ELSEVIER

Journal of Insect Physiology 50 (2004) 175–183

Journal of Insect Physiology

www.elsevier.com/locate/jinsphys

Bacillus thuringiensis toxin (Cry1Ab) has no direct effect on larvae of the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae)

Jörg Romeis *, Anna Dutton, Franz Bigler

Swiss Federal Research Station for Agroecology and Agriculture (FAL), Reckenholzstr. 191, 8046 Zurich, Switzerland

Received 14 July 2003; received in revised form 7 October 2003; accepted 6 November 2003

Abstract

Earlier studies have shown that larvae of the green lacewing predator *Chrysoperla carnea* are negatively affected when preying on lepidopteran larvae that had been fed with transgenic maize expressing the *cry1Ab* gene from *Bacillus thuringiensis*. To test whether the observed effects were directly caused by the Cry1Ab toxin, we have developed a bioassay which allows us to feed high concentrations of the toxin directly to the predator. The results of these feeding studies show no direct toxic effect of Cry1Ab on *C. carnea* larvae. The amount of toxin ingested by first instar *C. carnea* in the present study was found to be a factor 10,000 higher than the concentration ingested when feeding on *Bt*-reared lepidopteran larvae, a treatment that was previously shown to have a negative impact on the predator. In addition, feeding first instar *C. carnea* with the Cry1Ab toxin did not affect the utilisation of subsequently provided prey. Furthermore, the quality of the prey provided to first instars did not affect the sensitivity of second and third instar *C. carnea* to the *Bt*-toxin. The presented results strongly suggest that *C. carnea* larvae are not sensitive to Cry1Ab and that earlier reported negative effects of *Bt*-maize were prey-quality mediated rather than direct toxic effects. These results, together with the fact that lepidopteran larvae are not regarded as an important prey for *C. carnea* in the field, led us to conclude that transgenic maize expressing Cry1Ab poses a negligible risk for this predator.

Keywords: *Bt*; Feeding bioassay; Prey quality; Transgenic maize; Risk assessment

1. Introduction

One of the widely discussed environmental impacts of genetically modified (GM) crops is their potential effect on non-target organisms including biological control agents (Dale et al., 2002; Conner et al., 2003). All the current commercial transgenic insect-resistant crops express genes of the bacterium *Bacillus thuringiensis* (*Bt*) Berliner (Nap et al., 2003). Due to the experience with *Bt*-toxins as biopesticides, their known mode of action and specificity, as well as their fast degradability, they are generally regarded as safe for biological control agents and promoted in both organic and integrated pest management systems (Glare and O'Callaghan, 2000). However, in *Bt*-transgenic maize, the *cry1Ab* gene codes for a truncated and also more activated form of the original protein. In addition, the expression is continuous in most plant parts throughout plant growth. Thus, effects on non-target arthropods cannot be ruled out and have to be assessed as part of a risk assessment for *Bt*-transgenic crops (Federici, 2002). Selection of arthropods for pre-release risk assessment of transgenic plants should be done according to their economic/ecological importance in the crop and their potential exposure to the transgene product (Dutton et al., 2003b). In the case of *Bt*-transgenic maize, the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) should be selected as a test species since it is regarded as an important predator in maize, and likely to be exposed to the *Bt*-toxin (Dutton et al., 2002, 2003b). The test procedure for assessing the effects of an insecticidal protein on the selected

* Corresponding author. Tel.: +41-1-377-7299; fax: +41-1-377-7201.

E-mail address: joerg.romeis@fal.admin.ch (J. Romeis).

0022-1910/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2003.11.004

non-target arthropods should follow a tiered system that has been adapted from the ecotoxicological evaluation of plant protection products. In the first tier, worst case toxicity (sensitivity) studies should be performed using both the transgenic plant material and the pure toxin. For the latter, the test insect would ideally be exposed to a range of toxin concentrations in artificial diet feeding tests (Dutton et al., 2003b).

Earlier first tier studies revealed that *Bt*-maize (Event Bt11) expressing the *cry1Ab* gene from *B. thuringiensis* subsp. *kurstaki* causes negative effects on *C. carnea* larvae (Hilbeck et al., 1998a, b, 1999; Dutton et al., 2002). Significantly higher mortality, prolonged development and a decrease in weight were recorded for *C. carnea* larvae that were fed with lepidopteran larvae [*Ostrinia nubilalis* (Hübner) (Crambidae) or *Spodoptera littoralis* (Boisduval) (Noctuidae)] reared on either *Bt*-maize or on a Cry1Ab-containing artificial diet when compared to predators that were fed control prey reared on non-transformed maize or control diet. Additional studies in which the Cry1Ab toxin was incorporated into an artificial diet and fed directly to the *C. carnea* larvae indicated a direct toxic effect of the Cry1Ab toxin (Hilbeck et al., 1998b). However, recent results made us question the sensitivity of *C. carnea* larvae to this *Bt*-toxin (Dutton et al., 2002). *C. carnea* larvae were found not to be affected when fed with spider mites, *Tetranychus urticae* (Koch) (Acari: Tetranychidae), reared on *Bt*-maize (Event Bt11), even though ELISA tests revealed that spider mites contained the toxin at a level of 2.5 μg Cry1Ab/g fresh weight. For comparison, *S. littoralis* larvae contained significantly less of the *Bt*-toxin (0.7 μg Cry1Ab/g fresh weight) but nevertheless caused negative effects on *C. carnea* larvae that were fed exclusively with this prey (Dutton et al., 2002). Two facts appear to be of importance in this study. Firstly, spider mites were found to be a good quality prey, while *C. carnea* larvae fed with *S. littoralis* larvae reared on non-transgenic maize suffered a relatively high mortality. Secondly, studies of *S. littoralis* revealed that the lepidopteran larvae were negatively affected when reared on *Bt*-maize, indicated by an increased mortality and prolonged developmental time. This may have let to a further reduction in their quality as prey. In comparison, spider mites were not affected when reared on *Bt*-maize (Dutton et al., 2002). These results suggest that the reported effects of *Bt*-maize on *C. carnea* larvae are prey-quality mediated rather than direct effects of the Cry1Ab toxin.

To test whether the observed negative effects were directly or indirectly caused by the *Bt*-toxin, we have developed a bioassay which allows us to feed high concentrations of the Cry1Ab toxin to *C. carnea* larvae. Making use of the fact that chrysopid larvae feed on carbohydrate sources such as honeydew and (extra) floral nectar in order to extend longevity in the absence of prey (Downes, 1974; Limburg and Rosenheim, 2001), the toxin was provided dissolved in a sugar solution.

2. Materials and methods

2.1. Insect material

C. carnea were collected in Bolligen near Berne (Switzerland) in 1993 and since then maintained in the laboratory without any introductions of field-collected insects. Although the taxonomic status of *C. carnea* has been a topic of debate during recent years (Henry et al., 2001), our colony has been verified as *C. carnea* (Henry et al., 2002). Insects from the same laboratory colony were used in earlier studies to investigate the effect of *Bt*-toxins (Hilbeck et al., 1998a, b, 1999; Meier and Hilbeck, 2001; Dutton et al., 2002, 2003a). Larvae of *C. carnea* were maintained on pea aphids, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae), and adults were fed a diet containing honey, brewer's yeast and water (in the proportions 7:4:4). Rearing conditions were 22 ± 3 °C, $70 \pm 5\%$ r.h. with a 16 h light, 8 h dark photoperiod. For the sucrose utilisation experiment, the study on the food consumption and the experiment on the Cry1Ab uptake, 1-day old *C. carnea* larvae were used. For all other experiments, freshly emerged (up to 5 h old) larvae were used.

Egg masses of *S. littoralis* were provided by Syngenta (Stein, Switzerland), where the species is reared on an artificial diet. Freshly emerged larvae were kept on non-*Bt*-maize (N4640) for 24 h before being fed to *C. carnea*. Eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were received from Biotop (Valbonne, France) and stored for a maximum of 1 week at 4 °C. *O. nubilalis* eggs were obtained from INRA (Le Magneraud, France). The eggs were stored for a maximum of 2 days at 4 °C.

2.2. Bioassays

All experiments were conducted in a climatic chamber at 23 ± 1 °C, 80–85% r.h. with a 16 h light, 8 h dark photoperiod. If not stated otherwise, *C. carnea* larvae were kept individually in a Petri dish (5 cm diameter, 1 cm high) the lid of which contained a hole covered with a fine mesh-netting.

2.3. Food solutions and confirmation of Cry1Ab bioactivity

Lyophilised Cry1Ab toxin was purchased from M. Carey (Dept. Biochemistry, Case Western Reserve University, Cleveland, OH, USA). The Cry1Ab protoxin from *B. thuringiensis* subsp. *kurstaki* HD-1 was expressed as a single gene product in *Escherichia coli*.

Inclusion bodies containing Cry1Ab protoxin were dissolved and trypsinised and the Cry1Ab toxin was isolated using high-performance liquid chromatography (HPLC) (Pusztai-Carey et al., 1994). All experiments were done with protein from the same aliquot. In order to ensure that the Cry1Ab toxin had appropriate biological activity, *O. nubilalis* bioassays were conducted. Results from the sensitive insect bioassay conducted at Monsanto showed that the EC_{50} for neonate *O. nubilalis*, based on weight reduction of larvae at day 7 of the assay, of the Cry1Ab toxin from the aliquot used in the present study was 5 ng/ml artificial diet. This level of biological activity was comparable to that of a certified Cry1Ab protein standard obtained from Monsanto Company (Jian J. Duan, St. Louis, USA, personal communication).

Sucrose solutions (2 M) (Merck, Darmstadt, Germany) were prepared containing a range of Cry1Ab concentrations, reaching 1 mg/ml (0%, 0.0001%, 0.001%, 0.01% and 0.1% Cry1Ab, w/v). Within the climatic chamber, the concentration of the food solution increased within 1 h and stabilised at an approximate 10% higher concentration. Each *C. carnea* larvae received two 0.5 μl droplets of the food solutions that were changed every 2 days. The solutions were never completely consumed during this time period.

To confirm bioactivity of the Cry1Ab toxin in freshly prepared and 2-day old solutions, a sucrose solution containing 0.1% Cry1Ab was prepared. Fifteen droplets (5 μl each) were loaded into plastic Petri dishes and redissolved with 15 ml distilled water either immediately or after a 2-day period of exposure in the experimental climatic chamber. The extract was then diluted by a factor of 10 and 10 ml of the diluted extract were added to 90 ml *O. nubilalis* meridic diet, resulting in a diet that contained 50 ng Cry1Ab/ml diet. The control treatment consisted of redissolved fresh and 2-day old droplets of sucrose solution. The artificial diet was filled into small containers ($2 \times 2 \times 1.5$ cm) into which five *O. nubilalis* neonates were placed. The experiment was replicated 10 times per treatment leading to a total of 50 larvae per treatment. After 7 days, the weight of the surviving larvae was recorded. A larva was considered alive when it responded to mechanical stimulation by a fine hairbrush. Due to loss and death of larvae, the number of replications was reduced to 41–49 larvae per treatment. Data on *O. nubilalis* larval weight were analysed using three-way ANOVA with block (Petri dish), age of the food solution (freshly prepared, 2-day old) and treatment (sucrose solution, *Bt*-sucrose solution) as independent variables. Means were subsequently separated using Tukey HSD test. All statistical analyses were done using the software package Statistica.

2.4. Sucrose utilisation by C. carnea larvae

Neonate *C. carnea* were provided either a sucrose solution, water, or kept unfed ($n = 20$). Larval survival was recorded daily. Longevity data were compared among treatments using non-parametric statistics since data were not normally distributed and variances were not homogenous. Since data were uncensored, Kruskal–Wallis ANOVA was used and means were subsequently separated using Mann–Whitney-*U*-test (adjusted for ties) (Pyke and Thompson, 1986). The level of significance was Bonferroni corrected for two pairwise comparisons (no food vs. water; water vs. sucrose solution) leading to an adjusted $\alpha = 0.025$.

2.5. Effects of Cry1Ab on C. carnea food consumption

First instar (L_1) *C. carnea* were weighed individually on a microbalance (Mettler Toledo MX5, $d = 1$ μg; ±2 μg), transferred into a container ($2 \times 2 \times 1.5$ cm) and provided with either sucrose solution, or sucrose solution containing the highest concentration (0.1%, w/v) Cry1Ab ($n = 40$). An additional set of larvae was kept without food. After 30 min, the larvae were weighed again to determine the amount of food ingested. The weight change was established as the percentage of the initial larval weight. The weight of the control insects that were given no food changed on average (±SE) by $0.2 \pm 0.47\%$ with a maximum increase of 3 μg. Therefore, all larvae that had been provided with the food solutions and that had not gained more than 3 μg in weight were regarded as not having fed and were excluded from further analysis. Mean data on the percentage weight change were compared using Student's *t*-test.

2.6. Effect of Cry1Ab on first instar C. carnea longevity

C. carnea neonates were provided with either of the four different *Bt*-sucrose solutions or with a pure sucrose solution as control. Larval survival was recorded daily. The experiment was conducted six times with 10 larvae per treatment ($n = 60$). Longevity data were analysed using two-way ANOVA with experiment and *Bt*-concentration as independent variables.

2.7. Effect of Cry1Ab ingestion on prey utilisation in C. carnea

First instar *C. carnea* were provided with either the sucrose solution or a sucrose solution containing the highest concentration (0.1%) Cry1Ab for the first 6 days. Subsequently, the larvae, of which all were still L_1, were provided daily with fresh *E. kuehniella* eggs ad libitum until they reached the third instar (L_3). Larvae

were checked daily and parameters recorded were mortality, development (days required to reach the L_2 and L_3) and dry weight of freshly emerged L_3 (dried at 68 °C for 24 h). The experiment was conducted three times with 20 larvae per treatment (n= 60). Data on the development of L_1 and L_2 fed either sucrose solution or *Bt*-sucrose solution were compared using Mann–Whitney-*U*-test (adjusted for ties). Larval survival was compared between treatments using contingency table analysis (χ^2-test) with Yates correction for continuity. Mean data on L_3 dry weight were compared using Student's *t*-test on the log transformed data.

2.8. Effect of prey quality on C. carnea sensitivity to Cry1Ab

The experiment was conducted to determine whether the quality of prey provided during the L_1 has an effect on the sensitivity of older *C. carnea* larvae to the Cry1Ab toxin. The six treatments that were compared consisted of predator larvae that were fed ad libitum with either *E. kuehniella* eggs (high quality prey) or *S. littoralis* larvae (low quality prey) during the L_1 and were subsequently kept exclusively with water, sucrose solution, or sucrose solution containing the highest concentration (0.1%) Cry1Ab, once they had reached the L_2. *C. carnea* larvae were checked daily and parameters recorded were mortality, development (days required to reach the L_2), longevity of L_2/L_3 larvae fed with the different food solutions and proportion of larvae that reached the L_3. Once larvae had reached the L_2 and received the sucrose solution or *Bt*-sucrose solution, the L_2 and L_3 stages were not separated but the longevity of these older larvae was recorded.

The experiment was conducted three times with 10 larvae per treatment for L_1 *C. carnea* fed *E. kuehniella* eggs (n= 30), and with 20 larvae per treatment when the L_1 were fed *S. littoralis* larvae (n= 60). Data on the development of L_1 fed with either of the two prey species were compared using Mann–Whitney-*U*-test (adjusted for ties). Larval survival was compared between treatments using contingency table analysis (χ^2-test) with Yates correction for continuity. Data on longevity of L_2/L_3 were analysed using three-way ANOVA with experiment, prey (*E. kuehniella* eggs, *S. littoralis* larvae) and food (sucrose solution, *Bt*-sucrose solution) as independent variables. Longevity of water-fed larvae were analysed separately to detect differences in larvae fed the different prey species during the L_1 using Mann–Whitney-*U*-test.

2.9. Cry1Ab uptake by C. carnea

C. carnea larvae were weighed individually, transferred into a container (2 × 2 × 1.5 cm) and provided with either freshly prepared sucrose solution containing 0.1% Cry1Ab, or 2-day old sucrose solution containing 0.1% Cry1Ab (kept in the climatic chamber). After 30 min, the larvae were removed and weighed to determine the amount of food ingested. A 2-day old *Bt*-sucrose solution was tested as the provided food solutions in the earlier *C. carnea* feeding bioassays were renewed every 2 days. An additional treatment in which larvae were fed the freshly prepared *Bt*-sucrose solution for 30 min and subsequently kept on a pure sucrose solution for 6 days was performed in order to determine whether the protein could still be detected in the predator larvae after this period of time. Levels of Cry1Ab toxin for the *C. carnea* larvae were determined using a double sandwich ELISA kit (EnviroLogix Inc., Portland, ME, USA) with a limit of quantification of 0.25 ppm. Cry1Ab standards at concentrations 0, 0.5, 2.5 and 5 ppb were used as calibrators. Groups of 20 larvae were used per sample and dilution factors were calculated according to the dry weight of the larvae (see Dutton et al., 2002 for further details). The experiment was replicated four times. The amount of fresh *Bt*-sucrose solution consumed by the *C. carnea* larvae was compared to the amount consumed of the 2-day old solution using Student's *t*-test. The quantities of Cry1Ab detected in the larvae fed the different *Bt*-sucrose solutions were compared using Mann–Whitney-*U*-test.

2.10. Power analyses

In order to avoid committing type II errors, i.e. failing to reject a false null hypothesis, a retrospective power analysis was conducted on non-significant results (P> 0.05). Using the observed control means and variances, the power to detect an 'effect' corresponding to a 20% difference between treatment groups is given for each pairwise comparison (Student's *t*-test, Mann–Whitney-*U*-test) (Steidl et al., 1997; Marvier, 2002). For experiment 2.6, power was analysed for a one-way ANOVA with *Bt*-concentration as independent variable. The observed control mean and a 20% different mean were set as the extremes while the other treatment means were equally spaced between the extremes. For experiments 2.3 and 2.8, power analysis was done for a two-way ANOVA, again based on control means and 20% different means. Variances were taken from the observed controls. For all power analyses, the corresponding 'effect size' is given as d (Student's *t*-test, Mann–Whitney-*U*-test) or f (ANOVA) (Cohen, 1988). The 'effect size' is defined as the absolute difference between treatment groups in the parameter of interest, scaled by the within-population standard deviation (Steidl et al., 1997). For the contingency table analyses, a power analysis was conducted for a 'medium effect size' of w= 0.3 (Cohen, 1988). Cal-

culations were done using either Statistica or GPOWER (Erdfelder et al., 1996).

3. Results

3.1. Confirmation of Cry1Ab bioactivity

Mean larval weight (±SE) of the surviving *O. nubilalis* larvae was 5.5 ± 0.24 and 5.7 ± 0.26 µg for larvae where fresh or 2-day old sucrose solution was added to the diet, respectively. Larval weight was decreased to 1.1 ± 0.09 and 0.9 ± 0.08 µg when Cry1Ab toxin was added to the diet dissolved in a fresh or a 2-day old *Bt*-sucrose solution. The three-way ANOVA revealed no block effect (Petri dish) ($F_{9,140} = 1.36$, $P = 0.21$). This factor was therefore excluded from further analysis. While *O. nubilalis* larvae fed the *Bt*-sucrose solution were significantly lighter compared to the control ($F_{1,176} = 520$, $P < 0.0001$), larval weight was not affected by the age of the food solutions ($F_{1,176} < 0.001$, $P = 0.99$, Power = 0.94, $f = 0.26$).

3.2. Sucrose utilisation by C. carnea larvae

Longevity of neonate *C. carnea* was significantly affected by the food offered ($H_{2,60} = 42.8$, $P < 0.0001$). Unfed *C. carnea* larvae lived an average (±SE) of 1.6 ± 0.18 days, while longevity was significantly prolonged when water was provided (2.2 ± 0.15 days) ($U = 106$, $n = 20$, 20, $P = 0.006$). Longevity increased further by a factor of more than 4, to 10.1 ± 0.44 days, when larvae were fed a sucrose solution ($U = 0$, $n = 20$, 20, $P < 0.0001$). None of the larvae developed into L_2.

3.3. Effects of Cry1Ab on C. carnea food consumption

Weight gain did not differ between larvae provided the sucrose solution (mean ± SE; $15.7 \pm 1.18\%$, $n = 36$) and the *Bt*-sucrose solution ($14.7 \pm 0.93\%$, $n = 39$) ($t_{73} = 0.72$, $P = 0.47$, Power = 0.47, $d = 0.44$).

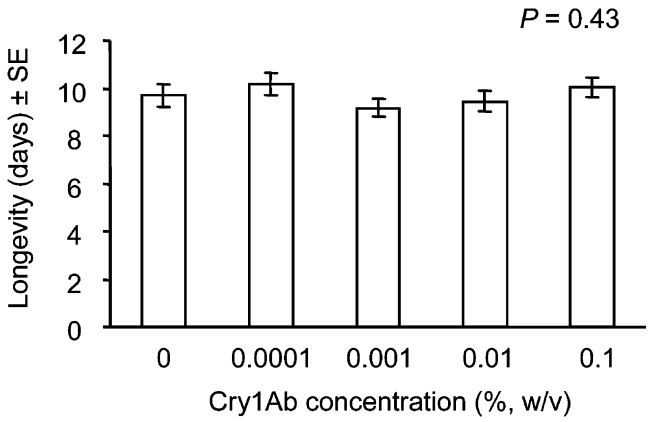

Fig. 1. Mean longevity (±SE) of first instar *C. carnea* fed with different concentrations of the Cry1Ab toxin dissolved in a 2 M sucrose solution ($n = 57$–60 per treatment).

3.4. Effect of Cry1Ab on first instar C. carnea longevity

Cry1Ab consumption had no significant effect on the longevity of L_1 *C. carnea* at the range of concentrations tested ($F_{4,264} = 0.96$, $P = 0.43$, Power = 0.77, $f = 0.20$) (Fig. 1). While a significant difference among the six experiments was detected ($F_{5,264} = 5.41$, $P < 0.0001$), there was no experiment × treatment interaction ($F_{20,264} = 0.74$, $P = 0.78$). None of the larvae developed into L_2.

3.5. Effect of Cry1Ab ingestion on prey utilisation in C. carnea

Neonate *C. carnea* larvae that were exclusively fed with either sucrose solution or *Bt*-sucrose solution for the first 6 days and subsequently provided with *E. kuehniella* eggs did not differ in their time required to reach the L_2 ($U = 1218$, $n = 50$, 51, $P = 0.58$, Power = 1, $d = 1.89$), the L_3 ($U = 1150$, $n = 47$, 50, $P = 0.83$, Power = 1, $d = 1.13$) or in their dry weight as L_3 ($t_{95} = 1.53$, $P = 0.13$, Power = 0.53, $d = 0.42$) (Table 1). Mortality during L_1 and L_2 was low and did not differ between larvae that were provided sucrose solution or *Bt*-sucrose solution (L_1: $\chi^2_1 = 0.05$, $P = 0.82$, Power $_{(w=0.3)} = 0.93$; L_2:

Table 1
Effect of *Bt*-consumption on prey utilisation in *C. carnea* larvae. Neonates were fed with either sucrose solution (2 M) or sucrose solution (2 M) containing Cry1Ab (0.1%, w/v) for 6 days and were subsequently provided *E. kuehniella* eggs as prey ($n = 59$, 58)

Food solution	L_1 development (days ± SE)[a]	L_1 survival (%)	L_2 development (days ± SE)	L_2 survival (%)	L_3 dry weight (µg ± SE)
Sucrose	5.1 ± 0.08[b]	84.7[c]	3.4 ± 0.09[b]	96.0[c]	1139 ± 77.8[d]
Bt-sucrose	5.1 ± 0.06	87.9	3.4 ± 0.08	96.1	1252 ± 68.6

[a] Days to reach the L_2, calculated from the day that larvae were provided prey.
[b] Differences between means within a column were not significant (Mann–Whitney-*U*-test, $P > 0.05$).
[c] Differences between percentages within a column were not significant (χ^2-test, $P > 0.05$).
[d] Differences between means within the column were not significant (Student's *t*-test, $P > 0.05$).

Table 2
Effect of *Bt*-consumption on the longevity of second/third instar *C. carnea* fed with different quality prey during the first instar. Second/third instars were provided either sucrose solution (2 M), sucrose solution (2 M) containing Cry1Ab (0.1%, w/v), or water

Prey provided during L_1 (*n*)	L_1 development (days ± SE)	L_1 survival (%)	Food provided from L_2 onwards (*n*)	Longevity of L_2/L_3 (days ± SE)	Development in L_3 (%)
E. kuehniella (90)	3.7 ± 0.05 a[a]	98.9 a[b]	Sucrose (29)	46.3 ± 2.70 a[c]	46.7
			Bt-sucrose (29)	46.6 ± 2.14 a	43.3
			Water (29)	5.6 ± 0.20 A	37.9
S. littoralis (169)	5.7 ± 0.10 b	72.2 b	Sucrose (41)	20.0 ± 1.54 b	0
			Bt-sucrose (41)	20.5 ± 1.60 b	2.4
			Water (40)	2.1 ± 0.13 B	0

[a] Different letters behind means indicate significant differences (Mann–Whitney-*U*-test, $P< 0.05$).
[b] Different letters behind percentages indicate significant differences (χ^2-test, $P< 0.05$).
[c] Different letters behind means indicate significant differences (small letters: two-way ANOVA; capital letters: Mann–Whitney-*U*-test, $P< 0.05$).

$\chi^2_1 = 0.24$, $P= 0.62$, Power $_{(w=0.3)}= 0.87$) (Table 1). The 'effect size' $w= 0.3$ of the power analysis corresponds to a 10% or 6% change in the survival rate for L_1 and L_2, respectively.

3.6. Effect of prey quality on C. carnea sensitivity to Cry1Ab

L_1 *C. carnea* fed *E. kuehniella* eggs developed significantly faster ($U= 160$, $n= 89, 122$, $P< 0.0001$) and suffered a significantly lower mortality ($\chi^2_1 = 26.0$, $P< 0.0001$) when compared to predator larvae that were fed *S. littoralis* larvae (Table 2). While L_2/L_3 *C. carnea* that had fed *E. kuehniella* eggs during the L_1 lived significantly longer when compared to those that had been reared with *S. littoralis* larvae ($F_{1,128}= 202$, $P< 0.0001$), longevity did not differ between larvae that were fed sucrose solution or *Bt*-sucrose solution ($F_{1,128}= 0.01$, $P= 0.91$, Power $= 0.86$, $f= 0.26$). There was no interaction between prey provided during L_1 and the food solution offered to the L_2/L_3 ($F_{1,128}< 0.001$, $P= 0.99$). While a significant difference among the three experiments was detected ($F_{1,128}= 5.13$, $P= 0.007$), none of the interactions of the factor experiment with any of the two other independent variables was significant ($P> 0.05$). Also for water-fed *C. carnea*, longevity was significantly higher in larvae fed with *E. kuehniella* eggs during the L_1 compared to those that were fed with *S. littoralis* larvae ($U= 8.50$, $n= 29, 40$, $P< 0.0001$). While between 38% and 47% of the larvae that were provided *E. kuehniella* eggs as L_1 also reached the L_3, only a single predator larvae fed with *S. littoralis* larvae did.

3.7. Cry1Ab uptake by C. carnea

Within a 30 min exposure period, *C. carnea* larvae consumed similar amounts of the fresh *Bt*-sucrose solution (mean ± SE; 12.4 ± 0.66 μg) as from the 2-day old *Bt*-sucrose solution (12.1 ± 0.88 μg) ($t_{151}= 0.31$, $P= 0.76$, Power $= 0.74$, $d= 0.42$). The ELISA did not show differences between the amount (±SE) of *Bt*-toxin in larvae fed freshly prepared (31.1 ± 1.51 μg Cry1Ab/g fresh weight) and 2-day old (25.1 ± 3.98 μg) *Bt*-sucrose solution ($U= 6$, $n= 4, 4$, $P= 0.56$). No Cry1Ab toxin was detected in larvae that were fed *Bt*-sucrose solution and subsequently provided pure sucrose solution for the consecutive 6 days.

4. Discussion

A novel bioassay has been developed that allows to expose *C. carnea* larvae to high concentrations of toxins in 'worst-case' toxicological studies. Results compiled using this method indicate that *C. carnea* larvae are not sensitive to Cry1Ab, a *Bt*-toxin expressed by commercial transgenic maize varieties.

Chrysopid larvae have been reported to feed on carbohydrate sources both in the laboratory and in the field and the positive effect of carbohydrate ingestion on larval longevity is well established (Downes, 1974; Limburg and Rosenheim, 2001). In the present study, longevity of neonate *C. carnea* was increased by a factor of more than 4 in larvae with access to a sucrose solution when compared to water-fed larvae. When *C. carnea* larvae were provided *E. kuehniella* eggs or *S. littoralis* larvae during the first instar and were subsequently fed a sucrose solution once they had reached the second instar, longevity increased by a factor of 8 and 10, respectively, when compared to larvae provided water. Given that *C. carnea* larvae ingested the sucrose solutions, the possible effect of dissolved toxins should be detectable using such a bioassay. In addition, feeding bioassays revealed that equal amounts of sucrose solution and sucrose solution containing 0.1% of the Cry1Ab toxin were ingested by neonate *C. carnea* larvae, showing that the addition of this *Bt*-

toxin did not affect the predator's gustatory response. This indicates that the predator larvae are unlikely to detect Cry1Ab when feeding on prey herbivores containing the toxin, thus leading to exposure (Dutton et al., 2003b). This is in contrast to various lepidopteran larvae which are known to be deterred from both diets and plants containing *Bt*-toxins (Gould and Anderson, 1991; Berdegué et al., 1996).

Our results show that first instar *C. carnea* are not affected by the Cry1Ab toxin at the range of concentrations tested, reaching 1 mg Cry1Ab/ml sucrose solution. Furthermore, ingestion of the toxin did not appear to have an effect on prey utilisation. Neonates that had been kept exclusively on a sucrose solution or a *Bt*-sucrose solution for 6 days continued their development similarly once they were provided *E. kuehniella* eggs as prey. Similarly, older *C. carnea* larvae were also not affected by Cry1Ab ingestion. Independent from the prey provided during the first instar, *E. kuehniella* eggs or *S. littoralis* larvae, longevity of second/third instar *C. carnea* was not decreased when the Cry1Ab toxin was included in the sucrose diet provided to these older instars. However, it was evident that *C. carnea* larvae that had been fed with *S. littoralis* larvae during their first instar lived significantly shorter when compared to those that had received *E. kuehniella* eggs. It is well established that larval development in chrysopids is prolonged when low quality food is provided (Principi and Canard, 1984) and the fact that *S. littoralis* larvae are a low quality prey for *C. carnea* is evident from earlier studies (Dutton et al., 2002, 2003a). The results presented here demonstrate that the effect of the Cry1Ab toxin on *C. carnea* larvae was not enhanced when the predator was set under nutritional stress by feeding on a low quality prey prior to the ingestion of the toxin.

In this study, it can be ruled out that the observed results are due to the fact that Cry1Ab was not biologically active or that the *C. carnea* larvae did not ingest the toxin. Bioassays with the sensitive target insect *O. nubilalis* showed that the dissolved protein was active in the sucrose solution, even after 2 days of having been dissolved. This is of importance as the food solutions where changed every 2 days in the *C. carnea* feeding bioassays. In addition, ELISA measurements of *C. carnea* larvae revealed that the larvae ingested similar amounts of toxin when feeding on either the freshly prepared or the 2-day old *Bt*-sucrose solutions. It is interesting to note that no toxin could be detected in *C. carnea* larvae that had been kept on a pure sucrose solution for 6 days after they had ingested Cry1Ab. Further studies revealed that the detectable amount of this toxin within the larvae was decreased by half within 24 h after feeding on the sucrose solution containing 0.1% Cry1Ab (unpublished results). Given that chrysopid larvae lack a connection between the mid- and hindguts, and defecation takes place only after imaginal moulting (Yazlovetsky, 2001), the toxin could not have been excreted. Excretion as a detoxification method has, for example, been described for larvae of the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Aphidiidae) that excret ingested lectin (*Galanthus nivalis* agglutinin) in their meconial pellets (Couty et al., 2001). In contrast, our results suggest that *C. carnea* larvae are able to digest Cry1Ab and possibly detoxify the protein. However, this has to be confirmed in more detailed digestion studies.

In the present study, we found that first instar *C. carnea* contained 31 μg Cry1Ab/g fresh weight when they had been left for 30 min to feed on a sucrose solution containing 0.1% Cry1Ab. In comparison, the amount of toxin consumed by first instar larvae was calculated to be 3.15 ng Cry1Ab/g fresh weight when fed exclusively with 1-day old *S. littoralis* larvae that had been reared on *Bt*-maize (Event Bt11). For this treatment, negative effects on different life-history parameters of *C. carnea* larvae have been reported before (Hilbeck et al., 1998a; Dutton et al., 2002). The above calculation is based on the fact that neonate *S. littoralis* larvae reared on *Bt*-maize for 1 day contain an average of 0.7 μg Cry1Ab/g fresh weight (Dutton et al., 2002) and the fact that a first instar *C. carnea* consumes a maximum of 30 *S. littoralis* larvae during its development, each of which has a fresh weight of about 0.15 mg (unpublished results). Thus, the amount of Cry1Ab toxin consumed by a neonate *C. carnea*, when dissolved in the sucrose solution, was therefore a factor 10,000 higher when compared to the amount ingested through *Bt*-reared *S. littoralis* larvae throughout the first instar development.

Based on the evidence available so far, it cannot be ruled out that the previously reported negative effects on *C. carnea* larvae when fed with *Bt*-reared *S. littoralis* could be due to the fact that the lepidopteran larvae process the *Bt*-toxin in a way that increases its toxicity to *C. carnea*. Alterations in *Bt*-specificity by the processing of different gut proteases have been previously reported (Haider et al., 1986; Rukmini et al., 2000). However, this seems to be unlikely since we have found that Cry1Ab extracted from *S. littoralis* and *T. urticae* did not differ in its activity on *O. nubilalis* (unpublished results).

The reported results are partially contradictory to an earlier study where a direct effect of Cry1Ab on the survival and development of second and third instar *C. carnea* was shown (Hilbeck et al., 1998b). In the earlier study, larvae were provided the toxin mixed in a complex artificial diet at a concentration of 100 μg Cry1Ab/ml diet. Similar to our study, Hilbeck et al. (1998b) found no effect of Cry1Ab on first instar *C. carnea*. However, toxin ingestion during the first instar enhanced the negative effects on the later instars.

In contrast, our study revealed no effect of Cry1Ab on second or third instar *C. carnea*. The two studies cannot be directly compared due to differences in the bioassay methodology. Possible reasons, as to why direct toxic effects of Cry1Ab on *C. carnea* were observed by Hilbeck et al. (1998b) but not in the present study are, firstly, *C. carnea* larvae might have ingested more of the toxin in the earlier study. However, this is rather unlikely given the high amount of toxin ingested in our feeding bioassays (see calculations above). Secondly, protease composition and/or activity may differ between the *C. carnea* larvae fed sucrose solution and those provided the artificial diet, with possible effects on further cleavage or degradation of the toxin. Thirdly, interactions between compounds in the *C. carnea* diet and the Cry1Ab toxin may have occurred. The artificial diet used by Hilbeck et al. (1998b) was of suboptimal quality for *C. carnea* larvae, indicated by a relative high mortality when compared to predator larvae fed with *T. urticae, R. padi* or *E. kuehniella* eggs (Hilbeck et al., 1998b; Dutton et al., 2002). Any changes in the physical or chemical properties of the artificial diet could have reduced its quality even further, causing the negative effects on *C. carnea*. This leads us to recommend that care must be taken when developing artificial diets for toxicological studies.

The results presented here suggest that the observed negative effects on *C. carnea* larvae when provided lepidopteran larvae reared on *Bt*-maize are most probably prey-quality mediated rather than direct effects of the *Bt*-toxin. Further evidence for this is the fact that *C. carnea* larvae also suffered a significantly increased mortality, prolonged development and decrease in weight when fed with *S. littoralis* larvae that had been reared on non-transformed maize plants sprayed with Dipel (Dutton et al., 2003a). This commercially available *Bt*-spray contains a number of different toxins (including Cry1Ab), and also affects *S. littoralis* larvae (Dutton et al., 2003a). A similar effect has been reported for *C. carnea* (most probably a sibling species; Henry et al., 2001) fed with larvae of *S. littoralis* that had ingested *B. thuringiensis* subsp. *entomocidus* (Salama et al., 1982). Again, the lepidopteran larvae were negatively affected by the *Bt*-toxin (Salama et al., 1982). These prey-quality mediated effects may be due to changes in the amino acid composition of the haemolymph of S. *littoralis* larvae as has been reported after ingestion of two *Bt*-strains (*kurstaki* HD-129 and *entomocidus* HD-635) (Salama et al., 1983).

The presented first-tier laboratory studies indicate that the observed negative effects on *C. carnea* larvae provided with Cry1Ab-fed lepidopteran larvae are due to a reduction in prey quality and not to a direct toxic effect. This is in agreement with the observation that no specific binding sites for Cry1Ab were detectable on midgut brush border membrane vesicles isolated from *C. carnea* larvae (Ruud de Maagd, Plant Research International, Wageningen, The Netherlands, unpublished results) since specific binding of the protein to midgut receptors is a prerequisite for *Bt*-toxicity (Schnepf et al., 1998; de Maagd et al., 2001). Since *C. carnea* larvae preferably feed on aphids rather than on lepidopteran larvae (Meier and Hilbeck, 2001), it can be concluded that *Bt*-maize expressing the Cry1Ab toxin poses a negligible risk for this important predator. This is confirmed by a number of higher tier field studies (Orr and Landis, 1997; Pilcher et al., 1997; Wold et al., 2001; Bourguet et al., 2002; Candolfi et al., in press), in which no negative effects of *Bt*-transgenic maize on chrysopids belonging to the *carnea*-group of *Chrysoperla* were detected.

Acknowledgements

We thank Heiri Klein and Marco D'Alessandro for help with the experiments and Dirk Babendreier (FAL), Thomas Hoffmeister (University of Kiel) and Jürg Hüsler (University of Berne) for discussions on the statistics. We are grateful to Jian J. Duan (Monsanto Company, St. Louis) for measuring the activity of the Cry1Ab toxin used for this study and to Ruud de Maagd (Plant Research International, Wageningen) for the permission to refer to unpublished data. We further thank J.J. Duan, William J. Moar (Auburn University) and Felix L. Wäckers (Netherlands Institute of Ecology, Heteren) for critical comments made on an earlier draft of the manuscript.

References

Berdegué, M., Trumble, J.T., Moar, W.J., 1996. Effect of *Cry*IC toxin from *Bacillus thuringiensis* on larval feeding behavior of *Spodoptera exigua*. Entomologia Experimentalis et Applicata 80, 389–401.

Bourguet, D., Chaufaux, J., Micoud, A., Delos, M., Naibo, B., Bombarde, F., Marque, G., Eychenne, N., Pagliari, C., 2002. *Ostrinia nubilalis* parasitism and the field abundance of non-target insects in transgenic *Bacillus thuringiensis* corn (*Zea mays*). Environmental Biosafety Research 1, 49–60.

Candolfi, M.P., Brown, K., Grimm, C., Reber, B., Schmidli, H., 2004. A faunistic approach to assess potential side-effects of genetically modified *Bt*-corn on non-target arthropods under field conditions. Biocontrol Science and Technology 14, in press.

Cohen, J., 1988. Statistical Power Analysis for the Behavioral Sciences, second ed. Lawrence Erlbaum Associates, Hillsdale.

Conner, A.J., Glare, T.R., Nap, J.-P., 2003. The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. The Plant Journal 33, 19–46.

Couty, A., Down, R.E., Gatehouse, A.M.R., Kaiser, L., Pham-Delègue, M.H., Poppy, G.M., 2001. Effects of artificial diet containing GNA and GNA-expressing potatoes on the development of the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Aphidiidae). Journal of Insect Physiology 47, 1357–1366.

Dale, P.J., Clarke, B., Fontes, E.M.G., 2002. Potential for the environmental impact of transgenic crops. Nature Biotechnology 20, 567–574.

De Maagd, R.A., Bravo, A., Crickmore, N., 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends in Genetics 17, 193–199.

Downes, J.A., 1974. Sugar feeding by the larva of *Chrysopa* (Neuroptera). Canadian Entomologist 106, 121–125.

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

Dutton, A., Klein, H., Romeis, J., Bigler, F., 2003a. Prey-mediated effects of *Bacillus thuringiensis* spray on the predator *Chrysoperla carnea* in maize. Biological Control 26, 209–215.

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

Erdfelder, E., Faul, F., Buchner, A., 1996. GPOWER: a general power analysis program. Behaviour Research Methods, Instruments and Computers 28, 1–11.

Federici, B.A., 2002. Case study: Bt crops—a novel mode of insect resistance. In: Atherton, K. (Ed.), Genetically Modified Crops: Assessing Safety. Taylor & Francis Group, London, pp. 164–200.

Glare, T.R., O'Callaghan, M., 2000. *Bacillus thuringiensis*: Biology, Ecology and Safety. John Wiley & Sons, Chichester.

Gould, F., Anderson, A., 1991. Effects of *Bacillus thuringiensis* and HD-73 delta-endotoxin on growth, behavior, and fitness of susceptible and toxin-adapted strains of *Heliothis virescens* (Lepidoptera: Noctuidae). Environmental Entomology 20, 30–38.

Haider, M.Z., Knowles, B.H., Ellar, D.J., 1986. Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal δ-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. European Journal of Biochemistry 156, 531–540.

Henry, C.S., Brooks, S.J., Thierry, D., Duelli, P., Johnson, J.B., 2001. The common green lacewing (*Chrysoperla carnea s. lat.*) and the sibling species problem. In: McEwen, P.K., New, T.R., Whittington, A.E. (Eds.), Lacewings in the Crop Environment. Cambridge University Press, Cambridge, pp. 29–42.

Henry, C.S., Brooks, S.J., Duelli, P., Johnson, J.B., 2002. Discovering the true *Chrysoperla carnea* (Insecta: Neuroptera: Chrysopidae) using song analysis, morphology, and ecology. Annals of the Entomological Society of America 95, 172–191.

Hilbeck, A., Baumgartner, M., Fried, M.P., Bigler, F., 1998a. Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). Environmental Entomology 27, 480–487.

Hilbeck, A., Moar, W.J., Pusztai-Carey, M., Filippini, A., Bigler, F., 1998b. Toxicity of *Bacillus thuringiensis* Cry1Ab toxin to the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae). Environmental Entomology 27, 1255–1263.

Hilbeck, A., Moar, W.J., Pusztai-Carey, M., Filippini, A., Bigler, F., 1999. Prey-mediated effects of Cry1Ab toxin and protoxin and Cry2A protoxin on the predator *Chrysoperla carnea*. Entomologia Experimentalis et Applicata 91, 305–316.

Limburg, D.D., Rosenheim, J.A., 2001. Extrafloral nectar consumption and its influence on survival and development of an omnivorous predator, larval *Chrysoperla plorabunda* (Neuroptera: Chrysopidae). Environmental Entomology 30, 595–604.

Marvier, M., 2002. Improving risk assessment for non-target safety of transgenic crops. Ecological Applications 12, 1119–1124.

Meier, M.S., Hilbeck, A., 2001. Influence of transgenic *Bacillus thuringiensis* corn-fed prey on prey preference of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). Basic and Applied Ecology 2, 35–44.

Nap, J.P., Metz, P.L.J., Escaler, M., Conner, A.J., 2003. The release of genetically modified crops into the environment. The Plant Journal 33, 1–18.

Orr, D.B., Landis, D.A., 1997. Oviposition of European corn borer (Lepidoptera: Pyralidae) and impact of natural enemy populations in transgenic versus isogenic corn. Journal of Economic Entomology 90, 905–909.

Pilcher, D.C., Obryski, J.J., Rice, M.E., Lewis, L.C., 1997. Preimaginal development, survival and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. Environmental Entomology 26, 446–454.

Principi, M.M., Canard, M., 1984. Feeding habits. In: Canard, M., Séméria, Y., New, T.R. (Eds.), Biology of Chrysopidae. Dr. W. Junk Publishers, The Hague, pp. 76–92.

Pusztai-Carey, M.P., Lessard, T., Yaguchi, M., Oct. 1994. US Patent 5356788.

Pyke, D.A., Thompson, J.N., 1986. Statistical analysis of survival and removal rate experiments. Ecology 67, 240–245.

Rukmini, V., Reddy, C.Y., Venkateswerlu, G., 2000. *Bacillus thuringiensis* crystal δ-endotoxin: role of proteases in the conversion of protoxin to toxin. Biochimie 82, 109–116.

Salama, H.S., Zaki, F.N., Sharaby, A.F., 1982. Effect of *Bacillus thuringiensis* Berl. on parasities and predators of the cotton leafworm *Spodoptera littoralis* (Boisd.). Zeitschrift für angewandte Entomologie 94, 498–504.

Salama, H.S., Sharaby, A., Ragaei, M., 1983. Chemical changes in the haemolymph of *Spodoptera littoralis* (Lep.: Noctuidae) as affected by *Bacillus thuringiensis*. Entomophaga 28, 331–337.

Schnepf, E., Crickmore, N., van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiology and Molecular Biology Reviews 62, 775–806.

Steidl, R.J., Hayes, J.P., Schauber, E., 1997. Statistical power analysis in wildlife research. Journal of Wildlife Management 61, 270–279.

Wold, S.J., Burkness, E.C., Hutchison, W.D., Vanette, R.C., 2001. In-field monitoring of beneficial insect populations in trangenic corn expressing *Bacillus thuringiensis* toxin. Journal of Entomological Sciences 36, 177–187.

Yazlovetsky, I.G., 2001. Features of the nutrition of Chrysopidae larvae and larval artificial diets. In: McEwen, P.K., New, T.R., Whittington, A.E. (Eds.), Lacewings in the Crop Environment. Cambridge University Press, Cambridge, pp. 320–337.