The detection and decay of Cry1Ab Bt-endotoxins within non-target slugs, *Deroceras reticulatum* (Mollusca: Pulmonata), following consumption of transgenic corn

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Abstract

With greater acreages being planted to transgenic crops, the exposure of non-target species to bioengineered material is increasing. Although the slug, *Deroceras reticulatum* (Müller), is a major agricultural pest throughout the world, *Bacillus thuringiensis* crops were not intended to target these species. Molluscs are readily consumed by many generalist predators; if these Cry1Ab-endotoxins are taken up by slugs during feeding on transgenic plants, predators would therefore be exposed to elevated endotoxin concentrations. Using a biochemical assay, we tested the hypothesis that slugs fed transgenic corn would accumulate detectable quantities of Cry1Ab-endotoxins for prolonged periods of time. Characterization indicated that at low dilution rates, Cry1Ab-endotoxins were detectable in slugs fed Bt-corn but no reactivity was elicited by specimens fed non-transgenic food. It was possible to detect Cry1Ab-endotoxins in slugs for 95.9 h after consumption of Bt-corn. Although quantities were small, these long detection periods indicated potential exposure of generalist predators to low concentrations of transgenic insecticidal toxins in the field.

Keywords: Bacillus thuringiensis, non-target effects, decay rates, ELISA, risk assessment, detection protocols

Introduction

In the United States, there has been a rapid increase in areas planted with transgenic insecticidal crops since the commercialization of *Bacillus thuringiensis* corn in the 1990s (Cannon 2000; Pray et al. 2002; Shelton et al. 2002). These crops include varieties of corn expressing the Cry1Ab endotoxin to control *Ostrinia nubilalis* (Hubner) (Lepidoptera: Pyralidae) and in some instances have been highly successful in protecting yield and reducing the requirement for traditional broad spectrum insecticides to be applied at high doses. This can confer significant advantages to many invertebrates in agroecosystems (Gould 1998; Hoy et al. 1998; Way & van Emden 2000). Despite the reduced yield losses and insecticidal inputs in some, but not all, crops (Obrycki et al. 2001; Shelton et al. 2002), concerns exist regarding the
potentially negative interactions between transgenic crops and non-target invertebrate food webs (Way & van Emden 2000; Wolfenbarger & Phifer 2000; Obrycki et al. 2001, 2004; Groot & Dicke 2002). Not only could insecticidal toxins be detrimental to species feeding directly upon transgenic plant material (Losey et al. 1999; Jesse & Obrycki 2000; Zwahlen et al. 2003a), but they could also affect predator fecundity if sufficient transfer of toxins occurs between herbivores and/or detritivores and their natural enemies. The transfer of toxins through the food chain sometimes (Hilbeck et al. 1999; Dutton et al. 2002), but not always (Dutton et al. 2003; Romeis et al. 2004) affects predator fitness. However, these reduced growth parameters may, in part, be due to exposure to poor quality prey items rather than direct effects caused by the presence of transgenic endotoxins. Alternatively, if certain life stages of beneficial arthropods feed on plant material to complete their development or supplement their food intake (Alomar & Wiedenmann 1996), exposure to endotoxins could be elevated, even though transgenic tissue was reported to have no effect on heteropteran longevity (Armer et al. 2000; Ponsard et al. 2002). It is therefore important to determine the concentration of insecticidal material taken up by different species in the food chain, and whether the flow of these toxins, at levels observed in the field, impacts natural enemy communities.

The field slug, *Deroceras reticulatum* (Müller) (Mollusca: Pulmonata), is a major pest of crops throughout the world, causing widespread damage in many agroecosystems (South 1992). Due to their pest status and diverse feeding habits, slugs, such as *D. reticulatum*, have been widely studied and form a significant proportion of the diet of many generalist invertebrate predators, such as carabids and spiders (Symondson et al. 1996, 2002; Nyffeler & Symondson 2001; Harper et al. 2005). It is the abundance of slugs in corn fields (Rollo & Ellis 1974; Byers & Calvin 1994; Hammond et al. 1999) that increases the likelihood of transferring Cry1Ab-endotoxins from transgenic corn to their natural enemies higher up the food chain. This is particularly important given the slow degradation rates of Bt-endotoxin in plant material during the autumn, winter and spring (Zwahlen et al. 2003b) and although *D. reticulatum* shows preference for fresh plant material (Lutman 1978), their diet from arable crops is particularly diverse (Hunter 1968). Similarly, earthworms constitute an important part of the diet of many of these invertebrate generalist predators (Symondson et al. 2000; Nyffeler & Symondson 2001; Harper et al. 2005). Potentially, this further exposes natural enemies to increased transgenic toxins if they are not rapidly broken down.

Using a quantitative, antibody-based assay, we characterized and optimized a system allowing the detection of small quantities of Cry1Ab-endotoxin within slugs. Addressing this issue significantly advances our understanding of the potential interactions between transgenic material and non-target species. The calculation of decay rates within slugs enables the application of these techniques (at optimized conditions) to study the potential interactions between transgenic plants and non-target components of complex food webs in the field. The hypothesis that the breakdown of Cry1Ab endotoxins in slugs would be sufficiently slow to indicate the potential for transfer of endotoxins to higher trophic level organisms (e.g., birds, hedgehogs, insect predators and spiders) was therefore tested. This hypothesis is based on the ability of antibody-based assay systems to detect target proteins in gut samples for significant periods after consumption of food (e.g., Symondson et al. 1999, 2000; Harwood et al. 2001, 2004; Schenk & Bacher 2004).
Materials and methods

Slug collection and maintenance

Adult slugs, *D. reticulatum*, were collected from margins surrounding alfalfa, *Medicago sativa* L., fields at the University of Kentucky Spindletop Research Station, Lexington, KY, USA, and transferred into Reynolds® Del-Pak® Plastic Containers (diameter 10.5 cm, height 4 cm) (Reynolds Metal Company, Richmond, VA, USA) containing a moistened absorbent cotton base (5 slugs/container). All slugs were maintained at 21°C and a 16:8 light:dark cycle. An *ad libitum* supply of food (a mixed diet of organic Russet Potatoes and organic Green Lettuce) was provided for approximately one month. Prior to the experiments, all food was removed; slugs were transferred into clean containers with a moistened absorbent cotton base and starved for 72 h.

Cry1Ab-endotoxin decay experiments

Following starvation, individual slugs were placed into separate triple-vented Petri dishes (9 cm diameter, 2 cm height) on a damp filter paper base. Slugs were allowed to feed for 3 h with an *ad libitum* supply of kernels (mean (±SE) Cry1Ab-endotoxin concentration = 0.72 ± 0.05 µg Cry1Ab g⁻¹ fresh weight) collected from fields of transgenic corn of the Bt hybrid N79-L3 (Bt-11 event, Syngenta Seeds, Golden Valley, MN, USA) located at the University of Kentucky Spindletop Research Station. Corn kernels are readily consumed by many slugs and are preferred food items compared to mature corn leaves (Harwood & Obrycki, unpublished). Although newly emerged corn plants are fed upon by *D. reticulatum*, kernels were selected as a representative food item due to their relatively high concentration of Cry1Ab endotoxin and likelihood of acceptance by slugs during feeding trials. The Bt-11 event expresses 65 kDa truncated Cry1Ab proteins with increased GC ratios for expression (Mendelsohn et al. 2003). Throughout the 3 h feeding period, continued monitoring of Petri dishes ensured that at least three feeding events occurred for all slugs; a “feeding event” is defined as slugs being visually observed to be consuming corn kernels. These observations were taken every 15 minutes. Any individuals with fewer than three feeding observations were rejected from the experiment.

After three hours, five slugs were frozen at −20°C (t = 0 h). All remaining slugs which were observed to feed on at least three occasions were transferred into clean, triple-vented Petri-dishes, on a damp filter paper base. These slugs were maintained at 21°C on a 16:8 light:dark cycle. Replicates of five slugs were then frozen at −20°C after a further 2, 4, 8, 16, 24, 36, 48, 60, 72, 84, 96, 120 and 144 h. As food items could have been consumed at any point during the 3 h feeding period, consumption was assumed to have taken place at the mid-point (after 1.5 h); 1.5 h has therefore been added to all analyses of Cry1Ab-endotoxin decay rates.

Slug preparation and screening protocol for detection of Cry1Ab-endotoxins

Slugs were weighed and individually homogenized 1:25 (mg µl⁻¹) in EnviroLogix Inc. Extraction Buffer Solution (EnviroLogix Inc., Portland, ME, USA) using an IKA® Ultra-Turrax T25 Homogenator (IKA® Works Inc., Wilmington, NC, USA). Between mixing different slugs, the homogenator head was disassembled and sequentially cleaned with distilled water, 70% EtOH followed by further washing.
with distilled water. This ensured that Cry1Ab-endotoxins were not transferred between samples. This was tested in two separate systems, by alternately homogenizing (1) seedlings of a Bt-11 even corn hybrid with its isoline \((n = 5\) for each treatment), and (2) slugs fed Bt-11 kernels alternated with slugs fed non-Bt food material \((n = 5\) slugs per treatment). No reactivity was elicited by the non-Bt isoline or slugs fed non-Bt food, whilst all Bt-11 event seedlings and slugs fed Bt-11 kernels screened positive versus ELISA.

The dilution factor \((1:25\ \text{mg} \mu\text{l}^{-1})\) was selected on the basis of preliminary optimization experiments which ensured positive controls (slugs fed with Bt-corn kernels from the hybrid N79-L3 (Bt-11 event)) screened positive, whilst negative controls at the same concentration (slugs maintained on a non-Bt diet) did not elicit a positive absorbance from the EnviroLogix Inc. Quantiplate Enzyme-Linked Immunosorbent Assay Kit. Following homogenization, samples were dispersed for approximately 30 s on a vortex mixer and centrifuged at 5000 \(\times\) \(g\) for 5 min to remove all particulate matter. Supernatants were removed (taking care not to disturb the solid pellet) and screened without further dilution following the protocols outlined below.

Slug supernatants were added, in duplicate, to ELISA plates at 100 \(\mu\text{l}\) per well. In addition to this experimental material, 100 \(\mu\text{l}\) of three EnviroLogix Inc. calibrators of known Cry1Ab endotoxin concentration (containing 0.5 ng g\(^{-1}\) Cry1Ab, 2.5 ng g\(^{-1}\) Cry1Ab and 5.0 ng g\(^{-1}\) Cry1Ab) and 100 \(\mu\text{l}\) of EnviroLogix Inc. Negative Control were added to one column of the plate (total 4 columns of EnviroLogix Inc. controls). Five additional negative slug-controls (slugs maintained on a non-Bt diet) were also coated (in duplicate), at 100 \(\mu\text{l}\) per well, to the ELISA plate to ensure continued non-reactivity to negative-control slugs. The contents of the wells were mixed by rapidly rotating the ELISA plate in a circular motion for 30 s, taking care to avoid cross-contamination between wells. Following thorough mixing, the plates were covered with a sterile acetate plate cover to prevent evaporation. After 15 min at room temperature, 100 \(\mu\text{l}\) of EnviroLogix Inc. Cry1Ab-Enzyme Conjugate was added to each well, the plate mixed by circular rotations as above and covered with a clean acetate sheet to avoid evaporation. The plate was incubated for 1 h at room temperature.

Upon completion of the incubation stage, contents of all wells were ejected and flooded three times with phosphate-buffered saline (pH 7.4), with Tween\(^\text{®} 20\). The upturned plate was then vigorously dropped onto paper towel to ensure all wash buffer solution was removed and 100 \(\mu\text{l}\) of EnviroLogix Inc. Substrate was added to all wells. The contents were mixed for 30 s by rapidly rotating the plate in a circular motion and incubated at room temperature for 30 min. After 30 min, 100 \(\mu\text{l}\) of 1.0 N hydrochloric acid was added to all wells to stop the reaction and absorbance recorded at 450 nm using a Thermo Labsystems Multiskan Plus\(^\text{®} \) spectrophotometer (Fisher Scientific Company L.L.C., Pittsburgh, PA, USA).

**Calculation of Cry1Ab endotoxin concentration in slug samples**

The mean absorbance readings of the EnviroLogix Inc. Negative Controls on each plate were subtracted from all wells (including calibrators). After subtraction, a linear regression was fitted to the three positive controls which contained concentrations of 0.5 ng Cry1Ab g\(^{-1}\) fresh weight, Cry1Ab, 2.5 ng g\(^{-1}\) and 5.0 ng g\(^{-1}\). The optical densities (at 450 nm) for test slugs (after subtraction of the mean \(\text{OD}_{450}\) for
EnviroLogix Inc. negative controls) were entered into the regression and multiplied by the dilution factor (25) to calculate parts per billion (ng g$^{-1}$) of detectable Cry1Ab-endotoxin within each slug sample.

**Results**

*Optimization of assay for the detection of Cry1Ab-endotoxin in slugs*

All ELISA plates, during optimization of the assay system and the determination of Cry1Ab-endotoxin decay rates (below), included eight replicates of three calibrators (0.5 ng Cry1Ab g$^{-1}$ fresh weight, 2.5 ng g$^{-1}$, 5.0 ng g$^{-1}$) to enable linear regression analysis (Figure 1) and determination of Cry1Ab-endotoxin concentration from optical densities of test material. Prior to regression, background absorbance was subtracted from colorimetric readings of the three calibrators. Although only three calibrators were used to construct a linear regression, this followed EnviroLogix Inc. protocols and the high $r^2$ value (0.995) indicated the validity of using so few standards in the construction of the calibrator regression.

Dilution of the positive-control slugs which were fed Bt-corn kernels revealed a rapid reduction in detectability of Cry1Ab-endotoxin (Figure 2a) at increased slug dilution factors, declining significantly according to a log-log regression. Despite the rapid decline of detectable Cry1Ab-endotoxins, at high slug concentrations (1:25 mg ml$^{-1}$) the strong reactivity of slugs fed with Bt-corn (Figure 2a) was not mirrored by false-positive reactivity of negative-control slugs (which were not fed with Bt material) (Figure 2b). Immediately after feeding, the absorbance of slugs fed with Bt-corn kernels was significantly greater than those not fed with Bt-corn ($t_3 = 8.11$, $P = 0.004$), whilst the OD$_{450}$ of slugs fed non-Bt material was statistically

![Figure 1. Regression of OD$_{450}$ for three calibrators (at 0.5 ng Cry1Ab g$^{-1}$ fresh weight, 2.5 ng g$^{-1}$ and 5.0 ng g$^{-1}$) used to calculate concentration of antibody-recognizable material during optimization of assay. Regression: $y = 0.297x + 0.203$; $r^2 = 0.99$. Dotted line represents OD$_{450}$ for EnviroLogix Inc. negative control.](image-url)
similar to the EnviroLogix Inc. Negative Control ($F_{1,10} = 0.02$, $P = 0.881$). This indicated no evidence for the reactivity of the ELISA to slug proteins at high concentrations.

**Determination of the decay rates of Cry1Ab-endotoxin in slugs**

Using the concentrations determined during optimization of the assay system (above), Cry1Ab-endotoxins within slugs decayed at an exponential rate over time (Figure 3). Using the regression equations from calibrators on each ELISA plate, Cry1Ab-endotoxins were present in *D. reticulatum* at detectable levels (above the absorbance recorded for the lowest calibrator of 0.5 ng Cry1Ab g$^{-1}$ fresh weight) for 95.9 h after consumption of transgenic material.
Discussion

This assay system, at optimized concentrations, was clearly capable of detecting very small quantities of Cry1Ab-endotoxin in *D. reticulatum*. Despite the very high concentration of test material coated onto ELISA plates, slugs fed with non-transgenic material did not react to the antibody (Figure 2b), whilst those “positive control” individuals fed with transgenic corn elicited very strong reactivity to the assay (Figure 2a). These characterization experiments illustrate the need for comprehensive optimization of different assay systems – some species may elicit different cross-reactivity and/or sensitivity levels. Each predator, herbivore or detritivore should therefore be optimized relative to the assay involved, following similar protocols to those described here, for maximizing sensitivity of the assay to detect very small quantities of Cry1Ab-endotoxin, whilst maintaining no reactivity to non-transgenic negative-control specimens.

The detection of Cry1Ab-endotoxin in slugs was possible for approximately four days after a single 3 h feeding event on kernels from Bt-corn. Although this time period was shorter than the detection of prey proteins in some predators (e.g. Harwood et al. 2001, 2004; Schenk & Bacher 2004), it was sufficiently long to allow potential transfer of insecticidal material along the food chain. The sensitivity of the assay, allowing the detection of very small concentrations (and therefore quantities) of Cry1Ab-endotoxin for this length of time, indicates its viability in field studies for measuring the uptake of insecticidal toxins by non-target species. Importantly, despite the long detection time of Cry1Ab-endotoxins in non-target slugs, antigenic decay of proteins in invertebrate guts can be influenced by many factors, including temperature (Sopp & Sunderland 1989; Hagler & Cohen 1990), level of starvation (Lövei et al. 1985; Symondson & Liddell 1995), feeding on alternative prey (Symondson & Liddell 1995) and meal size (Lövei et al. 1987). Furthermore, the rate at which target proteins

![Figure 3. Detection period for Cry1Ab-endotoxins following consumption of kernels from Bt-corn. Data are means for each time period ± SE. Decay rate is determined by regression of Concentration versus Time: Cry1Ab Concentration = -0.412 log<sub>10</sub> time + 2.38; r² = 0.80. Dotted line represents threshold for the concentration of 0.5 ng Cry1Ab g<sup>-1</sup> fresh weight, below which values are assumed to contain no significant quantities of Cry1Ab-endotoxin.](image-url)
Decay in different species can sometimes, but not always, vary (Harwood et al. 2001, 2004) making between-species comparisons difficult without the development of calibration models to factor out this variability. Clearly further research is required to quantify the extent to which these factors affect the breakdown of Cry1Ab-endotoxins within non-target species, following protocols designed in the 1980s and 1990s for characterizing antibody-based assay systems for predator-prey studies.

The exposure of predators to slugs which have fed upon Bt-corn may facilitate the transfer of toxins, but the low concentrations in slugs allowed to feed on kernels of Bt-corn are unlikely to pose any significant fitness reductions unless these arthropods sequester the toxins and continued exposure leads to changes in longevity or fecundity. However, long-term exposure and feeding on Bt-corn could occur in the field. Such exposure may impact non-target populations differently to laboratory trials reported here and lead to increased concentrations of Cry1Ab-endotoxins, especially if slugs feed on leaves or young seedlings which tend to have higher concentrations of endotoxin, even though Cry1Ab-endotoxins are expressed throughout the plant in many corn hybrids (e.g., Bt-11, MON-810, DBT-418 and CBH-351). To date, evidence is mixed with regard to the effect of invertebrate exposure to transgenic endotoxins. Some studies report reduced fitness parameters (e.g., Hilbeck et al. 1998; Losey et al. 1999; Jesse & Obrycki 2000), whilst others indicate no negative effects caused by exposure and feeding on transgenic material (e.g., Pilcher et al. 1997; Lundgren & Wiedenmann 2002; Anderson et al. 2004). However, the effect on molluscs has not been studied although it seems likely that long-term exposure to transgenic crops could affect their population structure given that earthworms are adversely affected by Bt-material (Zwahlen et al. 2003a). It is also likely that the concentration of Cry1Ab-endotoxins in the gut of slugs would be significantly higher than those levels reported here, where whole bodies were macerated on a weight/volume ratio, given that the food consumed contained high concentrations of Cry1Ab-endotoxins (0.72 ± 0.05 μg Cry1Ab g⁻¹ fresh weight). The screening of gut-contents in arthropod predators whose guts are easily extractable, such as carabids and coccinellids, could yield greater information on the uptake of these endotoxins, particularly at the low concentrations likely to occur higher in the food chain.

Carabids are important predators of slugs (Symondson et al. 1996, 2002) and earthworms (Symondson et al. 2000) in the field, exposing these natural enemies to potentially elevated levels of transgenic insecticidal material. In addition, their positive response (McKemey et al. 2004) and preferential feeding behavior towards dead slugs (Mair & Port 2001), and the ability of ELISA to detect proteins long after the death of target species (Calder et al. 2005), reveals the potential for Cry1Ab-endotoxin exposure to carabids through the mollusc/annelid food chain for significant periods of time. Given that higher order arthropod predators contain significant quantities of Cry1Ab-endotoxins in their guts (Harwood et al. 2005a), this is particularly relevant, especially in connection to long-term exposure where further experiments are clearly required to quantify the significance of transgenic crops to the structure of complex food webs. Although carabid-slug interactions have been widely studied and these natural enemies are the principle predators of slugs in agroecosystems, many other invertebrate and vertebrate species routinely feed on mollusc prey (South 1992). In terms of future risk assessment of the potential movement of transgenic endotoxins through the food chain, it is clear that all such trophic connections should be considered and examined in detail.
Applying quantitative assays to evaluate complex food webs in the field has been highly successful in studying interactions between generalist predator communities and their prey (Bohan et al. 2000; Symondson et al. 2000; Harwood et al. 2004, 2005b). Antibody-based technology is one of the most frequently applied methods of gut-content analysis used during field-evaluation of the biological control capacity of generalist predators (Harwood & Obrycki 2005). However, a prerequisite to utilizing these biochemical assays for the study of feeding behaviour in the field is the optimization of the antibody-based system involved in the analysis (e.g., Symondson & Liddell 1996; Harwood et al. 2001). To our knowledge, this was the first study optimizing an assay for use in non-target species which measures and quantifies the rate at which Cry1Ab-endotoxin decays following the consumption of transgenic material. In the laboratory, Raps et al. (2001) quantified the significant uptake of Cry1Ab endotoxins by *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) but concluded that the lack of uptake by *Rhopalosiphum padi* (L.) (Homoptera: Aphididae) was due to the very low concentrations of endotoxin with phloem sap of the N4640Bt hybrid. Dutton et al. (2002) also quantified the uptake of these toxins by *S. littoralis*, *Tetranychus urticae* (Koch) (Acari: Tetranychidae) and *R. padi*, providing further evidence for the movement of these toxins into some non-target invertebrates. Although the uptake of endotoxins may have little or no effect to non-target species, without measuring the rates of decay and the uptake of these endotoxins throughout the food chain, it is difficult to ascertain the consequence of exposure. This insecticidal material may be taken up at varying rates in the field and its breakdown in the gut is likely to vary given the large between-species differences that are sometimes (Symondson & Liddell 1993; Harwood et al. 2001), but not always (Harwood et al. 2004, 2005b), observed during quantitative ELISA’s. It is also necessary to consider Cry1Ab-endotoxin uptake on a hybrid-to-hybrid basis, given that expression and concentration of Bt-endotoxins varies between different hybrids. Therefore, detailed laboratory-based experiments are required to determine whether such variability exists in the detection of Cry1Ab-endotoxins in non-target arthropods.

Allowing the detection of small quantities of transgenic material in herbivores and detritivores will enable the future measurement of its flow through the food chain and exposure of predator communities to these endotoxins (which in the case of the slug-carabid trophic link is likely to be extremely small). The optimization of further invertebrate systems will therefore allow the detection of endotoxins in complex food webs (e.g., Harwood et al. 2005a) and, importantly, allow accurate assessment of possible exposure levels of non-target predators. This is especially important in terms of laboratory studies measuring the impact of transgenic exposure on natural enemies, as extreme care is needed when making assessments of fitness parameters (Lövei & Arpaia 2005). The measurement of Cry1Ab-endotoxin concentrations in “prey” species from the field will subsequently allow laboratory studies to accurately simulate natural levels of endotoxins and their impact on natural enemy communities to be reported relative to its recorded occurrence in the field. Such laboratory evaluation, in parallel with “worst-case” scenario risk assessments in a tiered approach (Hill & Sendashonga 2003), ultimately allow accurate simulation of “potential” effects of transgenic crops on non-target species in the field. However, caution should be exercised during the optimization of assay systems. Not only is it important to ensure high levels of sensitivity, but the ELISA should be specific to the target material at the
optimized concentrations. False-positive recordings could lead to inaccurate conclusions of the presence of endotoxins in non-target arthropod communities.

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