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Prey-mediated effects of Cry1Ab toxin and protoxin and Cry2A protoxin on the predator *Chrysoperla carnea*

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Abstract

Laboratory feeding experiments were carried out to study prey-mediated effects of artificial diet containing *Bacillus thuringiensis* proteins on immature *Chrysoperla carnea*. Activated Cry1Ab toxin and the protoxins of Cry1Ab and Cry2A were mixed into standard meridic diet for *Spodoptera littoralis* (Boisduval) larvae at the following concentrations; for Cry1Ab toxin, 25, 50, 100 μg g^{-1} diet were used; for Cry1Ab protoxin, the concentration was doubled (50 μg g^{-1} diet, 100 μg g^{-1} diet and 200 μg g^{-1} diet) to give relative comparable levels of toxin concentration. Cry2A protoxin was incorporated into the meridic diet at one concentration only (100 μg g^{-1} diet). For the untreated control, the equivalent amount of double distilled water was added to the meridic diet. Individual *C. carnea* larvae were raised on *S. littoralis* larvae fed with one of the respective treated meridic diets described above. The objectives were to quantify and compare the resulting effects on mortality and development time of *C. carnea* with those observed in two previous studies investigating prey-mediated effects of transgenic Cry1Ab toxin-producing corn plants and the other studying effects of Cry1Ab toxin fed directly to *C. carnea* larvae. Mean total immature mortality for chrysopid larvae reared on *B. thuringiensis*-fed prey was always significantly higher than in the control (26%). Total immature mortality of *C. carnea* reared on Cry1Ab toxin 100 μg g^{-1} diet-fed prey was highest (78%) and declined with decreasing toxin concentration. Cry1Ab protoxin-exposed *C. carnea* larvae did not exhibit a dose response. Prey-mediated total mortality of Cry1Ab protoxin-exposed chrysopid larvae was intermediate (46–62%) to Cry1Ab toxin exposed (55–78%) and Cry2A protoxin (47%) exposed *C. carnea*. In agreement with the previous studies, total development time of *C. carnea* was not consistently, significantly affected by the Bt-treatments except at the highest Cry1Ab toxin concentration. However, both highest mortality and delayed development of immature *C. carnea* raised on Cry1Ab toxin 100 μg g^{-1} diet – fed prey may have been confounded with an increased intoxication of *S. littoralis* larvae that was observed at that concentration. At all other *B. thuringiensis* protein concentrations *S. littoralis* was not lethally affected. Comparative analysis of the results of this study with those of the two previous studies revealed that in addition to prey/herbivore by *B. thuringiensis* interactions, also prey/herbivore by plant interactions exist that contribute to the observed toxicity of *B. thuringiensis* – fed *S. littoralis* larvae for *C. carnea*. These findings demonstrate that tritrophic level studies are necessary to assess the long-term compatibility of insecticidal plants with important natural enemies.

Introduction

Bacillus thuringiensis (Berliner) proteins are becoming ubiquitous, highly bioactive substances in the agroecosystems worldwide. This is due to an increase in the use of *B. thuringiensis*-based insecticides and the large scale release of various, transgenic crop plants expressing *B. thuringiensis* proteins conferring plant resistance to certain target insect pests. In the currently commercially available transgenic crop

plants, *B. thuringiensis* proteins are present throughout most of the plant during most of the growing period. Further, the *B. thuringiensis* protein is expressed in relatively high concentrations and, in contrast to *B. thuringiensis* insecticides, in a truncated, activated form. The current and future trends in plant molecular biology is to increase *B. thuringiensis* expression levels in plants with the most dramatic example being the expression of Cry1Ac in tobacco chloroplasts (McBride et al., 1995). Consequently, most, if not all, nontarget herbivores colonizing transgenic *B. thuringiensis* plants in the field are not lethally affected by *B. thuringiensis* proteins. However, they will ingest plant tissue containing *B. thuringiensis* protein which they may pass on to their natural enemies in a more or less processed form. The ubiquitous and temporally extended availability of *B. thuringiensis* proteins in the field in addition to its modified form of release, makes it necessary to verify and monitor the compatibility of this new pest management strategy with natural enemies. The longterm, agroecological safety of the combined use of transgenic crop plants and *B. thuringiensis* insecticides cannot simply be deduced from the past record of safe *B. thuringiensis* insecticide use when *B. thuringiensis* compounds were available in the field only during short periods. The activity of *B. thuringiensis* insecticides declines rapidly in the field within one week (Behle et al., 1997; Ignoffo & Garcia, 1978).

In previous studies, we demonstrated that Cry1Ab adversely affected *Chrysoperla carnea* (Stephens) larvae (Hilbeck et al., 1998a, b). Significantly higher mortality (59–66%) was observed for *C. carnea* larvae reared on *B. thuringiensis* corn-fed target and nontarget prey than when reared on prey larvae of both species that were fed *B. thuringiensis*-free corn plants (37%) (Hilbeck et al., 1998a). Similar results were obtained when the activated Cry1Ab toxin was incorporated into an artificial diet specifically designed for mass rearing of *C. carnea* larvae at 100 μg ml^{-1} diet (Hilbeck et al., 1998b). Fifty-six percent of *C. carnea* larvae died before reaching the adult stage when raised on Cry1Ab-incorporated diet while only 30% died in the control. However, when considering the differences between concentrations of Cry1Ab expressed in transgenic corn and the concentration used for the direct feeding study, this result was somewhat surprising. In *B. thuringiensis* corn, the concentration of Cry1Ab protein per gram fresh weight does not exceed 4 μg in leaves and was always lower in all other tissues analyzed (Fearing et al., 1997). Consequently, it is reasonable to assume that the amount of Cry1Ab toxin passed on to *C. carnea* by its herbivore/prey in the study using transgenic *B. thuringiensis* corn was much smaller than the amount present in the artificial diet containing Cry1Ab toxin at a concentration of 100 μg ml^{-1}. We speculated that this may be associated with further biochemical processing of Cry1Ab toxin inside the herbivores' gut compared with using exclusively trypsinized Cry1Ab toxin in the encapsulated diet, thereby, perhaps increasing its activity towards *C. carnea*.

This study was undertaken to test this hypothesis in tritrophic level experiments. The *B. thuringiensis* source was a well-defined meridic diet containing Cry1Ab toxin and protoxin at different concentration levels for the nontarget herbivore, the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval), also used in the previous study (Hilbeck et al., 1998a). In addition, another type of *B. thuringiensis* protein was included, the protoxin of Cry2A, because this protein exhibits relatively high toxicity to the European corn borer, *Ostrinia nubilalis* (Hübner), and therefore is a likely candidate for expression in transgenic corn (Dankocsik et al., 1990). Specific objectives were (1) to compare the prey-mediated effects of Cry1Ab toxin-containing diet on mortality and development time of *C. carnea* larvae with those of the previous studies discussed above (Hilbeck et al., 1998a, b) and (2) to test whether prey-mediated effects could also be observed when the protoxins of Cry1Ab and Cry2A were incorporated into the prey diet; and (3) to study whether differing concentrations in the artificial diet for the herbivorous prey would also result in differences of *C. carnea* mortality rates and development times.

Materials and methods

Insect species. Predaceous *Chrysoperla carnea* larvae are important natural enemies in many agricultural systems worldwide (New, 1975). Because of its importance in biological control, *C. carnea* is one of the most commonly tested species relative to pesticide side effects (Croft, 1990). *C. carnea* larvae from the permanent laboratory colony of our institute were used. These larvae have been maintained on pea aphids (*Acyrthosiphon pisum* Harris) for several years without introductions from the field. Adults were kept on a mixture of yeast, honey and water and were held at 22–25 °C, 70% r.h. and at L16:D8 h.

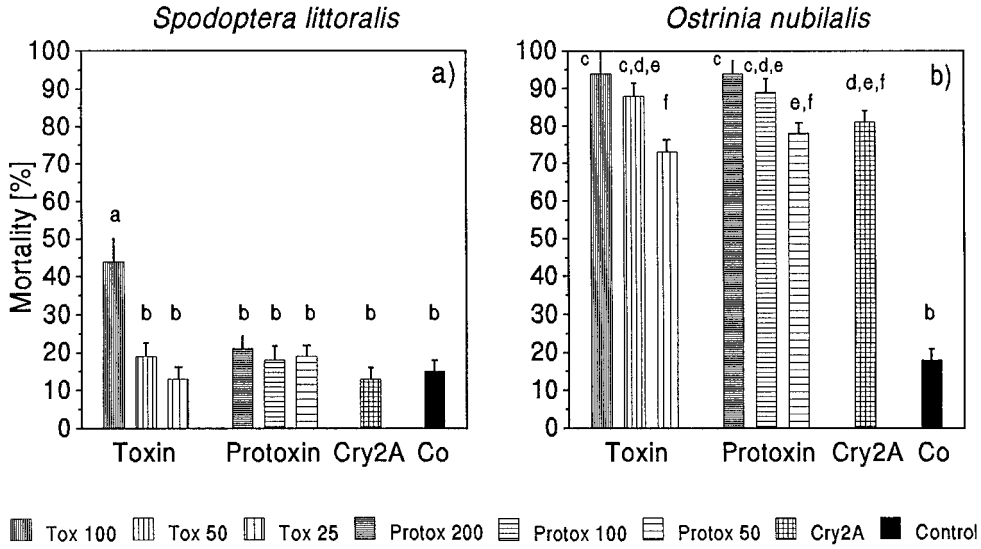

Figure 1. Stage-specific mean mortality (including ± SE) of *Chrysperla carnea* larvae raised on *Spodoptera littoralis* larvae that were fed various *Bacillus thuringiensis* protein – incorporated meridic diets. Columns with different letters represent treatment means that are significantly different at P = 0.05 (LSMEANS).

Spodoptera littoralis was used as the non-target herbivore/prey species that is not or only slightly affected by *Bacillus thuringiensis* proteins produced by the HD-1 strain of *Bacillus thuringiensis* var. *kurstaki* (Moore & Navon, 1973; Sneh & Gross, 1983; Salama & Matter, 1991; Salama et al., 1981; Sneh et al., 1981; Müller-Cohn et al., 1996; Höfte & Whiteley, 1989). Egg masses of *S. littoralis* were kindly provided by Norvartis (formerly CIBA), Basle, Switzerland, where they were also maintained as a laboratory colony for several generations. All egg masses were reared until eclosion in a growth chamber where the experiments were carried out.

Ostrinia nubilalis was obtained from French Agricultural Research, Inc., Lamberton, Minnesota. All egg masses were reared until eclosion in a growth chamber where the experiments were carried out.

B. thuringiensis *proteins.* The Cry1Ab protoxin from *B. thuringiensis* subsp. *kurstaki* HD-1 was expressed as a single gene product in *Escherichia coli* (Masson et al., 1990). Inclusion bodies containing Cry1Ab protoxin were solubilized and trypsinized and the Cry1Ab toxin was isolated using HPLC (Pusztai-Carey et al.,1994). The Cry2A protoxin from the NRD-12 isolate of *B. thuringiensis* var. *kurstaki* was expressed as a single gene product in *E. coli* by a modification of the procedure of Moar et al. (1994) as described by Moar et al. (1995a). All proteins were quantified using either Micro BCA (Pierce Chemical Co, Rockford, IL) or Bradford (Bradford, 1976) assay and protein purity was evaluated using SDS-PAGE. All materials were lyophilized.

Herbivore bioassays with B. thuringiensis-*proteins.* Bioassays were conducted using the nontarget species, *S. littoralis*, and the susceptible target species, *O. nubilalis*. This was done to determine whether the various *B. thuringiensis* proteins and concentrations incorporated into the meridic diet affected *S. littoralis* and to confirm the biological activity of the *B. thuringiensis* incorporated meridic diets on the susceptible, target species. Approximately 500 mg of the respective treated meridic diet was placed into each of ten vials (1.2 cm diameter × 7.5 cm length) per treatment (total of 8 treatments per species) and per species. Four neonate *S. littoralis* or *O. nubilalis* larvae were placed into each vial, and the vials were subsequently sealed with perforated plastic lids to allow air circulation (= 40 larvae per treatment in 10 vials). Bioassays were replicated four times over time and were performed concurrently with the *C. carnea* feeding trials. Therefore, a total of 1280 *S. littoralis* and 1280 *O. nubilalis* larvae were examined. Numbers of dead larvae were recorded after four days. In addition, larval weights of *S. littoralis* were measured.

Tritrophic feeding experiments. The experiments and the herbivore bioassays were carried out in a controlled-environment chamber at fluctuating temperatures (25 °C for 10 h during photophase and 20 °C for the remaining 14 h) averaging 22 °C per day, L16:D8 h and 70% r.h.

The appropriate amount of the respective *B. thuringiensis* protein was solubilized in a mixture of 5 ml 50 mM CAPS pH 10.5 and 13 ml double distilled water and mixed into 90 g standard meridic diet for *S. littoralis* larvae at the following concentrations. For Cry1Ab toxin, 100 μg g^{-1} diet, 50 μg g^{-1} diet and 25 μg g^{-1} diet were used. Because during activation the 130–140 kDa Cry1Ab protoxin will essentially be cleaved into half to produce the 65 kDa toxin, the amount of protoxin was doubled (200 μg g^{-1} diet, 100 μg g^{-1} diet and 50 μg g^{-1} diet) to give comparable levels of toxin concentration. Cry2A protoxin was incorporated into the meridic diet at one concentration only (100 μg g^{-1} diet). For the untreated control, only the equivalent amount of double distilled water and 50 mM CAPS pH 10.5 was added to the meridic diet. This resulted in a total of 8 treatments.

Neonate chrysopid larvae (approximately 24 h old) were placed individually in 150 ml clear plastic containers. Containers were closed with a lid that had a hole covered with fine mesh netting to allow air circulation. In each container, approximately 3 g of treated meridic diet was provided, and first, second or third instar *S. littoralis* larvae were added *ad libitum* according to size and instar of *C. carnea* larvae. Prior before *S. littoralis* were provided to *C. carnea*, they had fed on *B. thuringiensis* protein-containing diet for approximately 1 d. Twice a week, diet was replaced and new prey was added. Stage-specific mortality was monitored twice weekly. Developmental stage was recorded once a week until pupation. Developmental stage could reliably be identified by finding the exuvium after each molt. Thirty-one days after the beginning of the experiment, the number of *C. carnea* that had developed to the adult stage was determined. Thirty chrysopid larvae were used for each treatment resulting in 240 chrysopid larvae (8 treatments $\times$ 30) per replicate. The entire experiment was repeated four times independently over time resulting in a total of 960 chrysopid larvae tested.

Data analysis

Herbivore bioassays. For the analyses of herbivore mortality, an ANOVA was carried out on the arcsine-transformed mortality data, excluding the Cry2A data (see below). Because four *O. nubilalis* and *S. littoralis* larvae per experimental container were used in the herbivore bioassays, the observations were not independent and, therefore, the logistic regression was not appropriate. A model was used that tested for significant replication, vial, prey, *B. thuringiensis* type and concentration main effects and the interaction effects of the last three main factors. For the statistical analyses of larval *S. littoralis* weight, a regular two-way ANOVA was conducted for significant replication, vial, and treatment effects (excluding the Cry2A data). The model partitioned the treatments into two main effects, i.e., *B. thuringiensis* type and concentration, and their interaction effect.

All analyses were performed using the GLM procedure of the SAS statistical package (SAS Institute, 1996). In addition, mean mortality and their standard errors were determined, including the Cry2A data, and means were compared by carrying out the MEANS procedure and a least significant difference test (LSD, LSMEANS) of the SAS statistical package, respectively (SAS Institute, 1996).

Tritrophic studies. For the statistical analyses of the mortality of each *C. carnea* larval and pupal stage, a logistic regression was carried out calculating the proportion of individuals that died and accounting for the binomial probability distribution of mortality data. In addition, *C. carnea* mortality from first instar to adult (L1-A; entire immature) was determined and analyzed accordingly. For these analyses, the data set included only the 3 concentrations of both Cry1Ab toxin and Cry1Ab protoxin (i.e., 7 of 8 treatments), again excluding the Cry2A data. This was done because Cry2A protoxin was tested only at one concentration which was difficult to include meaningfully into the logistic regression model. The data were analyzed using a two-way factorial model that tested for significant replication effects and partitioned the treatments into two main effects (i.e., *B. thuringiensis* type and concentration), and their interaction effect. The analyses were performed using the GENMOD procedure of the SAS statistical package including a DSCALE and Type 3 statement producing the appropriate F-statistics (SAS Institute, 1996). In addition, mean mortality and standard errors were determined for all

treatments, including the Cry2A data, and means were compared by carrying out the MEANS procedure and a least significant difference test (LSD, LSMEANS) of the SAS statistical package.

As an indicator for *C. carnea* development time, we calculated the proportion of individuals that had molted to the next life stage (second and third instar, pupae, adult) on a given check date (day 3, 10, 17 and 31 after hatch) out of the total number of survivors through the particular instar. The stage-specific number of survivors was provided by the mortality data. For the statistical analyses, a regular two-way factorial analysis of variance (ANOVA) was conducted for significant replication and treatment effects, again excluding the Cry2A data (reasons see above). A model was used that partitioned the treatments into two main effects (i.e., *B. thuringiensis* type and concentration), and their interaction effect. Analyses were performed using the general linear model (GLM) procedure of the SAS statistical package (SAS Institute, 1996). Also mean developmental stage and standard errors were determined for all treatments, including the Cry2A data, and means were compared by carrying out the MEANS procedure and a least significant difference test (LSD, LSMEANS) of the SAS statistical package.

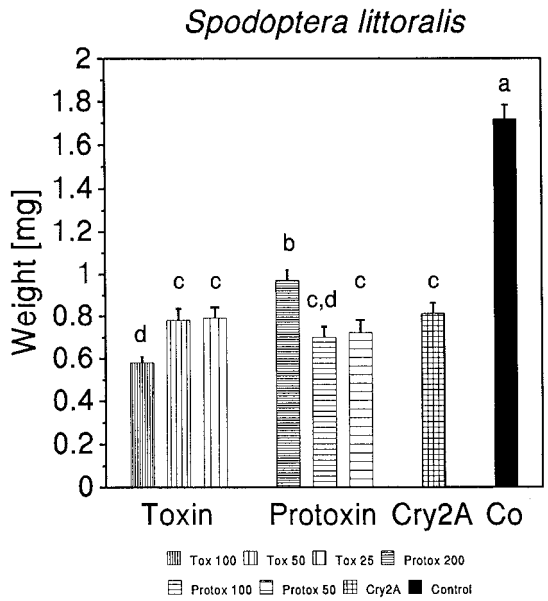

Figure 2. Mean percentage of immature *Chrysperla carnea* developed to the next life stage (second instar (L2), third instar (L3), pupa (P), adult (A), including ± SE) on days 3, 10, 17 and 31 after hatch that were raised on *Spodoptera littoralis* larvae fed various *Bacillus thuringiensis* protein – incorporated meridic diets. Columns with different letters represent treatment means that are significantly different at P = 0.05 (LSMEANS).

Results

Herbivore bioassays

Mortality of S. littoralis *and* O. nubilalis. Except for Cry1Ab toxin 100 μg g^{-1} diet-treated *S. littoralis*, mortality of *S. littoralis* larvae reared on Cry1Ab and Cry2A protein-containing diet ranged between 13 and 22% and was not significantly different from the *B. thuringiensis*-free control (15%) (Figure 1a). Only in the Cry1Ab-toxin 100 μg g^{-1} diet treatment, mortality of *S. littoralis* larvae was significantly higher than in the control (44%).

In contrast, mortality of *B. thuringiensis*-treated *O. nubilalis* larvae was always significantly higher than in the control (18%), ranging from 73 to 94% depending on the *B. thuringiensis* concentration (Figure 1b). In the ANOVA, this resulted in significant *B. thuringiensis* (Bt) × concentration and prey × *B. thuringiensis* × concentration interaction effects ($F_{Bt\times concentration}$ = 5.62, df = 2, 533, P = 0.004; $F_{Prey\times Bt\times concentration}$ = 3.16, df = 2, 533, P = 0.043). Mortality rates were similar for the corresponding Cry1Ab toxin and protoxin concentrations. And as the concentration declined, mortality of *O. nubilalis* decreased; again, at similar rates for the corresponding toxin and protoxin concentrations. No differences in mortality were observed between both species in the untreated controls.

S. littoralis *weight.* All *B. thuringiensis*-treated *S. littoralis* larvae exhibited significantly reduced weight compared to the control (Figure 2). Compared with the untreated control larvae, *S. littoralis* larvae exposed to the Cry2A 100 and Cry1Ab toxin 25 μg g^{-1} diets exhibited the second highest weight. In contrast to Cry1Ab protoxin exposed *S. littoralis* larvae, a dose response was observed for Cry1Ab toxin-fed larvae; as concentration of toxin increased larval weight declined. Weights of Cry1Ab protoxin-exposed larvae were similar across concentrations and intermediate to those at the highest and lowest Cry1Ab toxin concentration. This resulted in a highly significant *B. thuringiensis* × concentration interaction and also a significant replication effect was noted ($F_{Bt\times concentration}$ = 9.54, df = 2, 848, P < 0.001; F_{Rep} = 19.35, df = 4, 848, P < 0.001).

Table 1. Mean percentage mortality (± SE) of immature *C. carnea* raised on *S. littoralis* larvae that were fed various *B. thuringiensis* protein – incorporated meridic diets

Life stage[a]	Cry1Ab toxin[b]	Cry1Ab protoxin[b]	Cry2A protoxin	Control
L1	20.00 ± 4.36a	13.61 ± 3.56a,b	10.00 ± 3.60a,b	5.83 ± 2.50b
L2	18.14 ± 4.37a	16.69 ± 3.69a	13.72 ± 7.39a	2.71 ± 0.91b
L3	37.25 ± 5.50a	32.71 ± 4.36a	23.06 ± 8.79b	12.14 ± 4.58b
P	32.42 ± 6.68a	13.97 ± 3.06a	14.31 ± 4.22a	9.62 ± 1.92a
L1-A	66.94 ± 4.98a	54.44 ± 3.00a,b	46.67 ± 2.72b	25.83 ± 4.38c

Means with different letters between columns (Cry1Ab toxin versus Cry1Ab protoxin versus Cry2A versus control) are significantly different at $P < 0.05$ significance level (LSD). Data represent the mean of 4 replications; initial sample size was 30 *C. carnea* larvae per treatment.
[a]L1 = first instar; L2 = second instar; L3 = third instar; P = pupal stage; A = adult stage.
[b]Averaged across concentration.

Tritrophic studies

Mortality. Mean *C. carnea* larval mortality was generally higher when they were raised on *B. thuringiensis*-fed prey, but with various degrees of significance which will be explained in detail in the following (Table1, Figures 3a–f). Although elevated mortality values were observed for all *C. carnea* first and second instars larvae raised on *B. thuringiensis*-fed prey, these differences only were statistically significant between the highest Cry1Ab toxin concentration (100 μg g^{-1} diet) and the control (Figures 3a and 3b). This resulted in a significant *B. thuringiensis* x concentration interaction effect for first instars in the logistic regression analysis ($F = 4.425$, $df = 2, 18$, $P = 0.027$).

High mortality occurred during the third instar (L3) with mortality rates of more than 40% (Cry1Ab toxin 50 μg g^{-1} diet and Cry1Ab protoxin 50 μg g^{-1} diet). Except for Cry1Ab-protoxin 100 μg g^{-1} diet and Cry2A, always significantly more *C. carnea* third instars died when reared on *B. thuringiensis*-fed than on *B. thuringiensis*-free *S. littoralis* (Figure 3c). However, in the linear regression analysis only a marginally significant *B. thuringiensis* × concentration interaction effect was noted (L3: $F = 3.584$, $df = 2, 18$, $P = 0.049$). This was probably due to variability of the data.

During the first instar, a decline in *C. carnea* mortality with decreasing Cry1Ab toxin concentration was observed (Figure 3a). For all other larval stages and *B. thuringiensis* protein types, no response of mortality rates to *B. thuringiensis* concentration in the prey diet was detected.

During the pupal stage, at the two highest Cry1Ab toxin concentrations (100 and 50 μg g^{-1}), mortality of *C. carnea* raised on *B. thuringiensis*-fed prey was significantly higher than the control mortality (Figure 3d). Similar to the first instars, pupal mortality declined with decreasing toxin concentration when *C. carnea* were raised on Cry1Ab toxin-fed prey (Figure 3d). In the logistic regression analysis this resulted in a significant *B. thuringiensis* main effect and *B. thuringiensis* × concentration interaction effect ($F_{Bt} = 13.879$, $df = 1, 18$, $P = 0.002$; $F_{Bt\times concentration} = 3.632$, $df = 2, 18$, $P = 0.047$).

For the entire immature life stage (first instar until adult eclosion), mortality of *C. carnea* raised on *B. thuringiensis*-fed *S. littoralis* was always significantly higher than in the control (26%) regardless of the *B. thuringiensis* protein type used (Figure 3e). Mortality of *C. carnea* reared on Cry1Ab toxin 100 μg g^{-1} diet – fed prey was highest (78%) and declined with decreasing toxin concentration. Cry1Ab protoxin-exposed *C. carnea* larvae did not exhibit a dose response. Mortality rates of Cry1Ab protoxin-exposed *C. carnea* were intermediate to those of Cry 1Ab toxin-fed and Cry2A-fed *C. carnea*, and they did not exhibit a dose response (Figure 3e). The lowest mortality of *C. carnea* raised on *B. thuringiensis*-fed prey was detected for Cry1Ab protoxin 100 μg g^{-1} diet (46%) and Cry2A-exposed larvae (47%). This led to a significant *B. thuringiensis* main and *B. thuringiensis* × concentration interaction effect in the logistic regression analysis ($F_{Bt} = 6.163$, $df = 1, 18$, $P = 0.023$; $F_{Bt\times concentration} = 3.556$, $df = 2, 18$, $P = 0.049$).

In addition, significant differences in mortality between replications were detected (L1: $F = 7.929$, $df = 3, 18$, $P = 0.001$; L2: $F = 4.068$, $df = 3, 18$, $P = 0.023$; L3: $F = 15.544$, $df = 3, 18$, $P < 0.001$). However, relative differences between the treatments were consistent for all replications.

When averaged across concentrations of Cry1Ab-toxin and Cry1Ab-protoxin, overall mean mortality was always significantly higher than in the control, except for pupal mortality (Table 1). Although mortality was consistently higher for Cry1Ab toxin-exposed than Cry1Ab protoxin-exposed *C. carnea* larvae, this differences was never significant. Mortality rates of Cry2A-exposed *C. carnea* larvae were always lower than for both Cry1Ab toxin and Cry1Ab protoxin-exposed larvae but usually also higher than for *C. carnea* raised on *B. thuringiensis*-free prey (Table 1). However, this difference was only significant for total immature mortality between Cry2A-exposed and control *C. carnea* (L1-A). No significant differences in mortality were observed between treatments for the pupal stage.

Development time. Generally, *C. carnea* larvae raised on *B. thuringiensis*-fed prey developed to the next life stage at lower rates than did the *B. thuringiensis*-free control. However, due to large variability of the data the differences were often not significant (Figure 4a–d). After 10 days, a significantly lower percentage (50%) of *C. carnea* larvae had molted to third instars when they were raised on Cry1Ab 100 μg g^{-1} diet toxin – fed prey (Figure 4b) compared to the control (85%). After 17 days, only 27% and 32% of *C. carnea* had pupated that were raised on prey fed with Cry1Ab toxin 50 and Cry1Ab protoxin 200 μg g^{-1} diet, respectively, compared to 60% in the control. None had reached the pupal stage at the highest Cry1Ab toxin concentration (Figure 4c). This resulted in a significant concentration main effects ($F = 3.94$, $df = 2, 18$, $P = 0.038$) and a marginally significant *B. thuringiensis* main effect ($F = 4.01$, $df = 1, 18$, $P = 0.061$). On day 31 after hatch, none of the Cry1Ab 100 μg g^{-1} diet toxin-exposed *C. carnea* had developed to adult while 81% of the *C. carnea* in the control had emerged as adults already resulting in a significant *B. thuringiensis* and concentration main effect and a *B. thuringiensis* $\times$ concentration interaction effect ($F_{Bt} = 7.53$, $df = 1, 18$, $P = 0.013$; $F_{Concentration} = 6.48$, $df = 2, 18$, $P = 0.008$, $F_{Bt\times concentration} = 3.74$, $df = 2, 18$, $P = 0.044$). However, although percentages of adults emerged on day 31 were also lower in all other *B. thuringiensis* treatments compared with the *B. thuringiensis*-free control, these differences were not significant. This was partly due to the large variability of the data but also resulted because the differences in percent of *C. carnea* developing to adult between the treatments and the control were smaller than the differences observed until pupation (day 17) (Figure 4 c and d).

Except at day 10 after hatch, there were also significant replication effects (Day 3: $F_{Rep} = 12.29$, $df = 3, 18$, $P < 0.001$; Day 17: $F_{Rep} = 7.76$, $df = 3, 18$, $P = 0.002$; Day 31: $F_{Rep} = 8.974$, $df = 3, 18$, $P < 0.001$). However, relative differences between the treatments were consistent for all replications.

When averaged across concentrations of Cry1Ab toxin, mean percentage of individuals that developed to the next developmental stage on days 10, 17 and 31 was always significantly lower than in the control, except on day 3 (Table 2). No differences between treatments were observed on day 3. Except on day 17 (percent pupation), also no differences were observed between the control and both the Cry1Ab protoxin and Cry2A treatments. On day 17, however, only 22%, 37% and 40% had pupated in the Cry1Ab toxin, Cry1Ab protoxin and the Cry2A treatments, respectively, while 60% had reached the pupal stage in the control. For total development until the adult stage (day 31), significantly fewer Cry1Ab toxin – exposed *C. carnea* had reached the adult stage than in the control but no differences were observed between Cry1Ab protoxin or Cry2A protoxin exposed *C. carnea* and the control (Table 2).

Discussion

Herbivore bioassays

Data presented here confirm that *S. littoralis* is substantially less susceptible to Cry1Ab and Cry2A proteins than *O. nubilalis*. Against *S. littoralis*, no increased mortality compared to the controls was detected at 100 μg g^{-1} diet Cry2A, which is similar to results by Moar et al. (1994) using Cry2A against *Spodoptera exigua* (Hübner) where there was no detectable mortality at 500 μg g^{-1} diet. *S. littoralis* exhibited more than 2-fold higher mortality with Cry1Ab toxin at 100 μg g^{-1} diet compared to the equivalent concentration of Cry1Ab protoxin (200 μg g^{-1} diet). This suggests that *S. littoralis* may not efficiently process Cry1Ab protoxin to toxin. Similarly, *S. exigua* is greater than 2-times more susceptible to Cry1Ac and Cry1C toxins than their respective protoxins (Moar et al., 1990, 1995a, b). These findings indicate that the selectivity of transgenic Bt plants may be other than expected from previous experience when using Bt insecticides typically consisting of a mixture of crystals

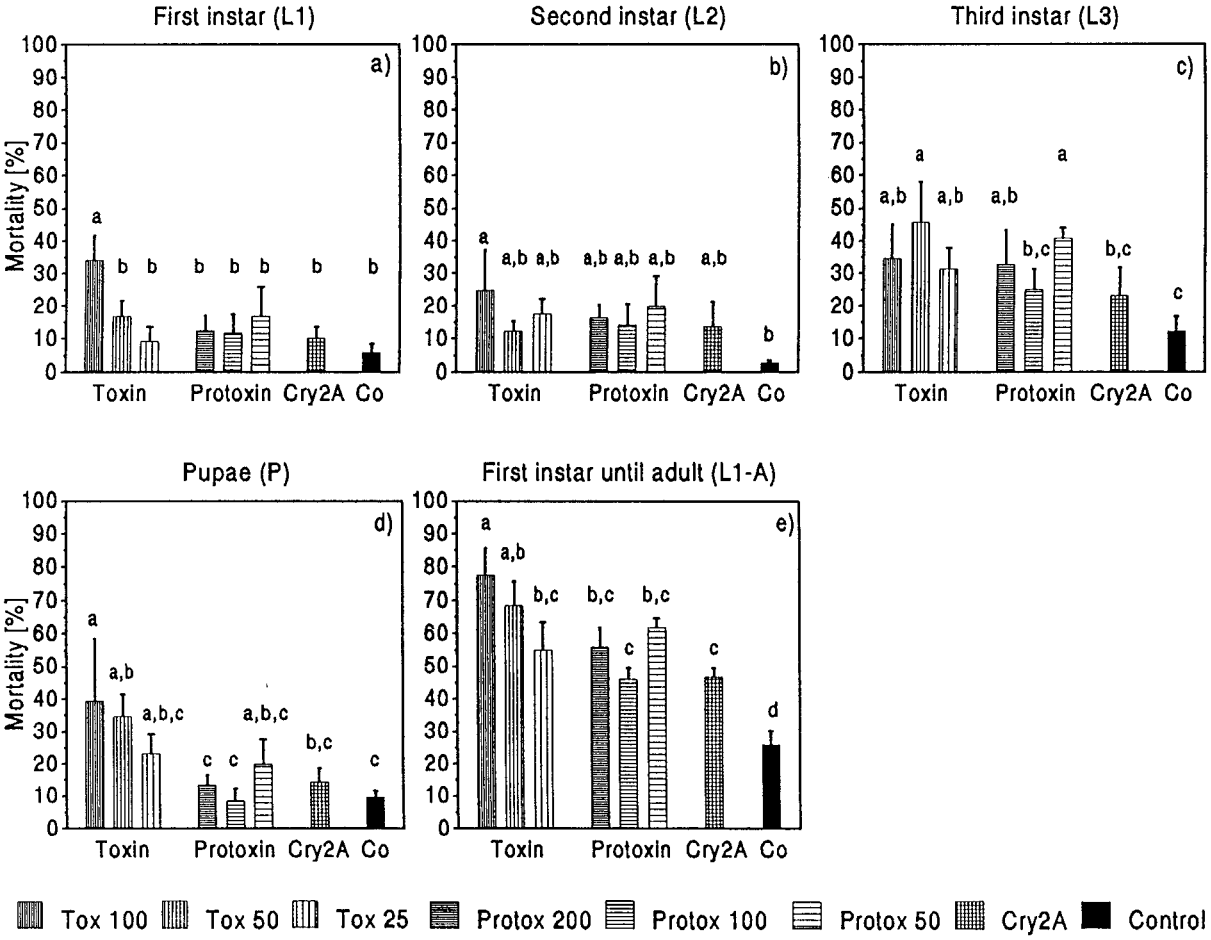

Figure 3. Mean mortality (including ± SE) of neonate *Spodoptera littoralis* larvae fed various *Bacillus thuringiensis* – incorporated diets for four days. Columns with different letters represent treatment means that are significantly different at P = 0.05 (LSMEANS).

Table 2. Mean percentage (± SE) of immature *C. carnea* developed to the next life stage (second instar (L2), third instar (L3), pupa (P), adult (A)) that were raised on *S. littoralis* larvae fed various *B. thuringiensis* protein-incorporated meridic diets

Days after hatch	Cry1Ab toxin[b]	Cry1Ab protoxin[b]	Cry2A protoxin	Control
Day 3 (to L2)	64.67 ± 7.42a	64.75 ± 6.34a	74.00 ± 15.80a	79.50 ± 6.85a
Day 10 (to L3)	61.17 ± 4.66a	69.58 ± 7.75a,b	84.50 ± 2.18a,b	85.00 ± 4.92b
Day 17 (to P)	22.17 ± 8.46a	36.50 ± 6.18a	39.50 ± 14.01a	60.25 ± 11.80b
Day 31 (to A)	42.92 ± 12.21a	65.67 ± 6.62a,b	61.25 ± 16.79a,b	80.50 ± 11.06b

Means with different letters between columns (Cry1Ab toxin versus Cry1Ab protoxin versus Cry2A versus control) are significantly different at P < 0.05 significance level (LSD). Data represent the mean of 4 replications; initial sample size was 30 *C. carnea* larvae per treatment.
[a]L2 = second instar; L3 = third instar; P = pupal stage; A = adult stage.
[b]Averaged across concentration.

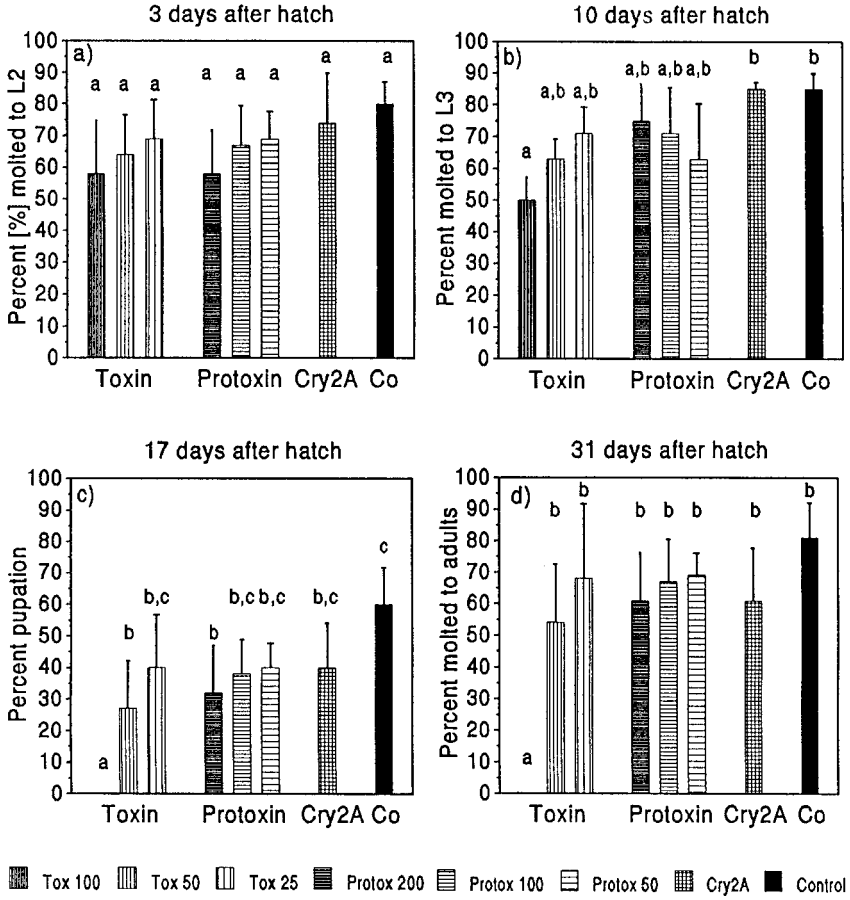

Figure 4. Larval weights of 4-day old *Spodoptera littoralis* (including ±SE) raised on various *Bacillus thuringiensis* – incorporated diets. Columns with different letters represent treatment means that are significantly different at P = 0.05 (LSMEANS).

and spores (Feitelson et al., 1992). However, reduced larval weight demonstrates that the Bt proteins did exert a sublethal effect on *S. littoralis*.

The data on *O. nubilalis* mortality confirmed the expected activity of the *B. thuringiensis* proteins. At 25 μg g^{-1} diet Cry1Ab toxin, 70–75% mortality against *O. nubilalis* was observed which is similar to the results by MacIntosh et al. (1990) who reported an LC_{50} of 3.6 μg ml^{-1} for *O. nubilalis* using purified CryIAb toxin.

To our knowledge, our report is the first that directly compared Cry1Ab with Cry2A against a target insect such as *O. nubilalis*. These results suggest that against *O. nubilalis*, Cry2A protoxin at 100 μg g^{-1} diet has comparable toxicity to Cry1Ab protoxin between 50 and 100 μg g^{-1} diet, respectively, making Cry2A protoxin 2–4 fold less active than Cry1Ab protoxin.

Tritrophic studies

Adverse prey-mediated effects were observed when immature *C. carnea* were raised on prey that had fed on diets containing Cry1Ab toxin, Cry1Ab protoxin and Cry2A protoxin. Total immature *C. carnea* mortality was highest when their prey had fed on Cry1Ab toxin containing diet (67% averaged across concentrations) (Table 1). Intermediate prey-mediated mortality was noted for Cry1Ab protoxin exposed *C. carnea* larvae. Lowest prey-mediated mortality was observed for Cry2A protoxin exposed chrysopid larvae. Yet, all total mortality values were significantly higher than in the control which makes this the first report of adverse, prey-mediated effects of Cry1Ab protoxin and Cry2A protoxin on immature *C. carnea*.

Total mean control mortality over a 31 day period was 26%, which is similar to the 23% control mortality for *C. carnea* after 9 days using a *Sitotroga*

cerealella egg-based bioassay as reported by Sims (1995). However, during the first 5–7 days of our bioassay period (in which we already observed a significant increase in *C. carnea* mortality when fed Cry1Ab toxin compared to the untreated control), total mean control mortality was less than 10% which is typical of many lepidopteran bioassays (MacIntosh et al., 1990, Moar et al., 1995a, b). These results suggest that increased control mortality can be expected when bioassay periods exceed 5-7 days.

Our studies also imply that *B. thuringiensis* by herbivore interactions exist that seem to enhance the impact of *B. thuringiensis* proteins. In previous studies, activated Cry1Ab toxin fed directly to *C. carnea* larvae at a concentration of 100 μg per ml artificial diet encapsulated into paraffin spheres resulted in 57% total immature mortality (Hilbeck et al., 1998b). In the study presented here, incorporating the same Cry1Ab toxin concentration into the meridic diet for *S. littoralis*, caused 78% prey-mediated total immature mortality in *C. carnea*, while *S. littoralis* died at a much lower rate (44%). This represents a 34% higher predator mortality than prey mortality. But because *S. littoralis* mortality was higher at the highest Cry1Ab toxin concentration compared with the control and other *B. thuringiensis* treatments, increased *C. carnea* mortality may have been confounded with increased intoxication of *S. littoralis*, possibly resulting in reduced nutritional quality as prey. In this study, prey-mediated total mortality of *C. carnea* (55%) similar to the rates observed in the direct feeding studies (57%) were noted at the 4-times lower Cry1Ab toxin concentration (25 μg g^{-1} diet) compared with the concentration used in the direct feeding study (100 μg ml^{-1} artificial diet) (Hilbeck et al., 1998b).

Interestingly, Cry1Ab protoxin-incorporated diet also caused significantly higher mortality in immature *C. carnea* than the untreated control, although to a lower degree than the Cry1Ab toxin-incorporated diet but it did not lethally affect *S. littoralis*. However, while a decreasing concentration of Cry1Ab toxin incorporated into the prey diet resulted in a simultaneous decline in the mortality for *C. carnea*, decreasing concentrations of Cry1Ab protoxin did not affect chrysopid mortality. Reasons for this are unknown but these findings suggest that *S. littoralis* may process the protoxin/toxin to a product where it lethally affects *C. carnea* but not itself. The bioassays with *S. littoralis* revealed no lethal effect of the *B. thuringiensis*-proteins incorporated into the artificial diet, except at the highest Cry1Ab toxin concentration (100 μg ml^{-1} artificial diet). However, *B. thuringiensis*-fed *S. littoralis* exhibited a sublethal effect (reduced weight) compared with the control. Regardless of the mechanism involved, these findings confirm our hypothesis that *B. thuringiensis* protein $\times$ herbivorous prey (*S. littoralis*) interactions increase toxicity of the prey to *C. carnea* while affecting the prey to a much lesser degree.

The findings of this study also support the results of the previous tritrophic level experiments using transgenic corn expressing Cry1Ab toxin at a concentration of less than 4 μg g^{-1} fresh weight (Hilbeck et al., 1998a). Total prey-mediated mortality of *C. carnea* larvae in these previous tritrophic studies was 59% and 66% when using *S. littoralis* or *O. nubilalis* as prey, respectively (Hilbeck et al., 1998a). In the studies reported here, prey-mediated total *C. carnea* mortality at the higher concentration of 25 μg Cry1Ab toxin/g diet was still slightly lower (55%). When comparing control mortalities of both studies, mortality in the trials using transgenic *B. thuringiensis* corn plants was approximately 10% higher (37%) than when using meridic diet (26%). This suggests that in addition to the *B. thuringiensis* protein $\times$ herbivorous prey interactions discussed above, herbivorous prey $\times$ plant interactions also occur that seem to contribute to the toxicity of *B. thuringiensis* plant – fed prey for *C. carnea* larvae although to a much lesser extent than the prey $\times$ *B. thuringiensis* interactions.

In agreement with the observations in both previous studies, overall development time was not consistently, significantly affected by the *B. thuringiensis* treatments, except at the highest Cry1Ab toxin concentration. The delayed development at this highest Cry1Ab toxin concentration (100 μg g^{-1} diet) may also have been confounded with the observed higher mortality of the *S. littoralis* larvae as discussed above. The development appeared to be slightly delayed for the other *B. thuringiensis* treatments as well but was confounded with overall variability of the data.

Our findings confirm that the selectivity of *B. thuringiensis* toxins and protoxins cannot generally be deduced from the long record of safe use of *B. thuringiensis* insecticides in the past that only were present in the field for relatively short periods of time after application. Transgenic *B. thuringiensis* plants produce *B. thuringiensis* proteins for many weeks or months. Further, several major crop plants expressing one or two *B. thuringiensis* protein types are now grown commercially and the introduction of more *B. thuringiensis* plants is imminent (Hoyle, 1995;

Niebling, 1995). In large areas of the United States, more than one of the currently commercially available *B. thuringiensis* crop plants are grown (*B. thuringiensis* corn and *B. thuringiensis* cotton in the southeastern United States and *B. thuringiensis* corn and *B. thuringiensis* potatoes in the northeastern and western United States). Hence, natural enemies, in particular those that are polyphagous and move between crop cultures, will encounter *B. thuringiensis*-containing, nontarget herbivorous prey in more than one crop most likely during the entire season.

The studies presented here also demonstrate that tritrophic level studies are essential to study the long-term interactions of transgenic, insecticidal plants with natural enemies. Similarly, Birch et al. (1999) reported adverse prey-mediated effects in adult ladybird beetles when their aphid prey had fed on transgenic lectin producing potato plants. While affecting their prey only sublethally (anti-feedant interfering with aphids' growth and reproduction), it caused significantly reduced longevity (lethal effect), fecundity and fertility in the ladybird beetles (Birch et al., 1999). Direct feeding studies with natural enemies are suitable for a first screening to determine direct toxicity at a high dose, but if herbivore-predator bioassays are not also conducted, processing of the insecticidal compound in the herbivores' gut is then ignored entirely and, thereby, important ecological information may be missed.

Field studies must be conducted to assess the ecological consequences of these results. Further, scouting programs should be established to monitor the compatibility of the long-term utilization of transgenic *B. thuringiensis* crop plants with natural enemies. If the introduction of transgenic insecticidal plants really is to represent a step toward a more sustainable and environmentally friendlier, while economically viable, agriculture, acceptance and success of this new form of pest management, aside from potential pest resistance concerns, will also depend on their long-term compatibility with naturally-occurring biological control.

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