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Effects of *Bt*-maize material on the life cycle of the land snail *Cantareus aspersus*

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ABSTRACT

Insect resistant *Bt*-maize (MON 810) expresses active Cry1Ab endotoxin derived from *Bacillus thuringiensis* (*Bt*). Snails constitute non-target soil species potentially exposed to *Bt*-toxin through consumption of plant material and soil in fields where transgenic plants have been grown. We studied the effect of the Cry1Ab toxin on survival, growth and egg hatchability of the snail *Cantareus aspersus*. From the age of 4–88 weeks, snails were fed either powdered *Bt*-maize or non-*Bt*-maize and exposed to soil samples collected after harvesting either the *Bt*-maize or non-*Bt*-maize. We applied four treatments: non-*Bt* soil + non-*Bt*-maize (MM); *Bt* soil + *Bt*-maize (BB), non-*Bt* soil + *Bt*-maize (MB), *Bt* soil + non-*Bt*-maize (BM). Eggs laid by snails not exposed to *Bt*-toxin were also exposed to the two types of soils (*Bt* and non-*Bt* soil).

At the end of growth (47 weeks of exposure), snails exposed to *Bt*-toxin in food and soil (BB) had a growth coefficient (GC) 25% lower than unexposed snails (MM). After the first period of reproduction (68 weeks) a significant difference remained for body mass GC between the BB and MM treatments. Differences in body mass were not significant at the end of exposure (88 weeks). For snails not previously exposed to *Bt* material, hatchability of eggs was similar in the soils tested. The outcome of the experiments indicates that, in growing snails, long-term exposure is needed to reveal an effect of *Bt*-maize. The hazard analysis of *Bt*-maize which we performed, based on a worst-case scenario, i.e. snails having no food choice, should now be complemented by other simple measurements, e.g. food intake, to understand the underlying mechanisms involved.

1. Introduction

The most successful transgenic crops have been those using the crystal protein endotoxin (cry) genes derived from *Bacillus thuringiensis*, especially those that produce Cry1A toxins (Roush, 1998). *Bt*-maize was developed mainly against *Ostrinia nubilalis*, the European corn borer whose larvae feed on the maize stalk and which are relatively unaffected by chemical pesticide application (Cannon, 2000). Ingestion of a lethal *Bt*-toxin by lepidopteran larvae results in a characteristic cessation of feeding that is followed by general paralysis or lack of movement, and death occurs within a few hours or days (Baines et al., 1997).

The toxin expressed by *Bt* crops is quite persistent in both plant residues and soil from the crop. Zwahlen et al. (2003a), studying in Switzerland degradation of the Cry1Ab toxin in leaves of transgenic *Bt* left in the maize field after harvesting in October, showed that the toxin could still be detected 8 months later. At the same time, a residual activity of *Bt*-maize against *O. nubilalis* larvae was still observed 6 months later (Zwahlen et al., 2003a). The main sources of *Bt* toxin in the soil are root exudates (Saxena and Stotzky, 2000; Saxena et al., 2002), incorporation of plant residues after harvest (Tapp and Stotzky, 1998) and probably pollen (Saxena et al., 2002). The toxin was also present in the rhizosphere soil of field grown *Bt*-maize plants throughout their growth and several months after their death and subsequent frost (Saxena and Stotzky, 2000). More recently, Cry1Ab was found by Griffiths et al. (2006) in the same soil and plant material as used in the present study, at concentrations of 2.87 to 15.8 $\mu g\ kg^{-1}$ soil and 1.36 to 8.51 $\mu g\ kg^{-1}$ dry weight leaves depending on the stage of the *Bt*-maize. Although a recent review by Clark et al. (2005) reports that, in general, there is a low level of hazard to most groups of non-target organisms, few data are available for some biological groups such as gastropod molluscs.

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Among soil fauna, land snails are possible non-target organisms that can be exposed through digestive and/or cutaneous routes to the *Bt*-protein released in soil by roots or by decomposing plant residues. In addition to soil, snails can eat fresh leaves of *Bt*-maize which are reported to contain high *Bt*-protein concentrations (Griffiths et al., 2006; de Vaufleury et al., 2007; Andersen et al., 2007). Thus, they are exposed to toxins from several sources and routes. Moreover, snails take part in nutrient turnover (Dallinger et al., 2001) and contribute to the diet of numerous predators (Laskowski and Hopkin, 1996; Scheifler et al., 2002). They are also used in biomonitoring of metal pollution (Dallinger et al., 2001) and for the evaluation of the effects of pollutants (ISO, 2006). Lastly, snails are long-lived non-target species that can be reared under controlled laboratory conditions and exposed throughout their entire lifecycle (Gomot-de Vaufleury, 2000, 2001; Gimbert et al., 2008).

The main aim of our study was to investigate the influence of *Bt*-maize (event MON810) on the snail *Cantareus aspersus* (synonyms: *Helix aspersa* or *Cornu aspersum*). *Bt*-maize has an inserted genetic fragment of the Cry1Ab gene from *B. thuringiensis* subsp. *kurstaki* strain HD-1 that produces an active delta endotoxin protein expressed in the maize tissue (Canadian Food Inspection Agency, 2002). We studied survival and growth of snails exposed to *Bt*-protein of plant origin. The snails were exposed *via Bt*-maize as food and/or via soil from *Bt*-maize crop fields (*Bt* soil). The effect of *Bt* soil on the hatchability of eggs from snails not previously exposed to *Bt* material was also assessed.

2. Materials and methods

2.1. Test soils

Three types of soil were collected in Foulum, Denmark, from fields cultivated for 1 year with either (a) non-*Bt*-maize (variety Monumental) or (b) *Bt*-maize (*Zea mays* L., variety MEB307, Cry1Ab event MON 810) and (c) a reference soil where maize was not cultivated. To check hatchability of snail eggs, we used all three types of soil. In experiments on snail growth and reproduction, we studied only soils (a) and (b). For each type of soil, composite soil samples were collected from the top 10 cm in plots planted with *Bt* or non-*Bt*-maize as previously described by Griffiths et al. (2005). Briefly, soil cores were collected within the rows of maize, between the plants. Soils were then dried at ambient temperature, sent from Denmark to France, sieved through a 3-mm mesh and dried again at 30 °C for 4 days. The soil at Foulum was a sandy loam (62.2% sand, 23.2% silt, 8.2% clay, a pH_{CaCl_2} of 5.7 and pH_{H_2O} of 6.2, 6.4% organic matter) and has been extensively described by Andersen et al. (2007). Water holding capacity (WHC) was assessed for each type of soil using gravimetric methods. Soils were then moistened to 50 to 60% of the total WHC before using.

2.2. Food

Animals were fed with either of the two types of maize material: *Bt*-maize (MON810, variety MEB 307) or non-*Bt*-maize (variety Monumental, near-isogenic to MEB 307). This material was harvested for silage in October 2002 and 2003 (two batches) and freeze-dried before crushing in a mill and passing through a 0.8 mm mesh. For both plant types, seeds were treated with Gaucho, a systemic insecticide containing the active substance imidacloprid used as a seed-coating/seed-dressing before sowing the maize. The dry, ground maize powders were received from the Department of Agroecology and Environment, University of Aarhus, Denmark.

2.3. Bt toxin analysis

After drying and before analysis, samples were stored at −80 °C. *Bt*-protein was assayed using the Quantiplate™ kit for Cry 1Ab/Cry 1Ac, AP003 (Envirologix). Three replicates were assayed for each sample. Freeze-dried maize powder (0.5 g) was extracted in 10 ml extraction buffer from the kit. For soils, two extraction ratios (either 2 or 8 ml of buffer was added to 1 g of freeze-dried soil) were tested with the extraction buffer used by Zwahlen et al. (2003a) (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, 3 mM NaN_3, 0.05% Tween 20, pH 7.4). After homogenisation using a Vortex agitator and incubation for 4 h at room temperature under agitation, the homogenate was centrifuged at 4000 × *g* at 4 °C for 10 min. The *Bt*-protein was quantified in the supernatant of samples by spectrophotometric measurements at 450 nm and reading on the calibration curve prepared with 0–5 μg kg^{-1} Cry1Ab calibrators. The detection limit of the kit was 0.14 μg kg^{-1} Cry1Ab in corn leaf extract and the quantification limit 0.25 μg kg^{-1}. Toxicity testing on snails and *Bt*-protein analysis were performed respectively after 2 and 12 months after soil collection.

2.4. Maize calorific content

The total calorific contents of the maize powders used for this experiment were estimated with a calorimetric bomb (Technoscience, Lyon, France) calibrated with benzoic acid. Three replicate samples were measured for each batch of maize.

2.5. Toxicity of Bt-maize and soils for the diamondback moth Plutella xylostella

The toxicity of the *Bt*-toxin in the materials used was checked with larvicidal activity tests performed on caterpillars of *P. xylostella*, a target species of the *Bt*-toxin (Talekar and Shelton, 1993). These tests were performed at the same time as the snails were exposed. The eggs used to establish the *P. xylostella* culture were obtained from INRA, Equipe Entomologie et Lutte biologique (Antibes, France). Freshly-hatched larvae were fed cabbage leaves. After emerging from pupae, adults (F1 generation) were fed a sugar solution as a source of food and water, and a piece of cabbage leaf was supplied as a substrate for egg laying.

At their final instar, larvae of the F_2 generation were used to check for *Bt* toxin activity. They were exposed to either untreated cabbage leaves (control) or cabbage leaves coated with an aqueous suspension of *Bt* or non-*Bt-maize* powder or soils used in this study, at a concentration of 0.1 g dry material per 1 ml distilled water. Three ml of the solution were spread on 5 g cabbage leaves and then fed to 10 final-stage moth larvae. We chose the final stage because it is the toughest and easiest to handle stage giving reasonable survival under control conditions. Mortality was checked after 24 h. Each treatment was replicated three times.

2.6. Influence of the Bt plant material on the life history of Cantareus asperses

The experiment consisted of four possible treatment combinations of two soils from either a non-*Bt*-maize field or a *Bt*-maize field and two plant powder types made from non-*Bt* or *Bt*-maize leaves (treatment abbreviations in parentheses):

- non-*Bt* soil and non-*Bt* powder (MM);
- *Bt* soil and non-*Bt* powder (BM);
- *Bt* soil and *Bt* powder (BB);
- non-*Bt* soil and *Bt* powder (MB).

Juvenile snails (ca. 1 g; 3 to 5 weeks old) were obtained from stock culture kept in the laboratory (University of Franche-Comté). When recording survival and growth rate, there were 10 replicates per treatment. Each replicate consisted of one individual snail per plastic box (24 cm × 10 cm × 8 cm deep; volume 1.6 l; Charles River Laboratories, ref E1DBBAC001) covered by a plastic lid. All boxes were kept in a climatic chamber with 18 L/6 D photoperiod, 20 °C and relative humidity > 80%. The bottom of the box had a 1 cm layer (200 g) of wet soil corresponding to the given treatment. The maize powder was given to the snail in a 3-cm diameter Petri dish lid. Snails were fed *ad libitum*, three times a week and at the same time, uneaten food and faeces were removed; cages were humidified with tap water to assure sufficient moisture. Twice a month, total snail fresh mass was determined and shell diameter measured using a calliper rule as described in ISO (2006).

The experiment started on 9 April 2003, and finished on 17 December 2004. From August until November 2003, and from August until October 2004, snails were transferred into separate wooden boxes placed in a dry room at a temperature of 20 °C to aestivate. After the periods of aestivation, the snails were activated by spraying with water and placed back in the plastic boxes to continue the experiment. Because of thinning of the snails' shells, from November, the snails were supplemented once a week with powdered marine alga (*Lithothamnium calcareum*) as a source of $CaCO_3$ (given in a 3-cm diameter Petri dish lid).

At the end of the growth phase (week 47, 1 March 2004), almost all snails had developed a lip on the aperture edge of their shells indicating sexual maturity and adult size (Gomot and Enée, 1980). All animals from a given treatment were then placed together in a box to allow mating. Survival, fresh mass, production of eggs and their hatchability were followed for 5 months until 25 July 2004. Then a second period of aestivation occurred for the next 2.5 months, until 18 October 2004 and following this reproduction was again monitored for a further 2 months, until 17 December 2004. As we only had one replicate of 10 snails per treatment, no statistical analysis was possible and raw data on fecundity and fertility are given for information in the results. For weeks 47 to 68, each reproducing box was filled with a 1 cm layer of wet soil as in the original treatment, whereas for weeks 83–88 the soil was put into a large Petri dish (15 cm diameter) inside the boxes, due to limited availability of test soil. Two 50 ml jars, filled with the test soil were placed in each box (50 cm × 20 cm × 40 cm) to enable egg laying. Three times a week, the jars were checked for the presence of an egg clutch. Each clutch was individually put into Petri dishes (9 cm in diameter) filled with the corresponding wet soil; eggs were counted as was the number of hatched individuals.

2.7. Effect of Bt plant exudates in soil on the hatchability of C. aspersus eggs

Three treatments were established: Foulum soil from non-*Bt*-maize, from *Bt*-maize, and from a fallow soil without maize cultivation. Because we were not able to obtain enough clutches of snail eggs during 1 day, two series with three replicates of each treatment were performed. In each series, three clutches of snail eggs were used; 20 eggs from each clutch were assigned to one replicate of a given treatment. Clutches were produced by adult *C. aspersus* reared under laboratory conditions using standard snail meal which had never been exposed to maize powder as the food source. To make exposure of each egg uniform, clutches were spread out and eggs put on a 1 cm wet soil layer in a round 100 ml glass container, covered by the next 1 cm wet soil layer. The container was closed by a Petri dish lid. To check the quality of egg-clutches, 20 eggs from each clutch were placed in similar glass containers lined with a layer of wet paper towel.

The eggs were checked every day for the next 4 weeks and watered with tap water, if necessary. The experiment was carried out in a climatic chamber at 20 °C and 18 L/6 D photoperiod.

2.8. Data analysis

For growing snails, the growth coefficient (GC) was calculated using the following equation:

$$\mathrm{GC} = \frac{M_{tn} - M_{t0}}{M_{t0}} \times 100$$

where M_{tn} is the body mass or shell diameter of the snail at time n, in g or mm and M_{t0} is the body mass or shell diameter of the snail at time 0 (start of the experiment), in g or mm.

At the end of the experiment, the change in body mass was calculated as:

$$\mathrm{Ch} = \frac{M_{ta} - M_{tb}}{M_{tb}} \times 100$$

where M_{tb} is the body mass before reproduction in g and M_{ta} is the body mass of the snails after reproduction in g.

All data were checked for normality (Kolmogorov–Smirnov's test) and homogeneity of variance (Bartlett's test). The data for body mass/shell growth were compared by one-way analysis of variance (ANOVA) with treatment as the factor, followed by the Tukey's post hoc multiple comparison test. If normality and homoscedasticity were not achieved, the non-parametric Kruskal–Wallis' test was used. A two-way ANOVA, with soil and plant as factors and two levels of each factor (with and without *Bt*) was also performed to analyse factor contributions and interactions. The free statistic software package R (version 2.7.0) was used (R Development Core Team, 2005).

Data for egg hatchability on different soil types were analysed with ANOVA (Statgraphics Centurion XV, StatPoint Inc.). Tukey's HSD test was used to compare treatments. When determining the effect of maize root exudates in soil on the hatchability of *C. aspersus* eggs, data on egg hatchability and time required to hatch were used in a two-factor ANOVA with treatment and series as the factors. Data were arcsine (square root) – transformed to obtain a normal distribution (Zar, 1999).

Outcomes of bioassays with *P. xylostella* were analysed (after arcsine transformation) by one-way ANOVA with maize treatment as the factor.

3. Results

3.1. Bt protein analysis and maize analysis

The average concentrations of *Bt*-protein in the maize powders (MEB 307) we used were (mean ± SD): 10.3 ± 3.14, 18.3 ± 1.68 and 16.8 ± 0.80 mg kg^{-1} maize dry mass, respectively, in the batches sampled in October 2002 and the two samples prepared in 2003. *Bt*-protein was lower than the detection limit in the three soils and non-*Bt*-maize powder.

The calorific contents did not differ between *Bt*- and non-*Bt*-maize (mean ± SD): 15.9 ± 0.472 and 15.1 ± 0.768 MJ/kg dry mass respectively in 2002 ($p = 0.34$) and 15.054 ± 0.285 and 15.429 ± 0.413 MJ/kg dry mass in 2003 ($p = 0.81$).

3.2. Toxicity of Bt-maize and soils with respect to the target species, P. xylostella

All *Bt* treatments caused significantly higher mortality to *P. xylostella* than control and non-*Bt*-treatments ($p < 0.001$). Mortality measured after 24 h for control soil was (mean ± SD) 3.3 ± 5.8% and for *Bt*-soil 40 ± 10%. For larvae exposed to non-*Bt*-maize powder,

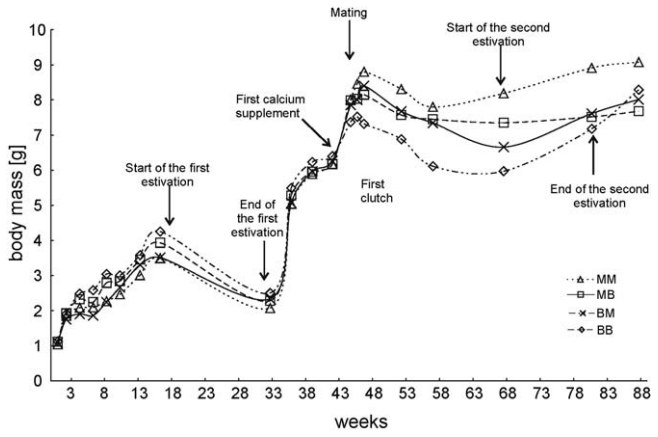

Fig. 1. Growth (average fresh mass in g) of the snail *Cantareus aspersus* exposed to *Bt* and non-*Bt* plant material. BB: soil from *Bt*-maize, *Bt*-maize powder; BM: soil from *Bt*-maize, non-*Bt*-maize powder; MB: soil from non-*Bt*-maize, *Bt*-maize powder; MM: soil from non-*Bt*-maize, non-*Bt*-maize powder.

mortality was 3.3 ± 5.8% and 6.7 ± 5.8% respectively for 2002 and 2003 samples whereas for *Bt*-maize powder, mortality reached 66.7 ± 15.3% and 40 ± 10%.

3.3. Influence of Bt plant material on life history of C. aspersus

The snails matured within about 1 year (including 4 months of aestivation) (Fig. 1). Only one snail died in treatment BM and time to reach sexual maturity as measured by development of the shell edge was not modified by exposure to *Bt*-material ($p > 0.8$).

At the end of the growth phase (i.e. 47 weeks) the growth coefficient (GC) differed significantly between treatments for shell diameter ($p = 0.043$) and to a lower extent for body mass ($p = 0.052$) (Fig. 2). A multiple comparison test of body mass showed that only the treatment BB led to a significantly lower GC than treatment MM ($p = 0.032$, Fig. 2) and revealed that shell diameter GC in BB was significantly lower than in MM ($p = 0.038$, Fig. 2). This effect seems to be related mainly to food exposure to *Bt*-protein (Table 1).

After the first period of reproduction (i.e. 68 weeks) there was still a significant difference between treatments for body mass GC ($p = 0.009$) and marginally for shell diameter ($p = 0.062$); the significant difference was between treatment BB and MM for body mass GC ($p = 0.005$) and marginally for shell diameter GC ($p = 0.064$). Soil and food were respectively responsible for the observed differences in GC mass and GC diameter (Table 1). The body mass loss during the first ($p = 0.086$, Fig. 3) and second period ($p = 0.39$) of reproduction was similar for all treatments, which is in

Table 1
Results of two-way analysis of variance for soil and food exposure to *Bt*-protein.

Stage	Two-way ANOVA		
	Source of variation	*F*-Value	*P*-Value
End of growth (week 47)			
Mass	Soil	1.615	0.212
	Food	2.879	0.098
	Soil × food	0.180	0.673
Shell diameter	Soil	4.651	0.037*
	Food	4.480	0.041*
	Soil × food	0.023	0.880
GC mass	Soil	3.018	0.091
	Food	5.463	0.025*
	Soil × food	0.004	0.094
GC shell	Soil	1.669	0.204
	Food	7.254	0.010*
	Soil × food	0.107	0.745
End of first period of reproduction (week 68)			
Mass	Soil	6.853	0.013*
	Food	1.881	0.179
	Soil × food	0.019	0.891
Shell diameter	Soil	2.384	0.131
	Food	3.307	0.077
	Soil × food	0.162	0.689
GC mass	Soil	9.186	0.004**
	Food	3.864	0.057
	Soil × food	0.0459	0.502
GC shell	Soil	1.117	0.297
	Food	6.841	0.013*
	Soil × food	0.090	0.765
GC change in body mass	Soil	7.090	0.011*
	Food	0.0026	0.959
	Soil × food	0.0522	0.820

GC = growth coefficient; see Fig. 2 legend for explanation.
* $p < 0.05$.
** $p < 0.01$.

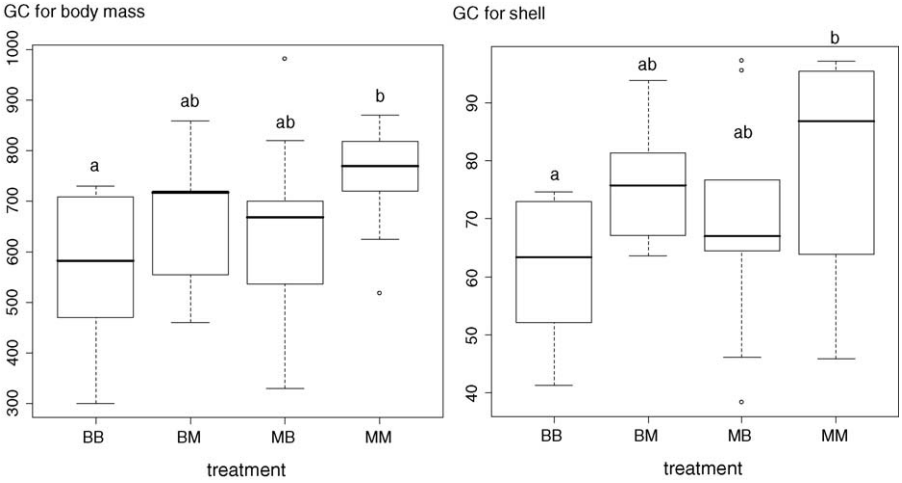

Fig. 2. Growth coefficient (GC: ((mass or shell diameter at time n − mass or shell diameter at $t0$)/mass or shell diameter at $t0$) × 100) of the snail *Cantareus aspersus* after 47 weeks exposure to *Bt* and non-*Bt* plant material in soil and/or food (before snails start to reproduce). The median and interquartile ranges are indicated by the horizontal line and box height, respectively. Whisker extremities are minimum and maximum values that are no more than 1.5 times the interquartile range from the median. Outliers (points further than 1.5 times the interquartile range from the median) are individually plotted. Values with different letters differ significantly (see *p* values in the text). BB, MB, BM, MM as in Fig. 1.

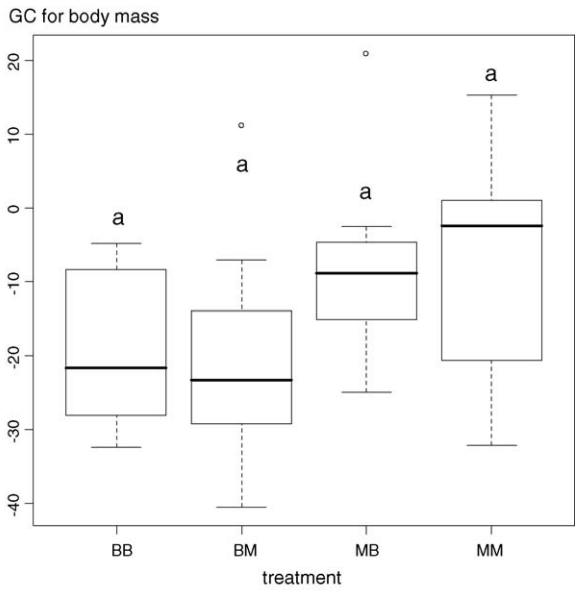

Fig. 3. Changes in body mass expressed in growth coefficient (GC: ((mass at time n − mass at $t0$)/mass at $t0$) × 100) after the first period of reproduction of the snail *C. aspersus* upon exposure to *Bt* and non-*Bt* plant material in soil and/or food. The median and interquartile ranges are indicated by the horizontal line and box height, respectively. Whisker extremities are minimum and maximum values that are no more than 1.5 times the interquartile range from the median. Outliers (points further than 1.5 times the interquartile range from the median) are individually plotted. Values with different letters differ significantly (see p values in the text). BB, MB, BM, MM as in Fig. 1.

accordance with the persistence of the difference in body mass GC observed both at 47 and 68 weeks of exposure. This effect was mainly explicated by soil exposure (Table 1). During reproduction shell diameter did not change in any treatment ($p = 0.18$, Kruskal–Wallis test), which is expected, because the shell usually stops growing, at least in length, when the snail reaches sexual maturity (i.e. at week 47 of the experiment). Differences in body mass after the second period of reproduction were statistically insignificant ($p = 0.43$).

From mating till the end of the experiment, survival was good with all treatments (1 snail died in BB, MM and BM treatments). Fecundity was 144, 140, 214 and 211 eggs per snail respectively for BB, MB, BM and MM treatments and appeared not related to change in body mass (Figs. 1 and 3). Fertility was 97, 116, 168 and 173 hatchlings per snail respectively for BB, MB, BM and MM treatments.

3.4. Determination of the effect of maize exudates in soil on hatchability of C. aspersus eggs

Hatchability of eggs incubated on paper was 75%. Hatchability was 67.4 (±27.3), 85 (±11.4), 75 (±20) and 81 (±11) % respectively for eggs from BB, MB, BM and MM treatments. No statistical analysis was performed as there was only one box with 9 or 10 snails for each treatment.

For snails not previously exposed to *Bt*-material, egg hatchability was similar for all soil types ($p = 0.96$) as was the time of hatching ($p = 0.21$).

4. Discussion

Results published on the toxicity of *Bt*-protein expressed in genetically modified crops, with respect to non-target species, are not consistent. No effect of *Bt*-maize (NK4640Bt) on soil-dwelling invertebrates was found by Saxena and Stotzky (2001a). They also did not observe significant differences in survival or weight of earthworms (*Lumbricus terrestris*) after 40 days in soil planted with *Bt* or non-*Bt*-maize, or after 45 days in soil amended with *Bt* or non-*Bt*-maize plant material (Saxena and Stotzky, 2001b). Similarly, in the framework of the ECOGEN program, using the same *Bt*-maize and control maize powders as we did, Vercesi et al. (2006) found that ground *Bt*-maize leaves added to soil had no effect on survival, growth (for 14 weeks) and reproduction (for 28 days) of the earthworm *Aporrectodea caliginosa*.

In contrast, Zwahlen et al. (2003b) observed in a laboratory trial, that after 200 days, adult *L. terrestris* lost 18% weight when fed *Bt*-maize litter, compared to a weight gain of 4% when fed non-*Bt*-maize. Probably, the higher lignin content in *Bt* than in non-*Bt*-maize made *Bt*-maize less nutritious for earthworms (Saxena and Stotzky, 2001b; Zwahlen et al., 2003b) and may explain the loss of weight, which required at least 28 weeks to appear. In another soil invertebrate, the nematode *Caenorhabditis elegans*, Höss et al. (2008) recently identified deleterious effects of rhizosphere and bulk soil samples from fields with *Bt* corn (MON 810, Novelis) on growth and reproduction. After 96 h exposure in *Bt* soils, mean body length of *C. elegans* was 55–93% of that in soils from a field with isogenic corn (Nobilis). *C. elegans* reproduction was also significantly lower in *Bt* soils (13 and 17% or 64 and 51% of that from isogenic plots). Reasons for the effects of soil from the *Bt*-field on *C. elegans* remain unclear (Höss et al., 2008).

In our study, *Bt*-maize influenced the growth of the snail *C. aspersus*, but only after long-term exposure (at least 47 weeks), when snails reached sexual maturity. This indicated that in the case of long-lived animals, like snails, the impact of *Bt*-material may be demonstrated in chronic tests. Indeed, neither exposure of snails to purified *Bt* protein, but only for 4 weeks (Kramarz et al., 2007a) or to growing *Bt*- and non-*Bt*-maize for 3 months in microcosms (de Vaufleury et al., 2007) revealed any negative effect. Comparison with other data is limited as this is the first time that *Bt*-maize has been tested in a long-term experiment with a terrestrial snail. Ester and Nijënstein (1995) reported a temporary reduction of feeding of the slug *Deroceras reticulatum* fed seeds of winter wheat treated with *Bt*-protein, at concentrations as high as 20 g active ingredient kg^{-1}.

C. aspersus appears to be vulnerable when exposed to *Bt*-protein in the food and in the soil for 47 and 68 weeks. Surprisingly, two-way ANOVA did not reveal interactions between food and soil exposure; growth inhibition seemed to be more related to food exposure (three of the four significant differences were observed for food exposure at 47 weeks) whereas at the end of the first reproduction, most of the difference was due to soil exposure. This may be due to modification of the metabolic requirement of snails depending on their age, being more dependent on food during growth and on soil during reproduction. In the maize, the *Bt*-protein concentration was higher than in the soil in which it was grown; however exposure to each source of *Bt*-protein separately did not have effects on snails. This suggests the Cry1Ab-protein in soil collected after harvesting of *Bt*-maize was still available for uptake by snails, even though we did not detect the protein by chemical analysis. This is also suggested by the toxicity of the *Bt*-soil to the target organism (*P. xylostella*) which we observed at the same time that snails were exposed. The lack of detection of *Bt*-protein in soil was unexpected as other studies for the same soils reported Cry1Ab concentrations of 2.87 to 15.8 μg kg^{-1} dry soil depending on the stage of growth of the *Bt*-maize (Griffiths et al., 2006) or from 0.97 to 2.2 μg kg^{-1} dry soil depending on the season and the tillage system in soils cultivated with MEB 307 (Andersen et al., 2007). The different results obtained for *Bt*-toxin may be the result of different storage conditions during which the toxin could

have been either degraded or become more adsorbed on soil particles and thus, less detectable when analysed.

Reasons for the decrease in the growth of snails exposed to *Bt*-material (especially those exposed to food and soil (BB), in which the GC was reduced by 25% at the end of growth and 38% after the first period of egg laying compared to non-exposed snails (MM)), as well as the trend of lower fertility and fecundity we observed, remain to be elucidated. This pattern does not seem to result from a lower quality of maize. Indeed, calorific content was similar for MEB307 and Monumental maize. Moreover other characteristics important for digestibility (e.g. crude fibre, enzyme digestible organic matter) were analysed on the same plant material and found to be similar by Vercesi et al. (2006) and Andersen et al. (2007). However, it cannot be excluded that subtle differences between transgenic and isogenic corn varieties may be responsible for the observed effects. Indeed, Rossi et al. (2003) found higher sugar and lower Neutral Detergent Fiber contents in Bt^+ plants producing the Cry1Ab protein (PI 33V08 and CA 7821 Bt corn) than in Bt^- maize (PI 3394 and CA 7770). However, they did not observe negative effects of *Bt*-protein on the rumen degradability of corn components for Holstein heifers. The digestive physiology of snails and cows is different, thus the impact of slight variations of *Bt*-maize on digestibility and thus the animal's growth may differ between these two organisms.

Other hypotheses can be formulated in relation to effects on snail growth of *Bt*-material. Firstly, the direct toxicity of *Bt*-protein is possible but remains to be elucidated because, to our knowledge, intestinal receptors specific to the protein Cry1Ab have not been described in gastropod molluscs. Secondly, we did not measure the food consumption in this experiment; thus, we cannot exclude that *Bt*-protein had an effect on this parameter. This remains to be measured, as done for example by Rossi et al. (2005) who showed that daily weight gain, feed intake and feed conversion ratio were similar for male broilers fed for 42 days with diet containing 50% *Bt* or Iso corn in their diet. Thirdly, exposure to *Bt*-protein may also have a fitness cost, e.g. by alteration of the energy budget, as suggested by Kramarz et al. (2007b) who founded that snails infected by nematodes and fed *Bt* maize grew more slowly than those fed with standard maize. Most likely this result indicates that less energy is available to cope with another stressor in animals exposed to the toxin. This energy could for example be necessary to produce new enzymes. Indeed it has been demonstrated that the slug *D. reticulatum* produced novel proteases when fed oilseed rape expressing an insecticidal cysteine protease inhibitor (OC-1 oryzacystatin-1) (Mulligan et al., 2006). The parameters measured during reproduction seem to reveal differences between *Bt* and non-*Bt*-exposed snails but these preliminary data need further investigation. Exposure of eggs to *Bt* material did not reduce hatchability. However, Vercesi et al. (2006) observed that cocoon hatching success of the worm *A. caliginosa* decreased from 97% for the control to 75% for cocoons exposed to a soil mixed with 4 g kg^{-1} of *Bt*-maize leaf powder. These concentrations were much greater than the more environmentally representative ones which we used.

5. Conclusions

In a semi-field exposure of juvenile snails for 3 months to *Bt*-maize in microcosms, growth and survival were not affected (de Vaufleury et al., 2007). In the present study, longer-term experiments (from juveniles to the end of reproduction over the course of more than 1.5 years) revealed non-negligible effects of maize expressing the insecticidal Cry1Ab protein. The growth coefficient was reduced by 25% in snails exposed for 47 weeks to *Bt*-material. This decrease was observed in the laboratory on snails having no other possible choice of food. This constitutes a worst-case scenario, which was counterbalanced by other environmental conditions (temperature, light, humidity) which remained constant and favourable. The hazard analysis of *Bt*-maize which we performed should now be completed by other simple measurements, e.g. food intake, to understand the underlying mechanisms.

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