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Effects of Bt maize on the herbivore *Spodoptera littoralis* (Lepidoptera: Noctuidae) and the parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae).

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Abstract

Recent studies have shown that transgenic insect resistant plants can have negative effects on non-target herbivores as well as on beneficial insects. The study of tritrophic interactions gives insight into the complex mechanisms of food webs in the field and can easily be incorporated into a tiered risk assessment framework. We investigated the effects of transgenic maize (*Zea mays*) expressing insecticidal proteins derived from *Bacillus thuringiensis* (Bt maize) on *Spodoptera littoralis*, a non-target herbivore, and on the hymenopteran parasitoid *Cotesia marginiventris*. In a laboratory study, *S. littoralis* larvae were reared for their whole lifespan on a mixture of leaves and stems from 2–4-week old Bt maize plants. *S. littoralis* survival, developmental times and larval weights were significantly affected by Bt maize diet. However, adult moths, which survived development on Bt maize, were the same size as the adults from the control group. *C. marginiventris* survival, developmental times and cocoon weights were significantly negatively affected if their *S. littoralis* host larva had been fed Bt maize. ELISA tests confirmed that *S. littoralis* larvae ingest high amounts of Cry1A(b) toxin while feeding on Bt maize. In *S. littoralis* pupae and in *C. marginiventris* cocoon silk, only traces of the toxin could be detected. No toxin was found in *S. littoralis* and *C. marginiventris* adults. Thus the toxin is not accumulating in the trophic levels and in fact appears to be excreted. Our results suggest that the effects on *C. marginiventris* when developing in susceptible *S. littoralis* larvae are indirect (host mediated). The biological relevance of those results and the significance of this study in risk assessment are discussed.

Introduction

Transgenic maize (*Zea mays*) expressing Cry1A(b) toxin derived from *Bacillus thuringiensis* (Bt maize) targets selectively the European Cornborer (*Ostrinia nubilalis,* Lepidoptera: Crambidae) and other stem boring Lepidoptera. As plants and their insect pests are part of a complex multitrophic system comprising a wide range of diverse organisms (Price et al., 1980; Poppy, 1997), growing transgenic plants in the field poses the unquantified risk of affecting non-target organisms, such as natural enemies. Parasitoids are very sensitive to changes in their hosts after toxin ingestion, as they usually complete their development on one single host individual. When Bt susceptible hosts are treated with Bt toxins, parasitoids are likely to be affected, more than predators, which are often generalists and feed on several different prey species (e.g. Salama & Zaki, 1983). Adverse effects on

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parasitoids have been reported in tritrophic studies with Bt transgenic plants and susceptible herbivores that were fed with Bt plant tissue (Bernal et al., 2002; Baur & Boethel, 2003; Meissle et al., 2004, Prütz & Dettner, 2004). However, no direct effects of Cry toxins have been shown for any parasitoid species. Observed adverse effects of Bt crops on parasitoids can be interpreted as indirect (host-mediated) effects due to changed host availability or nutritional quality of hosts.

Recent studies have shown that *Spodoptera littoralis* (Boisduval), a polyphagous lepidopteran pest, is partly affected by Cry1A(b) (Dutton et al., 2002) with potential implications for higher trophic levels. In our studies, *S. littoralis* larvae were reared for their entire developmental period on Bt maize and not only for one or a few days as was normal practice in previous work. Life long exposure to the Bt toxin informs more accurately about the susceptibility of the herbivore to the Bt toxin.

Cotesia marginiventris (Cresson) (Hymenoptera: Braconidae) is a polyphagous, solitary endoparasitoid and can be considered a generalist as it attacks many lepidopteran pest species. This makes the parasitoid potentially important for biological control (Tillman, 2001), especially for the control of secondary pests in Bt crops. Thus *C. marginiventris* is a useful model organism for the risk assessment of Bt crops.

This paper describes a worst-case (laboratory-scale) experiment to investigate potential lethal and sublethal effects of Bt maize on *C. marginiventris* via the host *S. littoralis*. The experiments were designed to address the following questions:

(1) Does Bt maize have lethal or sublethal effects on the non-target lepidopteran pest herbivore *Spodoptera littoralis*?
(2) Are *Cotesia marginiventris* larvae adversely affected when developing in Bt maize-fed *S. littoralis* larvae?
(3) Is Cry1A(b) transferred through different trophic levels and if so, in which quantities?

Materials and methods

Maize

Transgenic maize (*Zea mays*) plants (event Mon810, Monsanto: "Bt") expressing Cry1A(b) protein and the untransformed control cultivar Monumental ("Control") were used in all experiments. Plants were cultivated in 15 cm plastic pots (3 grains per pot) and kept in a greenhouse at a temperature of 22°C and L:D 12:12 h. New plants were sown weekly and were used for experiments when 2–4 weeks old (3–6 leaves stage and height of 25–75 cm). No fertilisers were applied.

Insects

Cultures and bioassays were maintained at a temperature of 25 ± 3°C, 60 ± 10% RH and a photoperiod of L:D 14:10 h. A culture of the herbivore *Spodoptera littoralis* on artificial diet (Beet Armyworm Diet F9219B, Bio-Serve, Frenchtown, USA) was established, using an initial population from Syngenta, Bracknell, UK. Adults were kept in 30 × 30 × 30 cm cages, fed with a honey and water solution with added vitamin mix and Streptomycin. Eggs were collected daily and sterilised in a sodium hypochlorite solution to minimize the risk of contaminating the culture, before they were placed in 250 mL plastic pots on the artificial diet. Ten-day-old larvae were transferred with their diet into 1.5 L plastic boxes, half-filled with vermiculite as substrate for pupation. For bioassays, sterilised eggs were placed in empty 1.5 L plastic boxes until hatching.

Cocoons of the endoparasitoid *Cotesia marginiventris* were obtained from the Laboratoire d'Ecologie, Université de Neuchâtel, Switzerland, where *C. marginiventris* has been reared on *S. littoralis* feeding on artificial diet. *C. marginiventris* prefers 3–5 day-old larvae of *S. littura* (Jalali et al., 1987), a species that is closely related to *S. littoralis* (Hill, 1983). For general culturing, 20–30 second instar caterpillars (3–5 days old) were offered to a single mated female parasitoid (2–6-days old) for 4–6 h. First instar *S. littoralis* larvae are too small to cope with parasitization (Cristina Tamo, personal comment). Parasitized caterpillars were reared individually in 30 mL plastic cups on artificial diet until cocoon formation. Emerging parasitoid adults were kept in plastic cylinders (9 cm in diameter, 20 cm high) with drops of honey on the wall and mineral water on cotton wool.

Effects of Bt maize on the herbivore Spodoptera littoralis

Survival of first instar larvae

To obtain data about how many *S. littoralis* larvae would survive through to the second instar and would be available for parasitization, newly hatched larvae were kept in groups of 20 individuals in 250 mL plastic cups (Roundstone Catering Equipment LTD, Melksham, Wiltshire, UK), closed with a punctured lid to allow ventilation. Fresh maize leaves and pieces of stem from different parts of Bt or Control plants were added and changed after 3 days. Larvae were counted after 3 and 4 days to examine survival. During the experimental period, the Control treatment was repeated three times with 6 cups each ($n = 18$). The Bt treatment was repeated seven times with 6 cups each ($n = 42$).

Sublethal effects and survival of later instars

Newly hatched *S. littoralis* larvae were kept in large groups in 1.5 L (22 cm × 15 cm × 8 cm) PE sandwich boxes, closed with a punctured lid to allow ventilation. They were fed a mixture of different parts (leaves and stem) from Bt or Control maize plants ad libitum.

The experiments started when larvae reached second instar (after 2 days on Control maize or 3 days on Bt maize) to make this experiment more comparable to the parasitization experiment (see below): 6 larvae per replicate were put together in 250 mL plastic cups and fed thereafter with either Bt or Control maize. As higher *S. littoralis* instars become cannibalistic, larvae were kept individually from day 8 in 30 mL plastic cups, covered with a piece of nappy liner (disposable adsorbent tissue used to line towelling nappies/diapers) and closed with a punctured plastic lid. As preliminary experiments have shown that fastest *S. littoralis* larvae start to build a pupation chamber after 12 days, the cup was filled with 1 cm of vermiculite on day 11 to avoid excessive moisture and to provide a substrate for pupation. Maize leaves and vermiculite were changed daily and cups cleaned when necessary. When larvae pupated they were not disturbed for 2 days, because of high sensitivity to disturbance during this stage. Pupae were placed on a layer of vermiculite in cleaned cups until emergence of adults. Survival was first recorded 2 days after the start of the experiment and then daily. Larvae were weighed on day 8 and day 11 and pupae were weighed 1 day after formation. Day 8 was chosen for measurements because that day marked the end of the first week of the experiment and day 11 was chosen to ensure that no larvae were disturbed after initiating pupation (stop feeding and empty their gut). The time taken until pupation and the emergence of the adult moth was recorded. Emerging moths were frozen, and wing length and width was measured with a pair of callipers, as wing size is a good index for the size of a lepidopteran species (Watanabe and Nozato, 1986) and the reproductive potential is usually correlated with the size of the adult in insects (e.g. Gage, 1998; Gilbert, 1984).

The experiment was initially conducted on 60 larvae per treatment (repetition 1) and repeated 2 months later on 48 larvae each (repetition 2).

Effects of Bt maize on the parasitoid Cotesia marginiventris

Two-to-five-day-old second instar *S. littoralis* caterpillars, weighing between 0.6 and 2.2 mg, which had been reared on maize leaves since their hatching, were offered individually to one mated *C. marginiventris* female (2–6-days old). Caterpillars reared on Control maize and caterpillars reared on Bt maize were offered alternately in 30 mL plastic cups until parasitization was observed. Each female stung up to 26 larvae, depending on her willingness to parasitize. Larvae were kept in 30 mL plastic cups covered with a piece of nappy liner and closed with a punctured plastic lid on a mixture of different maize parts (leaves and stem) until cocoon formation. Maize was changed daily and cups were cleaned when necessary. Survival was recorded daily. Larvae were weighed 3 and 6 days after parasitization to cover best the time between parasitization and the earliest day when parasitoid larvae would leave their host. Cocoons were weighed one day after formation, to prevent damage to the fragile freshly-spun cocoon. Cocoons were kept in 30 mL plastic cups until adult emergence. The time taken until cocoon formation and the emergence of the adult parasitoid and the sex of the emerged adult parasitoid were recorded. Altogether, 201 (Control) and 223 (Bt) larvae were parasitized by 55 females on 12 different days.

For statistical analysis of sublethal effects, only those *S. littoralis* larvae, in which a parasitoid cocoon or at least a parasitoid larva was observed, were included.

Transfer of Bt toxin through the different trophic levels

Five samples were taken from Bt and Control maize plants (19–28 days old) during the whole experimental period between August and December 2002 for Cry1A(b) toxin measurements. Each of these 5 replicates consisted of a mixture of stem and different leaf tissues from, on average, 5 maize plants, as each larva consumed a variety of maize tissue from a number of plants during its development.

Spodoptera littoralis individuals were taken from both feeding experiments between August and November 2002 on day 8 (5 from each experiment repetition) and day 11 (5 from the first and 3 from the second experiment repetition). Freshly formed pupae (5 from the first and 3 from the second experiment repetition) and newly emerged adults (5 from the first experiment repetition) were also taken. Thirteen freshly formed *C. marginiventris* cocoons and 50 newly emerged adults were taken throughout the whole experimental period between September and December 2002. All samples were stored in sealed plastic bags at −20°C until ELISA assays were conducted.

To extract soluble proteins, each sample was ground and homogenised in PBST extraction buffer at a ratio homogenised tissue (mg) : buffer (µl) 2:3. When necessary, additional buffer was added to assure that each sample was completely covered. Extraction took place overnight at 4°C on a platform shaker. The next day, after centrifugation, supernatants were collected, and total protein content (µg/µL sample extract) was measured with Bradford assays, using BSA (Bovine serum albumin) as standards.

To determine the presence of Cry1A(b) toxin, the PathoScreen kit for Bt-Cry1A(b)/1A(c) protein (Agdia Inc., USA) was used. Sample volumes containing 0.5 µg of total protein (maize), 5 µg (*S. littoralis* larvae), 50 µg (*S. littoralis* pupae and adults), 200 µg (*C. marginiventris* cocoons and empty cocoons) and 13 µg (*C. marginiventris* adults) were added to each test well and filled up to 100 µL with MEB (milk enhanced buffer). All samples were tested in triplicate. The lyophilised positive control was reconstituted in 2.5 mL of MEB to achieve a Cry1A(b) concentration of approximately 32 ng/mL (Agdia, personal comment) and dilutions of 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 ng/mL were used to obtain a standard curve, as well as pure MEB as a blank. Thereafter, standard procedures were followed as outlined in the kit. Optical density was measured at 620 nm with a microtiter plate reader (Anthos Reader 2001, Type 10 500; Anthos Labtech Instruments).

Corresponding tissue blanks (samples from the control treatment) were subtracted from Bt samples to eliminate unspecific binding effects of proteins to ELISA test wells. The limit of detection (LOD) was calculated for maize tissue and insect material separately. The standard deviation from all maize or insect control readings was multiplied by 3 to obtain $\text{Absorbance}_{\text{Limit}}$. The LOD was determined by interpolation at $\text{Absorbance}_{\text{Limit}}$ units from the corresponding Cry1A(b) standard curve. The resulting LOD for maize and insect material was 0.02 and 0.03 ng Cry1A(b) per ml protein solution, respectively. Results between 0.03 and 0.1 ng/ml are reported as traces of Cry1A(b). Mean amounts of Cry1A(b) toxin per fresh weight were calculated for Bt samples of maize plants, *S. littoralis* larvae on day 8 and day 11, pupae and adults, *C. marginiventris* cocoons, adults and cocoon silk (empty cocoons after adult emergence).

Statistical analysis

All data were analysed using SPSS for Windows version 11.0 (SPSS Inc., Chicago, USA). Mean values between treatments were compared with Mann–Whitney U Tests, as the data were generally not normally distributed and failed to normalise even after a wide range of transformations were attempted. Percentages were arcsinus transformed before mean value analyses were performed. Ratios were tested with a Chi Square Test and as only 2×2 tables were calculated, Fisher's Exact Test value is reported. Survival analyses of larvae until pupation were conducted using the Kaplan–Meier procedure and Breslow (Wilcoxon) Test. Survival data were recorded

until all *S. littoralis* or *C. marginiventris* larvae were either dead or pupated. All pupated individuals were considered as "surviving until the last recorded day".

Results

Effects of Bt maize on Spodoptera littoralis

Survival of first instar larvae

The survival of Monumental ("Control") maize-fed *S. littoralis* larvae was significantly higher than survival of Mon810 ("Bt") maize-fed larvae (Mann–Whitney U Test, $p < 0.001$). Ninety-eight percent of Control maize-fed larvae and 45% of Bt maize-fed larvae survived the first 3 days and 97.5% of Control maize-fed larvae and 40% of Bt maize-fed larvae survived the first 4 days after hatching.

Sublethal effects of later instars

On day 8 and day 11, Control maize-fed larvae weighed significantly more than Bt maize-fed larvae in both experiment repetitions (Mann–Whitney U Test, $p < 0.001$, Table 1). Bt maize-fed *S. littoralis* achieved only 16/22 % (experiment repetition 1/2) and 25/34 % of Control maize-fed *S. littoralis* larval weight on day 8 and day 11 respectively. Control maize-fed larvae developed significantly faster than Bt maize-fed larvae ($p < 0.001$). In the first repetition, weight of pupae was significantly higher in the Bt treatment ($p < 0.001$) and a similar trend was observed in the second repetition ($0.05 < p < 0.1$). Wing sizes of adults were not significantly different ($p > 0.05$).

Larvae in the second experiment repetition weighed significantly less (except Control maize-fed larvae on day 8; Mann–Whitney U Test, $p > 0.05$) and took longer to develop than larvae in the first experiment repetition ($p < 0.01$). In the Bt maize group, larvae in the second experiment repetition had body weights between 74% (day 8) and 55% (day 11) of larvae in the first experiment repetition. Pupae weighed 78% of the body weight of those in the first experiment repetition, and their developmental time was 10% longer. In the Control group, although they weighed 4% more on day 8 (no significant difference, see above), on day 11, larvae in the second experiment repetition weighed 76% and pupae 83% of the body weight of those in the first experiment repetition. Their developmental time was 5–7% longer. These differences between experiments demonstrate the importance

Table 1. Comparison of sublethal parameters of *Spodoptera littoralis* from second instar to adult. Mean values of larval and pupal weights, developmental times to pupation, time to adult emergence and wing sizes are presented with standard errors (SE). Control and Bt treatments within experiment repetition 1 and 2 are compared with Mann–Whitney U Tests

	Repetition 1			Repetition 2		
	Control	Bt	Significance	Control	Bt	Significance
Weight on day 8 (mg ± SE)	81.9 ± 3.59 ($n = 59$)	18.0 ± 0.91 ($n = 57$)	***	85.4 ± 4.82 ($n = 48$)	13.3 ± 0.98 ($n = 39$)	***
Weight on day 11 (mg ± SE)	454.0 ± 18.70 ($n = 54$)	153.0 ± 6.62 ($n = 49$)	***	345.9 ± 15.17 ($n = 45$)	84.8 ± 6.60 ($n = 36$)	***
Weight of pupae (mg ± SE)	186.0 ± 4.51 ($n = 48$)	210.7 ± 4.46 ($n = 44$)	***	155.0 ± 4.30 ($n = 38$)	163.7 ± 5.09 ($n = 29$)	$p = 0.08$
Time to pupation (days)	15.4 ± 0.20 ($n = 48$)	18.5 ± 0.14 ($n = 44$)	***	16.2 ± 0.28 ($n = 38$)	20.7 ± 0.27 ($n = 29$)	***
Time to adult emergence (days)	26.5 ± 0.28 ($n = 40$)	29.7 ± 0.20 ($n = 38$)	***	28.5 ± 0.22 ($n = 33$)	33.0 ± 0.34 ($n = 23$)	***
Wing width (mm)	6.4 ± 0.07 ($n = 40$)	6.5 ± 0.07 ($n = 38$)	$p > 0.1$	6.0 ± 0.10 ($n = 33$)	5.9 ± 0.13 ($n = 22$)	$p > 0.1$
Wing length (mm)	13.4 ± 0.13 ($n = 40$)	13.7 ± 0.15 ($n = 38$)	$p > 0.1$	12.8 ± 0.14 ($n = 33$)	12.5 ± 0.13 ($n = 22$)	$p > 0.1$

*** indicates significant differences at $p < 0.001$.

of repetition, and the problems which may be encountered if ring testing (conducting the same experiment in different laboratories) is adopted in risk assessment. However, Control maize-fed larvae of the second experiment repetition still weighed significantly more and developed faster than the Bt maize-fed larvae of the first experiment repetition. The latter, however, produced significantly heavier pupae and adults with larger wings (Mann–Whitney U Test, $p < 0.001$).

Survival of later instars

In the second repetition, survival from second instar until pupation was significantly higher in the Control group than in the Bt group (Figure 1; Kaplan–Meier, Breslow, $\chi^2 = 7.68$, df = 1, $p < 0.05$). This difference in survival was not observed in the first repetition (Kaplan–Meier, Breslow, $\chi^2 = 0.40$, df = 1, $p > 0.05$). Furthermore, survival in the Bt treatment of the second repetition was significantly lower than survival of Bt and Control larvae of the first repetition (Kaplan–Meier, Breslow, $\chi^2_{Bt2/Bt1} = 8.31$, $\chi^2_{Bt2/Control\ 1} = 11.56$, df = 1, $p < 0.05$). Pupal mortality in both repetitions was not significantly different between both treatments (χ^2, Fisher's Exact Test, df = 1, $p > 0.05$). In the Bt treatment, 0 (out of 39) and 3 (out of 26) pupae died in the first and second repetition, respectively. In the Control treatment, no adults emerged from 3 (out of 43, repetition 1) and 3 (out of 36, repetition 2) pupae.

Together with the experiment of first instar survival, the total mean survival of larvae when feeding exclusively on Bt maize for the whole life span from hatching to adult emergence was estimated as 37%, whereas the survival of Control maize-fed larvae was 86%.

Effects of Bt maize on Cotesia marginiventris

Sublethal effects

Parasitized Control maize-fed *S. littoralis* larvae weighed significantly more than Bt maize-fed *S. littoralis* larvae both 3 days and 6 days after parasitization. *C. marginiventris* cocoons were significantly heavier when developing in control maize-fed hosts (Figure 2; Mann–Whitney U Test, $p < 0.001$). Weight on parasitization day was equal (Mann–Whitney U Test, $p > 0.05$). *Cotesia marginiventris* developed faster in Control maize-fed *S. littoralis* than in control maize-fed *S. littoralis* (Figure 3; Mann–Whitney U Test, $p < 0.001$).

In the Bt maize-fed group, parasitized larvae weighed 62% and 82% of the body weight of

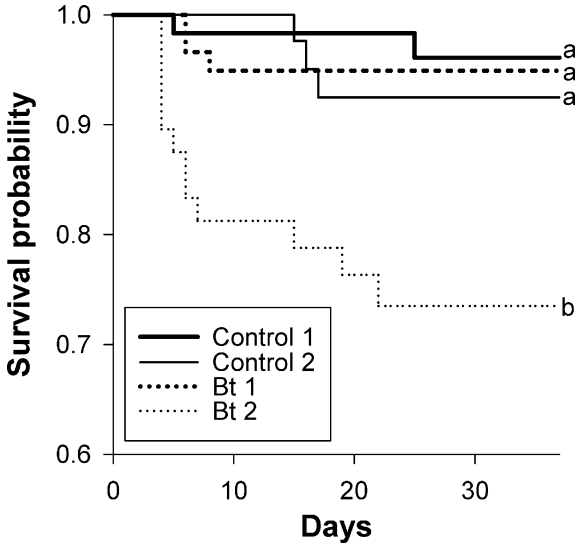

Figure 1. Survival of 2nd instar *S. littoralis* larvae until pupation as calculated in the Kaplan–Meier procedure. Larvae were reared either on transgenic ("Bt", $n_{Repetition\ 1} = 59$, $n_{Repetition\ 2} = 48$) or control maize ("Control", $n_{Repetition\ 1} = 60$, $n_{Repetition\ 2} = 48$). Small letters indicate significantly different survival curves ($p < 0.05$).

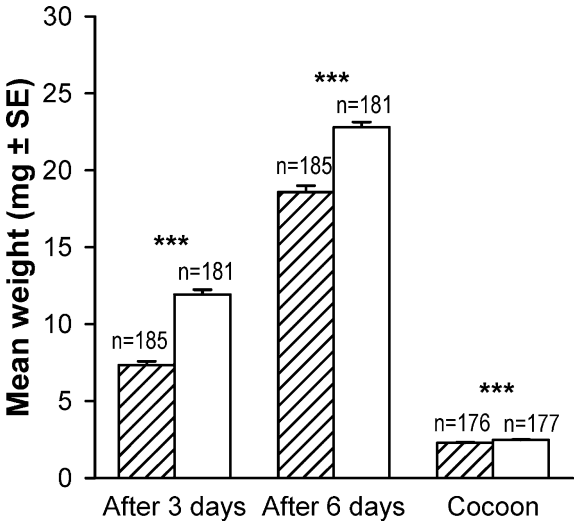

Figure 2. Mean weight (mg ± SE) of *C. marginiventris*-parasitized *S. littoralis* larvae 3 and 6 days after parasitization and weight of *C. marginiventris* cocoons. *S. littoralis* larvae were reared either on transgenic ("Bt", hatched) or control ("Control", white) maize ***$p < 0.001$.

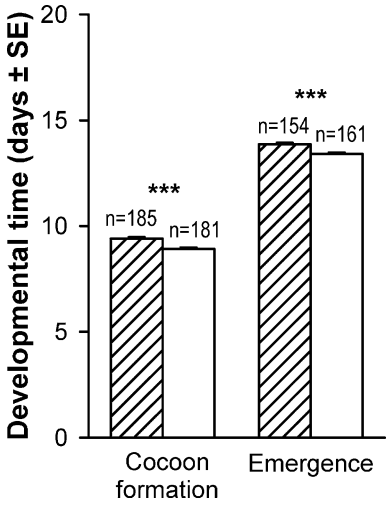

Figure 3. Developmental periods of *C. marginiventris* growing in *S. littoralis,* number of days (±SE) from parasitization until cocoon formation and emergence of the adult parasitoids. S. *littoralis* larvae were reared either on transgenic ("Bt", hatched) or control ("Control", white) maize ***$p < 0.001$.

parasitized Control maize-fed larvae three and 6 days after parasitization, respectively. Parasitized larvae were in general much smaller than non-parasitized larvae: Parasitized Bt maize-fed larvae achieved 46% and 15% of the weight of equivalent non-parasitized Bt maize-fed *S. littoralis* larvae 3 days 6 days after parasitization respectively. Control maize-fed larvae had 4% and 6% of the weight of equivalent non-parasitized Control maize-fed *S. littoralis* larvae 3 and 6 days after parasitization respectively. Weight data of non-parasitized larvae are from the experiments about sublethal effects on later instar *S. littoralis* larvae.

The sex ratio was different in the two treatments (χ, Fisher's Exact Test, df = 1, $p < 0.05$). In the Bt maize-fed group, 47% of emerging *C. marginiventris* adults were males, whereas in the Control maize-fed group, 61% were males. The development until emergence of females (13.75 ± 0.08 days) took significantly longer than that of males (13.54 ± 0.08 days), (Mann–Whitney U Test, $p < 0.05$). When both sexes were analysed separately, Control maize-fed larvae and *C. marginiventris* cocoons still weighed significantly more, and *C. marginiventris* developed significantly faster in Control maize-fed than in Bt maize-fed larvae (Mann–Whitney U Test, $p < 0.05$).

Survival

Survival of *C. marginiventris* until cocoon formation was significantly higher in the group exposed to Control maize-fed larvae than in the group exposed to Bt maize-fed larvae (Figure 4, Kaplan–Meier, Breslow, $\chi^2 = 8.77$, df = 1, $p < 0.05$). Mortality of parasitoid pupae was not different in both treatments (χ^2, Fisher's Exact Test, df = 1, $p > 0.05$) as no adults emerged from 11 (out of 165) and 12 (out of 173) cocoons in the Bt and Control treatment, respectively. Only two adult *S. littoralis* emerged in the Bt parasitization treatment and none in the Control treatment. This difference was not significant (χ^2, Fisher's Exact Test, df = 1, $p > 0.05$).

Transfer of Bt toxin through different trophic levels

Mon810 Bt maize plants contained a mean concentration of 1.597 µg Cry1A(b) toxin per gram fresh weight of plant tissue (Table 2). *Spodoptera littoralis* larvae feeding on such plants contained between 0.595 and 0.645 µg Cry1A(b) toxin per gram of fresh bodyweight. In *S. littoralis* pupae and *C. marginiventris* cocoon silk only traces of Cry1A(b) were detected. Adult *S. littoralis*,

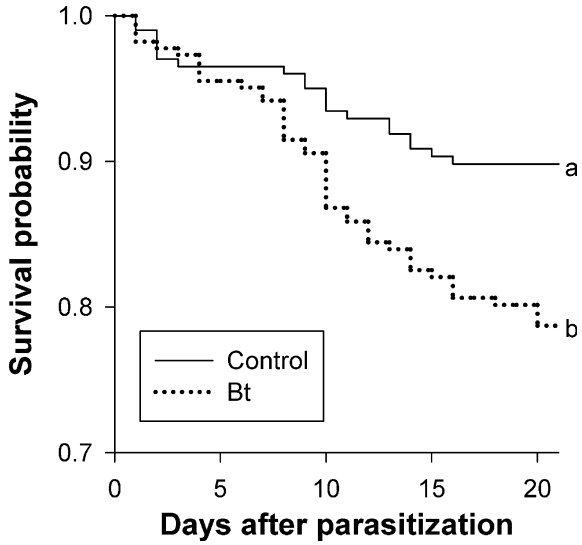

Figure 4. Survival of *C. marginiventris* developing in *S. littoralis* larvae from parasitization until cocoon formation as calculated in the Kaplan–Meier procedure. Parasitized larvae were reared either on transgenic ("Bt", $n = 223$) or control maize ("Control", $n = 201$). Small letters indicate significantly different survival curves ($p < 0.05$).

Table 2. Results of the ELISA tests of Bt maize tissue, the herbivore *S. littoralis* and the parasitoid *C. marginiventris*. The number of replicates and individuals in each replicate, the range of amount of material used, extraction buffer added and total soluble protein measured in sample extracts, the amount of total soluble protein added to test wells and the mean concentration ± SE of Cry1Ab (µg per g fresh tissue), are shown.

Sample	Number of individuals (and replicates)	Fresh weight homogenised tissue (mg; range)	Amount of buffer added (µl; range)	Amount of total soluble protein in sample extract (µg/µl; range)	Amount of total soluble protein added to well (µg)	Cry1Ab per fresh weight (µg/g; Mean ± SE)
Maize (MEB 307 Bt)	Ca. 25 (5)	284–5270	430–7985	0.26–1.33	0.5	1.597 ± 0.438
S. littoralis day 8	10 (2)	24–59	36–90	7.86–9.80	5	0.595 ± 0.225
S. littoralis day 11	8 (2)	107–355	160–540	6.83–8.95	5	0.645 ± 0.036
S. littoralis pupae	8 (2)	235–627	355–950	23.97–27.97	50	Traces
S. littoralis adults	5 (1)	378	850	25.67	50	Not detectable
C. marginiventris cocoons	13 (1)	29.9	100	13.79	200	Not detectable
C. marginiventris adults	Ca. 50 (1)	45.9	100	23.10	200	Not detectable
C. marginiventris empty cocoons	Ca. 50 (1)	32.5	150	0.85	13	Traces

C. marginiventris cocoons (including pupae) and adult parasitoids, contained no detectable amount of Cry1A(b) toxin.

Discussion

We have shown that *S. littoralis* larvae are significantly negatively affected by Bt maize (Mon810, Monsanto) in terms of developmental time and survival. This is not surprising as many lepidopteren species have been shown to be susceptible to Cry1A(b) (Glare & O'Callaghan, 2000). However, conflicting results have been reported regarding the susceptibility of *S. littoralis* to Cry1A(b). Similar to our findings, Dutton et al. (2002) reported significant susceptibility in bioassays with Cry1A(b) expressing Bt maize, in contrast to some workers who have found *S. littoralis* to be not or only marginally susceptible to purified Cry1A(b) toxin (Höfte & Whiteley, 1989; Escriche et al., 1998; Hilbeck et al., 1999). We observed the highest mortality in the first larval stage, where less than 50% of larvae survived the first 4 days on Bt maize. Other studies confirm that susceptibility of *S. littoralis* to Bt toxins is highest in the first larval stages and then decreases with progressed larval development (Sneh et al., 1981; Keller et al., 1996). In contrast to the study of Dutton et al. (2002), who investigated exposure of first instar larvae, our study is the first to look at continued exposure over the entire lifespan. As herbivores in a Bt maize field are likely to ingest toxic leaf material for their whole larval life rather than a few days only, long term exposure of hosts is necessary to obtain biologically relevant data when assessing the risk to parasitoids. Despite the longer development and higher mortality, those adults which did survive on a Bt maize diet reached the same size as adults from the control maize and had even heavier pupae. Peacock et al. (1998) also found no significant differences in pupal and adult weight of several lepidopteran species sensitive to Bt toxin, although they observed higher mortality and longer developmental periods. Moreau and Bauce (2001) reported a compensation of Bt effects with a longer developmental time and heavier pupa in *Choristoneura fumiferana* (Clemens) (Lepidoptera). Developmental polymorphism lead to supernumerary instars to com-

pensate for adverse effects of the Bt toxin (Moreau & Bauce, 2001). By studying host development, sensitive stages and compensation abilities to overcome toxin effects, host quality to parasitoids in different life stages can be concluded more easily.

In both Bt and Control treatments, weights were lower and developmental times were longer in the second repetition of the experiment. Such variability in larval performance might have been due to a deterioration of maize quality later in the year, greater temperature fluctuations, inbreeding of the caterpillars or variation in other biotic or abiotic factors that were not quantified. However, the performance of Control maize-fed larvae compared to Bt maize-fed larvae was still significantly better in both repetitions of the experiment. The differences between Bt and Control maize-fed larvae were more distinct under the apparently worse conditions of the second repetition, and the impact on Bt maize-fed larvae was higher than on Control maize-fed larvae. In addition to sublethal effects, Bt maize-fed larvae suffered significantly higher mortality in the second experimental repetition. In the apparently ''better'' conditions of the first experiment, the difference in survival between Bt and Control maize-fed larvae was very small, but under the ''worse'' conditions of the second experimental repetition, the mortality of Bt maize-fed larvae was significantly increased. The Bt toxin seems to act additively or even synergistically with negative environmental conditions on higher mortality of later instars. These findings show how important repetition over time and under different conditions can be when undertaking risk assessment studies. The effects of Bt toxin can be variable in different environments, and experiments conducted in different places at different times of the year and under different conditions can produce different results. The routine risk assessment of insecticides is conducted by ring testing (i.e. conducting the same experiments in different labs). Such ring testing for GM plants would be necessary, but perhaps more challenging, to assess if there is large variation between the datasets. It is imperative that risk assessment protocols developed are as robust as possible and that cause–effect relationships, endpoints and trigger values are developed, so that variability can be considered and assessments reliably made (Poppy, 2003). If the level of variability we observed is typical , then the trigger values and endpoints chosen will need to take this into consideration.

Our laboratory ''worst-case'' study showed negative effects on *Cotesia marginiventris* when developing in *S. littoralis* larvae that had fed on transgenic Bt maize. The cocoons of *C. marginiventris* were smaller and developmental times longer. In addition, *C. marginiventris* suffered greater mortality when parasitizing caterpillars feeding on Bt maize. Baur and Boethel (2003) showed that *C. marginiventris* needed longer to develop and had reduced fecundity and longevity when developing in *Pseudoplusia includens* (Walker) caterpillars feeding on transgenic Bt cotton (expressing lepidopteran specific Cry1A(c)). When developing in or on hosts fed with tissue from Bt crops, delayed development, higher mortality or reduced pupal weight have been reported for other host and parasitoid species (Bernal et al., 2002; Baur & Boethel, 2003; Meissle et al., 2004; Prütz & Dettner, 2004). Numerous studies with commercial Bt products have demonstrated longer development, reduced fertility, reduced longevity and skewed sex ratio, as reviewed by Flexner et al. (1986) and Vinson (1990). In contrast to most other studies, we used plant material instead of incorporating the toxin in artificial diets and we exposed the larvae continuously and not only for a few hours or days to the toxin. Consequently, our experiments give a more realistic level of exposure, which is essential for quantifiable risk assessment (Poppy, 2004). In addition, we not only assessed effects of Bt maize on the parasitoid but also on unparasitized hosts. Linking development data and mortality levels of unparasitized with parasitized hosts helps to interpret the nature of observed effects.

One of the major debates relating to GM biosafety is elucidating direct and indirect effects (Schuler et al., 1999; Poppy, 2000; Hails, 2002). In laboratory studies such as these, it is useful to try and resolve whether negative effects are caused indirectly, via a low quality host (''sick prey''), or directly via the Bt toxin. *Spodoptera littoralis* is clearly affected by Bt toxin and thus it is likely that the slower developing hosts cannot provide enough nutrients for ''normal'' development of parasitoid larvae. Further more, Bt

toxin is known to change the amino acid and ion composition in the haemolymph of herbivores such as *S. littoralis* (Salama et al., 1983). This may also lead to a suboptimal supply of nutrients for parasitoids. Most authors reporting effects of Bt toxins on parasitoids can explain their findings with indirect effects. (Flexner et al., 1986; Vinson, 1990; Bernal et al., 2002; Baur & Boethel, 2003; Meissle et al., 2004; Prütz & Dettner, 2004). Such indirect effects are not unique for Bt crops but also occur with traditionally bred insect resistant plants (Boethel & Eikenbary, 1986). However, *S. littoralis* larvae contained Cry1A(b) toxin and *C. marginiventris* larvae were exposed, as demonstrated in our ELISA results. Thus direct effects cannot be excluded, although very unlikely: Cry proteins need specific receptors in the target insect gut ephithelium and Cry1A(b) has been shown to be specific to Lepidoptera. So far, no direct effects on species from other insect orders have been demonstrated and receptors for Cry1A(b) have been found only within the order of Lepidoptera.

To which extent a parasitoid is affected by host mediated effects is dependent on parasitoid biology. In contrast to *C. marginiventris,* that feeds only on a part of its host's body, other parasitoids, such as *Campoletis sonorensis* (Carlson), consume the whole host body (Wilson & Ridgway, 1975). Therefore, they could be more affected by changes in both quality and quantity of nutrients in the host larva. They would also come into closer contact with the Bt toxin when consuming their host's gut. Preliminary studies have shown that *C. sonorensis* development was affected more by Bt treatment than that of *C. marginiventris* when developing in Bt maize-fed *S. littoralis* as they needed 3 days more (about 20%) for their development. In addition, the survival of parasitized larvae decreased from 58% to 40% and cocoons contained at least 0.1 μg Cry1A(b) per g fresh weight, which is 250 times more than the levels found in *C. marginiventris* cocoons (Meissle et al., 2004).

The mean Cry1A(b) level in our maize (event Mon810) was 1.6 μg per g fresh weight. Dutton et al. (2002) detected 3.4 μg Cry1A(b) per g fresh weight in their plant leaves (event Bt11) and Raps et al. (2001) (event Bt11) slightly lower levels (2.7 μg/g). However, we analysed a mixture of stem and leaf material and worked with another maize line, which might have lead to the lower toxin concentration in our study. ELISA results clearly showed that *S. littoralis* larvae ingest Cry1A(b) when feeding on Bt maize for 8 and 11 days. Around 0.6 μg Cry1A(b) per g fresh weight could be found, a similar value to what Dutton et al. (2002) found in first instar larvae after they had been feeding on Bt maize for ca. 3 days (0.72 μg/g). Both values exceed the toxin level found by Raps et al. (2001), where third/fourth instar S. *littoralis* had been fed on Bt maize for 24 h (0.32 g/g). If percentages are calculated, larvae in our experiment had as much as 40% of the Cry1A(b) content of our maize, whereas the levels were about 20% in the Dutton et al. study and only about 10% in the Raps et al. study. This may be due to the fact that Raps et al. (2001) and Dutton et al. (2002) exposed their larvae to only a short period and our larvae were exposed to the toxin continuously since hatching.

Shortly before pupation, *S. littoralis* larvae stop feeding and excrete large amounts of faeces. As only traces of the toxin could be detected in our *S. littoralis* pupae, the caterpillars seemed to excrete most of their gut content including the Bt toxin before pupation. Consequently, no toxin was found in adults.

No Cry1A(b) toxin was found in adult *C. marginiventris* and cocoons (which contain the pupae) and only traces in empty cocoon shells. Three explanations are possible for this low toxin content: Firstly, parasitoid larvae may not take up the toxin. As *C. marginiventris* consumes only a part of its host's body, the gut (where the toxin is concentrated) is most probably not consumed. Secondly, if toxin is ingested, it may be metabolised. Although Bt toxin was shown to be still detectable after passing through the gut of *S. littoralis* (Raps et al., 2001), it may be metabolized in the hymenopteran gut. Thirdly, hymenopteran parasitoids are known to excrete toxins from their body prior to pupation into meconial pellets (Viggiani, 1984; Couty et al., 2001a, b). Excretion of Cry1A(b) can explain why empty cocoons in our study contained measurable traces of Cry1A(b) whereas toxin levels in cocoons with pupae and adults were below the detection limit. Our ELISA tests show further that no accumulation of Cry1A(b) occurs in the Bt maize–*S. littoralis*–*C. marginiventris* food chain.

The question still remains as to how biologically relevant the negative effects found on parasitoids are for natural populations in the field. In other words, how can we relate "worst-case" lab studies to the ecological systems we wish to conserve. *Cotesia marginiventris* developing in Bt maize-fed *S. littoralis* needed not even half a day (about 5%) longer for their development than the control and their cocoons were less than 0.2 mg (8%) lighter. Although these differences are small, they were consistent and thus statistically significant as our sample size was relatively large. However, biological relevance instead of mathematical significance has to be determined to evaluate ecological impacts (McBride et al., 1993). For example, temperature alone can have a much stronger impact on *C. marginiventris* development (Sourakov & Mitchell, 2001).

One of the biggest challenges in developing powerful risk assessment frameworks is the establishment of ecological endpoints and thresholds (Poppy, 2003), which is the current focus of our research. We feel that studies like the one we have outlined, together with other studies, especially those at higher tiers, can help us begin to develop endpoints and thus offer a way forward in risk assessment.

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