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RESEARCH ARTICLE

Spiders from multiple functional guilds are exposed to Bt-endotoxins in transgenic corn fields via prey and pollen consumption

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ABSTRACT

A comprehensive assessment of risk to natural enemies from Bt-endotoxins from bioengineered crops must evaluate potential harm, as well as exposure pathways in non-target arthropod food webs. Despite being abundant generalist predators in agricultural fields, spiders (Araneae) have often been overlooked in the context of Bt crop risk assessment. Spiders and their prey were collected from transgenic corn fields expressing lepidopteran-specific Cry1Ab, coleopteran-specific Cry3Bb1, both proteins, and a non-transgenic near isoline. Spiders and prey were screened for Cry1Ab and Cry3Bb1 using qualitative enzyme-linked immunosorbent assay. Spiders from the three most common functional guilds, wandering sheet-tangle weavers, orb-weavers, and ground runners, tested positive for Cry1Ab and Cry3Bb1 proteins, with the highest per cent positive (8.0% and 8.3%) during and after anthesis. Laboratory feeding trials revealed that Bt-endotoxins were detectable in the *Pardosa* sp. (Lycosidae)-immature cricket-Bt corn pathway, but not in the *Tennesseellum formica* (Linyphiidae)-Collembola-Bt corn pathway. Additionally, direct consumption of transgenic corn pollen by *Pardosa* sp., *T. formica*, and *Cyclosa turbinata* (Araneidae) resulted in transfer of both Cry1Ab and Cry3Bb1 endotoxins. This study demonstrates that Bt-endotoxins are taken up by diverse members of a spider community via pollen and prey consumption and should be factored into future risk assessment.

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Risk assessment; Linyphiidae; Lycosidae; Araneidae

1. Introduction

In the nearly 20 years since transgenic *Bacillus thuringiensis* crops have been commercially available, a plethora of studies have examined their safety for non-target organisms. While the majority of these studies have found no significant negative impacts on non-target beneficial organisms (see meta-analyses by Marvier et al., 2007; Peterson et al., 2011; Wolfenbarger et al., 2008), risk assessment of genetically modified crops continues to be an important field of study. The impact of agricultural practices on vulnerable non-target organisms, such as monarch butterflies, honey bees, and other pollinators, has received

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increased attention in both the scientific and public arenas (Chagnon et al., 2015; Pleasants & Oberhauser, 2013). Given that genetically modified crops that confer herbicide tolerance and/or express insecticidal toxins have become ubiquitous in the agricultural landscape (USDA-NASS, 2015), understanding the ecological risks associated with this technology is therefore essential.

Risk can be partitioned into two key components: harm and exposure. Within the risk-assessment literature, more focus has been placed on harm than exposure, despite the fact that elucidating exposure pathways is essential in determining the likely impacts that beneficial organisms will experience in the field. Therefore, this study examined risk assessment for spiders (Araneae), a diverse taxon of non-target beneficial organisms, with a focus on Bt-endotoxin exposure pathways.

Within the predatory arthropods, spiders are common, abundant, and diverse in agroecosystems (Lundgren & Fergen, 2010; Lundgren et al., 2006; Nyffeler & Sunderland, 2003; Young & Edwards, 1990), including Bt crop fields (Duan et al., 2004; de la Poza et al., 2005; Sisterson et al., 2004). In addition to playing varied and essential roles in arthropod food webs (Wise, 1993), these generalist predators can be key predators of pests in crop fields (Greenstone, 1999; Harwood et al., 2004; Riechert & Lockley, 1984). For example, spiders inflicted mortality on 42% of cutworm larvae in tobacco (Nakasuji et al., 1973) and 49% of aphids in cereal crops (Chambers & Aikman, 1988) via both direct predation and non-consumptive effects.

Despite their prominent role in agroecosystems, spiders have frequently been overlooked in Bt crops' risk assessment or lumped into a single group at the order level (reviewed in Meissle, 2013; Peterson et al., 2011). Few studies have identified spiders at the species level (Habuštová et al., 2015; Svobodová et al., 2013), with several finding that there are significant differences in the abundance of certain spider species in Bt versus non-Bt crops (Lee et al., 2014; Naranjo, 2005; Řezáč et al., 2006; Toschki et al., 2007). While spiders as a whole are considered generalist predators, they are an incredibly diverse taxonomic group, with species occupying many different functional niches and displaying a diversity of hunting and feeding preferences (Foelix, 2011; Uetz, Halaj, & Cady, 1999). This diversity allows for the potential for Bt crops to affect spider species differentially, particularly as their routes to Bt-endotoxin exposure will vary. Several potential routes to Bt-endotoxin exposure for spiders were described by Peterson et al. (2011) and include (1) consumption of Bt-containing prey, (2) consumption of crop pollen, and (3) other forms of phytophagy.

Techniques using monoclonal and/or polyclonal antibodies, such as enzyme-linked immunosorbent assay (ELISA), have been successfully employed to detect the presence of Bt-endotoxins in field-collected arthropods, such as Coleoptera (Harwood et al. 2005, 2007; Peterson et al. 2009; Zwahlen & Andow 2005), Acari (Obrist et al. 2006; Torres & Ruberson 2008), and Araneae (Harwood et al. 2005). Ahmad et al. (2005) measured ground-dwelling arthropod abundance (including spiders) and, in parallel, used ELISA to quantify Bt-endotoxin concentration in the soil, but did not test for the uptake of proteins by the arthropods themselves. Recent work has also demonstrated that spiders are not strict carnivores; their diets may include plant-provided resources that contain Bt proteins, such as pollen (Peterson et al., 2010; Pfannenstiel, 2012; Schmidt et al., 2013), nectar (Patt & Pfannenstiel, 2008; Pfannenstiel & Patt, 2012), and other plant tissues (Meehan et al., 2009). Further study on the realistic exposure of spiders to Bt proteins in the field

is therefore needed to fully understand transgenic crop risk assessment for this important non-target group.

This study specifically examines the uptake of Bt-endotoxins by spiders from selected transgenic corn lines, to identify potential exposure pathways and the fate of Bt-endotoxins in the field. These objectives are achieved by collecting spiders from the field and testing them for the presence of Cry1Ab and Cry3Bb1 proteins, as well as conducting laboratory experiments to examine the movement of these proteins into higher trophic levels via prey or pollen ingestion. We hypothesise that both prey and pollen ingestion will be viable routes for Bt protein exposure to spiders in corn agroecosystems.

2. Methods

2.1. Field description and transgenic lines

Four 2500 m^2 fields (50 m × 50 m) of corn were planted on 6 May 2008, at the University of Kentucky Spindletop Research Station, Lexington, Kentucky, USA, and maintained under standard agronomic practices for Kentucky but with no insecticides. Herbicides (Lexar℠ – Syngenta Crop Protection, Greensboro, North Carolina, USA; Roundup® – Monsanto Company, St. Louis, Missouri, USA) were applied to all fields on 8 May 2008, followed by ammonium nitrate fertilisation on 6 June 2008 (approximately 300 kg/ha). The corn varieties planted were YieldGard Corn Borer™ (Bt-hybrid 4842S; MON810) (GPS coordinates at the centre of the field: 38°07.555N, 84°30.901W), which expresses lepidopteran-specific Cry1Ab protein, YieldGard Rootworm™ (Bt-hybrid 4843X; MON863) (38°07.667N, 84°30.636W), which expresses coleopteran-specific Cry3Bb1 protein, YieldGard Plus™ (Bt-hybrid 4846T; MON810 × MON863) (38° 07.703N, 84°30.440W), which expresses both Cry1Ab and Cry3Bb1, and a non-transgenic near isoline (isoline 4847) (38°08.141N, 84°30.206W) (Monsanto Company, St. Louis, Missouri, USA). These fields will henceforth be referred to as the Corn Borer, Rootworm, Plus, and Isoline fields. These crops were grown under Monsanto Academic Research License/Stewardship Agreement #50290588 with the University of Kentucky. In the immediately previous year (2007), experimental fields had been planted with the same varieties of Bt corn used in the current study and prior to 2007 had not been planted with Bt-crops. Distances between fields ranged from 150 to 800 m and non-Bt crops, including soybean, alfalfa, cucurbits, and sweet pepper, surrounded the corn.

2.2. Spider and prey collection

Spiders and any potential prey species were collected weekly from refuge traps, dry pitfall traps, and by visual searching with a hand-held aspirator between 21 May and 10 September 2008. Refuge traps consisted of twenty wooden boards (25 cm × 46 cm, 2.5 cm thick) aligned in transects between rows of corn (five refuge traps spaced 8 m apart in four rows 4 m apart) in each field. Pitfall traps consisted of a 500 mL plastic cup with a metal mesh insert (0.3 cm hardware cloth to separate spiders from potential prey items and reduce intraguild predation) flush with the soil surface with no liquid preservatives, which were similarly arranged in a grid of 20 traps per field. Pitfall traps were opened once a week at 22:00 h and checked the next morning at 6:00 h, ensuring that spiders had not remained in traps longer than 8 h.

All specimens were stored in 7 or 30 mL (depending on specimen size) Sterilin® plastic containers (Dynalab Corporation, Rochester, New York, USA) and frozen immediately in a portable Engel MT15 freezer (Engel, Jupiter, Florida, USA). Samples were transferred to a −20°C freezer until preparation for ELISA screening.

2.3. Plant tissue collection

Leaf tissue samples and pollen ($n = 10$ samples for both) were collected from each of the four corn varieties at the VT/R1 stage. To avoid contaminating samples with tassel material, pollen was passively collected by placing a brown paper bag over the entire tassel for a 48 h period during anthesis and sieving the collected pollen through a 170-mesh (90-µm) screen (following protocol by Hellmich et al., 2001).

2.4. ELISA sample preparation

Spiders, prey, and plant tissues were screened using AgDia Bt-Cry3Bb1 and Bt-Cry1Ab Multi-trait ELISA Kits (AgDia Inc., Elkhart, Indiana, USA), which are qualitative tests that screen for presence/absence of both Cry1Ab and Cry3Bb1 proteins. Spider and prey species were each washed to remove surface contamination prior to ELISA analysis by placing the arthropod in approximately 1 mL 1× phosphate buffered saline with Tween 20 (PBST buffer) in a 1.5 ml microcentrifuge tube, vortexing for 5 s, and centrifuging at 5000*g* for 30 s. The arthropod was then removed and the buffer discarded.

2.4.1. Spiders and prey

Whole body samples for spiders and small prey were used for sample preparation. The midgut of spiders contains branching diverticulae that may extend into the coxae of the legs (Foelix, 2011); it is therefore necessary to process the entire spider body. All samples were weighed and 1× PBST buffer was added to yield a 1:10 dilution (sample tissue weight in gram:buffer volume in mL). For very small prey (<0.022 g), 220 µL of buffer was added to allow for adequate volume to load ELISA plates. Samples were then homogenised by hand using a disposable polypropylene Kontes™ Pellet Pestle™ (Fisher Scientific Company LLC., Pittsburgh, Pennsylvania, USA) or the T25 Basic Ultra-Turrax® mechanical homogeniser (IKA® Works, Inc., Wilmington, North Carolina, USA) for large specimens, mixed on a vortex for 10 s and centrifuged at 5000*g* for 5 min. The resulting supernatant was removed to a clean microcentrifuge tube and later added to ELISA plate wells.

2.4.2. Corn tissue

Preparation of corn tissue followed the guidelines of the ELISA kit manufacturer (AgDia Inc.) for plant tissue screening. Leaf and pollen samples were weighed and diluted to 1:10 (sample tissue weight in milligram:buffer volume in millilitre) with 1× PBST buffer. Samples were homogenised with disposable pestles, centrifuged, and the resulting supernatant used for ELISA screening.

2.4.3. Negative controls

Five spider species (*Tennesseellum formica*, *Erigone autumnalis* and *Mermessus fradeorum* (Linyphiidae), *Cyclosa turbinata* (Araneidae), and *Pardosa* sp. (Lycosidae)) were collected from alfalfa fields using a hand-held aspirator, maintained in the laboratory at 21°C on a 16:8 L:D cycle and provided with a diet of *Sinella curviseta* (Collembola: Entomobryidae). The prey species *Myodocha serripes* (Hemiptera: Rhyparochromidae) was collected from non-transgenic corn and *S. curviseta* were obtained from the laboratory colony. In addition, non-transgenic corn plants were grown in the greenhouse (22 ± 2°C, 16:8 L:D cycle) for corn tissue negative controls. Sample preparation for these negative controls followed protocols described above for corn tissue.

2.5. ELISA screening

2.5.1. Arthropod and plant tissues

Samples were screened for both Cry1Ab and Cry3Bb1 Bt-endotoxins by double antibody sandwich ELISA using an AgDia Bt-Cry3Bb1 and Bt-Cry1Ab Multi-trait ELISA Kit. RUB6 enzyme conjugate diluent was added to the 100× enzyme conjugate to yield a 1× concentration; 100 µL of this solution was added to each test well. The sample supernatants previously described were coated into two ELISA plate wells each, at 100 µL per well. On each plate, positive controls (provided by manufacturer) and negative controls (described above) were loaded into eight wells each, at 100 µL per well. The ELISA plates were carefully rotated in a circular motion for 30 s to ensure mixing of samples within wells and placed in a humid chamber for a 2 h incubation period at room temperature. The samples were then ejected from the plate and all wells washed eight times with 1× PBST. To each well, 100 µL pNPP substrate solution was added and plates rotated as above. After 30 min incubation in darkness, the optical density at 405 nm was read using a Thermo Labsystems Multiskan Plus® spectrophotometer (Fisher Scientific Company LLC, Pittsburgh, Pennsylvania, USA), producing results for the presence/absence of Cry3Bb1 proteins. Following optical reading, the wells were ejected and washed eight times before adding 100 µL TMB substrate solution to each test well. The plate was rotated and incubated in darkness for 20 min before being read at 650 nm with the spectrophotometer to yield results for Cry1Ab proteins.

2.5.2. Determination of positive threshold for Cry1Ab and Cry3Bb1

A positive threshold for the presence of Bt protein was set for each plate reading. This was determined by calculating the mean absorbance of the eight negative control samples plus three standard deviations (after Peterson et al., 2009).

2.6. Laboratory feeding trials

To determine movement of Bt-endotoxins through multiple trophic levels, feeding trials were conducted. Corn leaf tissue and pollen from plants undergoing anthesis (growth stage VT/R1) from each of the four varieties were collected and fed *ad libitum* to two prey species: 2–4-day-old 'pinhead' crickets *Acheta domesticus* (Orthoptera: Gryllidae) (Petco.com, San Diego, California, USA) and springtails *S. curviseta* originally collected

from Spindletop Farm, Lexington, KY, and maintained in a laboratory colony. Prey insects were kept individually in plastic Petri dishes (60 mm×15 mm) with a moistened Plaster of Paris and charcoal base and allowed to feed for a 1 h period, during which time feeding was confirmed via observation using a stereomicroscope. A sub-set ($n = 10$ per prey species for each of the four corn varieties) of these insects was immediately frozen in microcentrifuge tubes and later screened by the AgDia ELISA kit for the presence/absence of Cry1Ab and Cry3Bb1 proteins (as described above). Remaining prey were fed to spider predators: *A. domesticus* were given to *Pardosa* sp. and *S. curviseta* were given to *T. formica*. Predators were allowed to feed for 1 h and predation events were confirmed by observation using a stereomicroscope. Spiders ($n = 10$ per spider species for each of the four corn varieties) were then immediately frozen in microcentrifuge tubes and screened by ELISA. Additionally, spiders from the species *Pardosa* sp., *T. formica*, and *C. turbinata* were placed individually into plastic Petri dishes with plaster and charcoal bases (as described above) and given approximately 2.5 mg corn pollen by dusting onto their webs or into the petri arena (for *Pardosa* sp. which do not spin prey-capturing webs) with a sterilised paint brush. Spiders were allowed to consume pollen for 1 h and feeding was confirmed by observation using a stereomicroscope during this time. Immediately following, spiders ($n = 10$ per spider species for each of the four corn varieties) were frozen for subsequent ELISA screening. All spiders for these trials had been collected by hand from non-transgenic corn and alfalfa fields at the Spindletop Research Station, maintained in a colony on diets of *S. curviseta* (for *Pardosa* and *T. formica*) or *Drosophila melanogaster* (Diptera: Drosophilidae) (for *C. turbinata*) and starved for one week prior to the feeding trials to ensure that Bt-endotoxins were not already present in their bodies.

2.7. Statistical analyses

Analyses were conducted using SAS® statistical software (SAS® Institute Inc., Cary, North Carolina, USA). For spiders and prey, χ^2 analysis was used to compare the proportion screening positive for Cry1Ab and Cry3Bb1 Bt-endotoxins from each of the four fields, as well as temporally during time periods that were determined based on corn phenology (after Harwood et al., 2007): pre-anthesis (21 May–10 July 2008), anthesis (11 July–31 July 2008), and post-anthesis (1 August–10 September 2008). Additionally, χ^2 analysis was used to compare the movement of Cry1Ab vs. Cry3Bb1 proteins during laboratory feeding trials.

3. Results

3.1. Spider collection

In 2008, 1108 spiders belonging to 29 genera and 12 families were collected (Table S1). Spiders were classified into ecological guilds, as defined by Uetz et al. (1999), with the most common taxa belonging to the wandering sheet-tangle weavers (*T. formica*, immature Linyphiidae, *Mermessus* spp., *E. autumnalis* and *Meioneta* sp.), ground runners (*Pardosa* sp., *Allocosa* sp. and immature Lycosidae), and orb-weavers (*C. turbinata*).

3.2. Bt-endotoxin uptake by spiders

Spiders tested positive for Cry1Ab Bt-endotoxins from the three transgenic fields, while positive results for Cry3Bb1 were limited to Rootworm and Plus fields, and no spiders screened positive for either protein from the non-transgenic near isoline (Table 1). Except for one immature Linyphiidae spider testing positive for Cry1Ab from the Cry3Bb1-expressing Rootworm field, ELISA results for uptake by spiders corresponded with the expression of Bt proteins in the corn lines from which they were collected. For the most commonly collected species, variation in the proportions screening positive for Bt-endotoxins were observed both between and within functional guilds from the Corn Borer (Figure 1), Rootworm (Figure 2), and Plus fields (Figure 3). Total per cent positive for Cry1Ab from the Corn Borer field was higher for ground runners (24%) and orb-weavers (29%) than for wandering sheet-tangle weavers (5%) ($\chi^2 = 46.08$, df = 2, $P < .001$). Total per cent positive for Cry3Bb1 from the Rootworm field was marginally significantly higher for ground runners (10%) compared to orb-weavers (4%) and wandering sheet-tangle weavers (3%) ($\chi^2 = 5.97$, df = 2, $P = .051$). From the Plus field, per cent positive for Cry1Ab was higher for ground runners (34%) and orb-weavers (19%) than for wandering sheet-tangle weavers (6%) ($\chi^2 = 26.72$, df = 2, $P < .001$) and per cent positive for Cry3Bb1 was also higher for ground runners (22%) and orb-weavers (24%) than for wandering sheet-tangle weavers (3%) ($\chi^2 = 22.35$, df = 2, $P < .001$). Per cent positive for Cry1Ab and Cry3Bb1 from the isoline field was 0% for all ecological guilds.

3.3. Prey collection

In 2008, 458 potential prey items belonging to 64 taxa were collected and screened by ELISA (Table S2). Some of the most dominant prey collected were millipedes (Diplopoda: Julida ($n = 39$) and Polydesmida ($n = 27$)), and centipedes (Chilopoda: Lithobiomorpha ($n = 35$)), as well as small dung beetles *Onthophagus* sp. (Coleoptera: Scarabaeidae) ($n = 34$), long-necked seed bugs *M. serripes* ($n = 30$), springtails (Collembola: Entomobryidae) ($n = 30$), and click beetles (Coleoptera: Elateridae ($n = 28$)).

Table 1. Per cent of total spiders screening positive for Cry1Ab and Cry3Bb1 Bt proteins from the YieldGard Corn Borer™ (Bt-hybrid 4842S; MON810), YieldGard Rootworm™ (Bt-hybrid 4843X; MON863), YieldGard Plus™ (Bt-hybrid 4846T; MON810 × MON863), and non-transgenic near isoline fields. Statistics given in the body of the table indicate Chi-square comparison between per cent positives for Cry1Ab and Cry3Bb1 within a given field.

		Per cent of spiders positive via ELISA for:				
Field	Protein(s) expressed	Cry1Ab (%)	Cry3Bb1 (%)	df	χ^2	*P*-value
Corn Borer	Cry1Ab	12.2[a,b]	0.0	1	75.62	<.001
Rootworm	Cry3Bb1	0.2	6.4[b,c]	1	23.94	<.001
Plus	Cry1Ab & Cry3Bb1	11.6 [a]	7.6[c]	1	3.19	.074
Isoline	None	0.0	0.0	–	–	–

[a]Per cent positive for Cry1Ab for the Corn Borer and Plus fields was not significantly different ($\chi^2 = 0.09$, df = 1, $P = .759$).
[b]Per cent positive for Cry1Ab was significantly higher for the Corn Borer field than per cent positive for Cry3Bb1 for the Rootworm field ($\chi^2 = 9.34$, df = 1, $P = .002$).
[c]Per cent positive for Cry3Bb1 for the Rootworm and Plus fields was not significantly different ($\chi^2 = 0.46$, df = 1, $P = .498$).

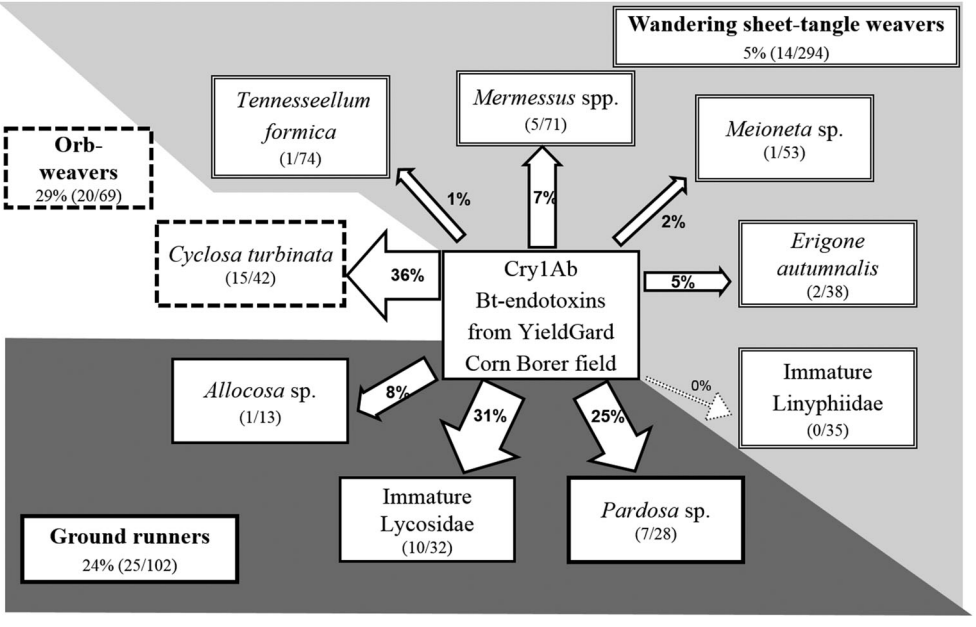

Figure 1. Uptake of Bt-endotoxins by spiders from the lepidopteran-specific YieldGard Corn Borer field. Species are separated by functional guild: wandering sheet-tangle weavers, ground runners, and orb-weavers; per cent positives for Cry1Ab shown with white arrows and number of positive individuals out of total collected given in parentheses.

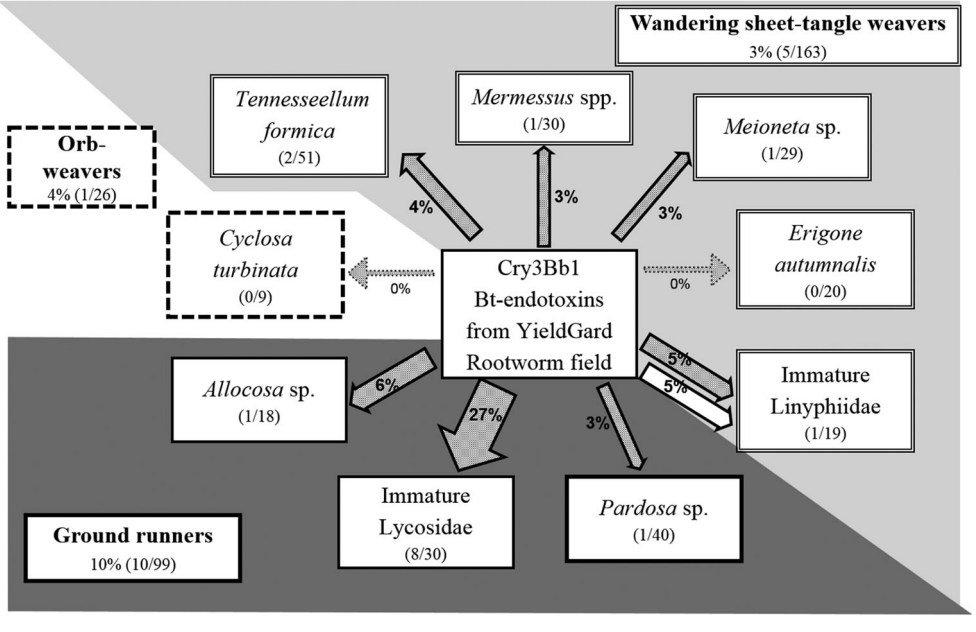

Figure 2. Uptake of Bt-endotoxins by spiders from the coleopteran-specific YieldGard Rootworm field. Species are separated by functional guild: wandering sheet-tangle weavers, ground runners, and orb-weavers; per cent positives for Cry1Ab shown with white arrows and Cry3Bb1 shown with grey arrows and number of positive individuals out of total collected given in parentheses.

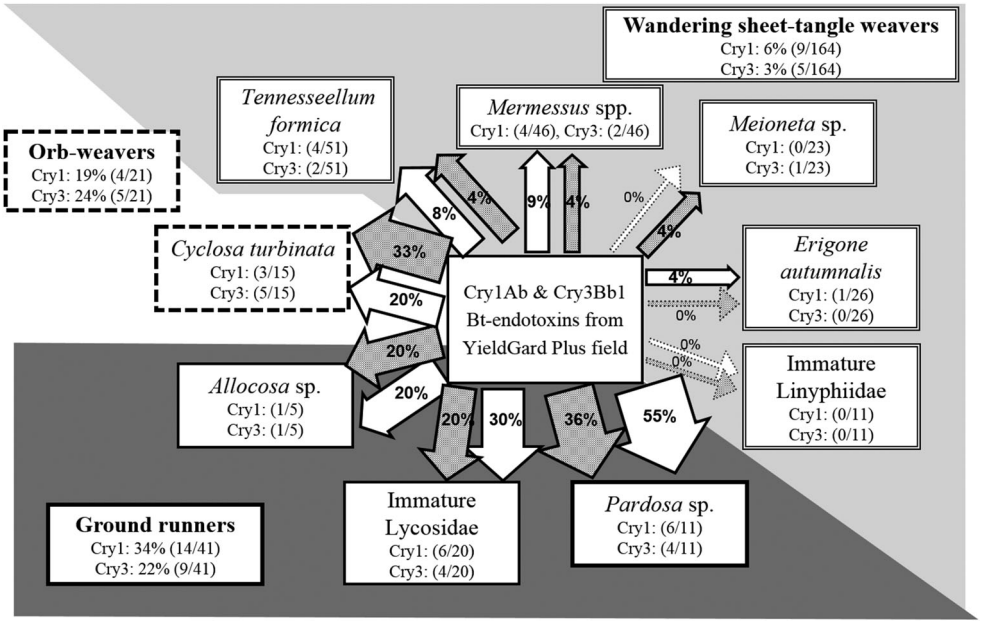

Figure 3. Uptake of Bt-endotoxins by spiders from the stacked YieldGard Plus field. Species are separated by functional guild: wandering sheet-tangle weavers, ground runners, and orb-weavers; per cent positives for Cry1Ab shown with white arrows and Cry3Bb1 shown with grey arrows and number of positive individuals out of total collected given in parentheses.

3.4. Bt-endotoxin uptake by prey

Prey tested positive for Cry1Ab Bt-endotoxins from Corn Borer and Plus fields and Cry3Bb1 from Rootworm and Plus fields, while no prey screened positive for either protein from the non-transgenic near isoline (Table 2). It is unlikely that the detection of Bt proteins by ELISA was impacted by the mass of spider or prey samples, as supported by results of Pearson Rank-Order tests for correlation between the mass of three of the most abundant spider species and ELISA results for Cry1Ab and Cry3Bb1 for these samples (Table S3).

3.5. Temporal uptake of Bt-endotoxins by spiders and prey

Uptake of Bt-endotoxins by spiders varied based on the time period during which they were collected ($\chi^2 = 8.52$, df = 2, $P = .014$), while uptake of Bt-endotoxins by prey did not vary across the season ($\chi^2 = 1.45$, df = 2, $P = .485$) (Figure 4). For spiders, the per cent positive for Bt proteins during the pre-anthesis time period (21 May–10 July 2008) (4.7%) was significantly lower than during anthesis (11 July–31 July 2008) (8.0%) ($\chi^2 = 6.97$, df = 1, $P = .008$) and post-anthesis (1 August–10 September 2008) (8.3%) ($\chi^2 = 6.01$, df = 1, $P = .014$). The percentages positive for spiders during (8.0%) and after anthesis (8.3%) were not different ($\chi^2 = 0.02$, df = 1, $P = .878$). During pre-anthesis, the per cent of spiders screening positive for Bt-endotoxins (4.7%) was not different from the per cent of prey screening positive (4.6%) ($\chi^2 = 0.001$, df = 1, $P = .981$). During anthesis the percentage of spiders screening positive (8.0%) was numerically greater than prey (6.5%), but this difference was not statistically significant ($\chi^2 = 0.647$, df = 1, $P = .421$). However, during

Table 2. Per cent of total prey items screening positive for Cry1Ab and Cry3Bb1 Bt proteins from the YieldGard Corn Borer™ (Bt-hybrid 4842S; MON810), YieldGard Rootworm™ (Bt-hybrid 4843X; MON863), YieldGard Plus™ (Bt-hybrid 4846T; MON810 × MON863), and non-transgenic near isoline fields.

		Per cent of spiders positive via ELISA for:				
Field	Protein(s) expressed	Cry1Ab	Cry3Bb1	df	χ^2	*P*-value
Corn Borer	Cry1Ab	12.2[a,b]	0.0	1	75.62	<.001
Rootworm	Cry3Bb1	0.2	6.4[b,c]	1	23.94	<.001
Plus	Cry1Ab & Cry3Bb1	11.6[a]	7.6[c]	1	3.19	.074
Isoline	None	0.0	0.0	–	–	–

[a] $\chi^2 = 0.02$, df = 1, $P = .883$.
[b] $\chi^2 = 1.39$, df = 1, $P = .238$.
[c] $\chi^2 = 0.001$, df = 1, $P = .982$.

the post-anthesis time period, spiders had a significantly higher per cent positive (8.3%) than prey (4.6%) ($\chi^2 = 3.918$, df = 1, $P = .048$).

3.6. Movement of Bt-endotoxins through trophic levels

Plant tissue collected from leaves and pollen of each of the four corn lines screened positive for the Bt-endotoxins that corresponded with their expected expression: Corn Borer plants were 100% positive for Cry1Ab and 0% for Cry3Bb1, Rootworm plants were 0% positive for Cry1Ab and 100% for Cry3Bb1, Plus plants were 100% positive for both Cry1Ab and Cry3Bb1, and isoline plants were 0% positive for both proteins (Figure 5(a) and Figure 6 (a)). When these plant materials were fed to gryllids, only 10% screened positive for Cry1Ab after being fed Corn Borer or Plus corn, while 100% were positive for Cry3Bb1 after being fed Rootworm or Plus corn (Figure 5(b)). Only 10% of Collembola fed Plus corn tested positive for Cry1Ab proteins and all others were 0% positive. When gryllids

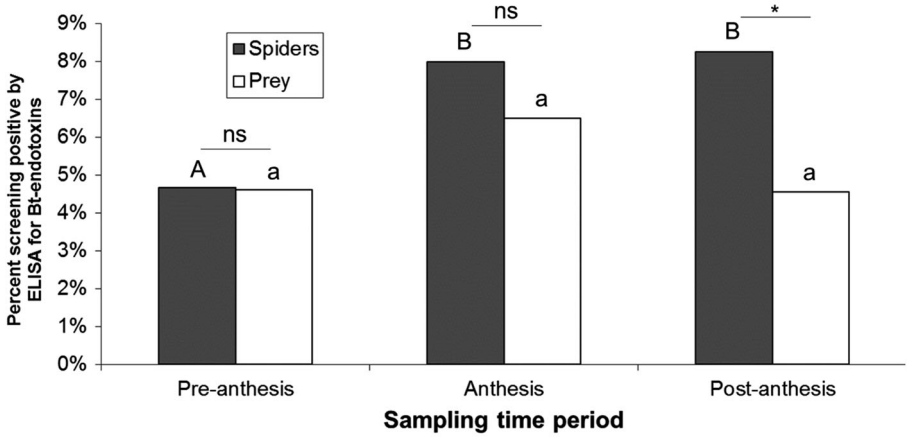

Figure 4. Temporal uptake of Bt-endotoxins by spiders and their prey during 2008. Pre-anthesis: 21 May–10 July 2008; Anthesis: 11 July–31 July 2008; Post-anthesis: 1 August–10 September 2008. Capital letters indicate statistical differences between spiders across the three time periods; lowercase letters indicate statistical differences between prey across the three time periods. Statistical comparisons between spiders and prey within each time period are given above the horizontal bar; ns = not significant, * = *P*-value < .05.

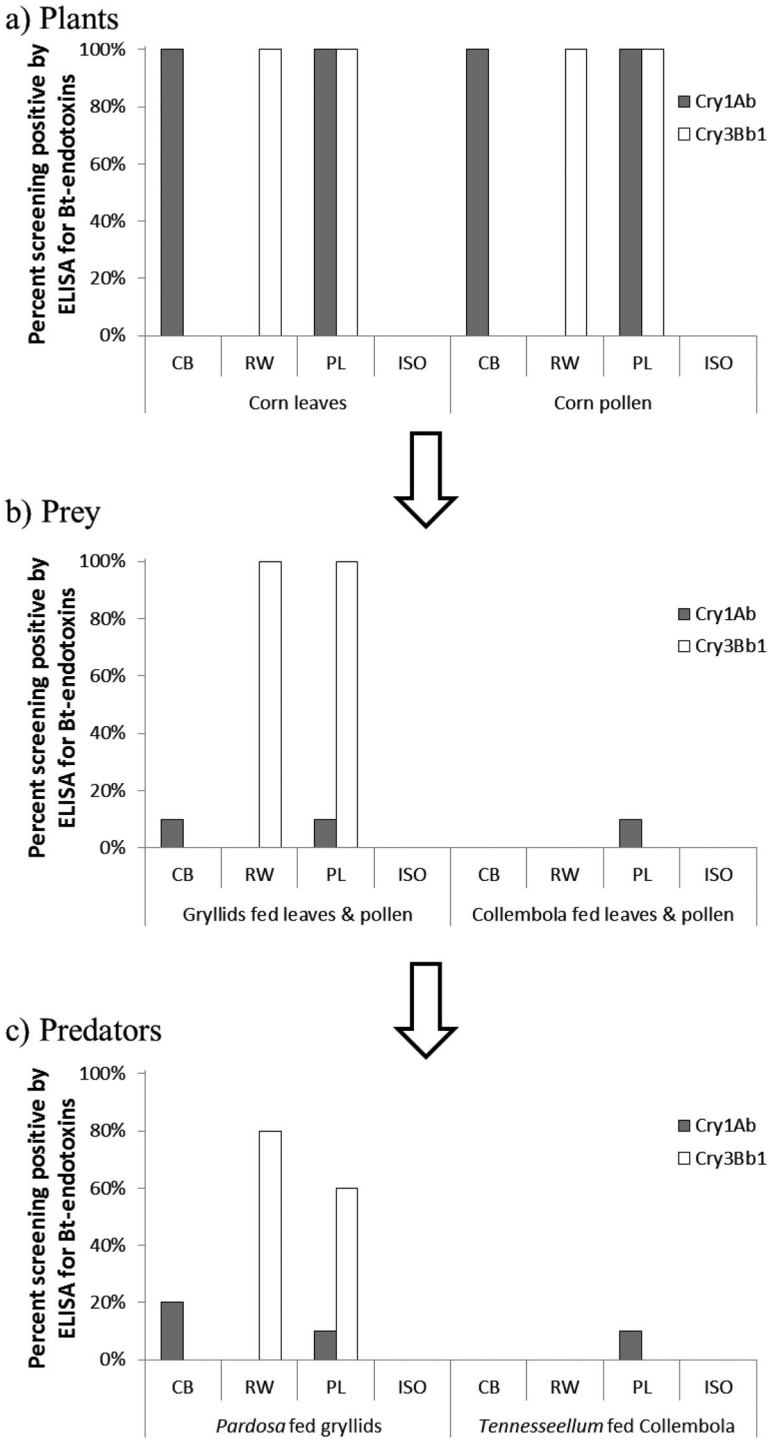

Figure 5. Movement of Bt-endotoxins through prey consumption in laboratory feeding trials. Per cent positives by ELISA for Cry1Ab and Cry3Bb1 for (a) plants, (b) prey, and (c) predators. Arrows indicate movement of Bt-endotoxins through the food chain. CB = YieldGard Corn Borer, RW = YieldGard Rootworm, PL = YieldGard Plus, and ISO = non-transgenic near isoline.

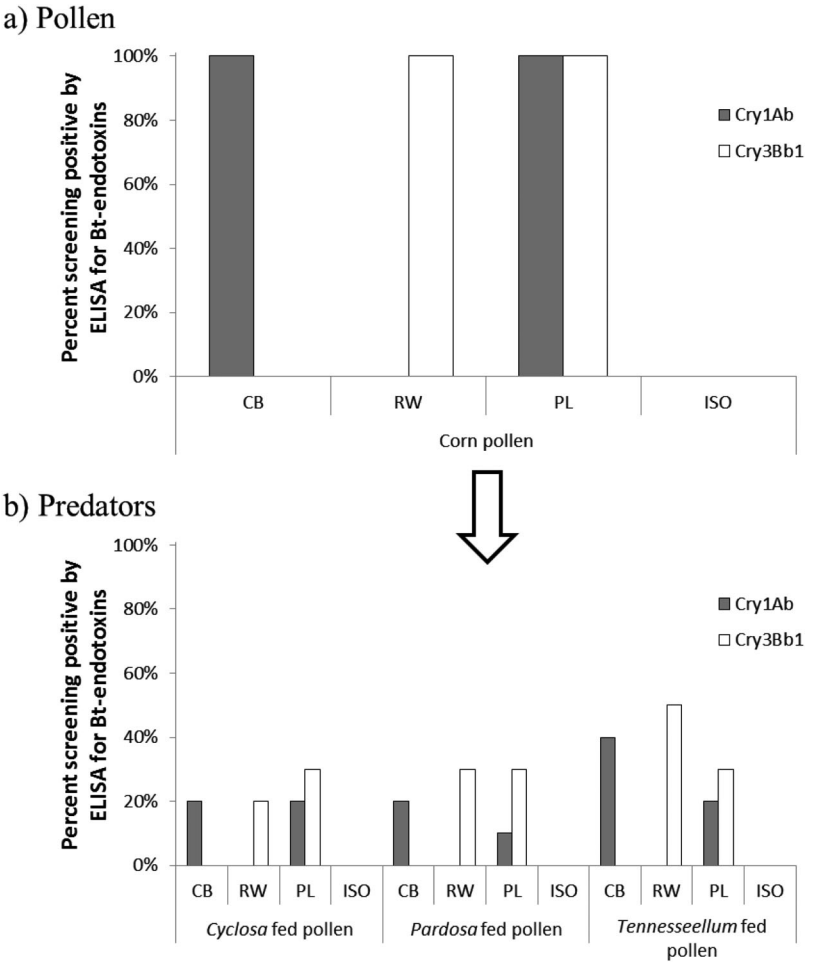

Figure 6. Movement of Bt-endotoxins through pollen consumption in laboratory feeding trials. Per cent positives by ELISA for Cry1Ab and Cry3Bb1 for (a) pollen and (b) predators. Arrow indicates movement of Bt-endotoxins through the food chain. CB = YieldGard Corn Borer, RW = YieldGard Rootworm, PL = YieldGard Plus, and ISO = non-transgenic near isoline.

were fed to *Pardosa*, 20% and 10% tested positive for Cry1Ab after consuming prey that had eaten plant material form the Corn Borer and Plus lines, respectively, and 80% and 60% tested positive for Cry3Bb1 after consuming prey that had eaten plant material from the Rootworm and Plus lines, respectively (Figure 5(c)). When Collembola were fed to *T. formica* spiders, only 10% tested positive for Cry1Ab after consuming prey that had eaten plant material from the Plus line. Three species of spiders that had fed directly on corn pollen also screened positive for the expected Bt-endotoxins that corresponded with the transgenic line consumed (Figure 6(b)). *C. turbinata* screened positive for Cry1Ab proteins in 20% of individuals after consuming pollen from Corn Borer and Plus lines and Cry3Bb1 proteins in 20% and 30% of individuals after consuming Rootworm and Plus pollen, respectively. *Pardosa* sp. screened positive for Cry1Ab proteins in 20% and 10% of individuals after consuming pollen from Corn Borer and Plus lines,

respectively, and Cry3Bb1 proteins in 20% and 10% of individuals after consuming Rootworm and Plus pollen, respectively. *T. formica* screened positive for Cry1Ab proteins in 40% and 20% of individuals after consuming pollen from Corn Borer and Plus lines, respectively, and Cry3Bb1 proteins in 50% and 30% of individuals after consuming Rootworm and Plus pollen, respectively. Chi-square analyses revealed no statistical differences when comparing per cent positives for Cry1Ab (Corn Borer vs. Plus) or Cry3Bb1 (Rootworm vs. Plus) between transgenic lines for all three species. Additionally, no differences were found between the uptake of Cry1 and Cry3 from the Plus line for all three species. There were also no differences in uptake of either protein based on spider species.

4. Discussion

The proportion of spiders screening positive for Cry1Ab and Cry3Bb1 varied depending on the transgenic line from which the spiders were collected, as well as the functional guild and species of the spider (Figures 1–3). Few studies have published data on the presence of Bt-endotoxins in field-collected spiders. Harwood et al. (2005) found that 7.7% (7 of 91) of spiders collected from lepidopteran-targeting transgenic fields tested positive for Cry1Ab, which is slightly less than the 12.2% positive for Cry1Ab reported in this study. Despite being collected from fields at the same research farm, the composition of spiders collected by Harwood et al. (2005) differed from that of the present study: their catch was dominated by Linyphiidae (59%) and Tetragnathidae (27%), with minor contributions from Thomisidae (7%), Theridiidae (3%), and Lycosidae (3%). In the current study, Linyphiidae (62%) and Lycosidae (24%) dominated the catch, while Araneidae (8%) and Tetragnathidae (5%) made minor contributions and all other families accounted for <1% each (Table S1). These differences in the composition of spider samples could be due to annual changes in arachnid communities, as well as the sampling method: Harwood et al. (2005) used visual searching and collection with a hand-held aspirator alone, while the current study used that method plus dry pitfall trapping and collecting from under refuge boards. Pitfall trapping and refuge boards are effective methods for collecting epigeal hunting spiders (functional group: ground runners), such as lycosids (Lang, 2000), which are an important part of the spider community and have received considerable attention in terms of their biological control potential (e.g. Carter & Rypstra, 1995; Halaj, et al. 2000; Nyffeler & Sunderland, 2003), yet this family is almost completely missed when visual searching alone is used in sampling. The current study found that the three most common taxa within the ground runners functional group (*Pardosa* sp., *Allocosa* sp. and immature Lycosidae) were positive for Bt-endotoxins from all but the near isoline field, with up to 55% screening positive; the increased dominance of this group in the spider catch may account for the higher overall per cent positives observed when compared to Harwood et al. (2005).

Numerous potential prey species for spiders tested positive for Bt-endotoxins from the field, including several beetles (Coleoptera), true bugs (Hemiptera), moth larvae (Lepidoptera: Noctuidae), adult and nymphal crickets (Orthoptera: Gryllidae), harvestmen (Opiliones), millipedes (Diplopoda: Julida), centipedes (Chilopoda: Lithobiomorpha, Geophilomorpha), and earthworms (Haplotaxida: Lumbricidae) (Table S2). Previous studies have also shown clear evidence for the uptake of Cry1Ab Bt-endotoxins from transgenic corn by potential prey items, including corn flea beetle *Chaetocnema pulicaria*

(Coleoptera: Chrysomelidae), Japanese beetle *Popillia japonica* (Newman) (Coleoptera: Scarabaeidae), pink spotted lady beetle *Coleomegilla maculata* (DeGeer) (Coleoptera: Coccinellidae), and damsel bug *Nabis roseipennis* Reuter (Hemiptera: Nabidae) (Harwood et al., 2005, 2007; Obrist et al., 2006; Wei et al., 2008; Zwahlen & Andow, 2005). These studies, as well as our own, found a large degree of variation in the uptake of Cry proteins by different prey species (see Table S2). This is not surprising, given that the collected prey belong to a wide variety of trophic guilds and ecological niches. In addition, some prey data may be skewed due to small sample size; due to the polyphagous nature of most spiders, effort was made to collect a variety of prey items rather than focusing on a limited number of prey species. The above-mentioned organisms could all be potential prey items but the soft-bodied insect larvae and hemipterans are most likely to be palatable for these predators. Spiders can consume millipedes (Foelix, 2011) and may also demonstrate high levels of intraguild predation (Wise, 1993) by preying upon other predatory arthropods such as centipedes, harvestmen, and other spiders (Jones, 1975; Lewis, 1981).

Temporal uptake of Bt-endotoxins peaked during and after anthesis for spiders, while the per cent of their prey screening positive for Bt-endotoxins did not increase (Figure 4). This is in contrast to the temporal detection of Cry1Ab in the carabid beetle *Harpalus pensylvanicus* (Peterson et al., 2009) and adult coccinellids (Harwood et al., 2007), which peaked during the post-anthesis phenological period (four to six weeks after the start of anthesis). These data suggest that tri-trophic movement via prey or consumption of other plant tissues, rather than direct pollen feeding is contributing to the uptake of Bt-endotoxins in these beetle species, whereas direct pollen consumption is indicated as a potential route for Bt-endotoxin movement in the field for spiders.

Spiders and prey sampled in 2008 did not vary significantly in their uptake of Cry1Ab between the Corn Borer and Plus fields. However, spiders did show a greater level of detection of Cry1Ab from the Corn Borer and Plus fields when compared to Cry3Bb1 uptake from the Rootworm and Plus fields. Differences in uptake between Cry1 and Cry3 proteins could be due to variable rates of breakdown and excretion by non-target arthropods, as well as differences in the expression of these proteins in the corn plants or differences in sensitivity of the ELISA test. In the present study, ELISA screening of leaf and pollen material from each of the four corn lines yielded identical results for Cry1Ab and Cry3Bb1 expression (Figures 5(a) and 6(a)); however, these data are non-quantitative. Reported concentrations of Bt-endotoxins in MON810 and MON863 events reveal that Cry1Ab proteins are expressed at nearly one order of magnitude lower than Cry3 proteins: 9.35 μg Cry1Ab/g fresh weight and 81 μg Cry3Bb1/g in young leaves (Monsanto, 2002, 2003). This expression profile is the opposite of what might be expected based on the current result that Cry1Ab uptake is higher than Cry3Bb1 for spiders in Kentucky corn fields.

Laboratory feeding trials showed that both Cry1 and Cry3 proteins can be transferred tri-trophically into wolf spider predators through cricket nymph prey; however, very little to no transfer of Bt-endotoxins was observed to be transferred through Collembola into linyphiid spiders. Collembola may be able to rapidly excrete the Cry proteins that they ingest in their food due to a rapid gut passage time (approximately 35 min, Thimm et al., 1998), lack of gut diverticula, and excretion of wastes stored in midgut cells during moulting (Fountain & Hopkin, 2005). However, Yang et al. (2015) detected

Cry1C and Cry2A in *Folsomia candida* Willem (Collembola: Isotomidae) after 14–28 days of feeding on Bt proteins. The concentration of Bt proteins was not measured prior to 14 days, although Cry1C concentration did increase after 28 days, indicating that a longer exposure period may be necessary for significant uptake of Cry proteins by Collembola. The presence of Cry1Ab in corn-fed crickets was low (10% for both Corn Borer and Plus) with similarly low presence in cricket-fed wolf spiders (20% and 10% for Corn Borer and Plus); the increase from 10% of crickets to 20% of wolf spiders positive for Cry1Ab is likely due to a small variation between the subsample of crickets screened for Cry1Ab and the subsample of crickets fed to wolf spiders. The presence of Cry3Bb1 in corn-fed crickets was much higher (100% for both Rootworm and Plus) with similarly high presence in cricket-fed wolf spiders (80% and 60% for Rootworm and Plus); this result was surprising given that a higher percentage of field-collected spiders from Corn Borer and Plus fields were positive for Cry1Ab compared to Rootworm and Plus fields for Cry3bb1. Tri-trophic movement studies involving spiders (reviewed in Peterson et al., 2011) also report movement of various Bt proteins into lycosid, linyphiid, and theridiid spiders from lepidopteran or hemipteran prey fed Bt rice and lacewing, spider mite or corn rootworm prey fed Bt corn (Chen et al., 2009; Han et al., 2015; Jiang et al., 2004; Meissle & Romeis, 2009; Tian et al., 2010). In addition, Meissle & Romeis (2012) found that although Cry3Bb1 was transferred to a theridiid spider via prey consumption, the Bt proteins were rapidly excreted, with Cry3Bb1 concentration decreasing by approximately 90% within five days of feeding. However, Tian et al. (2013) demonstrated that Cry1Ab proteins could accumulate in the wolf spider *Pardosa pseudoannulata* (Araneae: Lycosidae) at approximately 20x the concentration found in their brown planthopper prey.

Our study shows that direct consumption of corn pollen is an exposure pathway for Bt-endotoxin movement for all three of the spider species tested. This is consistent with other studies, which have shown that theridiid spiders screen positive for Cry3Bb1 (Meissle & Romeis, 2009) and araneid spiders screen positive for Cry1Ab (Ludy & Lang, 2006), both from transgenic Bt corn fields in Europe. However, pollen may not be a major route to Bt-endotoxin exposure for all types of Bt crops and all spiders. Yu et al. (2014) found that Cry1Ac was detected in thomisid and linyphiid spiders collected from Bt soybean, but concentrations did not spike during anthesis, indicating that pollen was not a significant exposure pathway in this particular scenario.

Araneae are a diverse taxon, whose role in agroecosystems should not be overlooked. Spiders possess unique traits that allow them to move into and persist in agricultural fields that undergo periodic disturbances. The immature stage of many spiders (Foelix, 2011) as well as the adults of certain groups such as Linyphiidae (Weyman et al., 1995) are capable of 'ballooning' by extruding silk from their spinnerets to catch air currents and 'float' up to several hundred kilometres (Okuma & Kisimoto, 1981). This allows spiders to enter agricultural fields soon after spring cultivation and planting (Riechert & Lockley, 1984; Sunderland et al., 1986). Once established in agricultural fields, spiders may be more likely than other, less polyphagous, predators to remain throughout the season; spiders can subsist on alternative non-pest prey or non-prey resources during periods of low pest abundance, allowing spider populations to 'lie in wait' for when pest prey do arrive (Greenstone, 1999; Harwood et al., 2003, 2004; Settle et al., 1996).

The majority of Bt risk-assessment literature reports no discernible negative effects of consumption of transgenic corn pollen or Bt-containing prey on spiders (reviewed in Peterson et al., 2011; Tian et al., 2010, 2012, 2013). In fact, linyphiid spiders that consume non-Bt corn pollen have enhanced survival (Schmidt et al., 2013) and miturgid spiders that consume cotton pollen have improved survival and development (Pfannenstiel, 2012). However, Zhou et al. (2014) reported that exposure to Cry1Ab can reduce activity of three key metabolic enzymes in a linyphiid and a lycosid spider species commonly found in Bt rice fields of China. Therefore, the effect of uptake of Cry1Ab, Cry3Bb1, and other Bt proteins by spiders from transgenic crop fields must be further studied.

Although the total percentage of Araneae screening positive for Bt-endotoxins in the field was relatively low, the results of this study have highlighted the consumption of Bt-containing prey and direct consumption of corn pollen as potential pathways for Bt-endotoxin uptake for spiders. This confirms two of the pathways proposed in Peterson et al. (2011) for exposure to spiders in the field and provides critical information for Bt risk assessment of Araneae in North America.

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