Toxicity of *Bacillus thuringiensis* Cry1Ab Toxin to the Predator *Chrysoperla carnea* (Neuroptera: Chrysopidae)

ANGELIKA HILBECK, WILLIAM J. MOAR,[1] MARIANNE PUSZTAI-CAREY,[2] AGATA FILIPPINI, AND FRANZ BIGLER

Swiss Federal Research Station for Agroecology and Agriculture, CH-8046 Zürich, Switzerland

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ABSTRACT Laboratory feeding studies were carried out to determine the effects of the *Bacillus thuringiensis* (Berliner) Cry1Ab toxin on developmental time and mortality of *Chrysoperla carnea* (Stephens) larvae. A bioassay technique was developed that allowed for incorporation of the Cry1Ab toxin into a liquid diet that was then encapsulated within small paraffin spheres. Because only 2nd and 3rd instars can penetrate the surface of the paraffin spheres, 2 different methods were used to rear chrysopid larvae through the 1st instar. The 1st method used small foam cubes soaked in non-encapsulated, liquid diet (with or without Cry1Ab). The 2nd method used *Ephestia kuehniella* (Hübner) eggs as prey during the first instar (no Cry1Ab exposure). After reaching the 2nd instar, all larvae received encapsulated, artificial diet with or without Cry1Ab, respectively. When reared only on artificial diet containing Cry1Ab toxin, total immature mortality was significantly higher (57%) than in the respective untreated control (30%). Also, significantly more chrysopid larvae died (29%) that received Cry1Ab later during their larval development compared with the respective control (17%). Although mortality was consistently higher, no or only small differences in developmental times were observed between Cry1Ab-treated and untreated *C. carnea* larvae. *C. carnea* larvae required significantly more time to complete larval development when reared on artificial diet only than when reared first on *E. kuehniella* eggs followed by encapsulated artifical diet or on only *E. kuehniella* eggs, regardless of exposure to Cry1Ab. These results demonstrate that Cry1Ab is toxic to *C. carnea* at 100 μmg/ml of diet by using encapsulated artificial diet.

KEY WORDS *Chrysoperla carnea, Bacillus thuringiensis,* Cry1Ab, risk assessment, natural enemies, biological control

THE USE OF *Bacillus thuringiensis* (Berliner) to control insect pests is expected to increase dramatically in agroecosystems of many countries in the coming years, primarily through transgenic plants expressing *B. thuringiensis* proteins. In 1997, nearly 3.65 million ha of *B. thuringiensis* crops were planted in the United States, ≈2.84 million ha of Bt corn, 0.7 million ha of Bt cotton and 10,000 ha of Bt potato (Mellon and Rissler 1998). Products containing *B. thuringiensis* have been used in agriculture for several decades and are commonly considered to have little or no effect on natural enemies of pest insects (Croft 1990, Flexner et al. 1986, Melin and Cozzi 1989). This opinion is primarily based upon previous studies that were designed to test for undesired side effects of *B. thuringiensis* compounds on beneficial insects when used as a foliar insecticide (Croft 1990). However, *B. thuringiensis* insecticides and transgenic plants expressing *B. thuringiensis* differ in a number of aspects. In most commercially available transgenic plants expressing *B. thuringiensis,* the *B. thuringiensis* proteins are produced in relatively high levels in a large proportion of the plants throughout most of their growing period until the plants senesce (Koziel et al. 1993, Perlak et al. 1990). Therefore, most if not all herbivores colonizing transgenic plants expressing *B. thuringiensis* during the season also will feed on and ingest plant tissue containing *B. thuringiensis* protein that they may pass on to their natural enemies in a more or less processed form. Furthermore, most *B. thuringiensis* proteins in transgenic plants are expressed in a truncated, activated form (Perlak et al. 1990, Fujimoto et al. 1993, Koziel et al. 1993) that differs from *B. thuringiensis* proteins found in *B. thuringiensis* crystals present in microbial *B. thuringiensis* insecticides (Feitelson et al. 1992). Hence, selectivity of *B. thuringiensis* proteins expressed in transgenic plants cannot simply be deduced from the long record of safety for the use of commercial *B. thuringiensis* insecticides. Recently, it has been suggested that current assessment methods do not adequately account for the modified, activated form of *B. thuringiensis* proteins expressed in transgenic plants and the extended duration of availability to herbivores and, consequently, to other members of the food chain beginning with the natural enemies of these herbivores (Goldburg and Tjaden 1990, Jepson et al. 1994,

[1] Department of Entomology, Auburn University, Auburn, AL 36849-5413.

[2] Department of Biochemistry, Case Western Reserve University, Cleveland, OH 44106-4936.

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Snow and Palma 1997). This study was intended as an initial step in this direction.

The predaceous larvae of *Chrysoperla carnea* (Stephens) are important natural enemies in many agricultural systems throughout the world (New 1975). Its immature life stage consists of 3 instars that all are voracious predators of many pest species, including aphids and lepidopterous pests, and a nonfeeding pupal stage. Because of its importance in biological control, *C. carnea* is one of the most commonly tested species for pesticide side-effects (Croft 1990). Hilbeck et al. 1998 demonstrated that *C. carnea* larvae raised on 2 different prey species that had fed on transgenic, Cry1Ab-expressing corn leaves, *Zea mays* (L.), had significantly higher mortality compared with *C. carnea* larvae fed prey species that had fed on nontransformed, Cry1Ab-free corn leaves. However, Croft (1990) noted that lethal and sublethal impacts of *B. thuringiensis* on natural enemies may not be directly related to the proteins but may result from the effect of the *B. thuringiensis* compounds on the nutritional quality of the pest insect. Additionally, potential modifications of a plant's chemistry cannot be ruled out when expressing a foreign gene. Therefore, to be able to differentiate direct effects of *B. thuringiensis* proteins from indirect effects on *C. carnea*, laboratory bioassays that use purified Cry1Ab protein (similar to the protein expressed in corn) and artificial diet are necessary.

A major obstacle in conducting feeding bioassays with *C. carnea* has been that the larvae typically suck their food from within a substrate. In previous studies investigating direct effects of *B. thuringiensis* proteins on *C. carnea*, the surface of *Sitotroga cerealella* (Olivier) eggs was coated with *B. thuringiensis* proteins and subsequently fed to *C. carnea* larvae (Croft 1990, Sims 1995). However, because *C. carnea* suck out the egg contents without ingesting the shells, they probably ingested little or no *B. thuringiensis* protein. In another study, *B. thuringiensis*-containing pollen of transgenic plants was provided to test for side effects of Cry1Ab on *C. carnea* larvae (Pilcher et al. 1997). However, concentrations of Cry1Ab in pollen are low (2.57–2.94 μmg/g dry weight) compared with that of leaves (Perlak et al. 1990, Koziel et al. 1993, Fearing et al. 1997) and predaceous *C. carnea* larvae feed only to a very limited extent if at all on pollen.

Mass-rearing techniques of *C. carnea* typically involve either live prey, such as aphids, or insect eggs such as *Ephestia kuehniella* (Hübner) or *S. cerealella* (Ridgway et al. 1970, Hassan 1975, Morrison et al. 1975, Morrison and Ridgway 1976). In an effort to improve efficient mass-production techniques for biocontrol purposes, attempts have been made to develop an artificial diet (AD) for chrysopid larvae. The larvae were either supplied with an artificial diet soaked into a sponge, droplets of an artificial diet, or an artificial diet encapsulated into paraffin spheres (Hagen and Tassan 1965, Vanderzant 1969, Bigler et al. 1976, Martin et al. 1978). The encapsulation technique of a liquid artificial diet into tiny paraffin spheres has been markedly improved and mechanized by a German company (STB Control, Aarbergen, Germany) and is being successfully used by this company for mass rearing of *C. carnea* as a biocontrol agent. Our study reports on testing a *B. thuringiensis* protein directly against *C. carnea* larvae by using an artificial diet (encapsulated in paraffin spheres). The specific objectives were to investigate the impact of Cry1Ab-toxin on mortality and development of *C. carnea* larvae and to determine the suitability of the paraffin encapsulated, artificial diet for feeding tests with *C. carnea* larvae of *B. thuringiensis* proteins that need to be ingested to be effective.

Materials and Methods

Insects. *C. carnea* larvae from the permanent laboratory colony of our institute were used. *C. carnea* larvae have been maintained using pea aphids *Acyrthosiphon pisum* (Harris) and *E. kuehniella* eggs since 1988 without any introduction of field-collected insects. Nonpredaceous adults were kept on a mixture of yeast, honey, and water. Rearing conditions were 22–25°C, 70% RH, and a photoperiod of 16:8 (L:D) h. Neonate larvae of *Ostrinia nubilalis* (Hübner) also were obtained from our permanent laboratory colony that has been maintained on a meridic diet for several generations under conditions similar to those described above for *C. carnea*.

***Bacillus thuringiensis* Proteins and Artificial Diet.** 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 dissolved and trypsinized and the Cry1Ab toxin was isolated using high-performance liquid chromatography (HPLC) (Pusztai-Carey et al. 1994). Isolated material was lyophilyzed. The artificial diet consisted of paraffin spheres of ≈1 mm in diameter containing a liquid diet specifically developed for optimal nutrition of *C. carnea* larvae (Schwenk and Tygges, STB Control, Aarbergen, Germany). Cry1Ab toxin was solubilized in 15 ml of double distilled water and added to the liquid diet before encapsulation to make a final concentration of 100 μg/ml of diet. The biological activity of the encapsulated Cry1Ab toxin-containing diet was verified by conducting a series of 5 bioassays during the course of the experiment by using a Cry1Ab-susceptible target pest species, *O. nubilalis*.

Feeding Trials. *C. carnea* larvae can use the encapsulated diet only after they have reached the 2nd instar and their mouthparts are strong enough to penetrate the skins of the paraffin spheres. Therefore, 2 different methods were used to rear chrysopid larvae through the 1st instar. Once chrysopid larvae had reached the 2nd instar, all of them received paraffin-encapsulated artificial diet with or without Cry1Ab toxin (±Bt/AD), respectively. Table 1 provides an overview of all applied treatments. For method 1, each 1st instar was supplied with one 0.5-cm^3 polyethylene foam cube soaked in nonencapsulated liquid diet either without *B. thuringiensis* (treatment 1, −Bt/AD) or with Cry1Ab toxin added at a concentration of 100

Table 1. Overview of treatments applied in feeding trials with *C. carnea* larvae

Treatment	Bt exposure	Diet
1	−Bt	Artificial diet (AD) only (method 1)
2	+Bt	Artificial diet (AD) only (method 1)
3	−Bt	*E. kuehniella* eggs/artificial diet (method 2)
4	+Bt	*E. kuehniella* eggs/artificial diet (method 2)
5	−Bt	*E. kuehniella* eggs only

μg/ml of diet (treatment 2, +Bt/AD). Hence, larvae raised according to treatment 2 were exposed to *B. thuringiensis* toxin during their entire larval stage (Table 1), whereas in the corresponding control (treatment 1), the equivalent amount of double distilled water was added. Diet-soaked foam cubes were replaced daily until larvae reached the 2nd instar. For method 2, *E. kuehniella* eggs attached to moistened pieces of cardboard paper (1 by 0.5 cm) were provided as food during 1st instars. One egg-covered cardboard piece was added to each rearing container with 1st instars. Consequently, *C. carnea* reared according to treatment 4 (eggs/+Bt/AD) were exposed to Cry1Ab toxin only during 2nd and 3rd instar (Table 1), whereas the corresponding control (i.e., treatment 3; eggs/−Bt/AD) did not receive any Cry1Ab toxin. In addition to the 4 treatments just described, a reference treatment was included where *C. carnea* larvae were raised exclusively on *E. kuehniella* eggs (treatment 5, eggs).

C. carnea larvae were kept individually in open-top plastic cups (5 by 6 cm diameter), the margins of which were coated with fluon (Whitford GmbH, Limburg, Germany) that prevented the larvae from escaping. Thirty 1-d-old *C. carnea* larvae were used per treatment. The experiment was repeated 5 times. Thus, a total of 750 chrysopid larvae was examined. Stage-specific mortality and developmental time were monitored daily. Developmental stage could reliably be identified by finding the exuviae after each molt. Experiments 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/d, 70% RH and a photoperiod of 16:8 (L:D) h.

For the statistical analyses of stage-specific mortality, a logistic regression was carried out calculating the proportion of individuals that died during each instar and accounting for the binomial probability distribution of mortality data. A model was used that tested for significant replication effects and partitioned the treatments into 2 main effects (i.e., *B. thuringiensis* treatment and type of diet, and their interaction effect). Analyses were performed using the GENMOD procedure of the SAS statistical package, including a DSCALE and Type 1 and 3 statement producing the appropriate *F*-statistics (SAS Institute 1996). In addition, mean mortality and standard errors were determined and means were compared by carrying out the MEANS procedure, including a LSMEANS statement (SAS Institute 1996).

Stage-specific developmental times were determined in numbers of days required to complete each instar. In addition, mortality and developmental time from 1st instar to adult (L1-A) (=entire immature) was determined. For the statistical analyses, a regular analysis of variance (ANOVA) was carried out testing for significant replication and treatment effects. A model was used that partitioned the treatments into 2 main effects (i.e., *B. thuringiensis* treatment and type of diet, and their interaction effect). Analyses were performed using the general linear model (GLM) procedure of the SAS statistical package, including a LSMEANS statement (SAS Institute 1996).

Bioassays of Cry1Ab with *O. nubilalis* Larvae. To determine if Cry1Ab toxin was still biologically active after being added into the artificial diet, 3 g of crushed diet containing Cry1Ab toxin or Cry1Ab toxin-free diet was each mixed into 3 g of standard meridic diet used for rearing the permanent laboratory colony of *O. nubilalis*. In addition, as a reference, freeze-dried Cry1Ab-toxin dissolved in double distilled water was mixed into meridic diet at a concentration of 100 μg of toxin per gram of meridic diet. For the control, an equivalent amount of double distilled water was mixed into the meridic diet. This resulted in a total of 4 treatments. Approximately 500 mg of the respective treated meridic diet was filled into each of 10 vials (1.2 cm diameter by 7.5 cm length) per treatment. Four neonate *O. nubilalis* larvae were placed into each vial and subsequently sealed with perforated plastic lids to allow air circulation (=40 larvae per treatment). Bioassays were replicated 5 times and were performed concurrently with the *C. carnea* feeding trials. Therefore, a total of 800 *O. nubilalis* larvae was examined. Numbers of dead larvae were recorded after 4 or 5 d. For the statistical analyses of mortality, a logistic regression was carried out calculating the proportion of individuals that died accounting for the binomial probability distribution of mortality data. A model was used that tested for significant replication and treatment effects. Analyses were performed using the GENMOD procedure of the SAS statistical package, including a DSCALE and Type 1 and 3 statement producing the appropriate *F*-statistics (SAS Institute 1996). In addition, mean mortality and standard errors were determined and means were compared by carrying out the MEANS procedure and a LSD test of the SAS statistical package, respectively (SAS Institute 1996).

Results

Cry1Ab Effects. Except during the 1st instar, overall mean mortality of *C. carnea* was consistently higher when raised on Cry1Ab toxin-containing than on *B. thuringiensis*-free diet (Fig. 1 b–f). Mortality during the 1st instar was low and no significant differences were observed, despite the early exposure of *C. carnea* larvae to Cry1Ab toxin when raised on artificial diet only (+Bt/AD) (Fig. 1a). Highest mortality occurred during 2nd instar and the pupal stage (Fig. 1 b and e). In the logistic regression analyses, this resulted in

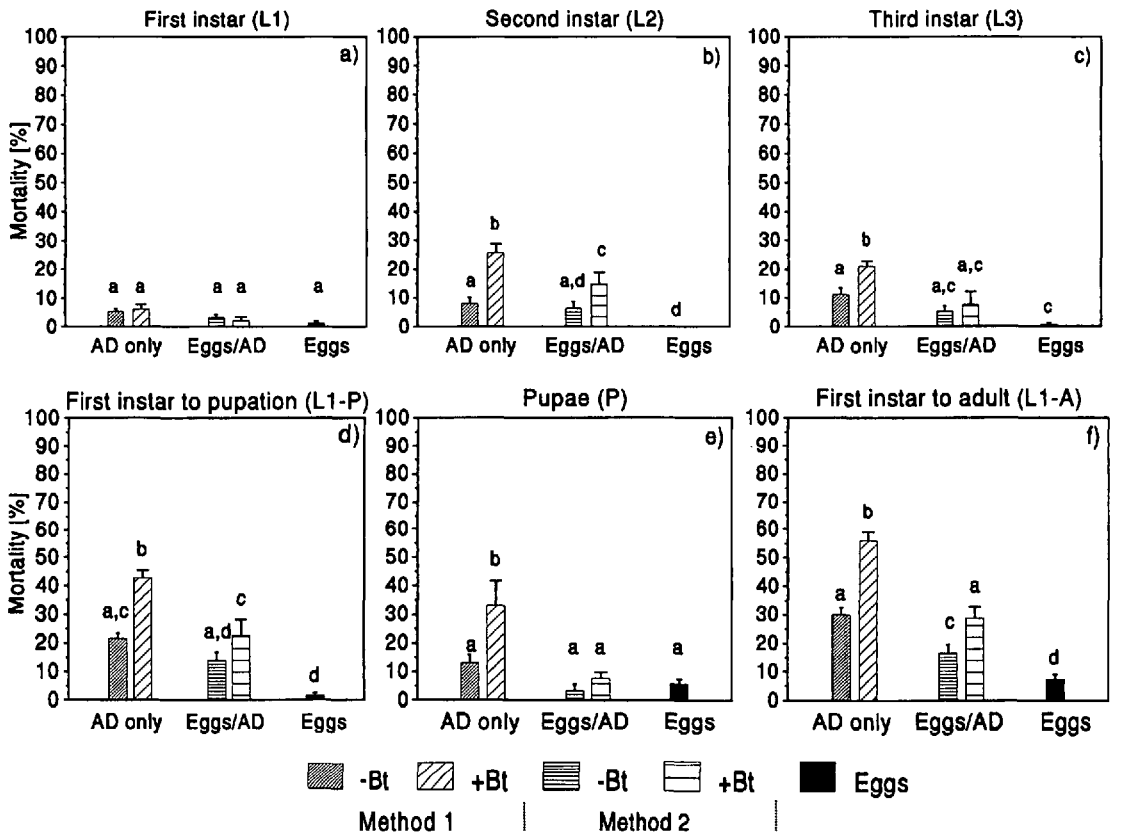

Fig. 1. Stage-specific mean mortality (%) and standard error of immature *C. carnea* feeding on different types of Cry1Ab toxin-containing and Cry1Ab toxin-free diets (a) 1st instars, (b) 2nd instars, (c) 3rd instars, (d) total larval stage (L1-P), (e) pupal stage, and (f) total immature life stage (L1-A). Means with different letters are significantly different at $P = 0.05$ significance level (LSMEANS), AD, artificial diet (method 1); eggs/AD, *E. kuehniella* eggs during 1st instar, artificial diet during 2nd and 3rd instar (method 2); eggs, *E. kuehniella* eggs only.

significant *B. thuringiensis* main effects for all stages, except 1st and 3rd instars ($F_{L2} = 23.87$; df = 1, 16; $P = 0.0002$; $F_{L1\text{-}P} = 19.10$; df = 1, 16; $P = 0.0005$; $F_P = 6.54$; df = 1, 16; $P = 0.0211$; $F_{L1\text{-}A} = 39.70$; df = 1, 16; $P = 0.0001$).

Detailed analyses revealed that mortality was highest for *C. carnea* larvae that received Cry1Ab toxin-containing diet since the 1st instar (+Bt/AD) (Fig. 1a–f). Total mortality of 43 and 57% was recorded for *C. carnea* continuously reared on Cry1Ab toxin diet (+Bt/AD) until pupation (L1-P) and adult eclosion (L1-A), respectively. Significantly fewer larvae died until pupation (21%) and adult eclosion (30%) in the respective Cry1Ab toxin-free control (−Bt/AD) (Fig. 1d and f). Larvae receiving Cry1Ab toxin diet after they had reached the 2nd instar (+Bt/Eggs/AD) exhibited significantly lower mortality than larvae continuously fed Cry1Ab toxin diet (+Bt/AD). Twenty-three and 29% of the larvae died before pupation (L1-P) and adult eclosion (L1-A), respectively, when exposed to Cry1Ab toxin later during larval development (+Bt/Eggs/AD) (Figs. 1d and f). Although mortality was consistently higher among Cry1Ab toxin-treated than untreated *C. carnea* larvae when they received artificial diet after reaching the 2nd instar (method 2), this difference was significant only for 2nd instars (Fig. 1b); and also resulted in a significant difference for total larval (L1-P) and entire immature life stage (L1-A) (Fig. 1d and f).

Similar to mortality, there were no significant differences during the 1st instar in developmental time between *C. carnea* larvae fed Cry1Ab toxin-containing and Cry1Ab toxin-free diet despite early exposure of *C. carnea* larvae to Cry1Ab toxin when raised continuously on artificial diet (+Bt/AD) (Fig. 2a). But during the 2nd and 3rd instar and the pupal stage, mean developmental times were significantly ($P < 0.05$) prolonged relative to the corresponding control (−Bt/AD) when larvae were exposed to Cry1Ab toxin-diet (+Bt/AD) since the 1st instar. This resulted in significant *B. thuringiensis* main effects in the ANOVA for second instars ($F_{L2} = 6.18$; df = 1, 16; $P = 0.024$) and pupae ($F_P = 10.51$; df = 1, 16; $P = 0.005$) and marginally significant *B. thuringiensis* main effect for third instars ($F_{L3} = 4.25$; df = 1, 16; $P = 0.055$) (Fig. 2b, c, and e). However, for total developmental time until pupation (L1-P) and adult eclosion (L1-A) these differences were insignificant (Fig. 2 d and f). No

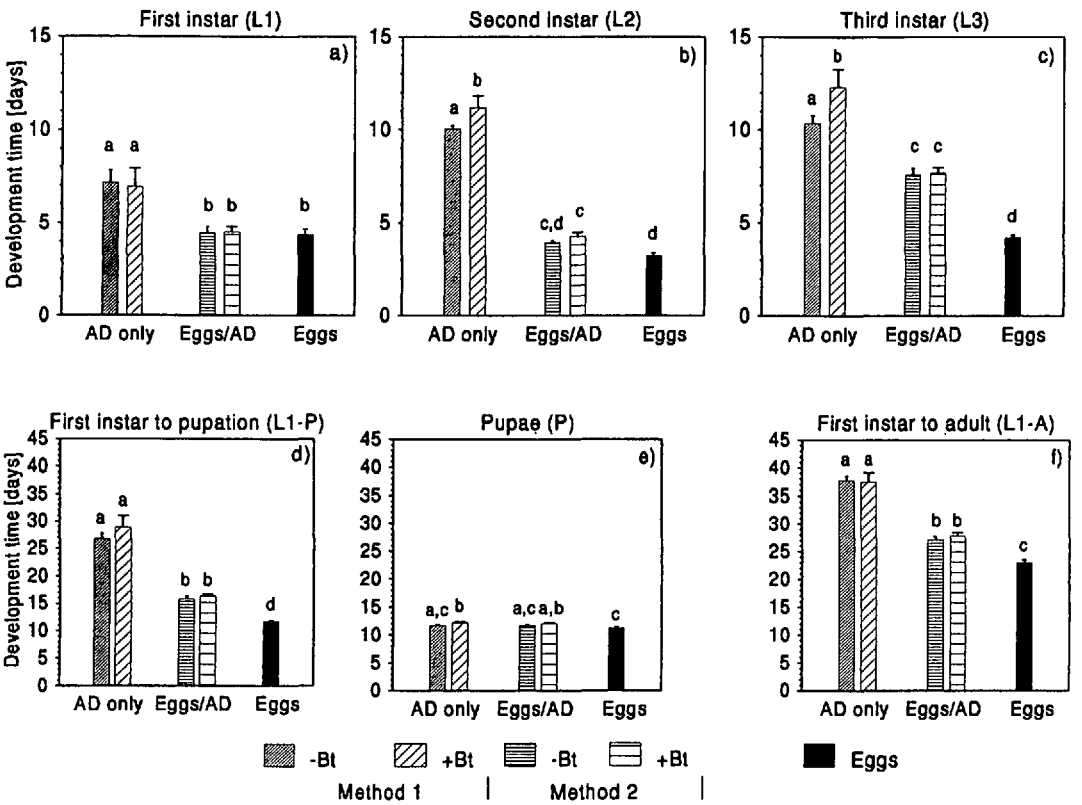

Fig. 2. Stage-specific mean development time (days) and standard error of immature *C. carnea* feeding on different types of CrylAb toxin-containing and CrylAb toxin-free diets (a) 1st instars, (b) 2nd instars, (c) 3rd instars, (d) total larval stage (L1-P), (e) pupal stage, and (f) total immature life stage (L1-A). Means with different letters are significantly different at $P = 0.05$ significance level (LSMEANS), AD, artificial diet (method 1); eggs/AD, *E. kuehniella* eggs during 1st instar, artificial diet during 2nd and 3rd instar (method 2); eggs, *E. kuehniella* eggs only.

significant differences in developmental time between larvae fed CrylAb toxin-containing or CrylAb toxin-free diet were observed at any time for *C. carnea* that received the artificial diet after they had reached the 2nd instar (method 2) (Fig. 2 b–f). For both mortality and developmental time, no interaction between CrylAb toxin treatment and type of diet provided could be observed.

Diet Effects. Except during first instar, there was always a significant diet main effect in the statistical analyses of mortality ($F_{L2} = 11.76$; df = 2, 16; $P = 0.0007$; $F_{L3} = 9.35$; df = 2, 16; $P = 0.002$; $F_{L1\text{-}P} = 24.19$; df = 2, 16; $P = 0.0001$; $F_{P} = 7.85$; df = 1, 16; $P = 0.0042$; $F_{L1\text{-}A} = 39.07$; df = 2, 16; $P = 0.0001$). Detailed analyses showed that until pupation, this was due to significantly lower mortality for larvae that were raised exclusively on *E. kuehniella* eggs. No statistical differences in levels of mortality were observed until pupation between larvae raised on CrylAb toxin-free diet according to method 1 (−Bt/AD) or method 2 (−Bt/E/AD) (Fig. 1a–d). But mortality of *C. carnea* pupae was higher when their larvae had been supplied untreated artificial diet since the 1st instar (−Bt/AD) than when they received artificial diet after reaching the 2nd instar (−Bt/E/AD) (Fig. 1e). This resulted in significantly higher total mortality from 1st instar to adult eclosion (L1-A) of *C. carnea* reared continuously on artificial diet (−Bt/AD) but did not exceed 30% (Fig. 1f). Lowest total mortality (L1-A) was observed for *C. carnea* raised exclusively on *E. kuehniella* eggs (8%) (Fig. 1f). Total immature mortality (L1-A) of *C. carnea* provided with a combination of *E. kuehniella* eggs and untreated artificial diet (method 2) was intermediate to the other 2 types of diet (17%).

There were also significant differences in developmental time for most life stages between larvae that were raised on the different types of diet, except pupae ($F_{L1} = 33.38$; df = 2, 16; $P = 0.001$; $F_{L2} = 288.97$; df = 2, 16; $P = 0.001$; $F_{L3} = 60.1$; df = 2, 16; $P = 0.001$; $F_{L1\text{-}P} = 141.06$; df = 2, 16; $P = 0.001$; $F_{L1\text{-}A} = 146.86$; df = 2, 16; $P = 0.001$). In contrast to mortality, only the developmental time of *C. carnea* pupae did not differ with respect to diet (Fig. 2e). *C. carnea* that received *E. kuehniella* eggs during the 1st instar and that were supplied untreated artificial diet after reaching the 2nd instar (−Bt/E/AD) developed significantly faster than larvae reared continuously on untreated artificial diet (−Bt/AD) who exhibited the slowest development. Larvae receiving untreated artificial diet after reaching the 2nd instar (−Bt/E/AD) required on av-

Table 2. Results of bioassays with 1st-instar *O. nubilalis* larvae monitoring activity of Cry1Ab-containing paraffine spheres

Run	Treatment			
	1	2	3	4
1	100 ± 0a	20 ± 8.16b	97.5 ± 2.5a	25 ± 7.45b
2	100 ± 0a	32.5 ± 8.38b	100 ± 0a	27.5 ± 8.70b
3	100 ± 0a	22.5 ± 6.92b	97.5 ± 2.5a	32.5 ± 11.21b
4	100 ± 0a	30 ± 8.98b	100 ± 0a	10 ± 5.53b
5	100 ± 0a	15 ± 5.53b	100 ± 0a	37.5 ± 10.04b

Treatments: 1, Cry1Ab toxin-containing encapsulated diet mixed into meridic diet; 2, Cry1Ab toxin-free encapsulated diet mixed into meridic diet; 3, purified Cry1Ab toxin-solution mixed into meridic diet (100 μg/g of diet); and 4, double distilled water mixed into meridic diet, $n = 10$. Means with the same letter in a row are not significantly different (LSD).

erage 16 and 27.5 d until pupation and adult eclosion, respectively (Fig. 2 d and f), whereas *C. carnea* raised on artificial diet since the 1st instar needed on average 28 d until pupation and 37.5 d until adult eclosion (Fig. 2 d and f). The shortest developmental time was noted for *C. carnea* that were exclusively fed *E. kuehniella* eggs. They required 12 and 23 d until pupation and adult eclosion, respectively (Fig. 2d and f).

***Ostrinia nubilalis* Bioassays.** The results of the bioassays using one of the primary target pests of Cry1Ab-expressing transgenic plants, *O. nubilalis*, confirmed the continuous biological activity of the paraffin-encapsulated artificial diet containing Cry1Ab toxin used in the experiments (Table 2). Mortality of *O. nubilalis* larvae fed with Cry1Ab toxin-containing diet was always significantly higher than when *O. nubilalis* larvae received Cry1Ab toxin-free diet, regardless whether crushed paraffin spheres were mixed into the meridic diet or into purified toxin and water ($F_{\text{Treatment}} = 2087.51$; df = 3, 192; $P = 0.0001$) (Table 2). Averaged across replications, mortality of 1st-instar *O. nubilalis* was 100 and 99% when crushed Cry1Ab toxin-paraffin spheres or purified, nonencapsulated Cry1Ab toxin-toxin solution was mixed into the meridic diet, respectively, compared with 24 and 26.5% mortality for the respective controls.

Discussion

Continuous exposure to Cry1Ab toxin resulted in significantly higher mortality of immature *C. carnea*. Fifty-seven percent of *C. carnea* exposed to the *B. thuringiensis* protein since the 1st instar died during immature development before adult eclosion whereas mortality was only 30% for untreated *C. carnea* in the respective control. This demonstrates a sensitivity of this species to Cry1Ab toxin at a concentration of 100 μg/ml of diet. The same pattern was observed when *C. carnea* was exposed to Cry1Ab toxin after reaching the 2nd instar, although overall mortality was lower and not always statistically significant. Total immature mortality was again almost twice as high in the Cry1Ab toxin treatment as in the untreated control. Despite this higher mortality, only very small to no effects due to Cry1Ab toxin could be observed for the total developmental time to pupation or adult eclosion. During the life stages in which mortality occurred (2nd and 3rd instar, and pupae), a slightly prolonged developmental time was observed only for *C. carnea* raised on Cry1Ab toxin-containing artificial diet since the 1st instar (method 1), but not for those exposed to Cry1Ab toxin later during larval development (method 2). As a result of the Cry1Ab toxin-treatment, *C. carnea* larvae and pupae that were affected by the Cry1Ab toxin died before completing their immature life stage. Surviving, unaffected *C. carnea* developed at rates similar to those in the untreated control.

These results support the findings from our previous study where we fed susceptible and nonsusceptible lepidopteran herbivores raised on transgenic, Cry1Ab-expressing corn plants to *C. carnea* larvae (Hilbeck et al. 1998). Mortality was also significantly higher for *C. carnea* raised on Cry1Ab toxin-fed prey whereas total immature developmental time was unaffected. From this, we concluded that total developmental time until pupation or adult eclosion is not an appropriate parameter for detecting Cry1Ab toxin effects.

Interestingly, mortality levels observed in the experiments described herein were similar to those observed in our previous study by using the transgenic *B. thuringiensis* corn variety by Novartis (Hilbeck et al. 1998). For this corn cultivar, the concentration of Cry1Ab protein did not exceed 4 μg/g of fresh weight in leaves and was substantially lower in all other tissues analyzed (Fearing et al. 1997). Consequently, perhaps the amount of Cry1Ab toxin passed on to *C. carnea* by its prey in our earlier studies using transgenic *B. thuringiensis*-corn was much smaller than the amount present in the paraffin-encapsulated artificial diet containing Cry1Ab toxin at a concentration of 100 μg/ml (Hilbeck et al. 1998). Yet, mortality levels in both studies were similar. Furthermore, most of the mortality of *C. carnea* occurred during the 1st and 2nd instar in our companion study whereas in the current study, increased mortality was observed for all immature life stages, except 1st instars. To determine the reasons for this, further investigations are required. It may be associated with the biochemical processing of Cry1Ab toxin inside the herbivore's gut, thereby, retaining and perhaps even increasing its activity toward *C. carnea*. Interestingly, Haider et al. (1986) reported that the insect specificity of *B. thuringiensis* var. *aizawai* IC1 could be altered depending on which digestive fluids processed the proteins. They showed that processing of *B. thuringiensis* proteins by *Pieris brassicae* gut enzymes resulted in a 55-kDa protein that only was toxic to lepidopteran cell lines, whereas with *Aedes aegypti* gut extracts, the resulting 52-kDa protein only was toxic to mosquito cell lines and a *Spodoptera frugiperda* cell line. Therefore, the hypothesis that the processing of Cry1Ab toxin within the herbivore host to a form more toxic to *C. carnea* has merit and warrants further investigation. Because a current and future trend in plant molecular biology is to increase expression levels of *B. thuringiensis* in plants, choosing a comparatively high concentration is justi-

fied. The most dramatic example of this trend is the expression of Cry1Ac in tobacco chloroplasts where McBride et al. (1995) reported that an unprecedented 3–5% of total soluble protein in tobacco leaves was Cry1Ac protoxin.

The observed effects may further be related to the modified form of *B. thuringiensis* protein in transgenic plants in contrast to *B. thuringiensis* insecticides that were mainly used for nontarget studies in the past (Croft 1990). In formulations, *B. thuringiensis* proteins are typically present as a mixture of crystals and spores (Feitelson et al. 1992), *B. thuringiensis* corn produces a 69-kDa portion of the native 130-kDa Cry1Ab protoxin. This is a relatively small protoxin that is comprised of ≈620–648 amino acids (Koziel et al. 1993). Inside the insect gut only a small fragment of ≈4 kDa must be further cleaved (e.g., by trypsin) to produce the fully activated, trypsinized 65-kDa toxin. Consequently, no crystal solubilization and almost no protoxin-toxin conversion is necessary within the insect gut. Both processes are important for the specificity of *B. thuringiensis* compounds (Goldburg and Tjaden 1990). Thus, selectivity and biochemical processing may be altered in a herbivore. For example, both *S. littoralis* and *S. exigua* are >2 times more susceptible to Cry1Ab and Cry1Ac toxins, respectively, than to their respective protoxins (MacIntosh et al. 1990; Moar et al. 1990; A.H., unpublished data).

No mortality or only sublethal side effects of *B. thuringiensis* proteins on *C. carnea* have been reported (Croft 1990, Sims 1995, Pilcher et al. 1997). One reason may be that there was a lack of an appropriate bioassay system whereby *C. carnea* larvae could actually ingest *B. thuringiensis* proteins. Previous studies investigating direct effects of *B. thuringiensis* on *C. carnea* were carried out, for example, by coating *S. cerealella* eggs with the respective *B. thuringiensis*-preparation (Croft 1990, Sims 1995). But due to the sucking feeding behavior of chrysopid larvae, it is likely that only little or no *B. thuringiensis* preparation actually was ingested in these trials. The paraffin-encapsulated diet used in our studies represents a method for studying the direct effects of purified, water-soluble *B. thuringiensis* proteins on immature *C. carnea*.

Although developmental time was prolonged for *C. carnea* larvae reared on artificial diet only in our study, control mortality over a mean of 37 d remained acceptably low (AD/−Bt, 30%). Sims (1995) reported 23% control mortality for *C. carnea* after only 9 d by using a *S. cerealella* egg-based bioassay and 30% control mortality for another hymenopteran natural enemy after only 9 d. However, during the first 7 d of our bioassay period (end of 1st instar) total mean control mortality was well below 10% in all treatments, which is typical of many lepidopteran bioassays (MacIntosh et al. 1990, Moar et al. 1995). Mortality was particularly low in the *E. kuehniella* treatment where in contrast to Sims (1995), total mortality over a period of 20 d was only 8%. Our data showed that exposure to Cry1Ab toxin during the 1st instar was important for total mortality. Information on overall mortality and sensitivity of *C. carnea* was lost when exposing *C. carnea* larvae to Cry1Ab toxin after they had reached the 2nd instar. Therefore, we conclude that providing artificial diet continuously according to method 1, including 1st instars, is a suitable method to detect the direct effects of *B. thuringiensis* or other insecticidal substances (affecting intestinal processes of insects) on immature *C. carnea* fitness. But it is acknowledged that a population suffering 30% mortality may be stressed, which may result in potentially enhanced responses.

Another reason for not finding effects of *B. thuringiensis* on *C. carnea* in the past may be that in previous studies chrysopid larvae were exposed to *B. thuringiensis* proteins only for a portion of their whole larval period. For example, Pilcher et al. (1997) and Sims (1995) exposed *C. carnea* larvae to Cry1Ab-pollen or Cry1Ac-coated *S. cerealella* eggs for a total of 3 and 9 d, respectively. Similarly, Salama et al. (1982) provided neonate *B. thuringiensis* var. *entomocidus*-fed *S. littoralis* larvae to *C. carnea* larvae only for 7 d. In all studies, untreated food was supplied afterwards, which may have allowed them to recover from a potential long-term effect. As our data demonstrate, increased mortality occurred during all stages, except the 1st instar. If our experiments had been terminated after 3 and 9 d, much if not all of the mortality would have been missed depending on what period of the larval development would have been chosen. In the past, when *B. thuringiensis* formulations were used as an insecticide, the short-term approach in nontarget trials was appropriate. However, in the future, *B. thuringiensis* proteins will be common in the agroecosystem where they may be present for several weeks or months. Hence, natural enemies will encounter *B. thuringiensis*-containing prey or hosts frequently, in particular, in areas where different *B. thuringiensis* crop plants are grown either next to each other or following each other. Therefore, long-term bioassays with natural enemies are more realistic indicators of possible population-level effects in a system with transgenic plants. But obviously, trials investigating predation efficiency and predator performance under field conditions are necessary before conclusions regarding the potential ecological relevance of the results presented in our paper can be drawn.

The results of our current laboratory experiments and those of Hilbeck et al. (1998) support the hypothesis that the selectivity of *B. thuringiensis* plants cannot simply be deduced from the record of safety of *B. thuringiensis* insecticides but needs to be verified in trials that account for their modified form of release in transgenic plants and their prolonged presence in the plants. For the long-term use of these new plants, their agroecological safety is as essential as is pest resistance development, particularly because performance and fitness of natural enemies in *B. thuringiensis* crop fields also may affect pest resistance development (Gould et al. 1991, Johnson et al. 1997). *B. thuringiensis*-based insecticides and transgenic plants are still more environmentally friendly than most if not all chemical insecticides. However, our results suggest that a closer look at the impact of *B. thuringiensis* on beneficial insects may be necessary before a complete IPM-

biocontrol system that is based on *B. thuringiensis* transgenic plants is initiated.

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