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ORIGINAL PAPER

Ingestion and excretion of two transgenic Bt corn varieties by slugs

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Abstract The release of transgenic *Bacillus thuringiensis* (Bt) corn expressing various Cry endotoxins has raised concern that these endotoxins are disseminated in the food web and may adversely affect non-target beneficial organisms, such as predators and organisms of the decomposer food web. We therefore investigated in a laboratory study, whether the Cry1Ab and Cry3Bb1 protein from Bt corn could potentially be transferred to such organisms by measuring the Cry protein content in the two common agricultural slug pests *Arion lusitanicus* and *Deroceras reticulatum* and their feces. We measured Cry1Ab and Cry3Bb1 protein concentration in leaves, intestines, and feces of corn leaf-fed slugs using ELISA and determined how much of the ingested protein is excreted by the slugs. Cry3Bb1 concentration in leaves of DKC5143Bt corn was significantly higher than Cry1Ab concentration in leaves of N4640Bt corn. While slugs were feeding on corn leaves, the Cry3Bb1 and Cry1Ab proteins were found in intestines and feces of both slug species. Bt protein concentrations in intestines of Cry3Bb1 corn-fed slugs were in both slug species higher than in Cry1Ab corn fed slugs, whereas no differences between Cry3Bb1 and Cry1Ab protein in feces were found. After slugs had ceased feeding on Bt corn, Cry1Ab was detectable in fresh slug feces for a significantly longer time and often in higher amounts than the Cry3Bb1. Our results indicate that both Cry proteins are likely to be transferred to higher trophic levels and to the decomposer food web. Since different Bt proteins seem to vary in their degradation, they have different transfer probabilities. This should be considered in risk assessments for non-target arthropods.

Keywords Enzyme-linked immunosorbent assay · Genetically modified maize · Mollusca · Decomposer food web · Natural enemies · Trophic interactions

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Introduction

The release of insect resistant transgenic corn may provide many economic and agronomic benefits (Shelton et al. 2002). The worldwide most commonly used insect resistant corn varieties express Cry1Ab proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) subsp. *kurstaki* against stem boring lepidopterans, particularly the European corn borer (*Ostrinia nubilalis*). Since 2003, new Bt corn varieties expressing Cry3Bb1 proteins against herbivorous chrysomelids (Coleoptera), particularly the corn rootworm complex (*Diabrotica* spp.), have been

commercialized. These varieties express the protein in higher concentrations than varieties containing the *cry1Ab* gene. Although Bt crops can prevent yield loss, reduce insecticide use and provide a high target selectivity, their potential effects on non-target species have to be evaluated as part of the environmental risk assessment. If Bt proteins are taken up by widespread herbivores, they may disseminated in the food web and could hence also expose non-target species with important roles in agroecosystems, such as natural enemies of pests and organisms associated with the decomposition of dead organic material.

Slugs are important pests of corn throughout the world and can cause defoliation between 2–59% (South 1992; Byers and Calvin 1994; Hammond et al. 1999). Particularly, the two slug species *Arion lusitanicus* Mabille (Mollusca: Arionidae) and *Deroceras reticulatum* Müller (Mollusca: Agriolimacidae) are major pests in young corn fields in Europe and slug problems have even increased due to conservation tillage practices (Hammond et al. 1999). Slugs are a common prey of carabids, spiders, birds and hedgehogs (Symondson et al. 2006). Since they are abundant and an important element in the food web of cornfields, it is likely that they play a role in transferring Bt proteins from transgenic corn to higher trophic levels (Harwood et al. 2006) or via feces to the decomposer food web.

Field and laboratory research have shown that the Cry1Ab from Bt corn is present in relatively high levels in many non-target arthropod herbivores and predators (e.g., Raps et al. 2001; Head et al. 2001; Dutton et al. 2002; Harwood et al. 2005; Zwahlen and Andow 2005; Harwood and Obrycki 2006; Obrist et al. 2006b), which indicates Bt protein movement between the trophic levels in live- and residue-based food webs. However, Cry1Ab concentrations varied in both, herbivores and predators and were dependent on the species, their diet, the amount protein in the food source, and the amount of food source consumed (Head et al. 2001; Obrist et al. 2006a; Torres et al. 2006). Bt proteins can also be transferred via plant residues, dead bodies or feces of herbivores to the decomposer food web. Several studies have shown that only part of the acquired Cry1Ab was digested by several investigated arthropod groups, such as lepidopterans, woodlice and diplopods (Head et al. 2001; Raps et al. 2001; Pont and Nentwig 2005; Weber and Nentwig 2006). The remaining Cry1Ab was found in feces where it was still insecticidally active (Pont and Nentwig 2005; Weber and Nentwig 2006). If Bt proteins are readily accessible within the feces, they may become ingested by decomposers. Thus, not only natural enemies of herbivores, but also organisms living or feeding on feces, such as microorganisms or woodlice, may be exposed to such Bt proteins.

It now needs to be established whether and to what extent the Cry3Bb1 from Bt corn is transferred to predators of herbivores and decomposer communities associated with animal feces. This is particularly important since Cry3Bb1 Bt corn varieties contain much higher Bt protein concentrations than Cry1 expressing varieties, which might lead to a higher exposure of non-target organisms to Cry3Bb1 than Cry1Ab. Besides Bt protein concentration, several other processes, such as assimilation, accumulation and digestion influence the protein transfer to predators and to the decomposer food web. All these processes also depend on the chemical and physical properties of the specific Cry protein. While little is known about the persistence of Cry3Bb1 within the live and residue plant matrix, studies on the degradability of Cry3Bb1 protein indicate a rapid degradation and no accumulation of the Cry3Bb1 protein in soil (Ahmad et al. 2005; Icoz and Stotzky 2007). On the other hand Cry1Ab protein could be detected for a long period in soil and within the plant matrix (Saxena and Stotzky 2000; Zwahlen et al. 2003), indicating that these two Cry proteins vary in their chemical and physical properties.

The aim of this study was to investigate the transfer of both the Cry1Ab and Cry3Bb1 from Bt corn to the two slugs species *A. lusitanicus* and *D. reticulatum* and their feces to assess the exposure of non-target predators and decomposer organisms to these proteins. First, we examined whether the two slug species fed the same amount of transgenic Cry1Ab and Cry3Bb1 corn and their corresponding near isolines. Secondly, we measured the amounts of Cry1Ab and Cry3Bb1 in leaves, the intestines, and feces of the slugs, determined ingestion and excretion ratio, and in which quantities and for how long the different Bt proteins were detectable in slug intestines and feces. Third, we examined whether the Cry1Ab protein in feces was still insecticidally active. Since slugs convert large amounts of biomass, we hypothesized that they may be able to channel considerable

amounts of Bt proteins to other trophic levels. To our knowledge this is the first study to compare the degradation patterns of these two different Bt proteins and to study Cry3Bb1 contents in herbivores. This study will provide an assessment of how these two widely used Cry proteins could potentially be transferred to organisms of higher trophic levels and to the decomposer food web.

Material and methods

Plant cultivation

We used two genetically modified corn (*Zea mays* L.) cultivars, N4640Bt (transformation event Bt11, Syngenta, Stein am Rhein, Switzerland) and DKC5143Bt (event Mon88017, Monsanto, St. Louis, USA) and their corresponding non-transformed isolines N4640 and DKC5143, respectively. N4640Bt expresses the Cry1Ab against lepidopteran pests, and DKC5143Bt the Cry3Bb1 against chrysomelid pests. Plants were grown in a climate chamber at 25°C during the photophase of 16 h, and at 20°C for the remaining 8 h. Plants were grown in 20 L plastic pots filled with geranium and balcony plant soil (Mioplant, Zurich, Switzerland). We used four plants per pot and five pots per variety. Before sowing, 35 g of long term fertilizer (14% N, 7% P, 14% K, 1.5% Mg, Hauert, Grossaffoltern, Switzerland) was added to each pot. Additionally, plants were fertilized once a week with 0.5 L of 0.2% liquid fertilizer per pot (10% N, 10% P, 7.5% K, 1.24% B, Maag Agro, Dielsdorf, Switzerland). Two separate feeding experiments were carried out, one with *A. lusitanicus* and one with *D. reticulatum.* Since they were carried out one after the other, the corn used for the experiments was also planted at two different dates. Fresh leaves were collected at the 8-leaf stage and at the 7-leaf stage for the first and second experiment, respectively, and they were immediately used for the experiments. For both experiments, only leaf pieces of the fifth leaf counted bottom-up of one of the four corn varieties were used (the two germination leaves were not included). For each slug, three leaf parts from the same leaf and of the same size and shape (about 1 g fresh weight each) were used for the experiments as described below. The first leaf part was fed to the slugs. The second leaf part was used for Cry protein quantification. The third leaf piece was used to estimate the dry weight of the leaves fed to the slugs. Both the second and third leaf parts had no slug contact.

Feeding experiment with *Arion lusitanicus* and *deroceras reticulatum*

Eighty *A. lusitanicus* of about 5 cm in size and 80 *D. reticulatum* of about 2 cm in size were collected in the field and brought to the lab where they were kept in plastic boxes (20 × 30 × 15 cm) in a climate chamber at 20°C during the photophase of 16 h and 15°C for the remaining 8 h. During five days they were fed with fresh dandelion leaves (*Taraxacum officinale*) ad libitum. Two days prior to the experiment the slugs were starved and then weighed. They were placed individually in smaller plastic boxes (11 × 15 × 6 cm). The first part of each leaf piece of one of the four corn varieties was added to each box, resulting in 20 replicates per treatment and feeding experiment. Each individual received the first leaf piece from a different corn plant. A piece of moist cotton wool was added to each box to keep the air humid within the closed boxes. Slugs were allowed to feed on the corn leaves for three days. Feces were collected daily beginning on day 2 after the beginning of the experiment and stored at −25°C until used for later ELISA and sensitive insect bioassays. After three days feeding on corn leaves, all 80 slugs per feeding series were weighed to determine their weight gain/loss. Ten individuals per treatment and feeding series were frozen immediately at −25°C to later dissect the intestines. Leaf parts not fed by slugs were removed, dried for 72 h at 40°C and reweighed. The amount of corn leaf dry weight consumed by each slug was then calculated as described below. The remaining ten individuals per treatment and feeding series were fed with dandelion leaves for further five days and feces were collected daily to calculate how long the Bt protein was still remaining in the feces. After five days, all slugs were frozen to later dissect the intestines. At the same time as the experiments were started, twenty plastic boxes per treatment and feeding series were set up without any slugs. The second leaf part was put into these boxes in order to quantify Cry1Ab degradation in leaves over time. Part of each leaf piece was removed daily and immediately frozen until further analysis. The

third leaf piece was not put into the boxes but was dried at 40°C for 72 h to estimate the initial dry weight of the leaves provided to the slugs.

Calculation of leaf consumption

The leaf consumption was calculated as follows: Fresh weight of the first leaf part, which was provided to each slug was multiplied with the ratio dry weight/fresh weight of corresponding leaves put into the dry oven (leaf part three). This is called estimated dry weight. The dry weight of the remains of the leaves slugs fed on was then subtracted from the estimated dry weight.

Bt protein concentrations in leaves, feces and intestines

Cry1Ab concentrations in leaves, feces and intestines were quantified using ELISA as described by Gugerli (1979, 1986) and Zwahlen et al. (2003). Briefly, lyophilized leaf pieces of about 15 mg dry weight and intestines of *A. lusitanicus* were put into extraction bags (type universal, Bioreba, Switzerland) together with 5 ml extraction buffer (Zwahlen et al. 2003). Samples were homogenized with a hand model homogenizer (Faust Laborbedarf AG, Schaffhausen, Switzerland), fixed to an electric drill for laboratory use to extract the Bt proteins. The extract was centrifuged for 10 min at 600 *g*. Supernatant of leaf extracts and intestines of *A. lusitanicus*, were further diluted 20- and 5-fold, respectively, in extraction buffer. In contrast, intestines of *D. reticulatum* were homogenized in 3 mL extraction buffer, centrifuged and the supernatant was used without being further diluted. Lyophilized feces of both species were homogenized in 3 mL extraction buffer and centrifuged. Supernatants of *A. lusitanicus* feces were then further diluted 5-fold in extraction buffer while *D. reticulatum* feces were used without further dilution. In order to determine the calibration curve for the Cry1Ab in leaf samples on each ELISA plate, reference samples of purified Cry1Ab protein were suspended in pooled extracts of control leaves (N4640) at concentrations between 0.01 and 1000 ng protein/mL. For the quantification of Cry1Ab in feces and intestines, reference samples of purified Cry1Ab were suspended in pure extraction buffer instead. Optical density was measured at 405 nm. The Cry3Bb1 protein concentration in leaves, feces and intestines was quantified using a PathoScreen kit for Bt Cry3Bb1 protein (Agdia, Indiana, USA) following the Agdia product documentation. Samples were extracted in PBST washing buffer (Agdia, Indiana, USA) and diluted as described above, except for leaf-extracts, which were diluted 50-fold in PBST washing buffer (Agdia, Indiana, USA). Calibration curves were determined using reference samples of purified Cry3Bb1 that was suspended in PBST washing buffer and diluted to concentrations between 0.313 and 20 ng protein/mL. Optical density was measured at 630 nm. For the quantification of Cry1Ab and Cry3Bb1, the concentration of the calibration curve and the optical density of all samples of each plate were log-transformed and a linear regression was carried out to calculate the Bt protein concentration in the samples (GraphPad Software Inc. 2000). All Cry protein concentrations were calculated as µg Cry protein per g dry weight.

Susceptible herbivore bioassays

The insecticidal activity of Cry1Ab in slug feces was tested in a bioassay using larvae not older than one day of *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae). We performed the bioassay only with feces of *A. lusitanicus* since *D. reticulatum* produced very little feces during the experiment. Slug feces were ground in an ultra centrifugal mill (ZM 1, Retsch Technology GmbH, Germany). Ten percent of slug feces (based on dry weights) from slugs fed with Cry1Ab corn or its control were mixed into an artificial diet for *O. nubilalis*. The diet is based on agar-agar, corn semolina, wheat germs, torula yeast and water (Bathon et al. 1991). Since the diet contained corn semolina we tested it for the presence of Cry1Ab using ELISA to ensure it did not contain any Cry proteins that might interfere with our bioassay. The diet was put into 10 vials (53 mm height, 22 mm diameter) per treatment. Ten larvae were placed into each vial resulting in a total of 100 larvae per treatment. Vials were closed with parafilm and kept in a climate chamber at 25°C and a photophase of 16 h. Mortality and weight of larvae were recorded after six days.

To test for the insecticidal activity of Cry 3Bb1 in slug feces a similar bioassay using neonate larvae of the Colorado potato beetle (*Leptinotarsa*

decemlineata Say, Coleoptera: Chrysomelidae) was carried out. However, the sensitivity of the beetle larvae to Cry3Bb1 is much lower than of the Cry1Ab assay with *O. nubilalis*. Our preliminary experiments revealed that a maximum of 20% (control) slug feces can be incorporated into the diet with acceptable mortality. However, at least 2 µg Cry3Bb1/g dry weight diet is necessary to detect differences between beetles fed with Cry3Bb1-containing diet and the control (M. Meissle, pers. comm.). Protein measurements of the ground up slug feces of *A. lusitanicus* revealed that the maximal Bt protein concentration which can be reached in the artificial diet, is only ca. 1/10 of the concentration necessary for detectable effects on *L. decemlineata* larvae. Thus, the biological activity of Cry3Bb1 in slug feces could not be estimated.

Data analyses

Analyses were conducted in R, version 2.3.1 (R Development Core Team 2006), using the package "nlme" (Pinheiro et al. 2006).

Slug weights and leaf consumption

Differences in slug weights among the four corn variety treatment groups were analyzed by an oneway ANOVA (for both slug species separately). Differences in consumption among the four corn varieties were analyzed using a two factorial ANOVA with slug species and corn variety as explanatory variable and corn leaf consumption as dependent variable. The interaction term between slug species and corn variety was excluded as it was not significant.

Bt concentration in leaves

To evaluate differences in Bt protein concentration in leaves of the two corn varieties over the three days, we applied linear mixed effects models (LME) with the "lme" function for each feeding series separately. Day, Bt corn variety and the interaction of both were fitted as explanatory variables. We controlled for the repeated sampling of the same leaves with leaf replicate fitted as random variable. The dependent variable Cry protein concentration in leaves was log-transformed to meet model assumptions. Non significant interaction terms ($P > 0.1$) were excluded from the model. Since Bt protein concentrations in both corn varieties differed significantly between the two feeding experiments, Bt protein concentrations in slug feces were also analyzed separately for the two feeding experiments.

Bt concentration in intestines

Differences in Cry1Ab and Cry3Bb1 protein concentration in intestines were analyzed using a two factorial ANOVA with slug species and corn variety as explanatory variables and log-transformed Cry protein concentration in intestines as dependent variable.

Bt concentration in feces

Two days after removing transgenic corn leaves (day 5) the Cry3Bb1 could not be detected anymore in feces of *D. reticulatum*. In *A. lusitanicus* feces, the Cry3Bb1 could be detected longer until day 5. Thus, we included in the *D. reticulatum* feeding experiment only day 2–4 and in the *A. lusitanicus* feeding experiment day 2–5 in the statistical analysis. For both feeding experiments we performed separate LMEs with day, Bt corn variety and day*Bt corn variety as explanatory variables, slug individual as random variable and log-protein concentration as dependent variable. We also calculated the excretion ratio for each individual (proportion of excreted Bt protein on day X to ingested Bt protein on day X−1) and we applied a LME for both feeding experiments together. Day, slug species and Bt corn variety were fitted as explanatory variables, slug identity as random variable. Interaction terms were stepwise excluded when they were not significant. The dependent variable excretion ratio was arcsin-transformed to meet model assumptions.

Susceptible herbivore assay

Differences in mortality and average weight gain of *O. nubilalis* in the bioassays were tested with an independent sample *t*-test. Mortality was arcsin-, larval weight log-transformed to obtain normal distribution of data and equality of variance. A significance level of 0.05 was used for all statistical analyses.

Results

Slug weights and food consumption

Mean (±SE) weight of *A. lusitanicus* before the experiment was 1197.6 ± 374.6 mg and 570.13 ± 220.83 mg for *D. reticulatum*. For both slug species, mean weight of the four treatment groups was similar (*A. lusitanicus*: $F_{3,79} = 1.9$, $P = 0.132$; *D. reticulatum*: $F_{3,79} = 1.1$, $P = 0.367$). During the feeding experiment, *A. lusitanicus* gained 225.74 ± 124.74 mg and *D. reticulatum* lost 40.1 ± 47.3 mg of the initial weight. We found no differences in weight gain or loss among the four treatment groups. Our analysis of leaf consumption showed that the heavier species *A. lusitanicus* consumed more than *D. reticulatum* ($F_{1,128} = 255.2$, $P < 0.0001$). No significant differences in leaf consumption were found among the four corn varieties for each species ($F_{3,128} = 2.1$, $p = 0.2$) (Fig. 1).

Bt protein concentrations in leaves, intestines, and feces

The Cry3Bb1 protein concentration in leaves was significantly higher than the Cry1Ab protein concentration in both feeding series. Furthermore, Bt protein concentrations varied over the three days tested in both feeding series. The highly significant interaction between day and variety in the *A. lusitanicus* feeding series indicated a decrease of the Cry1Ab protein concentration over three days whereas the Cry3Bb1 protein concentration remained stable (Fig. 2, Table 1).

In intestines, both the Cry1Ab and the Cry3Bb1 were detected in both slug species after three days of consecutive feeding on Bt corn (Fig. 3). The Cry3Bb1 was detectable in significantly higher amounts than the Cry1Ab. No significant differences in Bt protein concentrations could be observed between the two slug species (Table 1). The maximum concentration of Cry3Bb1 found in *A. lusitanicus* and *D. reticulatum* intestines was 17.8 µg and 10.7 µg/g DW, minimum concentration 0.9 µg and 0.4 µg/g DW, respectively. The maximum concentration of Cry1Ab found in *A. lusitanicus* and *D. reticulatum* intestines was 2.9 µg and 9.5 µg/g DW, minimum concentration 0.7 µg and 1.2 µg/g DW, respectively. No Cry1Ab or Cry3Bb1 could be detected anymore in slug intestines five days after removing transgenic leaves (day 8).

In the *A. lusitanicus* feeding experiment we found no significant differences in Bt protein in feces between the two Bt corn varieties during the four days tested (Fig. 4a). Bt protein concentrations varied significantly among days. Furthermore, we recorded a significant interaction between day and corn variety (strong decrease of Cry3Bb1 and slight decrease of Cry1Ab protein in feces after removing transgenic

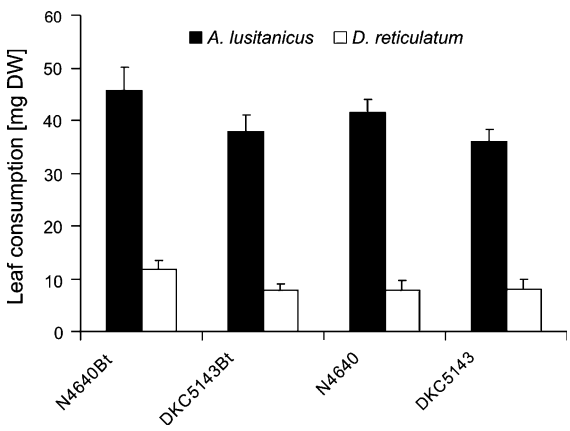

Fig. 1 Total corn leaf consumption (mean + SE) by *A. lusitanicus* and *D. reticulatum* of the two transgenic (N4640Bt, DKC5143Bt) and non-transgenic (N4640, DKC5143) corn varieties within the first three days of the feeding experiment. $N = 20$ per slug species and corn variety

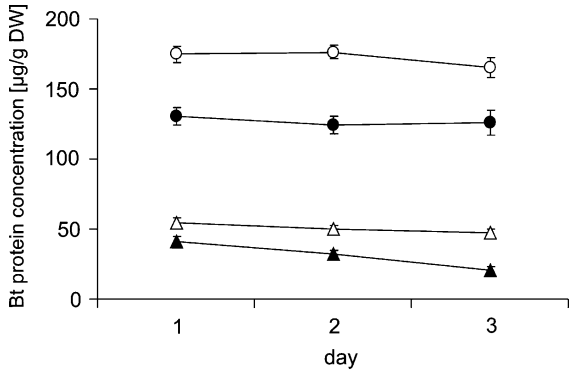

Fig. 2 Bt protein content (mean ± SE) over the three experimental days in leaves of N4640Bt (Cry1Ab) and DKC5143Bt (Cry3Bb1) used for the feeding experiment. $N = 20$ per corn variety and feeding experiment. Black symbols show Bt protein concentrations for the *A. lusitanicus* feeding experiment, white symbols for the *D. reticulatum* feeding experiment. Triangles show concentration in N4640Bt, circles in DKC5143Bt

Table 1 Effects of different explanatory variables on Cry protein concentrations in leaves, feces (for both slug species separately) and intestines and factors affecting excretion ratios. Shown are the results of LME's (leaves, feces, excretion ratio) and two-factorial ANOVA (intestines). Non significant interaction terms ($P > 0.1$) were excluded from the model. Given are model and error *df*-, *F*- and *P*-values

Explanatory variables	Model *df*, error *df*	*F*-value	*P*-value
Leaves			
A. lusitanicus			
Day (d)	1,78	23.1	<0.0001
Corn variety (cv)	1,38	611.2	<0.0001
d * cv	1,78	13.6	<0.0001
D. reticulatum			
Day (d)	1,79	8.7	0.0042
Corn variety (cv)	1,38	424.0	<0.0001
Feces			
A. lusitanicus			
Day (d)	1,49	47.8	<0.0001
Corn variety (cv)	1,15	3.4	0.08
d * cv	1,49	9.4	0.0035
D. reticulatum			
Day (d)	1,23	33	<0.0001
Corn variety (cv)	1,17	0.2	0.67
d * cv	1,23	4.9	0.037
Intestines			
Corn variety (cv)	1,24	6.2	0.02018
Slug species (ss)	1,24	0.2	0.66
cv * ss	1,24	4.2	0.052
Excretion ratio			
Day	1,72	30	<0.0001
Slug species	1,18	2.7	0.1172
Corn variety	1,72	12.9	0.0006

corn leaves, Table 1). Cry3Bb1 concentrations in feces decreased significantly from 14.3 ± 3.7 μg/g DW on day 3 to 3.7 ± 1.2 μg on day 4 (one day after removing transgenic corn leaves, Tukey HSD, $P = 0.031$), but was not significantly different between day 2 and 3 and any of the days after day 4 (Fig. 4a). In contrast, there was no significant decrease in Cry1Ab concentration among different dates, neither after removing corn leaves (day 3: 3.4 ± 0.5 μg; day 4: 2.6 ± 0.9 μg/g DW; Tukey HSD, $P = 0.74$) nor before or after. In the *D. reticulatum* feeding experiment, there was no significant difference between Cry1Ab and Cry3Bb1 concentrations during the three days tested (days 2–4), but concentrations

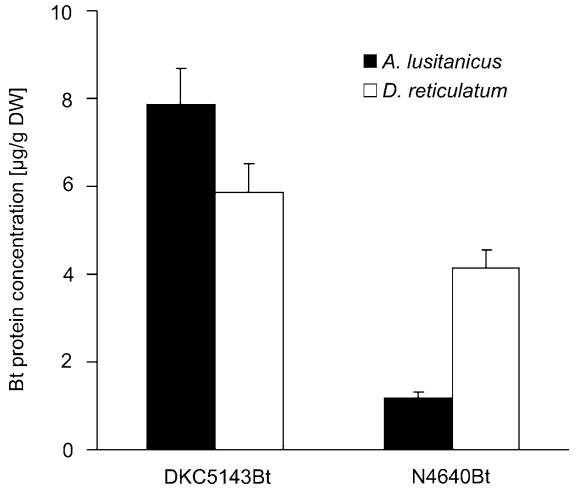

Fig. 3 Protein concentrations (mean + SE) in intestines of *A. lusitanicus* and *D. reticulatum* after three days of consecutive feeding on N4640Bt or DKC5143Bt. Sample size was 7 per slug species when feeding on N4640Bt, 8 for *A. lusitanicus* and 6 for *D. reticulatum* feeding on DKC5143Bt

differed significantly among days (Fig. 4b; Table 1). We recorded again a significant interaction between day and corn variety (stronger decrease of Cry3Bb1 than Cry1Ab protein in feces after removing transgenic corn leaves). After transgenic corn leaves had been removed (day 3), Cry3Bb1 concentrations in feces decreased significantly from 10.5 ± 2.9 μg to 0.7 ± 0.1 μg/g DW (Tukey HSD, $P = 0.003$) and Cry1Ab concentrations from 7.4 ± 0.8 μg to 1.7 ± 0.4 μg (Tukey HSD, $P < 0.0001$). In the *A. lusitanicus* feeding experiment, Cry1Ab protein concentration in feces was still 3.6 ± 0.6 μg, in the *D. reticulatum* series 0.9 ± 0.1 μg five days after removing transgenic corn leaves (day 8). No Cry3Bb1 protein was detectable in feces of *A. lusitanicus* three days after removing transgenic corn leaves. In the feces of *D. reticulatum*, no Cry1Ab protein was detectable two days after removing transgenic corn leaves.

The excretion ratio over the three days measured (days 2–4) was higher for Cry1Ab than for Cry3Bb1 with about 10–18% of ingested Cry1Ab and 5–11% of Cry3Bb1 being excreted each day. Excretion ratios varied among days and were higher on day 2 and 3 than on day 4, one day after corn leaf removal (Tukey HSD, $P < 0.0001$). No differences in excretion ratio between slug species were found ($F_{1,18} = 2.7$, $P = 0.12$, Table 1).

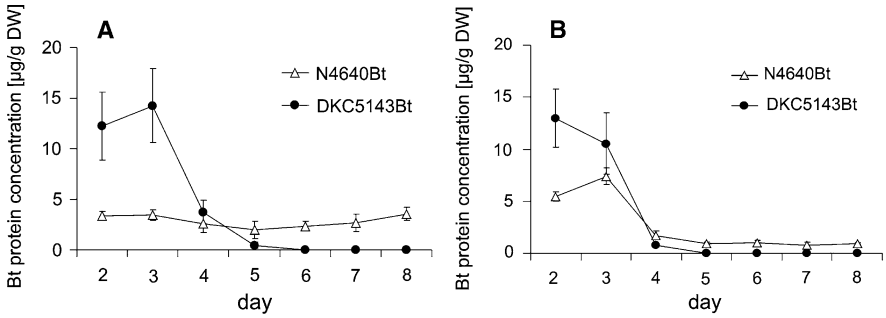

Fig. 4 Bt protein content (mean ± SE) in feces of (**a**) *A. lusitanicus* and (**b**) *D. reticulatum*. On day two and three slugs were feeding on transgenic corn. Leaves were replaced afterwards by dandelion. $N = 10$ per slug species and corn variety

Susceptible herbivore bioassays

Mortality of *O. nubilalis* fed with diet containing feces of Cry1Ab feces-fed slugs was 28.5 ± 8.79% and higher than mortality of larvae fed with diet containing non-Bt feces (11.0 ± 6.57%). However, differences were not significant ($t_{17} = -1.6$, $P = 0.124$). The weight of surviving larvae was significantly lower when feeding on diet containing Bt feces (0.1 mg ± 0.01) compared to the control (0.7 mg ± 0.05) ($t_{9.2} = 10.3$, $P < 0.0001$).

Discussion

This laboratory study shows that slugs and their feces can be a source of exposure to biologically active Cry proteins for the decomposer food web and species of higher trophic levels, such as slug predators. Both Cry proteins were detectable in the intestines of both species after having been fed Bt corn for three days in a row. These results suggest that, although there are species-specific differences, slugs in Bt cornfields may be a source of exposure to Bt proteins for predators. Similarly, the feces contain the Bt proteins for at least as long as the slugs feed on corn leaves. In the case of Cry3Bb1, it seems that concentrations in feces decrease rapidly once slugs switch to a different food source. In contrast, the Cry1Ab was detectable until the end of the experiment five days after the last ingestion of Bt corn. Our results indicate that Cry3Bb1 will be transferred from slugs and their feces to the food webs in higher amounts but during a shorter time than Cry1Ab.

Slugs are known to be a food source for predators, such as carabids, birds, and hedgehogs (e.g., Bohan et al. 2000; Toft and Bilde 2002; Symondson et al. 2006). Since the abundance of slugs in corn fields may be high (South 1992), the likelihood of transferring Cry proteins from transgenic corn to their natural enemies of higher trophic levels may be high. We detected 0.4–17.8 µg/g DW of the Bt protein in intestines of *A. lusitanicus* and *D. reticulatum*. Our findings are similar to those of Harwood and Obrycki (2006) who found Cry1Ab concentrations over 0.5 ng/g fresh weight in the slug *Deroceras laeve* Müller (Mollusca: Limacidae) when fed with Bt corn kernels for three hours. The Cry1Ab protein was detected for approximately four days after feeding, which indicates potential exposure of generalist predators for an extended time. Slugs allowed to feed on Bt corn leaves contained about 58 ng/g fresh weight Cry1Ab protein. When these slugs were fed to the large carabid *Scarites subterraneus* Fabricius (Coleoptera: Carabidae), the Cry1Ab protein was not detectable in their guts (Harwood et al. 2006). On the other hand, Zwahlen and Andow (2005) have found that seven out of eight species of Carabidae were exposed to the Cry1Ab when found in fields containing either Bt corn residues or live Bt corn and Bt corn residues. Harwood et al. (2005) detected significant quantities of Cry1Ab protein in field-collected arthropod predators of three orders (Coccinellidae, Araneae, Nabidae) indicating protein transfer to higher trophic levels. We assume that although only little protein was left in slug intestines in our study, this protein could reach higher trophic levels.

Between five and eighteen percent of the Bt proteins ingested were also not digested or adsorbed by the two slug species *A. lusitanicus* and *D. reticulatum* and were present in their feces. Thus, the Cry proteins could be transferred to the decomposer food web through the feces. This is in accordance with earlier studies that have shown that Cry1Ab was present in feces of snails (de Vaufleury et al. 2007), woodlice (Pont and Nentwig 2005), diplopods (Weber and Nentwig

2006) and lepidopteran larvae (Raps et al. 2001). Our sensitive insect bioassays with Cry1Ab showed that *O. nubilalis* larvae were at least sublethally affected by the Cry1Ab protein, indicating that the Cry1Ab was still insecticidally active. The effects, however, of the Bt protein excreted by slugs on non-target species remain to be demonstrated.

Interestingly, after removing transgenic corn leaves, only a slight, but not significant decrease of Cry1Ab concentration could be observed in feces whereas Cry3Bb1 concentration decreased strongly. Cry1Ab protein was still detectable in feces for at least four days after corn removal. In contrast, the Cry3Bb1 was not detectable anymore in feces two days after leaves were removed. This indicates that the Cry3Bb1 protein within slugs either degrades or adsorbs faster than the Cry1Ab protein. Although it is unlikely that whole proteins will be adsorbed into the body of a slug since the specificity of Bt endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts (Hofmann et al. 1988; Van Rie et al. 1990), we cannot exclude that this could happen since we measured Cry protein concentration only in the slug intestines and not within the whole slugs. Another reason for the differences in degradation between Cry1Ab and Cry3Bb1 proteins in our study could be structural differences between the two proteins.

Although Cry3Bb1 concentration in slug feces was higher than Cry1Ab concentration and correlated with the about five times higher Cry3Bb1 expression in corn leaves compared to the Cry1Ab expression, the excretion ratio of Cry1Ab was about twice as much as for Cry3Bb1. Our data are in accordance with other studies (Pont and Nentwig 2005; Weber and Nentwig 2007) showing that about 20% of the Cry1Ab ingested was not digested and could be detected in the feces of the isopod *Porcellio scaber* Latreille (Isopoda: Oniscidae) and the diplopod *Allajulus latestriatus* Curtis (Diplopoda: Julidae). Raps et al. (2001) found that the Cry1Ab concentration in feces of the herbivore *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) was comparable to the one in their food source, fresh corn leaves, suggesting that the food and hence Cry1Ab passes the gut quickly and is poorly digested. Bt proteins are degraded rapidly in simulated gastric fluid (Herman et al. 2003). The digestive system (crop, digestive gland, salivary gland) of *D. reticulatum* and the gut of three other investigated slug species are slightly acidic (Misra and Shrivastava 1994; Walker et al. 1996). This could be a reason for the relatively high but not complete digestion of Bt proteins by slugs. Other reasons for the incomplete digestion of Bt proteins in the gastro-intestinal tract of slugs and lepidopterans could be an insufficient amount of digestion enzymes available, that the Bt proteins were not readily accessible to the animals, or a combination of these reasons. Whether the remaining protein in feces is readily accessible to the decomposer food web needs to be determined.

Besides demonstrating in our study that slugs do contribute to the transfer of Cry proteins to the decomposer food web, we show here that slugs themselves acquire Cry1 and Cry3 proteins. Cry3Bb1 concentration in intestines was higher than Cry1Ab concentration and was about 6% of the ingested proteins regardless of the type of Bt protein. Our consumption studies did not show any evidence that *A. lusitanicus* or *D. reticulatum* would avoid Bt corn leaves. *A. lusitanicus* fed four times more than *D. reticulatum* and gained weight while the majority of *D. reticulatum* lost weight. Maybe leaves of the corn provided were too tough, particularly for *D. reticulatum*, which is known to feed on younger corn seedlings than the ones offered in our experiment. Since weight gain or loss was similar among the four treatment groups, we suppose that the nutritional quality of the investigated corn varieties was poor but rather equal. Our findings are in agreement with earlier studies by Escher et al. (2000) and Weber and Nentwig (2006), which showed that consumption rates of woodlice *P. scaber* and diplopods *A. latestriatus* did not significantly differ between Bt Cry1Ab and non-Bt leaves. Wandeler et al. (2002), who compared consumption rate by *P. scaber* of two Bt and six non-Bt corn varieties, found that differences in consumption among corn varieties could not be attributed to the presence of the Cry1Ab protein. Other corn variety properties such as C/N ratio, lignin content, or amount of soluble sugars may be more important for consumers than presence or absence of Cry proteins.

Conclusions

The laboratory experiment showed that slugs may contribute to the transfer of Bt proteins to higher

trophic levels and to the decomposer food web, even though there are differences among the type of Bt protein ingested and slug species that lead to consequences regarding the exposure of other species. Although most of the Cry proteins seem to be digested or adsorbed by the slugs, there is still insecticidally active Cry1Ab protein left in the intestines and feces, indicating that slug predators and organisms associated with the feces may ingest insecticidally active protein. If we assume continuous feeding of slugs on transgenic corn leaves, the exposure for slug predators and the decomposer food web could be high. The potential adverse effects on these organisms and the situation in the field, however, need further investigation.

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