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Bitrophic toxicity of Cry1Ac to *Cycloneda sanguinea*, a predator in Brazilian cotton

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Abstract

Insect predators are exposed to the Cry1Ac toxin in Bt cotton fields through several pathways. In this study, we investigated the effects of activated Cry1Ac added to a diet on *Cycloneda sanguinea* (L.) (Coleoptera: Coccinellidae), which is one of the main predators of non-target pests in Brazilian cotton. Direct bitrophic exposure of *C. sanguinea* to Cry1Ac was done by feeding beetles with *Aphis gossypii* (Glover) (Hemiptera: Aphidae) sprayed with 500 μg per ml Cry1Ac solution. Larval and pupal survival, development time, aphid consumption, and adult longevity were recorded daily. Couples within the same experimental treatment were paired and numbers of eggs laid and hatched per female were recorded daily. Net replacement rate was calculated for each female. During development, a *C. sanguinea* larva consumed on average 1.8 μg of activated Cry1Ac. No significant differences due to Cry1Ac were observed for any of the response variables, except aphid consumption. Larvae receiving Cry1Ac consumed more aphids than larvae receiving distilled water alone. Additional statistical analyses were conducted to evaluate independence of responses, and for the independent responses, a simple meta-analysis was conducted to test the null hypothesis that all responses were zero. Nearly all of the response variables were statistically independent. Two pairs of responses were not independent, but the associated multivariate tests were not significant. The meta-analysis suggested that all effects were not different from random variation around zero and no cumulative effects could be detected. Our results indicated that bitrophic exposure to activated Cry1Ac is likely to have little or no adverse ecological effect on *C. sanguinea*.

Introduction

Brazil is the fifth largest cotton producer in the world. In 2011–2012, 4.89 million tons were produced on approximately 1.4 million ha, with an average productivity of 3 500 kg ha^{-1} (CONAB, 2012). However, the occurrence of a large number of herbivore pests causes considerable damage and constrains productivity, efficiency, and sustainability of this crop in Brazil. This constraint can only be overcome with improved and more environmentally friendly methods of pest control. Transgenic Bt cotton varieties, which have been developed to protect the cotton against pest Lepidoptera, are one of the possibilities.

Presently, Bt cotton varieties engineered to continuously produce truncated (activated) Cry1Ac that originates from the soil bacteria *Bacillus thuringiensis* Berliner (Bt) are being commercially grown in Brazil. Bollgard® I (event 531) cotton has been available since 2006. In 2009, Bollgard® II (event 531 re-transformed to also express Cry2Ab2), MON531 × MON1445 and Widestrike©, which was obtained by crossing lines 281-24-236 (expressing Cry1F) and 3006-210-23 (expressing Cry1Ac), were approved for commercial use. These varieties can effectively suppress three of the four most serious lepidopteran pests of cotton in Brazil: *Alabama argillacea* (Hübner), *Heliothis virescens* (Fabricius), and *Pectinophora gossypiella*

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(Saunders) (CTNBio, 2009). Bollgard® I is ineffective against *Spodoptera frugiperda* (FE Smith), also a serious pest of cotton in the Brazilian Midwest, the main cotton production area. Bollgard® II provides slightly better control, and Widestrike© is quite effective against this pest. Other primary pests such as cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) (Sujii et al., 2008, 2013) and the boll weevil, *Anthonomus grandis* Boheman (Sujii et al., 2013) are unaffected by Cry1Ac and may increase in numbers due to a potential reduction in the use of pesticides. Bollgard® II and Widestrike© also do not control the non-lepidopteran primary pests of cotton in Brazil, which has limited their acceptability.

The use of Bt cotton can result in an increase in non-target pests. For example, mirid bugs have become more abundant in Bt cotton fields in China (Lu et al., 2010), and stink bugs have increased in southeastern USA (Greene et al., 2006). Although these published changes are probably not related to release from natural enemies, it is still possible that Bt cotton could reduce natural enemies resulting in greater crop loss from non-target pests in Brazil.

In commercially available Bt cottons in Brazil, Cry toxins are continuously expressed in all vegetative tissues and most reproductive tissues (Grossi-de-Sá et al., 2006). Consequently, non-target herbivores and subsequently their natural enemies will be exposed to these toxins. For example, on cotton Torres & Ruberson (2008) found about 16 times more Cry1Ac in *Tetranychus urticae* Koch than in the Bt cotton cultivar DPL 555. Similarly, an accumulation of Cry1Ab/Ac fusion toxin was observed in *Spodoptera littoralis* (Boisduval) (Zhang et al., 2006a) and in the aphid *A. gossypii* in China (Zhang et al., 2004). The Cry toxin present in the phytophagous mites and Lepidoptera was passed on to the predaceous bugs *Geocoris punctipes* (Say), *Nabis roseipennis* Reuter, and *Orius insidiosus* (Say) (Torres & Ruberson, 2008), and the predacious coccinellid *Propylaea japonica* (Thunberg) (Zhang et al., 2004, 2006a,b). Under controlled conditions or even in the field, predators are exposed to the Cry1Ac protein produced by the Bt cotton through herbivores, particularly those herbivores that are not the main targets of control (Torres et al., 2006).

Testing potential effects of Cry toxins on coccinellids has led to contrasting results, perhaps because there are no standard protocols. For example, Porcar et al. (2010) reported that oral ingestion of activated Cry1Ab incorporated into artificial diet at 50 μg μl^{-1} did not cause any effects on mortality in short-term experiments with *Adalia bipunctata* (L.). In longer term experiments, however, Schmidt et al. (2009) described adverse effects on the same insect, when either Cry1Ab or Cry3Bb were sprayed over its diet, eggs of *Ephestia kuehniella* Zeller, at lower concentrations (maximum of 50 μg ml^{-1}). On the other hand, indirect exposure by feeding *A. bipunctata* with spider mites reared on Cry1Ab- or Cry3Bb-expressing plants, and direct exposure using toxins diluted in a sucrose solution were also evaluated (Alvarez-Alfageme et al., 2011). In neither case did the authors detect any detrimental effects on the ladybird. Furthermore, they questioned whether insects in Schmidt et al.'s (2009) study did in fact consume the toxins, as *A. bipuctata* larvae sucked the contents of *E. kuehniella* eggs, rather than ingesting whole eggs or shells. This issue was addressed by Hilbeck et al. (2012). They showed intake of Cry toxins by *A. bipunctata* when using the same methods as in Schmidt et al. (2009), and argued that Alvarez-Alfageme et al. (2011) did not see any effects due to the use of different protocols. Due to the distinctly different results, it is not clear whether effects of Cry toxins in the field, if any, would impact this predator. In cases where toxicity can be demonstrated, it would be scientifically relevant to understand the mechanisms by which test substances work. For instance, Ping et al. (2008) have verified toxicity of Cry7Ab3 toward a coccinellid pest, *Henosepilachna vigintioctomaculata* (Motschulsky). Additional experiments by Song et al. (2012) demonstrated midgut cell lysis and binding of Cry7Ab3 to a cadherin protein.

Although Bt cotton has already been commercialized in Brazil, there has been no evaluation of the effect of Cry1Ac on the ladybeetle *Cycloneda sanguinea* (L.) (Coleoptera: Coccinellidae), one of the main predators of non-target pests in Bt cotton in Brazil. Ladybeetles are widely regarded as important biological control agents in agricultural habitats. Five species dominate the ladybeetle community associated with cotton in Brazil (Faria et al., 2006), and an analysis of the behavioral and physiological traits of these species (Oliveira et al., 2004), and the community composition on cotton in Central Brazil (EMG Fontes, CSS Pires & ER Sujji, unpubl.) has indicated that *C. sanguinea* is the most significant coccinellid predator and one of the priority candidates for ecological non-target risk assessment of genetically modified (GM) cotton (Faria et al., 2006). This species is abundant in the tropical and subtropical range of the New World (Gordon, 1985) and is reported to be a dominant predator in several crops in Florida (USA) (Michaud, 2002), Central and South America (Vandenberg & Gordon, 1988), and in Brazil (Santa-Cecilia et al., 2001; Oliveira et al., 2004). The main prey for *C. sanguinea* in cotton in Brazil is the cotton aphid, *A. gossypii*, but this predator also feeds on coccids, pseudococcids, diaspidids, whiteflies, insect eggs, small caterpillars, and virtually any other soft-bodied insect (Michaud, 2002). It is a voracious predator (Oliveira et al.,

2004); the larvae and adults consume whole aphids, and in the laboratory at 30 °C one individual consumes, on average, 1 066 *A. gossypii* during the entire immature stage (Isikber & Copland, 2001).

In this study, we investigated the effects of activated Cry1Ac sprayed on *A. gossypii* aphids on *C. sanguinea* life history parameters and reproductive performance. We expect that the results will contribute to broaden the existing knowledge about the ecological effects of Bt crops in Brazilian agroecosystems.

Materials and methods

Insect sources

All insects used in this study originated from colonies established from cotton fields in the Federal District, Brazil (15°56′S, 48°08′W), and maintained in the Laboratory of Ecology and Biosafety of Embrapa Genetic Resources and Biotechnology (Brasilia). Larvae and adults of *C. sanguinea* were fed with different species of aphids, but mainly *A. gossypii*, supplemented with fresh pollen of *Bidens pilosa* (L.) (Asteraceae), honey, and water. Aphids fed to the *C. sanguinea* colony were field collected or laboratory reared on non-transgenic cotton (*Gossypium hirsutum* L. var. *latifolium*), grown in 0.45-l plastic pots. Larvae of *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) used in the Cry1Ac activity test were maintained on solid artificial diet based on beans (*Phaseolus vulgaris* L., Fabaceae) and wheat germ (Schmidt et al., 2001). These colonies were kept under controlled environment at 25 ± 2 °C and L12:D12 h light regime.

Production of Cry1Ac

The Cry1Ac protoxin (133 kDa) of *B. thuringiensis* was obtained by heterologous overexpression in *Escherichia coli* (Migula) Castellani & Chalmers host strain ECE53 transformed by recombinant plasmid pOS4201, which contains the *cry1Ac* gene from *B. thuringiensis* var. *kurstaki* HD73. Briefly, cells were grown in Luria-Bertani broth containing ampicillin (0.1 μg ml^{-1}) at 37 °C and 300 r.p.m. until OD_{600} = 0.8. The induction was made by 0.001 M isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h and cells were harvested by centrifugation (20 min, 10 °C, 2 600 ***g***). After resuspending in 100 ml of lysis buffer [15% sucrose (wt/vol), 0.05 M ethylene-diamine-tetraacetic acid, 0.05 M Tris-HCl pH 8.0, containing 1 mg ml^{-1} lysozyme] for 45 min at 4 °C, the cell suspension was sonicated on ice (5 × 300 s, 70% cycle) and centrifuged (15 min, 4 °C, 5 000 ***g***). The pellet was then washed by centrifugation at 10 000 ***g*** for 30 min in each condition: three times in 2% (vol/vol) Triton X-100 plus 0.5 M NaCl, five times in 0.5 M NaCl, and two times in distilled water. All Cry1Ac protoxin produced was solubilized in 0.05 M Na_2CO_3, pH 10.5 and 0.01 M dithiothreitol (DTT) at 37 °C for 2 h. After dialysis against 10 l of distilled water (15 kDa membrane, under agitation, at 4 °C, two swaps after 4 h and the last one overnight), the solubilized Cry1Ac was digested in a trypsin/protoxin ratio of 1:50 (wt/wt) at 37 °C for 18 h. The proteolysis was stopped by phenyl-methane-sulfonyl-fluoride at 0.001 M and the toxin activation monitored in 10% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The activated toxin was used in all of the bioassays.

Toxin activity test

The biological activity of the activated Cry1Ac obtained by heterologous over-expression in *E. coli* was verified by conducting a bioassay using second instars of the Cry1Ac susceptible *A. gemmatalis*. The experiment consisted of two treatments: (T1) 1 ml of artificial diet containing 150 μl of water, and (T2) 1 ml of artificial diet containing 150 μl of water with 50 μg of activated Cry1Ac. Each treatment consisted of four repetitions containing 20 *A. gemmatalis* caterpillars. The larvae were monitored for 7 days and their survivorship was recorded. Student's t-test for homocedastic independent samples was used to compare the treatment means.

Bioassay protocol

Direct (bitrophic) exposure of *C. sanguinea* to activated Cry1Ac was performed by feeding beetles with laboratory-reared *A. gossypii* sprayed with a solution of activated Cry1Ac. Individual beetles were tested in cages consisting of transparent 350-ml plastic pots containing a 40-ml layer of 2% (wt/vol) autoclaved agar. Newly harvested, young non-Bt cotton leaves were sterilized in 0.25% (wt/vol) NaClO for 10 min, dried with towel paper, and immersed in the warm liquid agar solution in a way to have the petiole and part of the leaf edges immersed. The leaves were positioned with the undersurface up to expose the preferred leaf surface to aphids. The agar maintained leaf turgor. The temperature of the agar solution was <40 °C when the leaves were immersed in it.

The experiment consisted of daily transferring a known number of aphids to each bioassay cage using a thin brush. Preliminary studies were conducted to determine the number of apterous aphids consumed daily by each larval stage, and they were offered accordingly, as described in Table 1. Two treatments with four experimental replicates were conducted: (T0) negative control consisting of spraying the aphids with pure distilled water (water was the solvent used to prepare the protein solution), and (T1) spraying the aphids with 500 μg ml^{-1} activated Cry1Ac in

Table 1 Daily number of aphids offered to *Cycloneda sanguinea* larvae

Larval age (days)	No. aphids
1	20
2	40
3	60
4	80
5	100
6	120
7	140
8	160
9	100
10	100
11	80

distilled water. Aphids were transferred to fresh cages and allowed to commence feeding. The aphids were sprayed with 235 ± 15 µl per cage of protein solution or distilled water and then let dry at room temperature. Newly sprayed aphids were offered within 24 h of spraying.

Each replicate experiment had 20 individual beetle larvae in each treatment. One neonate (24 h) *C. sanguinea* larva was put inside a cage with aphids. All cages were then kept in environmentally controlled rearing rooms at 25 ± 2 °C, and 12 h light regime. Young coccinellid larvae usually suck out the inside of their prey (Hagen, 1962; Hodek & Honêk, 1996), and first instar *C. sanguinea* also did this. This means that first instars were minimally exposed to the Cry1Ac applied to the aphid cuticle. Beetles were transferred to new cages daily, because of the high rate of consumption of aphids (Isikber & Copland, 2001). Larval survival, stage of development, and the number of aphids consumed were recorded daily.

Adult survivorship and fecundity

After emergence, the adults were sexed, divided into couples and transferred to the adult cages, fed with aphids, pollen, honey and water, as described for the *C. sanguinea* colony above. Unlike larvae, adults move and change habitats, so adults were not fed Cry1Ac food to investigate the effects of larval consumption on adult traits. Couples from the different treatments were kept separated and adult mortality and life span, number of eggs laid, and number of eggs hatched per female were recorded daily. Net replacement rate, λ, was calculated from these data for each individual female beetle.

Quantification of the amount of Cry1Ac protein consumed

An assay was conducted to measure the amount of toxin consumed daily by each *C. sanguinea* larva in the bioassays. The replications consisted of 10 Petri dishes with 110 aphids each. The same treatments as in the bioassays were applied: (T0) aphids sprayed with distilled water (control), and (T1) aphids in cages sprayed with 235 µl of 500 µg ml^{-1} of activated Cry1Ac in distilled water. After spraying, the solution sprayed on the aphids was let dry at room temperature for 20 min and then the aphids were transferred to 1.5-ml microtubes containing 350 µl of PBS (phosphate-buffered saline: 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, 0.014 M NaCl, 0.003 M KCl, pH 7.6). After vortex agitation for 20 min, the microtubes were centrifuged (10 min, 4 °C, 2 600 ***g***) and the supernatants collected to be frozen at −20 °C until analysis. The estimation of Cry1Ac protein sprayed on aphids was made by indirect enzyme-linked immunosorbent assay. Standards of purified Cry1Ac at concentrations 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 100 ng per well were used to build an optical density calibration curve for estimating toxin content of tested material. The purified Cry1Ac was purchased from Dr. Marianne Pusztai-Carey (Department of Biochemistry, Case Western Reserve University, Cleveland, OH, USA). The samples (100 µl per well in triplicate) were incubated in 96-well plates at 37 °C for 2 h and the plate washed 3 × 5 min with 100 µl per well of PBS added with tween 20 (0.5% vol/vol) (1 × PBST). The residual sites of binding were blocked adding 100 µl per well of non-fat dried milk (0.5% wt/vol) and incubated overnight at 4 °C. The washing step was repeated and 100 µl per well of the anti-Cry1Ac monoclonal antibody was added (diluted 1:1 000 in 1 × PBST) with an incubation time of 2 h at room temperature. The anti-Cry1Ac monoclonal antibody was produced in mouse by Celula B Laboratory from Federal University of the Rio Grande do Sul (UFRGS, Brazil). The washing step was repeated and 100 µl per well of the secondary antibody (diluted 1:1 000 in 1 × PBST) of goat anti-mouse IgG (H+L) horseradish peroxidase-conjugated (HRP; BioRad™, Hercules, CA, USA) was added in an incubation time of 2 h at room temperature. Following another washing step, 100 µl per well of tetra-methyl-benzidine substrate solution (TMB; BioRad™) was added and incubated for 20 min at room temperature. The reaction was stopped with 100 µl per well of 1 N H_2SO_4 solution and the absorbance read at 450 nm.

Statistical analysis

Independence of responses. Although previous studies investigating the effect of Cry toxin on natural enemies have measured several response variables, none of these studies examined whether the responses were independent of each other. Tests of association (Sokal & Rohlf, 1973) were conducted among the response measures of *C. sanguinea* to determine whether, for example, higher aphid consumption resulted in lower pupal mortality or,

for example, whether shorter development time resulted in lower mortality. If such associations existed, then the response measures would not be independent, and multivariate analysis methods might be necessary. For continuous, ordinal variables, Pearson' correlation coefficient, r, was calculated among individuals within each experimental replicate, treatment, and sex separately so that correlation coefficients did not include contributions from the fixed and random effects of the experimental design. The many correlation coefficients were combined across sex and experiment within treatments, by transforming the correlation coefficients to standard normal variates (z-scale) using $\ln[(1 + r)/(1 - r)]$, which has a standard deviation of $1/(n - 3)$, where n is the number of beetles in each subgroup (SAS, 2009). The combined correlation coefficient and P-value were reported. For categorical variables, the Cochran–Mantel–Haenszel (CMH) test was used with Proc Mianalyze, which enables a test of association in 2×2 tables across multiple strata (experimental replicates, treatments, and sex) (SAS, 2009). The CMH odds ratio and P-values were reported. For correlations between categorical and ordinal variables, the point biserial correlation coefficient (Glass & Hopkins, 1995) was calculated and combined similar to the ordinal variables.

Analysis of responses to Cry1Ac. Aphid consumption, larval development time, pupal development time, total eggs laid by an individual female, proportion of eggs that hatched, and net replacement rate (λ) were tested for homoscedastic error variance using Levene's test, and net replacement rate was transformed by $\ln(\lambda + 0.5)$ to stabilize the error variance (Sokal & Rohlf, 1973). Transformed data were analyzed by ANOVA with treatments paired within replicate experiments. Means were calculated on the untransformed scale except for λ, which was calculated on the transformed scale. Error terms were the interactions with experimental replicate (not the number of beetles tested). Proportion of females ovipositing and stage-specific larval and pupal survival were analyzed using log-linear models (reporting g^2) (Bishop et al., 2007) and means were calculated on the logit scale. Instantaneous larval and adult survival rates were analyzed using the log-rank test, a non-parametric test for Kaplan–Meier survival rates (reporting χ^2) (Cox & Oakes, 1984), using error terms that were the interactions with experimental replicate. The mean difference between the treatments and corresponding SEMs were calculated and reported on the transformed scale. Because of the paired experimental design, the mean difference in the treatments is the appropriate statistic for significance testing. Consequently, the difference in the raw treatment means is not necessarily equal to the mean difference in the treatments. When possible, the effect of sex (male, female, or undetermined) was included in the statistical model. For the non-independent categorical response variables, we conducted a 4-way log-linear analysis to investigate the effect of the Cry1Ac on each variable, because this analysis controls for the association (non-independence) between the variables (Bishop et al., 2007). Analyses were conducted with PC-SAS, using Proc GLM, Proc CATMOD, and Proc LIFETEST (SAS, 2009).

Meta-analysis. For non-significant tests of association, treatment (e.g., Cry1Ac vs. control) effects, or treatment*sex interactions, the joint null hypothesis that there was no association/treatment effect/treatment*sex effect across all of the non-significant comparisons. Because this hypothesis can be rejected by either positive or negative effects, it was tested using Fisher's sum of logs test for the independent response variables. Under the null hypothesis, the distribution of P-values is uniform on the interval [0,1]. The uniformity of the distribution of P-values can be tested with the statistic $-2\sum_{i=1}^{I}\ln(p_i)$, which is distributed as a χ^2 distribution with I degrees of freedom, where I is the total number of P-values. This simple meta-analysis evaluates whether there were cumulative or non-zero responses hidden among the statistically non-significant effects.

Results

Toxin production and activity

Most Bt proteins are expressed in transgenic plants in a truncated, activated form (Perlak et al., 1990; Fujimoto et al., 1993) that differs from Cry proteins found in crystals present in *B. thuringiensis* microbial insecticides. The efficient heterologous production (0.92 mg ml^{-1}) of the Cry1Ac protoxin (133 kDa) can be visualized (Figure 1, lanes 2 and 3). All of the protoxin was hydrolyzed to an activated 66 kDa toxin (Figure 1, lanes 4 and 5). This activated toxin was used in all bioassays.

The mortality rate of *A. gemmatalis* was significantly higher when the second instars were fed with artificial diet containing 50 μg of activated Cry1Ac compared to artificial diet only (93.0 ± 6.7 vs. $8.0 \pm 4.5\%$; $t = 18.22$, d.f. = 3, $P<0.0005$), indicating that the activated heterologous Cry1Ac produced had biological activity as expected.

Quantification of activated toxin consumption by *Cycloneda sanguinea* larvae

The estimated toxin sprayed on the aphids in a bioassay cage containing 110 aphids was 0.29 ± 0.04 μg (coefficient of variation = 13.9%), using a calibration curve of

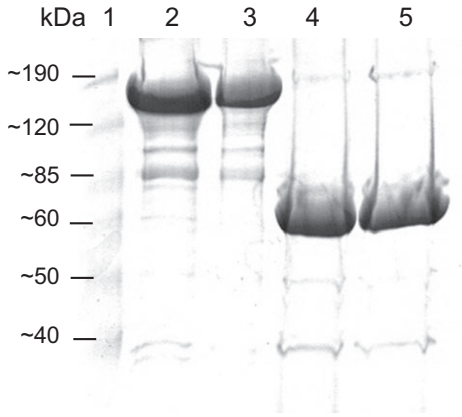

Figure 1 SDS-PAGE 10% gel of Cry1Ac protoxin (133 kDa) obtained by heterologous expression and its activation by trypsin proteolysis (66 kDa). Lane 1: BenchMark ladder (Invitrogen™, Carlsbad, CA, USA); lanes 2 and 3: 100 and 50 μg of protoxin Cry1Ac, respectively; lanes 4 and 5: 100 and 50 μg of trypsinized Cry1Ac, respectively.

$y = 0.009x + 0.027$ ($r^2 = 0.94$). This was equivalent to 18.39 ± 2.53 μg of activated Cry1Ac g^{-1} fresh weight of aphid, and gives an average amount of 1.80 ± 0.29 μg of Cry1Ac consumed by each surviving larvae, keeping in mind that the first instars had minimal exposure through the single aphid they consumed before molting.

Independence among *Cycloneda sanguinea* response variables

Association among the nine *C. sanguinea* response variables is shown in Table 2 for each Cry1Ac bioassay treatment separately. The associations among response variables were similar in the two treatments. Nearly all of the associations were not significant, which implies that the responses were independent and can be analyzed as univariate responses.

The only significant associations were between percentage females ovipositing and both larval and pupal mortality. These two associations were significant and in the same direction for both Cry1Ac bioassay treatments. When larval or pupal mortality was high, the percentage of surviving females that oviposited was high (Table 2).

Effect of Cry1Ac on *Cycloneda sanguinea* performance

There was significantly greater aphid consumption in the T1 (Cry1Ac) than in the T0 (control) treatment. Otherwise, no significant differences on larval, pupal, and adult mean performances were found, as shown after univariate analyses for continuous responses (Table 3). Furthermore, larval and pupal survival, and adult longevity did not differ between treatments (Table 4). Specifically, there was no difference in the net replacement rate, i.e., the average number of adult offspring per individual adult (Table 5).

Table 2 Combined correlation coefficients (and P-values in parenthesis) for correlation of response variables within treatments, controlling for variation among experiments and sex using the Mianalyze procedure and Fisher's z transformation of Pearson correlation coefficients. Association tests were calculated with Cochran–Mantel–Haenszel odds ratios (in italics) and P-values for categorical data, controlling for experimental replicate (strata) using the FREQ procedure (SAS, 2009). Coefficients for the control treatment (T0) are above the diagonal and coefficients for the Cry1Ac treatment (T1) are below the diagonal

	Control treatment (T0)									
Cry1Ac treatment (T1)	AP	LD	PD	AL	Ovip	% hatch	% ovip	LM	PM	
AP	–	0.41 (0.59)	0.04 (0.96)	−0.23 (0.82)	−0.45 (0.43)	−0.79 (0.53)	0.42 (0.58)	0.76 (0.14)	0.33 (0.46)	
LD	0.62 (0.51)	–	−0.18 (0.77)	−0.51 (0.55)	−0.38 (0.58)	0.38 (0.66)	0.00 (1.0)	0.73 (0.26)	0.45 (0.42)	
PD	−0.15 (0.86)	−0.65 (0.36)	–	0.01 (0.99)	−0.09 (0.92)	−0.41 (0.54)	–	–	–	
AL	0.10 (0.91)	0.42 (0.74)	0.27 (0.84)	–	0.29 (0.69)	−0.16 (0.86)	–	–	–	
Ovip	−0.34 (0.71)	0.33 (0.68)	−0.23 (0.86)	0.52 (0.56)	–	−0.30 (0.89)	–	–	–	
% hatch	0.90 (0.60)	0.08 (0.94)	0.04 (0.98)	−0.10 (0.92)	−0.09 (0.93)	–	–	–	–	
% ovip	0.18 (0.77)	0.15 (0.82)	–	–	–	–	–	*20.80* (<0.0001)	*255.24* (<0.0001)	
LM	0.44 (0.29)	0.31 (0.46)	–	–	–	–	*18.67* (<0.0001)	–	*13.66* (0.48)	
PM	0.36 (0.16)	0.43 (0.35)	–	–	–	–	*10.34* (0.0002)	*0.81* (0.66)	–	

AP, aphid consumption; LD, larval development time; PD, pupal development time; AL, adult longevity; Ovip, oviposition; LM, larval mortality; PM, pupal mortality.

Table 3 Effect of spraying of prey cotton aphids with activated Cry1Ac protein on (male and female) *Cycloneda sanguinea*: aphid consumption, larval and pupal development time, total number of eggs laid, percentage of egg hatch, and net replacement rate

Response variable	Source	F	Error MS	d.f.	P
Aphid consumption	Trt	17.80	210.63	1,3	0.02
	Sex	0.42	16158.99	2,12	0.67
	Trt*sex	0.03	16158.99	2,12	0.97
Larval development time	Trt	0.10	0.60	1,3	0.77
	Sex	8.73	0.46	2,12	0.004
	Trt*sex	0.81	0.46	2,12	0.47
Pupal development time	Trt	3.65	0.82	1,3	0.15
	Sex	1.85	0.39	1,6	0.22
	Trt*sex	2.46	0.39	1,6	0.17
Total no. eggs laid	Trt	0.77	5896.40	1,2	0.47
% eggs hatched	Trt	0.50	1662.89	1,2	0.55
Net replacement rate (λ)	Trt	4.78	1.51	1,2	0.16

Trt, treatment; F, calculated statistic from Fisher distribution.

Table 4 Effect of spraying of prey cotton aphids with activated Cry1Ac protein on (male and female) *Cycloneda sanguinea*: survival in stage-specific and instantaneous larval, pupal, and adult longevity

Response variable	Source	Test statistic	g^2 or χ^2	d.f.	P
Stage-specific larval survival (log-linear model)	Trt	–	0.15	1	0.70
Instantaneous larval survival (log-rank test)	Trt	1.57	0.14	1	0.71
Stage-specific pupal survival (log-linear model)	Trt	–	0.22	1	0.64
	Sex	765.30	3.58	2	0.17
	Trt*sex	763.7	3.55	2	0.17
Adult longevity (log-rank test)	Trt	−1.28	0.18	1	0.67
	Sex	−3.34	1.31	1	0.25
	Trt*sex	−4.62	2.22	1	0.14

Trt, treatment; g^2, log-linear model; χ^2, log-rank test.

The 4-way log-linear models indicated that the significant association between percentage females ovipositing and both larval and pupal mortality did not affect the statistical inference given for the individual univariate responses above. When two correlated response variables were analyzed simultaneously, they have three degrees of freedom: one for each variable separately and one for their interaction. For larval mortality and % oviposition, the effects of Cry1Ac were not significant ($\chi^2 = 0.53$, d.f. = 3, P = 0.91). The effects of Cry1Ac were also not significant for pupal mortality and % oviposition ($\chi^2 = 0.44$, d.f. = 3, P = 0.93).

Larval development time differed slightly, but significantly among the sexes (Tables 3 and 6). Some individuals died in the pupal stage before their sex could be determined (sex unknown), and these had a slightly longer larval development time than those that did not die (Table 6). Sex and the interaction of sex and Cry1Ac treatment were not significant for any of the other responses.

Meta-analysis

Examination of all of the non-significant association tests with Fisher's sum of logs method, gave a test statistic of 10.06 for the control treatment and 8.94 (both d.f. = 22, P = 0.99) for the Cry1Ac treatment. These results suggest that all of the remaining tests of association were not different from random variation around zero, and no cumulative associations could be detected among the nine response variables.

Examination of the non-significant effects for the Cry1Ac treatment and the treatment*sex interaction using Fisher's sum of logs method, gives a test statistic of 6.09 (d.f. = 10, P = 0.81) for the non-significant treatment effects and 5.26 (d.f. = 5, P = 0.38) for the treatment*sex effects. These results suggest that all treatment or treatment*sex effects were not different from random variation around zero and that no cumulative effects could be detected in the data.

Discussion

Our results suggest that bitrophic exposure of *C. sanguinea* to activated Cry1Ac on the external surfaces of aphids has no detectable effect on larval or adult development, survival, or reproduction. For these, the measured effect of Cry1Ac was not different from random variation around no effect. There was an effect on the number of aphids consumed by the larvae, with more aphids consumed in the Cry1Ac treatment than in the control. If this result were to persist in the field, it would suggest that the level of biological control associated with *C. sanguinea* would be higher in Cry1Ac Bt cotton than in conventional cotton. However, this result requires confirmation from the field. Together, our data indicate that direct consumption of activated Cry1Ac is likely to have little or no ecological effect on *C. sanguinea*.

Our study adds three analyses that strengthen these conclusions. First, we tested whether the multiple response variables were statistically independent. All previously

Table 5 Means, standard error of the difference in means, and n (in parenthesis) for the number of aphids consumed during immature period, larval and pupal development time, and lifetime oviposition, percentage of egg hatch, net replacement rate, survival, and adult longevity of *Cycloneda sanguinea* fed on cotton aphids either sprayed with distilled water (T0) or with activated Cry1Ac (T1)

Response variable	Pure water (T0)	Cry1Ac solution (T1)	Difference in means (T1–T0)
No. aphids consumed	667.6	682.2	14.6 ± 3.4 (4)*
Larval development time (days)	9.9	10.0	0.06 ± 0.16 (4)
Pupal development time (days)	4.7	5.2	0.45 ± 0.24 (4)
Lifetime oviposition (no. eggs laid)	84.9	58.0	−26.9 ± 26.5 (3)
% eggs hatched	47.6	35.2	−12.4 ± 18.2 (3)
Net replacement rate [ln(λ + 0.5)]	0.61	0.26	0.35 ± 0.17 (3)
Larval survival (logit)	1.45	1.56	0.11 ± 0.44 (4)
% larval survival	80	76	
Pupal survival (logit)	1.06	1.66	0.60 ± 0.54 (4)
% pupal survival	74	82	
Adult longevity (days)	64.8	65.4	−0.6 ± 0.6 (3)

*P<0.05.

Table 6 Means, standard error of the difference in means, and n (in parenthesis) of the number of aphids consumed, larval and pupal development time, and adult longevity for male and female *Cycloneda sanguinea*

Response variable	Females	Males	Unknown	SE difference among means
No. aphids consumed	679.4	655.9	689.5	31.8 (4)
Larval development time (days)	9.6	9.8	10.2	0.2 (4)**
Pupal development time (days)	4.9	5.1	–	0.2 (4)
Adult longevity (days)	58.7	53	–	12.2 (4)

Unknown: individuals who died in the pupal stage before their sex could be determined.
**P<0.01.

published studies have implicitly assumed that the multiple measured responses were independent because they all used univariate statistical methods for the multivariate responses (Lövei et al., 2009). A lack of independence would compromise the many meta-analyses of laboratory responses of predators to Cry toxins (Lövei et al., 2009). We found that only four of 48 possible tests of association were statistically significant (Table 2). This implies that it is appropriate to conduct univariate analyses of aphid consumption, larval and pupal development time, adult longevity, number of eggs laid, and proportion of eggs that hatched. The three remaining response variables had significant correlations and could not be considered statistically independent. When larval or pupal mortality was high, the proportion of surviving females that laid eggs was high. However, the multivariate analyses associated with these two pairs of variables were not statistically significant, indicating that the univariate analyses of these three variables provided sufficiently accurate statistical inference.

Second, we used a meta-analysis and tested whether the observed effects were the same as random variation around a zero effect size (no effect) or whether the effects might combine in some way to give a cumulative significant effect. We found that, excluding the statistically significant effect, all measured effects were not different from random variation around a zero effect size and that no cumulative effects could be detected. Neither of these two analyses has been used before in published studies on the effects of Cry proteins or proteinase inhibitors on arthropods in the lab. The meta-analysis reinforced our conclusion that direct consumption of Cry1Ac had no detectable effects on the performance of *C. sanguinea*. The tests for statistical independence and associated multivariate tests justify our univariate statistical analyses and demonstrate further that direct consumption of Cry1Ac was unlikely to affect interactions among the response variables.

Third, we evaluated the purity of activated Cry1Ac used in our bioassays and the quantity of Cry1Ac that *C. sanguinea* would be exposed to during the bioassays. The SDS-PAGE gel shows that our methods produced purified activated Cry1Ac. Although many studies use trypsin to activate Cry1Ac (e.g., Schmidt et al., 2009; Porcar et al., 2010; Song et al., 2012), rarely do they state the purifica-

tion methods or provide evidence of the purity of the Cry toxin (for exceptions see Dhillon & Sharma, 2009; Alvarez-Alfageme et al., 2011). Our bioassays were conducted at an extremely high exposure to activated Cry1Ac, even when compared with levels expressed in Cry1Ac plants commercialized in Brazil. Beetles consumed on average 1.80 ± 0.29 μg of activated Cry1Ac. In comparison, Cry1Ac is expressed at 11.5 ng g^{-1} fresh weight in Bollgard I® cotton plants (Monsanto Company, 2002), and in event 3006-210-23, expression levels reached 1.44 ng mg^{-1} dry weight (USDA, Animal & Plant Health Inspection Service, 2003). Cry1Ac is not always detectable in *A. gossypii* fed on Bt cotton (Torres & Ruberson, 2008; Lawo et al., 2009), although a toxin accumulation of 6 ng g^{-1} fresh weight was reported by Zhang et al. (2006b). In another aphid, Burgio et al. (2011) detected low levels of Cry1Ac in *Myzus persicae* (Sulzer) fed on Cry1Ac-expressing transgenic *Brassica napus* L., ranging from 4.8 ± 0.6 to 7.1 ± 1.2 ng g^{-1} fresh weight. In our bioassays, the aphids carried from ca. 2 600- to ca. 3 000-fold the quantity of Cry1Ac reported by Zhang et al. (2006b) and Burgio et al. (2011), respectively.

Several studies have been conducted to evaluate the effect of Bt proteins on insect predators in the laboratory (Fontes et al., 2003; Lövei & Arpaia, 2005; Lövei et al., 2009). One of the main predator taxa targeted by these studies is Coccinellidae, with the focus being placed on *P. japonica* and *Coleomegilla maculata* DeGeer (Lövei et al., 2009). Assessment of the environmental risks of Bt cotton to non-target natural enemies in Brazil has been largely based on studies related to predatory species that are numerically and ecologically insignificant in Brazilian cotton habitats. The assessment of potential adverse effects of a GM crop on non-target species should take into consideration the choice of an appropriate non-target species that exists in the specific environment in which the GM crop will be released (Andow & Hilbeck, 2004; Hilbeck et al., 2006). This study uses a non-target species known to be an important predator in Brazilian cotton.

Laboratory tests should seek, as much as possible, to approximate the conditions in the field (Lövei & Arpaia, 2005), so in this study we used prey preferred by *C. sanguinea* on cotton leaves to deliver Cry1Ac to the predator. These experimental conditions were favorable for the predator, i.e., good quality preferred food provided in abundance. Adverse effects of Bt plants on predatory coccinellids may arise via several exposure pathways, including pollen or other plant parts (a food source for larvae and adults), modified products of the introduced Bt gene (Obrist et al., 2006), and/or accumulation on the herbivore prey (Dutton et al., 2002; Zhang et al., 2004; Obrist et al., 2006; Torres & Ruberson, 2008).

Stephens et al. (2012) reported uptake of Cry3Bb in aphids fed on transgenic maize, and fewer *Harmonia axyridis* (Pallas) observed in Cry3Bb maize plots than in controls. Additionally, the authors demonstrated reduced life span when *H. axyridis* were fed aphids collected from Cry3Bb maize fields. Previous studies using pollen from transgenic maize expressing Cry3Bb did not, however, affect another coccinellid, *C. maculata* (Duan et al., 2002; Lundgren & Wiedenmann, 2002). Thus, the results of the present study can be interpreted as indicative of no effects on *C. sanguinea*, but do not give a complete picture of what will happen in the field, or of potential effects on other coccinellids.

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