Taylor & Francis
Taylor & Francis Group

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

J. D. HARWOOD & J. J. OBRYCKI

Department of Entomology, University of Kentucky, Lexington, KY, USA

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* (Hübner) (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 ($\pm$SE) Cry1Ab-endotoxin concentration $= 0.72 \pm 0.05$ 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 mg μl^{-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 µl per well. In addition to this experimental material, 100 µl 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 µl 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 µl 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 µl 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® 20. The upturned plate was then vigorously dropped onto paper towel to ensure all wash buffer solution was removed and 100 µl 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 µl 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® 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 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 μl^{-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
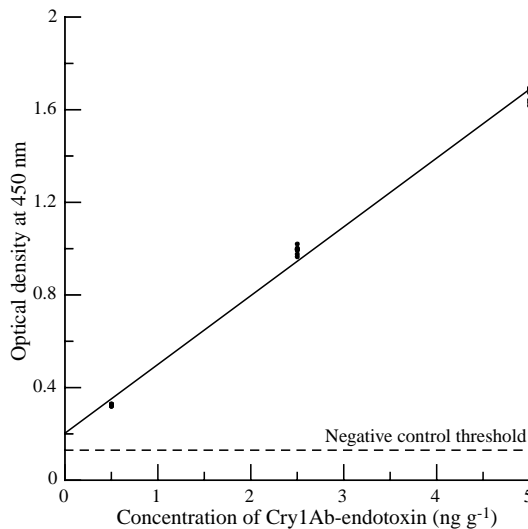

Figure 1. Regression of OD$_{450}$ for three calibrators (at 0.5 ng Cry1Ab g^{-1} fresh weight, 2.5 ng g^{-1} and 5.0 ng g^{-1}) used to calculate concentration of antibody-recognizable material during optimization of assay. Regression: $y = 0.297x + 0.203$; r$^2 = 0.99$. Dotted line represents OD$_{450}$ for EnviroLogix Inc. negative control.
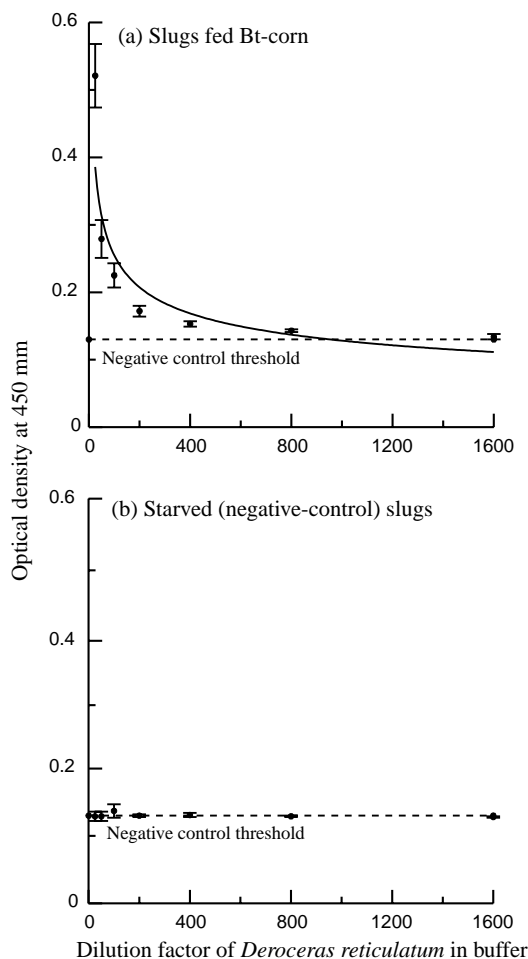
Figure 2. Optical density (at 450 nm) $\pm$SE of different concentrations of (a) *D. reticulatum* fed with Bt-kernels (positive controls) and (b) *D. reticulatum* not exposed to any transgenic material (negative control slugs). Regression (solid line) for OD_{450} of positive-control slugs versus concentration: $\log_e y = -0.298 \log_e x + 0.009$; $r^2 = 0.86$. Dotted line represents OD_{450} for EnviroLogix Inc. negative control.

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.
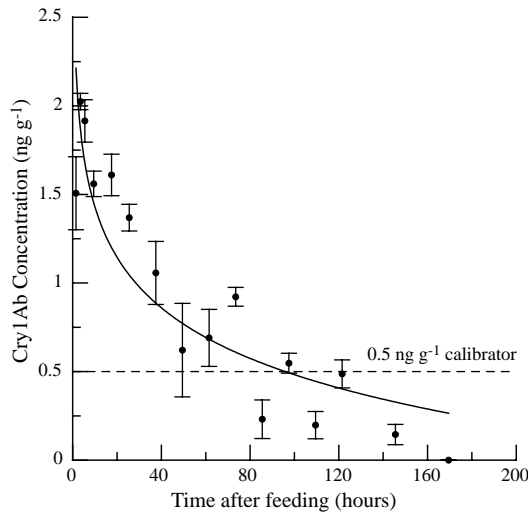
Figure 3. Detection period for Cry1Ab-endotoxins following consumption of kernels from Bt-corn. Data are means for each time period ± SE. Decay rate is determined by regression of Concentration versus Time: Cry1Ab Concentration = $-0.412 \log_e$ time + 2.38; $r^2 = 0.80$. Dotted line represents threshold for the concentration of 0.5 ng Cry1Ab g^{-1} fresh weight, below which values are assumed to contain no significant quantities of Cry1Ab-endotoxin.

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

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.

Acknowledgements

References

Alomar O, Wiedenmann RN, editors. 1996. Zoophytophagous Heteroptera: Implications for Life History and Integrated Pest Management. Thomas Say Publications in Entomology: Proceedings. Entomological Society of America, Lanham, MD.

Anderson PL, Hellmich RL, Sears MK, Sumerford DV, Lewis LC. 2004. Effects of Cry1Ab-expressing corn anthers on monarch butterfly larvae. Environmental Entomology 33:1109–1115.

Armer CA, Berry RE, Kogan M. 2000. Longevity of phytophagous heteropteran predators feeding on transgenic Btt-potato plants. Entomologia Experimentalis et Applicata 95:329–333.

Bohan DA, Bohan AC, Glen DM, Symondson WOC, Wiltshire CW, Hughes L. 2000. Spatial dynamics of predation by carabid beetles on slugs. Journal of Animal Ecology 69:367–379.

Byers RA, Calvin DD. 1994. Economic injury levels to field corn from slug (Stylommatophora, Agrolimacidae) feeding. Journal of Economic Entomology 87:1345–1350.

Calder CR, Harwood JD, Symondson WOC. 2005. Detection of scavenged material in the guts of predators using monoclonal antibodies: a significant source of error in measurement of predation? Bulletin of Entomological Research 95:57–62.

Cannon RJC. 2000. Bt transgenic crops: risks and benefits. Integrated Pest Management Reviews 5:151–173.

Dutton A, Klein H, Romeis J, Bigler F. 2002. Uptake of Bt-toxin by herbivores feeding on transgenic maize and consequences for the predator *Chrysoperla carnea*. Ecological Entomology 27:441–447.

Dutton A, Romeis J, Bigler F. 2003. Assessing the risks of insect resistant transgenic plants on entomophagous arthropods: Bt-maize expressing Cry1Ab as a case study. BioControl 48:611–636.

Gould F. 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annual Review of Entomology 43:701–726.

Groot AT, Dicke M. 2002. Insect-resistant transgenic plants in a multi-trophic context. The Plant Journal 31:387–406.

Hagler JR, Cohen AC. 1990. Effects of time and temperature on digestion of purified antigen by *Geocoris punctipes* (Hemiptera: Lygaeidae) reared on artificial diet. Annals of the Entomological Society of America 83:1177–1180.

Hammond RB, Beck T, Smith JA, Amos R, Barker J, Moore R, Siegrist H, Slates D, Ward B. 1999. Slugs in conservation tillage corn and soybeans in the eastern corn belt. Journal of Entomological Science 34:467–478.

Harper GL, King RA, Dodd CS, Harwood JD, Glen DM, Bruford MW, Symondson WOC. 2005. Rapid screening of invertebrate predators for multiple prey DNA targets. Molecular Ecology 14:819–828.

Harwood JD, Obrycki, JJ. 2005. Quantifying aphid predation rates of generalist predators in the field. European Journal of Entomology 102:335–350.

Harwood JD, Phillips SW, Sunderland KD, Symondson WOC. 2001. Secondary predation: quantification of food-chain errors in an aphid-spider-carabid system using monoclonal antibodies. Molecular Ecology 10:2049–2057.

Harwood JD, Sunderland KD, Symondson WOC. 2004. Prey selection by linyphiid spiders: molecular tracking of the effects of alternative prey on rates of aphid consumption in the field. Molecular Ecology 13:3549–3560.

Harwood JD, Wallin WG, Obrycki JJ. 2005a. Uptake of Bt-endotoxins by non-target herbivores and higher-order arthropod predators: molecular evidence from a transgenic corn agroecosystem. Molecular Ecology 14:2815–2823.

Harwood JD, Sunderland KD, Symondson WOC. 2005b. Monoclonal antibodies reveal the potential of the tetragnathid spider *Pachygnatha degeeri* (Araneae: Tetragnathidae) as an aphid predator. Bulletin of Entomological Research 95:161–168.

Hilbeck A, Baumgartner M, Fried PM, Bigler F. 1998. Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). Environmental Entomology 27:480–487.

Hilbeck A, Moar WJ, Pusztai-Carey M, Filippini A, Bigler F. 1999. Prey-mediated effects of Cry1Ab toxin and protein and Cry2A protoxin on the predator *Chrysoperla carnea*. Entomologia Experimentalis et Applicata 91:305–316.

Hill RA, Sendashonga C. 2003. General principles of risk assessment of living modified organisms: lessons from chemical risk assessment. Environmental Biosafety Research 2:81–88.

Hunter PJ. 1968. Studies on slugs of arable land. III. Feeding habits. Malacologia 6:391–399.

Jesse LCH, Obrycki JJ. 2000. Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly. Oecologia 125:241–248.

Losey JE, Rayor LS, Carter ME. 1999. Transgenic pollen harms monarch larvae. Nature 399:214.

Lövei GL, Arpaia S. 2005. The impact of transgenic plants on natural enemies: a critical review of laboratory studies. Entomologia Experimentalis et Applicata 114:1–14.

Lövei GL, Monostori E, Ando I. 1985. Digestion rate in relation to starvation in a predaceous ground beetle larva, *Poecilus cupreus* (Coleoptera, Carabidae). Entomologia Experimentalis et Applicata 37:123–127.

Lövei GL, Sopp PI, Sunderland KD. 1987. The effect of mixed feeding on the digestion of the carabid *Bembidion lampros*. Acta Phytopathologica et Entomologica Hungarica 22:403–407.

Lundgren JG, Wiedenmann RN. 2002. Coleopteran-specific Cry3Bb1 toxin from transgenic corn pollen does not affect the fitness of a nontarget species, *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae). Environmental Entomology 31:1213–1218.

Lutman J. 1978. The role of slugs in an Agrostis-Festuca grassland. In: Heal OW, Perkins DF, editors. Ecological Studies: Analysis and Synthesis. vol 27. Production Ecology of British Moors and Montane Grasslands. New York: Springer-Verlag. pp 332–347.

Mair J, Port GR. 2001. Predation by the carabid beetles *Pterostichus madidus* and *Nebria brevicollis* is affected by the size and the condition of the prey slug *Deroceras reticulatum*. Agriculture, Ecosystems & Environment 3:99–106.

McKemey AR, Glen DM, Symondson WOC. 2004. How does a carabid predator find aggregations of slugs in the field? Electroantennograms and behavioural assays suggest chemical cues. Bulletin of Entomological Research 94:235–244.

Mendelsohn M, Kough J, Vaituzis Z, Matthews K. 2003. Are Bt crops safe? Nature Biotechnology 21:1003–1009.

Nyffeler M, Symondson WOC. 2001. Spiders and harvestmen as gastropod predators. Ecological Entomology 26:617–628.

Obrycki JJ, Losey JE, Taylor OR, Jesse LCH. 2001. Transgenic insecticidal corn: beyond insecticidal toxicity to ecological complexity. BioScience 51:353–361.

Obrycki JJ, Ruberson JR, Losey JE. 2004. Interactions between natural enemies and transgenic insecticidal crops. In: Ehler LE, Sforza R, Mateille T, editors. Genetics, Evolution and Biological Control. London: CAB International. pp 183–206.

Pilcher CD, Obrycki JJ, Rice ME, Lewis LC. 1997. Preimaginal development, survival, and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. Environmental Entomology 26:446–454.

Ponsard S, Gutierrez AP, Mills NJ. 2002. Effects of Bt-toxin (Cry1Ac) in transgenic cotton on the adult longevity of four heteropteran predators. Environmental Entomology 31:1197–1205.

Pray CE, Huang J, Hu R, Rozelle S. 2002. Five years of Bt cotton in China – the benefits continue. The Plant Journal 31:423–430.

Raps A, Kehr J, Gugerli P, Moar WJ, Bigler F, Hilbeck A. 2001. Immunological analysis of phloem sap of *Bacillus thuringiensis* corn and of the nontarget herbivore *Rhopalosiphum padi* (Homoptera: Aphididae) for the presence of Cry1Ab. Molecular Ecology 10:525–533.

Rollo CD, Ellis CR. 1974. Sampling methods for slugs, *Deroceras reticulatum* (Müller), *Deroceras laeve* (Müller) and *Arion subfuscus* Nilsson in Ontario corn fields. Proceedings of the Entomological Society of Ontario 105:89–95.

Romeis J, Dutton A, Bigler F. 2004. *Bacillus thuringiensis* toxin (Cry1Ab) has no direct effect on the larvae of the green lacewing *Chrysoperla carnea* (Stephens). Journal of Insect Physiology 50:175–183.

Schenk D, Bacher S. 2004. Detection of shield beetle remains in predators using a monoclonal antibody. Journal of Applied Entomology 128:273–278.

Shelton AM, Zhao JZ, Roush RT. 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. Annual Review of Entomology 47:845–881.

Sopp PI, Sunderland KD. 1989. Some factors affecting the detection period of aphid remains in predators using ELISA. Entomologia Experimentalis et Applicata 51:11–20.

South A. 1992. Terrestrial Slugs: Biology, Ecology and Control. London: Chapman & Hall.

Symondson WOC, Liddell JE. 1993. Differential antigen decay rates during digestion of molluscan prey by carabid predators. Entomologia Experimentalis et Applicata 69:277–287.

Symondson WOC, Liddell JE. 1995. Decay rates for slug antigens within the carabid predator *Pterostichus melanarius* monitored with a monoclonal antibody. Entomologia Experimentalis et Applicata 75:245–250.

Symondson WOC, Liddell JE. 1996. A species-specific monoclonal antibody system for detecting the remains of field slugs, *Deroceras reticulatum* (Müller) (Mollusca: Pulmonata), in carabid beetles (Coleoptera: Carabidae). Biocontrol Science and Technology 6:91–99.

Symondson WOC, Erickson ML, Liddell JE, Jayawardena KGI. 1999. Amplified detection, using a monoclonal antibody, of an aphid specific epitope exposed during digestion in the gut of a predator. Insect Biochemistry and Molecular Biology 29:873–882.

Symondson WOC, Glen DM, Erickson ML, Liddell JE, Langdon CJ. 2000. Do earthworms help to sustain the slug predator *Pterostichus melanarius* (Coleoptera: Carabidae) within crops? Investigations using monoclonal antibodies. Molecular Ecology 9:1279–1292.

Symondson WOC, Glen DM, Ives AR, Langdon CJ, Wiltshire CW. 2002. Dynamics of the relationship between a generalist predator and slugs over five years. Ecology 83:137–147.

Symondson WOC, Glen DM, Wiltshire CW, Langdon CJ, Liddell JE. 1996. Effects of cultivation techniques and methods of straw disposal on predation by *Pterostichus melanarius* (Coleoptera: Carabidae) upon slugs (Gastropoda: Pulmonata) in an arable field. Journal of Applied Ecology 33:741–753.

Way MJ, van Emden HF. 2000. Integrated pest management in practice – pathways toward successful application. Crop Protection 19:81–103.

Wolfenbarger LL, Phifer PR. 2000. Biotechnology and ecology – the ecological risks and benefits of genetically engineered plants. Science 290:2088–2093.

Zwahlen C, Hilbeck A, Howald R, Nentwig W. 2003a. Effects of transgenic Bt corn litter on the earthworm *Lumbricus terrestris*. Molecular Ecology 12:1077–1086.

Zwahlen C, Hilbeck A, Gugerli P, Nentwig W. 2003b. Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. Molecular Ecology 12:765–775.