline (Mycogen 2A496) were obtained from Dow AgroSciences (Indianapolis, IN) and grown in the greenhouses at NYSAES. The two maize varieties were grown in Ray Leach Cone-tainer cells (diameter 3.8 cm; depth 21 cm; volume 164 ml) (Stuewe & Sons, Tangent, OR) with Cornell Mix and 500 ml of Power-Gro liquid fertilizer was applied weekly. When maize reached the V5 stage, leaves were used to feed *S. frugiperda*. All maize plants were grown in the same greenhouse at 21 ± 2°C under a photoperiod of 16:8 (L:D) h.

Insects. A Bt-susceptible *T. ni* strain was maintained on an artificial diet in the laboratory for >20 yr without exposure to Bt toxin. The Cry1Ac/Cry2Ab-resistant strain (GLEN-BGII) was originally collected from commercial greenhouses in British Columbia, Canada, and further selected on Bollgard II foliage in the laboratory (Kain et al. 2004).

A Cry1 F-resistant strain of *S. frugiperda* was obtained from Dow AgroSciences and maintained on an artificial diet. This strain developed resistance to Cry1 F maize in Puerto Rico (Storer et al. 2010) and is able

to complete its life cycle on Cry1 F maize (Tian et al. 2012).

Eggs of *G. punctipes* and *O. insidiosus* were obtained from the U.S. Department of Agriculture–Agricultural Research Service (USDA–ARS), Maricopa, AZ (SEN laboratory), where they had been reared on eggs of *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) and *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) and green bean pods for >20 yr with annual introductions of wild stock. Insects were reared on eggs of Bt-susceptible *Plutella xylostella* L. (Lepidoptera: Plutellidae) reared on an artificial diet (Shelton et al. 1991) and green bean pods for several generations before testing. Newly hatched first-instar nymphs were used to initiate bioassays.

All insects were maintained in a climatic chamber at 27 ± 1°C and 50 ± 10% RH, under a photoperiod of 16:8 (L:D) h. All experiments were conducted under these conditions as well.

Prey-mediated Effects of Cry1Ac/Cry2Ab Cotton on *G. punctipes*. Newly hatched first-instar *G. punctipes* were individually kept in 30-ml cups with a piece of a non-Bt cotton leaf and supplied with Bt-susceptible *T. ni* eggs. A water-saturated cotton ball was provided on the bottom of each cup to maintain humidity and provide moisture for the predator. Eggs were supplied every 2 d and *G. punctipes* mortality and molt were checked daily. When *G. punctipes* reached the fourth instar, they were supplied with Bt-susceptible *T. ni* fed non-Bt cotton, Cry1Ac/Cry2Ab-resistant *T. ni* fed non-Bt cotton, or Cry1Ac/Cry2Ab-resistant *T. ni* fed Cry1Ac/Cry2Ab cotton. Newly hatched *T. ni* larvae were allowed to feed on their respective cotton plants for 2 d before providing them as prey. In preliminary studies, we determined that fourth-instar *G. punctipes* were able to consistently attack and eat 2-d-old prey. A piece of a non-Bt cotton leaf was placed in each cup to provide food for the larvae before they were consumed by the predator. We used non-Bt leaf material because we did not want to confound any tri-trophic effect by using Bt plants during this stage. Prey and cotton leaves were changed daily and *G. punctipes* mortality and molting were recorded daily. The gender of newly emerged adults was determined and insects were weighed. The experiment was initiated with 30 *G. punctipes* nymphs for each treatment.

To assess fecundity, 10 mating pairs of newly emerged *G. punctipes* adults from each treatment were kept individually in petri dishes (diameter 9 cm). Adults were supplied with Bt-susceptible *T. ni* fed non-Bt cotton, Cry1Ac/Cry2Ab-resistant *T. ni* fed non-Bt cotton, or Cry1Ac/Cry2Ab-resistant *T. ni* fed Cry1Ac/Cry2Ab cotton and a piece of a non-Bt cotton leaf for 20 d. Their prey were larvae that had fed on their respective cotton plants for 2 d following eclosion. Small cotton pads (5 mm by 5 mm), provided as an oviposition substrate, were collected daily. The number of eggs was counted under a microscope. The number of adults surviving the 20-d oviposition period also was noted. To estimate egg-hatching rates, 30 eggs from each treatment were separated from a cotton pad and transferred into individual 30-ml cups where they were monitored until they hatched. Three replications were used.

The offspring (F2 of *G. punctipes*) underwent another generation of testing, as described earlier.

Prey-mediated Effects of Cry1 F Maize on *G. punctipes*. Bioassays were carried out as described earlier but using the Cry1 F-resistant *S. frugiperda* strain and Cry1 F maize and nontransformed maize plants.

Prey-mediated Effects of Cry1Ac/Cry2Ab Cotton on *O. insidiosus*. Newly emerged first-instar *O. insidiosus* were kept individually in 30-ml cups with a piece of a non-Bt cotton leaf and supplied with *T. ni* eggs. A water-saturated cotton ball was provided on the bottom of each cup to maintain humidity and provide moisture for the predator. Eggs were supplied every 2 d and *O. insidiosus* mortality and molting were checked twice daily. When *O. insidiosus* reached the fifth instar, they were supplied with Bt-susceptible *T. ni* fed non-Bt cotton, Cry1Ac/Cry2Ab-resistant *T. ni* fed non-Bt cotton, or Cry1Ac/Cry2Ab-resistant *T. ni* fed Cry1Ac/Cry2Ab cotton. Newly hatched larvae were allowed to feed on their respective cotton plants for 2 d before providing them as prey. In preliminary studies, we determined that fifth-instar *O. insidiosus* were able to consistently attack and eat 2-d-old prey. A piece of control cotton leaf was placed in each cup to provide food for the prey and additional nutrition for the predator. Again, we used non-Bt leaf material because we did not want to confound any tri-trophic effect by using Bt plants during this stage. Prey and cotton leaves were changed daily and *O. insidiosus* mortality and molting were recorded twice daily. The gender of newly emerged adults was determined and the insects were weighed. The experiment was initiated with 30 *O. insidiosus* nymphs for each treatment.

For assessing fecundity, 10 mating pairs of newly emerged *O. insidiosus* adults from each treatment were kept individually in 30-ml cups. A green bean pod section was present in all cups as an oviposition substrate. Adults were supplied with Bt-susceptible *T. ni* fed non-Bt cotton, Cry1Ac/Cry2Ab-resistant *T. ni* fed non-Bt cotton, or Cry1Ac/Cry2Ab-resistant *T. ni* fed Cry1Ac/Cry2Ab cotton and a piece of a non-Bt cotton leaf for 14 d. As before, these prey larvae were allowed to first feed on their respective treatment plants for 2 d before exposure to predators. Green beans were changed daily, and *O. insidiosus* eggs were counted under a microscope. The number of adults surviving the 14-d oviposition period was noted.

To estimate egg-hatching rates, 10 green bean sections from each treatment were randomly selected and transferred into individual 30-ml cups. Newly hatched nymphs were recorded until hatching ceased; unhatched eggs were then counted under a microscope.

The offspring (F2 of *O. insidiosus*) underwent another generation of testing, as described earlier.

Prey-mediated Effects of Cry1 F Maize on *O. insidiosus*. Bioassays were carried out as described earlier but using the Cry1 F-resistant *S. frugiperda* strain and

Cry1 F maize and nontransformed near-isogenic maize plants.

Bt Protein Residue in Insects. For each bioassay, another 50 first-instar *G. punctipes* and *O. insidiosus* were reared for each treatment as described earlier. Emerging adults were supplied with treatment prey for 10 additional d. Three samples units (10–15 insects per sample unit) from each treatment were collected. Three sample units of Bt and non-Bt crop leaves (20 mg per sample unit) and prey (*T. ni* and *S. frugiperda*, 20 mg per sample unit) that were used in bioassays were also collected. Bt protein concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using Cry1Ac and Cry2Ab detection kits from EnviroLogix (Portland, ME) and Cry1 F detection kits from Agdia (Elkhart, IN). Kits were identified as QualiPlate Kit for Cry1Ab/Cry1Ac - AP 003 CRBS, QuantiPlate Kit for Cry2A - AP 005, and Bt-Cry1 F ELISA Kit (Quantitative) PSP 11700.

Before analysis, all insects were washed with phosphate-buffered saline with Tween 20 (PBST) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.05% Tween-20, pH 7.4) four times to remove any Bt protein from the surface. Leaf samples were diluted at a rate of 1:1,000 (mg sample: μl PBST buffer) and fully ground with a mortar and pestle. Insect samples were diluted at a rate of 1:20 (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. Because no purified Cry1Ac protein was provided in the Cry1Ac kit, Cry1Ab was purchased from M. Carey (Department of Biochemistry, Case Western Reserve University, Cleveland, OH). Purified Cry1Ab protein samples at concentrations of 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 ng/g were used as calibrators. We ran the negative controls (samples from non-Bt treatment) and absorbance readings double the negative control were considered positive.

Statistical Analyses. Data on nymphal and adult survival of *G. punctipes* and *O. insidiosus* were analyzed using the Wilcoxon test for homogeneity. Data on other life-history parameters of *T. ni*-fed *G. punctipes* and *O. insidiosus* were subjected to one-way analysis of variance (ANOVA) and the Tukey honestly significant difference test was used to separate means. Data on life-history parameters of *S. frugiperda*-fed *G. punctipes* and *O. insidiosus* were analyzed using the Student *t*-test. Bt protein levels in plant tissues and insects were analyzed using one-way ANOVA and Tukey test or Student *t*-test, as appropriate. Before analysis, all proportional data were arcsine square root transformed, as necessary, but untransformed means are presented. All statistical analyses were performed with SAS version 9.1 (SAS Institute 2001). For all tests, $\alpha = 0.05$.

Results

Prey-mediated Effects of Cry1Ac/Cry2Ab Cotton on *G. punctipes*. Newly hatched *G. punctipes* reached the fourth instar feeding on *T. ni* eggs for 12–14 d. Then Bt-susceptible *T. ni* that were fed non-Bt cotton, Cry1Ac/Cry2Ab-resistant *T. ni* fed non-Bt cotton, or Cry1Ac/Cry2Ab-resistant *T. ni* fed Cry1Ac/Cry2Ab cotton were supplied to *G. punctipes*. No significant differences were detected for any life-history parameter of *G. punctipes* (including survival, development time, adults weight, fecundity, and fertility) among the three treatments over two generations (Table 1). In this experiment, as with the others listed later, we observed the predators eating the prey and the prey were dead after being consumed by the predator.

Prey-mediated Effects of Cry1 F Maize on *G. punctipes*. Cry1 F-resistant *S. frugiperda* that fed on non-Bt maize or Bt maize were supplied to *G. punctipes* when they reached the fourth instar feeding on *S. frugiperda* eggs. As before, there were no significant differences observed for any of the life-history parameters of *G. punctipes* between the control (non-Bt) maize treatment and the Cry1 F maize treatment over two generations (Table 2).

Prey-mediated Effects of Cry1Ac/Cry2Ab Cotton on *O. insidiosus*. *O. insidiosus* nymphs reached the fifth instar feeding on *T. ni* eggs for 5.5–7 d whereupon they were provided Bt-susceptible *T. ni* fed on non-Bt cotton, Cry1Ab/Cry2Ab-resistant *T. ni* fed on non-Bt cotton, or Cry1Ab/Cry2Ab-resistant *T. ni* fed on Cry1Ab/Cry2Ab cotton. The different prey provided did not have an effect on any of the life-history parameters of *O. insidiosus* over two generations (Table 3).

Prey-mediated Effects of Cry1 F Maize on *O. insidiosus*. Newly hatched *O. insidiosus* nymphs supplied with *S. frugiperda* eggs reached the fifth instar in ≈6 d. The predators were then provided Cry1 F-resistant *S. frugiperda* that fed on non-Bt maize or Cry1 F maize. No significant differences were detected for any life-history parameters in the first or second generation (Table 4).

Bt Proteins Levels in Bt Crops, Prey, and Predators. Bt cotton leaves contained high levels of Cry1Ab and Cry2Ab (Table 5). *T. ni* contained 20-fold lower levels of Cry1Ac and 23-fold lower levels of Cry2Ab compared with Bt cotton leaves. Only trace amounts were detected in *G. punctipes* and *O. insidiosus* for Cry1Ac (levels below the limit of quantification of 0.1 ng/g), and the average concentrations of Cry2Ab protein in *G. punctipes* and *O. insidiosus* were 47- and 37-fold lower than those in *T. ni*, respectively.

Similar results were found in Bt maize treatments (Table 5). Cry1 F protein levels in *S. frugiperda* were 7-fold lower than in Cry1 F maize leaves. Average concentrations of Cry1 F protein in *G. punctipes* and *O. insidiosus* were 54- and 27-fold lower compared with *S. frugiperda*, respectively.

As expected, no Bt proteins were detected in non-Bt plants, prey fed non-Bt plants, or predators fed prey from non-Bt plants.

Discussion

Our results demonstrate that neither *G. punctipes* nor *O. insidiosus*, two widely distributed generalist predators inhabiting cotton and maize, are affected by

Table 1. Tri-trophic effects on life-history parameters (mean ± SE) of *Geocoris punctipes* when fed *Trichoplusia ni* larvae that were reared on Cry1Ac/Cry2Ab or non-Bt isoline cotton leaves over two generations

Parameters	Control cotton Susceptible *T. ni*	Control cotton Resistant *T. ni*	Cry1Ac/Cry2Ab cotton Resistant *T. ni*	Statistics
First generation				
Survival (%)[a]	96.7	93.3	100.0	$\chi^2 = 1.99$; df = 2; $P = 0.37$
Development time (d)[b]				
First–third instar	13.1 ± 0.1 (30)	13.1 ± 0.2 (30)	13.2 ± 0.2 (30)	$F = 0.08$; df = 2, 89; $P = 0.93$
Fourth–fifth instar[c]	13.1 ± 0.1 (29)	13.2 ± 0.2 (28)	13.2 ± 0.2 (30)	$F = 0.04$; df = 2, 86; $P = 0.96$
Nymph to adult	26.2 ± 0.2 (29)	26.3 ± 0.3 (28)	26.4 ± 0.3 (30)	$F = 0.12$; df = 2, 86; $P = 0.89$
Male fresh wt (mg)[b]	32.8 ± 0.1 (15)	34.0 ± 0.1 (12)	32.9 ± 0.1 (15)	$F = 0.74$; df = 2, 41; $P = 0.49$
Female fresh wt (mg)[b]	46.8 ± 0.1 (14)	45.1 ± 0.1 (16)	43.3 ± 0.1 (15)	$F = 2.81$; df = 2, 44; $P = 0.07$
Adult survival (%)[a,d]	75.0	75.0	80.0	$\chi^2 = 0.22$; df = 2; $P = 0.89$
Total fecundity (20 d)[b]	51.6 ± 2.4 (10)	46.3 ± 4.6 (10)	48.3 ± 1.7 (10)	$F = 0.73$; df = 2, 29; $P = 0.49$
Egg hatch (%)[b]	74.4 ± 2.9 (3)	83.3 ± 5.1 (3)	82.2 ± 4.8 (3)	$F = 1.21$; df = 2, 8; $P = 0.36$
Second generation				
Survival (%)[a]	86.7	83.3	90.0	$\chi^2 = 0.62$; df = 2; $P = 0.73$
Development time (d)[b]				
First–third instar	14.6 ± 0.2 (28)	14.9 ± 0.2 (28)	14.5 ± 0.2 (29)	$F = 0.84$; df = 2, 84; $P = 0.43$
Fourth–fifth instar[c]	13.0 ± 0.2 (26)	13.2 ± 0.2 (25)	12.8 ± 0.2 (27)	$F = 1.40$; df = 2, 76; $P = 0.25$
Nymph to adult	27.7 ± 1.3 (26)	28.0 ± 1.5 (25)	27.4 ± 1.2 (27)	$F = 1.76$; df = 2, 76; $P = 0.18$
Male fresh wt (mg)[b]	34.2 ± 0.1 (15)	35.1 ± 0.1 (12)	36.1 ± 0.1 (17)	$F = 1.37$; df = 2, 43; $P = 0.27$
Female fresh wt (mg)[b]	43.5 ± 0.1 (11)	46.7 ± 0.2 (13)	46.0 ± 0.2 (10)	$F = 1.25$; df = 2, 33; $P = 0.30$
Adult survival (%)[a,d]	85.0	80.0	80.0	$\chi^2 = 0.24$; df = 2; $P = 0.89$
Total fecundity (20 d)[b]	57.7 ± 8.2 (10)	51.8 ± 7.6 (10)	52.4 ± 7.3 (10)	$F = 0.18$; df = 2, 29; $P = 0.84$
Egg hatch (%)[b]	86.7 ± 3.9 (3)	85.6 ± 2.9 (3)	87.8 ± 3.9 (3)	$F = 0.13$; df = 2, 8; $P = 0.88$

Number of replications is given in parentheses; exp initiated with 30 nymphs in each treatment.
[a] Wilcoxon test.
[b] One-way ANOVA.
[c] Predators were exposed to *T. ni* larvae at the beginning of the fourth instar.
[d] Based on survival during the first 20 d of adult life.

three common Bt Cry proteins found in many Bt cotton and Bt maize crops in the United States and elsewhere when exposed through intoxicated prey. The predators were chronically exposed to these three Cry proteins via a natural pathway in realistic worst-case exposure conditions over the course of two consecutive generations. The ability to eliminate any potential prey-quality-mediated effects through the use of Bt-resistant target prey was a major advantage and allowed us to focus evaluation

Table 2. Tri-trophic effects on life-table parameters (mean ± SE) of *Geocoris punctipes* when fed Cry1F-resistant *Spodoptera frugiperda* larvae that were reared on Cry1F maize leaves or non-Bt maize leaves over two generations

Parameters	Non-Bt maize	Cry1F maize	Statistics
First generation			
Survival (%)[a]	86.7	86.7	$\chi^2 = 0.00$; df = 1; $P = 1.00$
Development time (d)[b]			
First–third instar	13.9 ± 0.1 (30)	13.9 ± 0.2 (30)	$t = 0.00$; df = 58; $P = 1.00$
Fourth–fifth instar[c]	14.6 ± 0.3 (26)	14.7 ± 0.3 (26)	$t = 0.26$; df = 50; $P = 0.80$
Nymph to adult	28.5 ± 0.3 (26)	28.7 ± 0.3 (26)	$t = 0.49$; df = 50; $P = 0.62$
Male fresh wt (mg)[b]	35.4 ± 1.0 (14)	35.7 ± 0.8 (13)	$t = 0.20$; df = 25; $P = 0.84$
Female fresh wt (mg)[b]	42.4 ± 1.6 (12)	43.7 ± 2.0 (13)	$t = 0.63$; df = 23; $P = 0.54$
Adult survival (%)[a,d]	60.0	75.0	$\chi^2 = 0.18$; df = 1; $P = 0.67$
Total fecundity (20 d)[b]	52.5 ± 4.6 (10)	48.9 ± 5.4 (10)	$t = 0.51$; df = 18; $P = 0.62$
Egg hatch (%)[b]	87.8 ± 2.9 (3)	90.0 ± 1.9 (3)	$t = 0.63$; df = 4; $P = 0.56$
Second generation			
Survival (%)[a]	90.0	86.7	$\chi^2 = 0.13$; df = 1; $P = 0.72$
Development time (d)[b]			
First–third instar	14.1 ± 0.1 (30)	14.3 ± 0.1 (30)	$t = 1.34$; df = 58; $P = 0.19$
Fourth–fifth instar[c]	12.3 ± 0.1 (27)	12.4 ± 0.1 (26)	$t = 0.45$; df = 51; $P = 0.65$
Nymph to adult	26.3 ± 0.2 (27)	26.5 ± 0.2 (26)	$t = 0.70$; df = 51; $P = 0.49$
Male fresh wt (mg)[b]	38.7 ± 1.2 (14)	39.2 ± 0.6 (15)	$t = 0.41$; df = 27; $P = 0.69$
Female fresh wt (mg)[b]	47.5 ± 1.7 (13)	49.4 ± 2.0 (11)	$t = 0.76$; df = 22; $P = 0.45$
Adult survival (%)[a,d]	75.0	75.0	$\chi^2 = 0.01$; df = 1; $P = 0.96$
Total fecundity (20 d)[b]	57.4 ± 4.8 (10)	54.0 ± 3.6 (10)	$t = 0.56$; df = 18; $P = 0.58$
Egg hatch (%)[b]	84.4 ± 2.2 (3)	83.3 ± 1.9 (3)	$t = 0.38$; df = 4; $P = 0.72$

Number of replications is given in parentheses; exp initiated with 30 larvae in each treatment.
[a] Wilcoxon test.
[b] Student *t*-test ANOVA.
[c] Predators were exposed to *S. frugiperda* larvae at the beginning of the fourth instar.
[d] Based on survival during the first 20 d of adult life.

Table 3. Tri-trophic effects on life-table parameters (mean ± SE) of *Orius insidiosus* when fed *Trichoplusia ni* larvae that were reared on Cry1Ac/Cry2Ab or non-Bt isoline cotton leaves over two generations

Parameters	Control cotton Susceptible *T. ni*	Control cotton Resistant *T. ni*	Cry1Ac/Cry2Ab cotton Resistant *T. ni*	Statistics
First generation				
Survival (%)[a,b]	93.3	96.7	96.7	$\chi^2 = 0.52$; df = 2; $P = 0.77$
Development time (d)[c]				
First–fourth instar	6.1 ± 0.1 (30)	6.0 ± 0.1 (30)	6.1 ± 0.1 (30)	$F = 0.20$; df = 2, 89; $P = 0.81$
Fifth instar[b]	3.3 ± 0.1 (28)	3.3 ± 0.1 (29)	3.2 ± 0.1 (29)	$F = 0.17$; df = 2, 85; $P = 0.84$
Nymph to adult	9.4 ± 0.1 (28)	9.3 ± 0.1 (29)	9.3 ± 0.1 (29)	$F = 0.07$; df = 2, 86; $P = 0.93$
Male fresh wt (mg)[c]	2.7 ± 0.2 (15)	2.3 ± 0.2 (16)	2.7 ± 0.2 (16)	$F = 1.16$; df = 2, 46; $P = 0.32$
Female fresh wt (mg)[c]	4.2 ± 0.3 (13)	4.3 ± 0.3 (13)	4.1 ± 0.3 (13)	$F = 0.16$; df = 2, 38; $P = 0.85$
Adult survival (%)[a,b]	80.0	85.0	75.0	$\chi^2 = 0.03$; df = 2; $P = 0.98$
Total fecundity (14 d)[c]	35.3 ± 4.2 (10)	32.3 ± 3.3 (10)	37.9 ± 3.6 (10)	$F = 0.56$; df = 2, 29; $P = 0.58$
Egg hatch (%)[c]	80.6 ± 5.0 (10)	82.9 ± 5.3 (10)	83.5 ± 4.8 (10)	$F = 0.16$; df = 2, 29; $P = 0.85$
Second generation				
Survival (%)[a]	83.3	86.7	93.3	$\chi^2 = 1.33$; df = 2; $P = 0.51$
Development time (d)[c]				
First–fourth instar	6.5 ± 0.1 (27)	6.7 ± 0.1 (28)	6.6 ± 0.1 (28)	$F = 1.42$; df = 2, 82; $P = 0.25$
Fifth instar[b]	3.0 ± 0.1 (25)	3.2 ± 0.1 (26)	3.1 ± 0.1 (28)	$F = 1.83$; df = 2, 78; $P = 0.17$
Nymph to adult	9.5 ± 0.1 (25)	9.9 ± 0.1 (26)	9.7 ± 0.1 (28)	$F = 2.09$; df = 2, 78; $P = 0.13$
Male fresh wt (mg)[c]	2.7 ± 0.2 (13)	2.5 ± 0.2 (14)	2.6 ± 0.1 (15)	$F = 0.40$; df = 2, 41; $P = 0.67$
Female fresh wt (mg)[c]	4.3 ± 0.3 (12)	4.6 ± 0.3 (12)	4.4 ± 0.2 (13)	$F = 0.17$; df = 2, 36; $P = 0.84$
Adult survival (%)[a,d]	85.0	75.0	85.0	$\chi^2 = 0.78$; df = 2; $P = 0.68$
Total fecundity (14 d)[c]	41.9 ± 4.2 (10)	38.0 ± 4.7 (10)	40.0 ± 4.0 (10)	$F = 0.20$; df = 2, 29; $P = 0.82$
Egg hatch (%)[c]	79.1 ± 5.0 (10)	80.9 ± 4.3 (10)	81.3 ± 5.2 (10)	$F = 0.08$; df = 2, 29; $P = 0.93$

Number of replications is given in parentheses; exp initiated with 30 nymphs in each treatment.
[a] Wilcoxon test.
[b] Predators were exposed to *T. ni* larvae at the beginning of the fifth instar.
[c] One-way ANOVA.
[d] Based on survival during the first 14 d of adult life.

on the Cry proteins, as the predators would be exposed to them in the field.

Our results are consistent with other studies of *O. insidiosus* exposed to Bt proteins through various routes. Al-Deeb et al. (2001) found no effects on development, survival, and adult mass when predators were exposed to *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) larvae feeding on an artificial diet spiked with Dipel, a commercial formulation containing multiple Cry1 and Cry2 Bt proteins, compared

Table 4. Tri-trophic effects on life-table parameters (mean ± SE) of *Orius insidiosus* when fed Cry1F-resistant *Spodoptera frugiperda* larvae that were reared on Cry1F maize leaves or non-Bt maize leaves over two generations

Parameters	Non-Bt maize	Cry1F maize	Statistics
First generation			
Survival (%)[a,b]	90.0	93.3	$\chi^2 = 0.28$; df = 1; $P = 0.60$
Development time (d)[c]			
First–fourth instar	6.2 ± 0.1 (30)	6.1 ± 0.1 (30)	$t = 0.75$; df = 58; $P = 0.46$
Fifth instar[b]	3.4 ± 0.1 (27)	3.3 ± 0.1 (28)	$t = 0.66$; df = 53; $P = 0.51$
Nymph to adult	9.6 ± 0.1 (27)	9.4 ± 0.1 (28)	$t = 1.30$; df = 50; $P = 0.20$
Male fresh wt (mg)[c]	2.5 ± 0.2 (14)	2.6 ± 0.1 (16)	$t = 0.13$; df = 28; $P = 0.89$
Female fresh wt (mg)[c]	4.3 ± 0.2 (13)	4.4 ± 0.3 (12)	$t = 0.25$; df = 23; $P = 0.81$
Adult survival (%)[a,d]	70.0	80.0	$\chi^2 = 0.23$; df = 1; $P = 0.63$
Total fecundity (14 d)[c]	38.1 ± 2.5 (10)	36.5 ± 3.0 (10)	$t = 0.42$; df = 18; $P = 0.68$
Egg hatch (%)[c]	81.6 ± 5.0 (10)	85.1 ± 5.4 (10)	$t = 0.48$; df = 18; $P = 0.63$
Second generation			
Survival (%)[a]	93.3	96.7	$\chi^2 = 0.34$; df = 1; $P = 0.56$
Development time (d)[c]			
First–fourth instar	6.5 ± 0.1 (29)	6.6 ± 0.1 (30)	$t = 0.64$; df = 57; $P = 0.53$
Fifth instar[b]	3.1 ± 0.1 (28)	3.2 ± 0.1 (29)	$t = 0.84$; df = 55; $P = 0.40$
Nymph to adult	9.6 ± 0.1 (28)	9.8 ± 0.1 (29)	$t = 1.06$; df = 55; $P = 0.29$
Male fresh wt (mg)[c]	2.6 ± 0.1 (15)	2.8 ± 0.2 (15)	$t = 0.80$; df = 28; $P = 0.43$
Female fresh wt (mg)[c]	4.5 ± 0.2 (13)	4.3 ± 0.2 (14)	$t = 0.70$; df = 25; $P = 0.49$
Adult survival (%)[a,d]	75.0	80.0	$\chi^2 = 0.01$; df = 1; $P = 0.93$
Total fecundity (14 d)[c]	38.4 ± 3.5 (10)	39.0 ± 4.5 (10)	$t = 0.11$; df = 18; $P = 0.92$
Egg hatch (%)[c]	84.6 ± 5.1 (10)	82.4 ± 4.5 (10)	$t = 0.32$; df = 18; $P = 0.75$

Number of replications is given in parentheses; exp initiated with 30 larvae in each treatment.
[a] Wilcoxon test.
[b] Predators were exposed to *S. frugiperda* larvae at the beginning of the fifth instar.
[c] Student *t*-test ANOVA.
[d] Based on survival during the first 14 d of adult life.

Table 5. Bt protein levels (μg/g fresh wt) in Bt crops (cotton and maize), prey (*Trichoplusia ni* and *Spodoptera frugiperda*), and predators (*Geocoris punctipes* and *Orius insidiosus*)

Sample	Cotton		Maize
	Cry1Ac[a]	Cry2Ab[b]	Cry1F[b]
Leaves	1.37 ± 0.21a	23.4 ± 2.1a	3.12 ± 0.19a
Prey	0.067 ± 0.07b	1.03 ± 0.14b	0.43 ± 0.04b
G. punctipes	Trace[c]	0.022 ± 0.002c	0.008 ± 0.001c
O. insidiosus	Trace[c]	0.028 ± 0.005c	0.016 ± 0.002c
Statistical analysis	$t = 6.32$; df = 4; $P = 0.02$	$F = 1024$; df = 3,11; $P < 0.01$	$F = 586$; df = 3, 11; $P < 0.01$

Prey: *T. ni* for cotton, *S. frugiperda* for maize.
[a] Means (±SE) within a column followed by different letters are significantly different (Student *t*-test, $P < 0.05$); $N = 3$.
[b] Means (±SE) within a column followed by different letters are significantly different (one-way ANOVA, $P < 0.05$); $N = 3$.
[c] Levels detected were below the level of quantification (0.1 ng/g).

with a control diet containing no Bt proteins. There were also no effects on *O. insidiosus* development or survival when predators were provided pollen from Cry1Ab Bt maize (Pilcher et al. 1997). Duan et al. (2008) reported a similar lack of effect using Cry3Bb1 protein in a bee pollen diet. Studies with other species of *Orius* also have reported neutral effects of various Cry proteins (Cry1Ac, Cry1Ab, Cry2Ab, Cry3A) on various life-history traits using direct exposure through spiked diets (Gonzalez-Zamora et al. 2007), plant foliage and pollen (Armer et al. 2000, Pons et al. 2004, Lumbierres et al. 2012), and intoxicated prey (Zwahlen et al. 2000, Obrist et al. 2006, Gonzalez-Zamora et al. 2007, Lumbierres et al. 2012). Zhang et al. (2008) reported subtle negative effects on some life-history traits and prey consumption for *Orius sauteri* (Poppius) (Hemiptera: Anthocoridae) using intoxicated aphids as prey. However, it is questionable if the predator was exposed to Cry proteins, given that aphids in general contain no or only trace amounts of Cry protein when feeding on Bt plants (Romeis and Meissle 2011) and exposure in neither the aphids nor predators was confirmed. In contrast, Lumbierres et al. (2012) demonstrated that Cry1Ab consumption, directly from the plant or through intoxicated prey, actually increased development rates and fecundity in *Orius majusculus* Reuter.

Fewer studies have examined *G. punctipes* and *Geocoris* spp. in general. Ponsard et al. (2002) reported that longevity of field-collected adult *G. punctipes* and *Orius tristicolor* (White) (Hemiptera: Anthocoridae) of unknown age was reduced by 27 and 28%, respectively, when they were exposed to Cry1Ac via a tritrophic exposure using susceptible *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) feeding on Bt cotton leaves. However, they did not assess the effects of Bt cotton on the prey larvae. *Spodoptera* spp. are known to be at least partially susceptible to this Cry protein (Ramirez-Romero et al. 2007, Torres and Ruberson 2008) and very likely suffered some ill effects from feeding on Bt cotton. In addition, Ponsard et al. (2002) used a mixture of two unrelated non-Bt cotton cultivars as controls, potentially introducing other plant-mediated qualities into their study system. Finally, their results varied greatly between trials, in part due to the use of a laboratory colony source in the fourth trial for *G. punctipes* and the fact that essential moisture was not provided to either insect in the first two trials. In contrast, Torres and Ruberson (2006) found no differences in development, survival, adult mass, or fecundity of *G. punctipes* fed on *S. exigua* larvae that had been reared on Bt (Cry1Ac) cotton compared with larvae fed on a non-Bt control, even though they did show that *S. exigua* larvae were a poorer host for this predator compared with eggs of *Helicoverpa* (Boddie) (Lepidoptera: Noctuidae). In both the (Ponsard et al. 2002, Torres and Ruberson 2006,) studies, *G. punctipes* were provided access to both intoxicated prey and to Bt plant material, but their individual contributions were not assessed. However, Armer et al. (2000) showed no effect of Cry3A on *Geocoris* spp. when predators were provided access to Bt potato foliage. Our findings clearly show the lack of any negative effects of multiple Cry proteins on a broad range of life-history traits, including adult survival, when potential prey quality effects are removed. This suggests that the average 17% reduction in *G. punctipes* on Bt cotton reported by Naranjo 2005 over a 5-yr field study was likely due to other factors such as reductions in caterpillar prey, as originally suggested by the author, rather than Bt toxicity.

We found that the concentration of various Cry proteins declined rapidly as they moved through the food chain. Titers of Cry1Ac, Cry2Ab, and Cry1 F declined 7–23-fold from plant tissue to prey and then another 27–54-fold in the predators. Levels of Cry1Ac in the two predators were below the level of quantification. Similarly, Torres and Ruberson (2006) and Torres et al. (2006) failed to detect Cry1Ac in *G. punctipes* exposed through intoxicated *S. exigua*, even though the prey contained levels of the protein corresponding to >70% of that found in the plant. Armer et al. (2000) also failed to detect Bt proteins in *Geocoris* spp. and *O. tristicolor* feeding directly on Bt potato foliage. Torres and Ruberson (2008) were able to detect titers of Cry1Ac in both *G. punctipes* and *O. insidiosus* from tri-trophic exposures through both spider mites and thrips feeding on Bt cotton and these levels represented only a 6–11-fold reduction from the prey. Finally, it has been shown in other species of *Orius* that these predators readily pick up Cry1Ab from plant or prey sources, albeit at declining levels compared with plant or prey titers (Obrist et al. 2006, Meissle and Romeis 2009, Lumbierres et al. 2012).

While different prey species appear to influence the transfer of certain Cry proteins, we believe that the predators in our study were definitely exposed to all the Bt proteins we tested based on ELISA results for Cry1Ac, Cry2Ab, and Cry1 F. Furthermore, our previous studies have demonstrated that these proteins were biologically active after *T. ni* had fed on Cry1Ab/Cry2Ac cotton (Li et al. 2011) and *S. frugiperda* had fed on Cry1 F maize (Tian et al. 2012).

As with any study, questions remain. The current study did not include time-series analyses of retention of the Cry toxins in the guts of the prey larvae, so that we could quantify the actual exposure level of the Bt proteins to the predator. Because risk to the predator is a function of hazard (inherent toxicity of the protein) × exposure, we cannot be sure of the importance of each in these tri-trophic studies. Table 5 indicates that both predators contained Bt proteins when they had preyed on caterpillars that fed on Bt plants expressing these proteins (albeit in trace amounts for Cry1Ac). Whether the proteins were inherently nontoxic to the predators or whether the dose of the proteins was not sufficient to cause harm is impossible to determine from these studies. However, the important conclusion is that predators were exposed to prey that had consumed Bt proteins in what we consider realistic worst-case exposure conditions and the development, survivorship, and reproduction of the predators were not affected.

There is some controversy about the concept of direct and indirect effects of Bt proteins in the literature. The indirect effects of Bt proteins, acting through deleterious effects on the target prey or hosts that a natural enemy may consume, may occur in the field. Such 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 direct mortality of the prey or host or induce sublethal effects that would alter their quality as food for a predator or parasitoid. Indirect effects also are manifested by the absence of target prey or hosts of specialist natural enemies when the control technology is highly effective (see Wolfenbarger et al. 2008). Outside of instances where the target prey or hosts are largely absent from the crop—and thus too are specialist natural enemies—the impacts of indirect effects are unclear. Analyses of extant data in multiple Bt crops suggest a general lack of changes in natural enemy abundance (Marvier et al. 2007, Wolfenbarger et al. 2008, Naranjo 2009) and biological control function (Naranjo 2009) in Bt crops.

However, indirect prey quality-mediated effects observed in laboratory studies are sometimes incorrectly assumed to represent direct toxic effects (Lövei et al. 2009). Based on the meta-analyses of Naranjo (2009), Shelton et al. (2009) estimated that ≈63% of all studies examining Bt protein effects on natural enemies via tri-trophic exposures used susceptible prey or hosts that were potentially compromised and could interfere with a valid assessment of direct toxic effects. Our prior analyses (Romeis et al. 2006, Naranjo 2009) further suggest that arthropod predators are less susceptible to prey quality effects than parasitoids. Nonetheless, the use of Bt-resistant prey here, as in other studies (Ferry et al. 2006; Chen et al. 2008; Lawo et al. 2010; Li et al. 2011; Tian et al. 2012, 2013), eliminates any such confounding effects and allows for a more accurate assessment of direct exposure to and hazard of Bt Cry proteins.

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