Toxic and Behavioral Effects to Carabidae of Seed Treatments Used on Cry3Bb1- and Cry1Ab/c-Protected Corn

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ABSTRACT Most transgenic corn seed is now treated with systemic neonicotinoid insecticides. To address potential direct nontarget effects of these combined technologies, 16 Carabidae species from 10 genera (*Agonum*, *Amara*, *Anisodactylus*, *Bembidion*, *Chlaenius*, *Harpalus*, *Patrobus*, *Poecilus*, *Pterostichus*, and *Scarites*) field-collected from corn were directly exposed to *Bacillus thuringiensis* (*Bt*) Cry toxin-laden pollens and seed treatments in feeding and defined-dose bioassays. All adults readily fed on field or sweet corn pollens that expressed coleopteran-specific Cry3Bb1 or lepidopteran-targeting Cry1Ab/c, and no significant toxicity was observed. Adult survivorship ranged from 47 d for the predator *Pterostichus melanarius* (Illiger) to a year for the more omnivorous *Scarites quadriceps* Chaudoir, feeding solely on pollen containing 30–90 μg Cry3Bb1/g and water. In contrast, commercial doses of neonicotinoid seed treatments (imidacloprid, thiamethoxam, or clothianidin) elicited nearly complete mortality for 18 carabid species in 4-d bioassays containing corn seedlings. Carabid consumption of fungicide-only (fludioxonil plus mefenoxam) seed treatments was generally observed within 1 d, compared with a 2-d latency on neonicotinoid treatments, suggesting an antifeedant effect of the insecticide. In microcosm bioassays containing a corn seedling and five prey, clothianidin seed treatments killed adult western corn rootworm, *Diabrotica virgifera virgifera* LeConte and *S. quadriceps*, although the smaller *Harpalus pensylvanicus* (DeGeer) was more tolerant. We conclude that the neonicotinoid/fungicide seed treatments, and not Cry3Bb1 or CryIAb/c, are a major direct mortality factor for ground beetles. Field studies are needed to determine population and community level effects on Carabidae when these transgenic and seed-treatment technologies are combined.

KEY WORDS Bt Cry3B toxin, ground beetles, nontarget effects, neonicotinoid seed treatments

SEED TREATMENTS DIRECTED PRIMARILY against soil-borne pathogen and insect pests constitute a greatly expanding global market valued at $1.2 billion in 2003 (Maude 1996, Dansby 2005). Increasingly the use of seed treatments focus on genetically modified seed, notably corn, cotton, and soybeans, which in 2004 represented 45, 76, and 85%, respectively, of the total acres of these crops planted in the United States (NASS 2004). Corn receives more total pesticide inputs than any other North American crop (Smith et al. 2004). Historically, larval and adult control of the western corn rootworm (*Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae), a major pest of corn in North America and Europe with accrued costs of $1 billion per year in the United States alone, has been highly dependent on nonselective, potently neurotoxic soil insecticides. In 2003, corn expressing *Bacillus thuringiensis* (*Bt*) Cry3Bb1 toxins was launched as a new biotechnology to target this and other diabroticites (Dale et al. 2002, Al-Deeb and Wilde 2003), and replace the human and ecotoxic nerve poisons such as organophosphates and pyrethroids. Previously released events include transgenic corn expressing Cry1Ab/c toxins to protect against the European corn borer, *Ostrinia nubilalis* (Hübner), and other lepidopterous corn pests (Shelton et al. 2002, Yue et al. 2003) and herbicide-tolerant cultivars. Most U.S. corn production now incorporates transgenes that yield glyphosate-, corn borer-, and/or rootworm-ready seeds (Smith et al. 2004), and transgenic technologies are being combined with seed-treatment technologies.

In 60 AD, Pliny noted that crushed cyanogenic cypress, onion sap, or wine were used to protect seeds against insects and pathogens (Jeffs 1986). Brine and dung treatments of seeds during the Reformation were replaced by treatment with arsenicals, mercurials, and copper salts in the 18th century (Leukel 1948). In recent years, systemic pesticides, including broad-spectrum fungicides and novel neonicotinoid insecticides, have replaced the older persistent contact synthetics and inorganics. Now seed enhancement technologies include biological, physical, and chemical agents. Seeds are rapidly becoming the delivery system for many materials including seed protectants

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(fungicides, insecticides, herbicides, elicitors), biological inoculants, nutrients, and genetics (Halmer 2000). Advancements also have been made in providing organic seed dressings with a long shelf life (Taylor et al. 1994).

All commercial Cry-protected field corn seed is currently treated with clothianidin, thiamethoxam, or imidacloprid, which are systemic neonicotinoid insecticides that protect against root and seedling feeding (Smith et al. 2004). These treatments are also very effective against Stewart's wilt vectored by corn flea beetles (Kuhar et al. 2002, Pataky et al. 2005), which renders the seed unsuitable for export to countries like South Africa that do not have this disease. All insecticidal corn seed treatments are incorporated with a multicomponent fungicidal seed dressing broadly used on other crop seeds to prevent diseases (Taylor and Harman 1990). Seed treatments are an excellent strategy to minimize above-ground exposures and environmental loadings of pesticides (Lange 1959, Jeffs 1986, Martin 1988).

While these inputs aid in stand establishment, there is a need to optimize the treatment doses so that beneficial populations of arthropods are not harmed. Carabids are soil-dwelling arthropods susceptible to insecticides and other environmental pollutants, and reductions in carabid abundance and diversity have been observed in many crops after insecticide applications (Humphrey and Dahm 1976, Ellsbury et al. 1998, Albajes et al. 2003). Incorporation of Cry1Ab/c or other lepidopteran-targeting *Bt* toxins may also alter ground-beetle communities (French et al. 2004) but more often seems to influence population levels of above-ground nontarget species (Betz et al. 2000, Groot and Dicke 2002, Jasinski et al. 2003, Andow and Hilbeck 2004, Dively et al. 2004).

Incorporation of the coleopteran-specific Cry3Bb1 toxins into corn has provoked the need to assess its impact on nontarget Coleoptera such as coccinelids and carabids. For the former pollen-feeding predator group, ingestion of Mon 863 laden corn pollen has resulted in low or negligible impact (Duan et al. 2002, Lundgren and Wiedenmann 2002, Al-Deeb and Wilde 2003). Some adult Carabidae, mostly from the genus *Harpalus*, feed incidentally on pollens (Larochelle and Larivière 2003), whereas many others are seed predators (Lund and Turpin 1977, Tooley and Brust 2002). Pollen is an excellent diet component for enhancing fecundity in many adult beetles including western corn rootworm (Kim and Mullin 2003), and our experience in developing optimal bioassays for pollen feeding studies with diabroticites led us to adapt corn pollen as a vehicle to study the toxic impact of chronic ingestion of *Bt* Cry toxins on carabid beetles.

Laboratory protocols are needed to evaluate risk or predict nontarget effects of *Bt* Cry toxins and associated seed-treatment technologies on key indicator arthropods in the corn agroecosystem. Here *Bt* field corn and sweet corn expressing Cry3Bb1 and Cry1Ab/c, respectively, in field plots that included alfalfa and snap beans, were the source of all Carabidae and some western corn rootworm used in laboratory experiments. The primary goal of these studies was to develop bioassays to assess the individual and combined toxicities or behavioral effects of corn-expressed Cry3Bb1 toxin and representative commercial corn seed treatments on the ground beetle community.

Materials and Methods

Carabidae and Adult Western Corn Rootworm. Ground beetle adults were collected alive from pitfall traps placed in low- or no pesticide input sections of a split-plot field experiment at the R. E. Larson Field Research Center at Rock Springs, PA. Twelve acres were planted in a split plot design: four 1-acre main plots consisted of a 2 by 2 factorial arrangement of genotype (transgenic versus isoline) and insecticide management (managed versus unmanaged) and was replicated three times. Main plots were split among field corn, sweet corn, and snap beans (for rotational purposes) and were surrounded by alfalfa to establish a common adjacent boundary. Carabid species selected were those common in pitfall sampling and included members from the *Agonum*, *Amara*, *Anisodactylus*, *Bembidion*, *Chlaenius*, *Harpalus*, *Patrobus*, *Poecilus*, *Pterostichus*, and *Scarites* genera (Table 1). Before bioassay, ground beetles were maintained for at least 2 d on crushed Dad's kitten food (Meadville, PA) and water ad libitum in plastic rearing chambers (32 by 24 by 11 cm) containing a moistened 2.5-cm layer of Scotts Metro-Mix 200 soil previously sieved through a 1-mm screen. Voucher specimens are deposited in the Frost Entomological Museum, Pennsylvania State University.

A nondiapausing laboratory strain of western corn rootworm was obtained from French Agricultural Research (Lamberton, MN) as pupae in soil, and the emerged adults were placed into a plastic rearing chamber (30 by 17 by 9 cm). Adult beetles were fed isoline corn pollen and water ad libitum (Kim and Mullin 2003) for at least 2 d before bioassay. All beetles were kept at 22 ± 1°C and a 14 L:10 D photoperiod under cool-white fluorescent lights in a Revco model RIXX555 Incubator (Thermo Electron, Asheville, NC).

Corn Pollens and Seeds. Field or sweet corn pollen for adult corn rootworm and Carabidae feeding studies were obtained from cut corn tassels collected from the unmanaged (no foliar insecticide) portions of the field plot. *Zea mays* L. variety DeKalb DKC60-12 (YGRW) and DKC60-17 (RR2) field corns from Monsanto (St. Louis, MO), and Rogers (Syngenta Seeds, Boise, ID) Attribute WSS 0984 and Boreal sweet corns were the sources for Cry3Bb1, Cry1Ab/c, and near isoline pollens, respectively. Inflorescences were cut with ≈30 cm of stem, placed in water, and allowed to shed pollen into an aluminum tray (Kim and Mullin 2003). Pollen was sieved consecutively through 710-μm brass mesh followed by 256- and 153-μm Nitex screens (Tetko, Briarcliff Manor, NY) using vortex agitation to remove plant and animal debris. Pollen was stored at −20°C until use. Corn seeds and respec-

Table 1. Weights and longevity of adult Carabidae feeding on transgenic corn pollen

Carabidae species	Average weight (mg ± 95% CI)	*n*	Survival time (days ± 95% CI)[a] Cry1Ab/c	Cry3Bb1	All pollens	*n*
Agonum cupripenne (Say)	23.0 ± 3.1	6	171 ± 98	106 ± 17	141 ± 56abc	11
Agonum muelleri (Herbst)	21.7 ± 1.6	6	112	—	112	1
Agonum placidum (Say)	22	1	—	82	82	1
Amara pennsylvanicus (Hayward)	82	1	—	137	137	1
Anisodactylus sanctaecrucis (Fabricius)	43.4 ± 4.5	5	3	—	3	1
Bembidion quadrimaculatum oppositum Say	1.5 ± 0.2	3	12	—	12	1
Chlaenius tricolor tricolor Dejean	71.2 ± 3.1	56	91 ± 83	129 ± 144	110 ± 76abc	6
Harpalus affinis (Shrank)	48.3 ± 7.9	3	133	37	85	2
Harpalus caliginosus (Fabricius)	347 ± 39	7	—	91	91	1
Harpalus herbivagus Say	27	1	8	—	8	1
Harpalus pensylvanicus (DeGeer)	139.2 ± 7.1	73	180 ± 104	249 ± 160	215 ± 92ab	8
Patrobus longicornis (Say)	70.0 ± 7.8	2	—	46	46	1
Poecilus chalcites (Say)	60.7 ± 3.1	58	73 ± 54	134 ± 110	103 ± 61bc	6
Poecilus lucublandus lucublandus (Say)	81	1	145 ± 108	151 ± 94	148 ± 64abc	6
Pterostichus melanarius (Illiger)	145.7 ± 7.6	60	25 ± 17	70 ± 74	47 ± 39c	8
Scarites quadriceps Chaudoir	519 ± 20	63	177	246 ± 191	223 ± 119a	3

[a] ANOVA based on those seven species where we had replication. There were no significant differences between Cry1Ab/c and Cry3Bb pollens. Differences among species in survival time on all pollens are noted by different letters (LSD test at $P < 0.05$).

tive commercial pesticide treatments used in bioassays are presented in Table 2.

Pollen Feeding Bioassays. Filtered pollens from field-collected *Bt* corn tassels and their near isolines were presented to individual beetles in a 100 by 15-mm petri dish arena with water ad libitum. Pollen (5–25 mg depending on size of beetle) was placed on a 7.5-cm Whatman No. 2 filter paper, and distilled water (1 ml) was added as a pool on the edge of the chamber. New water and pollen were added every 2–3 d (three times per week), and the beetle was transferred to a new dish with filter paper every 2–4 wk, depending on the size and fecal output of the carabid. Dishes were kept in the darkness of a closed laboratory bench drawer at room temperature. We conducted pollen feeding bioassays on six species of adult Carabidae, replicated three to six times per species, using two primary treatments: (1) Coleoptera-selective Cry3Bb1 pollen and (2) Lepidoptera-selective Cry1Ab/c. We also conducted limited pollen feeding tests with 10 other carabid species. We compared adult survivorship on pollen, and each specimen was archived for confirmation of identification.

Seed Treatment Bioassays. Commercial corn seeds with pesticidal treatments (Table 2) were germinated between moistened Whatman No. 2 filter papers in dark cabinets at room temperature over a 2- to 3-d period. Newly emerged corn seedlings were presented to individual carabid beetles for at least 4 d in a petri dish with distilled water added as needed as in the pollen-feeding bioassay, except that a 5.5-cm filter paper was used. Five fungicide/insecticide treatments with or without Cry3Bb1 (Table 2) were presented individually to members of five species of Carabidae, replicated 10–18 times. For each replicate, date of first seedling consumption, date of mortality, and poison symptoms were recorded daily for at least 4 d. A beetle was scored as dead if unable to move an appendage after prodding or if on the last test day it was unable to right itself from its back and had been on its back for at least 48 h. The initial dry seed weight, final fresh beetle weight (dead or alive), and final dried seedling weight were noted for each dish, and the beetle specimen was archived. Tests using fewer seed treatments were conducted on 13 additional carabid species depending on availability, with up to 10 replicates per treatment.

Microcosm Bioassays. One seed each of DKC60-17 (near isoline) or DKC60-12 YGRW (Poncho 250; 250 μg clothianidin/seed) was planted 2 cm deep in 250 ml of Metro-Mix 200 sieved through a 1-mm screen and placed in a 0.95-liter wide-mouth Mason jar. One carabid and five adult *D. v. virgifera* were introduced to the spiked seedling that emerged under sunlight after

Table 2. Seed and seed treatments used in bioassays[a]

Corn seed	Seed treatment Code	Fungicide	Neonicotinoid (μg/seed)	Average seed weight (mg ± 95% CI)
DKC60-17 (isoline)	F	Maxim xl	0	245 ± 11
DKC60-15 (isoline)	F	Maxim xl	0	279 ± 15
DKC60-12 (Cry3Bb1)	G	Maxim xl	160 imidacloprid	280 ± 8
DKC60-12 (Cry3Bb1)	P	Maxim xl	250 clothianidin	230 ± 16
34F41 (Cry3Bb1)	C	Maxim xl	800 thiamethoxam	281 ± 4

[a] DKC60 (DeKalb), 34F41 (Pioneer Hi-bred, Johnston, IA): F, fungicides only; G, Gaucho; P, Poncho (Gustafson, now Bayer CropScience, Research Triangle Park, NC); C, Cruiser (Syngenta Crop Protection, Greensboro, NC); Cry3Bb1, Yieldgard Ready Rootworm (Monsanto Co., St. Louis, MO); Maxim xl, fludioxonil/mefenoxam (2.5/1; Syngenta).

daily watering over a 6-d period. A 1.5-ml Eppendorf watering vial was added, and the jar capped with shading screen. *Scarites quadriceps* Chaudoir and *Harpalus pensylvanicus* (DeGeer) were each tested in 8–13 replications of two treatments. Beetle mortality/neurotoxicity symptoms and plant feeding were scored daily over at least 4 d at 22°C and under a 14 L:10 D photoperiod in a Revco RIXX555 Incubator (Asheville, NC). The remaining corn seedling was washed free of soil particles and dried, and the total plant height above the crown, maximum root length, and total dry biomass above and below the crown were recorded.

Cry3Bb1 Immunoassay for Corn Pollen. Ten to 30 mg fresh weight of corn pollen from storage at −20°C were mixed with 1 ml of pH 10.5 extraction buffer (50 mM sodium borate decahydrate, 0.75 M potassium chloride, 0.075% Tween 20, and 10 mM ascorbic acid; Palm et al. 1994) in a 16 by 100-mm culture tube. The mixture was vortexed for 30 s, sonicated for 30 s, and centrifuged at 3,000 rpm for 5 min. The supernatant was pipetted into a 1.5-ml microcentrifuge tube, and the pollen pellet was re-extracted as before with 0.5 ml of buffer. The pooled extract was stored at −20°C until immunoanalysis was performed.

YGRW Cry delta endotoxin was measured using the Pathoscreen Bt-Cry3Bb1 enzyme-linked immunosorbent assay (ELISA) kit (Agdia, Elkhart, IN), employing a sandwich ELISA format, peroxidase reporter, and 96-well microplate precoated with anti-Cry3Bb1 antibodies. Sample extracts were thawed and vortexed. Twenty microliters or less of extract was pipetted into each well containing phosphate-buffered saline (PBS), Tween 20, and horseradish peroxidase–conjugated anti-Cry3Bb1antibodies, according to the manufacturer's protocol. Plates were incubated at 25°C for 2 h. After plate washing with PBS, antibody-bound Cry residues were detected by incubation with the 3,3′,5,5′-tetramethylbenzidine substrate, and absorbance at 650 nm was recorded using a Molecular Devices SpectraMax 190 plate reader (Sunnyvale, CA). Standard curves for quantitation were generated using the negative controls and the *Bt* Cry toxin standards available from Agdia, with a detection limit of 1 ng/ml extract for Cry3Bb1.

Statistical Analysis. The influence of seed treatment and carabid species on adult survivorship, latency-to-mortality, carabid predation on western corn rootworm, and all seedling measurements were analyzed with analysis of variance (ANOVA), and means were separated with the least significant difference (LSD) test, using PROC GLM in SAS version 9.1.3 (SAS Institute, Cary, NC). Proportion mortality of carabids or corn rootworms was analyzed as a categorical variable using PROC CATMOD in SAS 9.1.3. The concentration of Cry3Bb1 in corn pollen was calculated using simple linear regression of absorbance against dose (inverse prediction). Analyses were performed on the linear response range (at least four doses) of the dose–response curves. All error bars represent the mean ± 95% confidence interval (CI). The significance of a treatment or species effect was evaluated at $\alpha = 0.05$.

Results

Carabid Pollen Feeding. All 16 adult carabid species tested readily fed on corn pollen (Table 1), which therefore provided a convenient vehicle for oral exposure of beetles to relatively high doses of Cry toxins. Coleopteran-selective *Bt* pollen was statistically compared with Rogers WSS 0984 sweet corn pollen expressing the lepidopteran-targeting Cry1Ab/c toxins. For the seven species where we had replication (species with 95% CI; Table 1), there was no species by pollen interaction ($F = 0.38$; df = 6,33; $P = 0.89$). No significant carabid toxicity, as measured by adult survivorship, was attributable to the pollens ($F = 0.90$; df = 1,33; $P = 0.35$): Carabid survival on Cry3Bb1 pollen (136 ± 38 d) was equivalent or somewhat better than on Cry1Ab/c (111 ± 35 d) over all species. Indeed beetles survived, on average, 123 ± 26 d (95% CI) over all species/treatments (Table 1) solely on pollen and water in these small arenas. A species difference was apparent ($F = 2.53$; df = 6,33; $P = 0.04$) in that the more dedicated carnivore *Pterostichus melanarius* (Illiger) survived a significantly shorter time (47 d) on both *Bt* corn pollen diets than the more omnivorous *H. pensylvanicus* (215 d) and *S. quadriceps* (223 d). Larger beetles like *Harpalus caliginosus* (F.) consumed up to 50 mg pollen per day, and *S. quadriceps* and *H. pensylvanicus* lived up to 1 yr on Cry3Bb1 pollen.

YieldGard Rootworm Corn (DKC60-12) from the MON 863 event expressed from 30 to 90 μg Cry3Bb1/g pollen based on immunoassay results.

Germinated Seed Treatment Bioassays. To assess the acute toxicities caused by commercial corn seed treatments, newly germinated corn seedlings from fungicide- and insecticide-treated seeds were presented to individual carabid beetles for at least 4 d in a petri dish arena (Fig. 1). Mortality rates in these bioassays were significantly influenced by both species ($\chi^2 = 13.8$; df = 4; $P < 0.008$) and seed treatment ($\chi^2 = 88.7$; df = 4; $P < 0.001$). The 95% CIs for 4-d percentage mortality overlapped among *Chlaenius tricolor tricolor* Dejean, *H. pensylvanicus*, and *P. melanarius* on imidacloprid, clothianidin, and thiamethoxam, respectively. *Poecilus chalcites* (Say) had some tolerance to imidacloprid at 160 μg/seed (Fig. 1). An additional 13 taxa were bioassayed on a subset of the seed treatments dependent on availability of live beetles from the field. For these species and seed treatment bioassays, *Agonum cupripenne* (Say), *A. muelleri* (Herbst), *A. placidum* (Say), *Amara pennsylvanicus* (Hayward), *Amara* spp. (*aenea* or *impuncticalis*), *Anisodactylus sanctaecrucis* (F.), *Bembidium rapidum* (LeConte), and *Harpalus affinis* (Shrank) were all killed by the thiamethoxam corn seed treatment, except for a 90 ± 20% 4-d mortality in the case of *A. muelleri* ($n = 10$). *A. muelleri*, *Amara* spp., *A. sanctaecrucis*, *H. affinis*, and *Harpalus herbivagus* Say were all killed by the clothianidin treatment, whereas

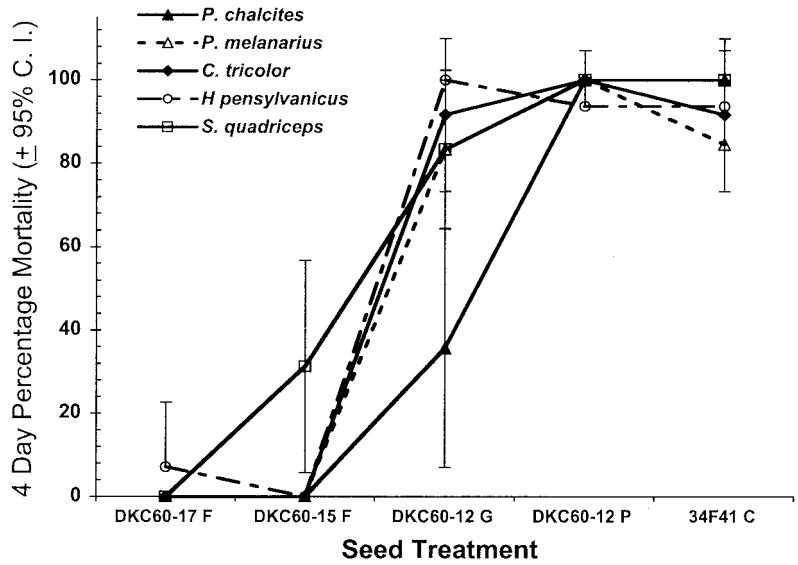

Fig. 1. Acute toxicty (as percentage mortality) of corn seed treatments on five species of Carabidae after 4 d of exposure in a germinated seed bioassay. DKC60-17 and DKC60-15 are near isolines, whereas DKC60-12 and 34F41 express Cry3Bb1. F, fungicides only, Maxim xl (21% fludioxonil, 8.4% mefenoxam); G, Gaucho (160 μg imidacloprid/seed) plus F; P, Poncho (250 μg clothianidin/seed) plus F; C, Cruiser (800 μg thiamethoxam/seed) plus F.

A. pennsylvanicus, *A. sanctaecrucis*, *Bembidium quadrimaculatum oppositum* Say, *H. herbivagus*, *H. caliginosus* ($n = 6$), *Patrobus longicornis* Say, and *Poecilus lucublandus lucublandus* (Say) were all killed by imidacloprid. Overall, except for *P. chalcites* on the 160-μg rate of imidacloprid, the insecticide treatments caused nearly 100% mortality in 17 of the 18 Carabidae species or taxa tested.

Susceptibility to fungicide treatments was lower, or absent, and varied among species. Surprisingly, larger species such as *S. quadriceps* (Table 1) tended to be more susceptible to the fludioxonil and mefenoxam fungicide blend (Maxim xl; 31 ± 26% 4-d mortality on DKC60-15 F; $n = 16$) than smaller species like *P. chalcites* (0 ± 0%; $n = 28$ for DKC60-15 and -17 F), which also had some tolerance to imidacloprid as reported above (Fig. 1). Little or no mortality was observed for *C. tricolor*, *H. pensylvanicus*, *P. chalcites*, and *P. melanarius* on any fungicide treatments (Fig. 1). There was also no acute toxicity found for any of the other carabid species tested (Table 1) on the fungicide-only treatments.

The rate at which seed treatments caused mortality varied among species ($F = 3.0$; df = 17,322; $P < 0.001$). Among the neonicotinoid seed treatments applied at commercial doses (Table 2), the clothianidin and thiamethoxam treatments generally acted more rapidly on ground beetles than imidacloprid (Table 3) at its low-

Table 3. Mean no. of days surviving and feeding period for adult Carabidae in a seedling bioassay using transgenic and isoline seeds treated with neonicotinoid plus fungicide seed treatments or fungicide-only controls

	Carabidae species	Seed source, transgene, and seed treatment				
		34F41 Cry3Bb1 thiamethoxam	DKC60-12 Cry3Bb1 clothianidin	DKC60-12 Cry3Bb1 imidacloprid	DKC60-15 isoline fungicide	DKC60-17 isoline fungicide
Days surviving[a]	*Chlaenius tricolor tricolor*	2.4c	2.4c	4.0b	5.0a	5.0a
	Harpalus pensylvanicus	2.6c	3.3bc	3.7b	5.0a	5.0a
	Poecilus chalcites	2.8b	2.7b	5.5a	5.0a	5.0a
	Pterostichus melanarius	2.5c	2.1c	3.9b	5.0a	5.0a
	Scarites quadriceps	2.1c	2.1c	4.7ab	4.4ab	5.5a
Feeding period[b]	*Chlaenius tricolor tricolor*	1.3bc	0.6c	1.3bc	2.8a	2.2ab
	Harpalus pensylvanicus	1.4c	1.9c	2.1bc	3.6a	2.9ab
	Poecilus chalcites	0.8c	0.4c	1.6b	3.2a	3.2a
	Pterostichus melanarius	1.2b	0.6b	1.3b	2.9a	3.0a
	Scarites quadriceps	0.7c	1.5c	2.7b	2.6b	3.8a

Means for species among seed treatments followed by the same letter are not significantly different (LSD test, $\alpha = 0.05$).

[a] The 4-d standard bioassay period was prolonged up to 8 d for some imidacloprid treatments to determine day of death for tolerant carabids. Only beetles showing acute symptoms of toxicity 2 d before final observation were scored as dead; survival in most fungicide treatments was at least 5 d.

[b] Based on a 4-d standard bioassay.

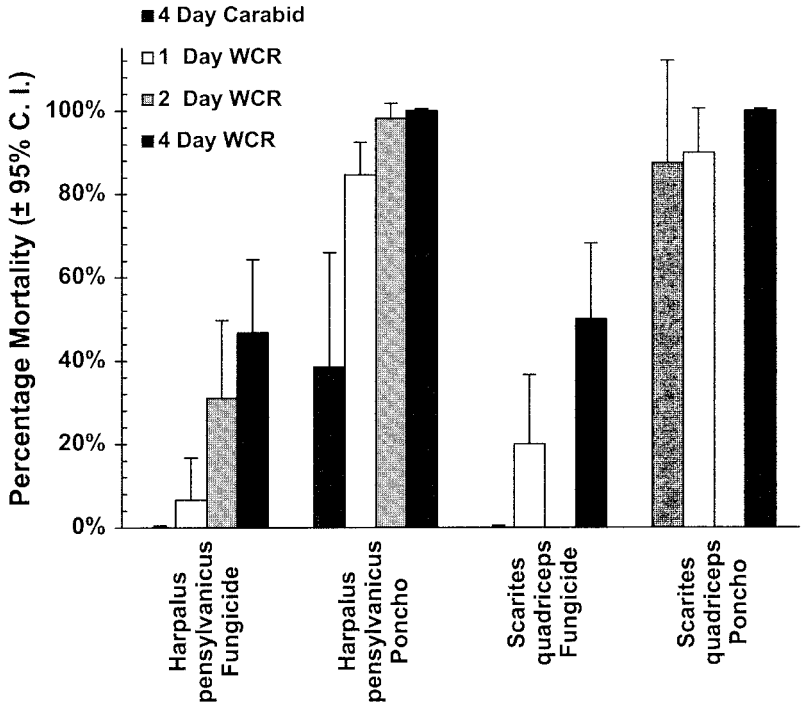

Fig. 2. Acute toxicity (as percentage mortality) of seed treatments on adult Carabidae and western corn rootworms in a corn seedling microcosm bioassay. Fungicide, Maxim xl (21% fludioxonil, 8.4% mefenoxam) on the near isoline DKC60-17; Poncho, 250 μg clothianidin/seed plus Maxim xl on the Cry3Bb1-expressing DKC60-12.

est EPA-registered use rate of 160 μg/seed. Average survival time of *S. quadriceps* and *P. chalcites* on imidacloprid-treated corn was about twice as long as that on clothianidin and thiamethoxam (Table 3). Similar significant differences in latency of toxicity for imidacloprid were found with *C. tricolor* and *P. melanarius*, whereas survival times for *H. pensylvanicus* were more similar across treatments (Table 3). *P. chalcites* and even the more susceptible *H. pensylvanicus* recovered more from imidacloprid paralysis than the other carabid species tested here. However, 20% at most of individuals for these tolerant species exhibited early signs of recovery from paralysis to the newer generation neonicotinoids, clothianidin and thiamethoxam, only to die later within the 4-d bioassay. Because of some beetle species recovering from toxication, bioassays with imidacloprid were sometimes prolonged to 6 d of assessment, and beetles were scored as dead only if paralyzed for at least 2 consecutive d.

Consumption of fungicide treatments in these seedling toxicity bioassays was generally observed within 24 h, with an average total feeding period (Table 3) of 3.05 ± 0.15 d (n = 131). In contrast, there was usually a 1 d or more latency to feeding in the neonicotinoid treatments with an average total feeding period of 1.88 ± 0.16 d (n = 71) for imidacloprid, 1.07 ± 0.35 d (n = 67) for clothianidin, and 1.06 ± 0.37 d (n = 66) for thiamethoxam. Days of feeding were similar across species for each treatment, although *S. quadriceps* was more sensitive to feeding inhibition by the fungicide treatment of isoline DKC60-15 than the other carabids species tested (Table 3).

Treated Seedling Microcosm Bioassays. In seedling microcosms with treated seed, western corn rootworm prey, and carabid adults, corn rootworm mortality from predation was not affected by carabid species (F = 0.86; df = 1,37; P = 0.35), seed treatment (F = 0.68; df = 1,37; P = 0.42), or their interaction (F = 2.5; df = 1,37; P = 0.12). Over the 4-d bioassay, carabids consumed an average of 2.3 of the 5 corn rootworm adults (46%) in the fungicide-only treatments compared with 1.9 (38%) in the neonicotinoid seed treatment. Thus, in the fungicide-only treatment, essentially all the rootworm mortality was caused by carabid predation. However, total corn rootworm mortality was caused by both predation and intoxication. This total mortality (Fig. 2) was influenced by the seed treatment (χ^2 = 79; df = 1; P < 0.0001), but not carabid species (χ^2 = 3.6; df = 1; P < 0.06), within 24 h, and this pattern continued for the 2- and 4-d analysis of mortality rates. Clothianidin at 250 μg/seed (Poncho 250) rapidly killed western corn rootworm (>90% in 1 d regardless of which carabid species was present) compared with the fungicide fludioxonil plus mefenoxam (Maxim xl) control treatment.

No carabid mortality for either species was seen in the fungicide-alone treatment; however, microcosm assays with clothianidin-treated corn were lethal to

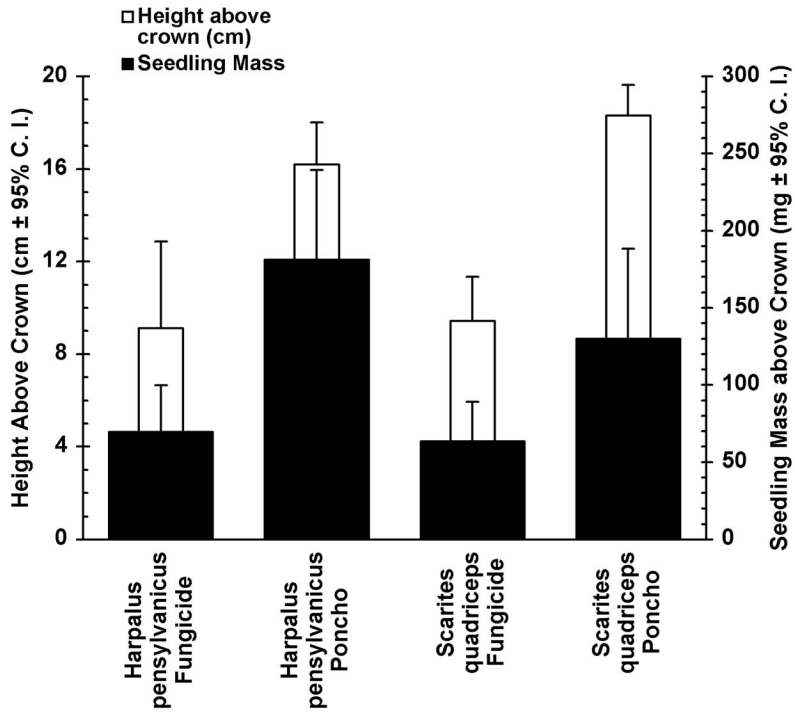

Fig. 3. Impact of seed treatments on corn seedling height and mass in a microcosm bioassay containing adult Carabidae and western corn rootworms. Fungicide, Maxim xl on the near isoline DKC60-17; Poncho, 250 μg clothianidin/seed plus Maxim xl on the Cry3Bb1-expressing DKC60-12.

carabids and caused greater mortality to *S. quadriceps* ($88 \pm 24\%$; $n = 8$) than *H. pensylvanicus* ($38 \pm 27\%$; $n = 13$) over the 4-d bioassay ($\chi^2 = 79$; df = 1; $P < 0.03$). Carabid exposure to clothianidin was at least partly through ingestion of toxified prey. Among the Carabidae, intoxication from this nerve poison required more time than for *D. v. virgifera.* Generally a 4-d latency was required for *S. quadriceps* mortality, and there was less acute toxicity for *H. pensylvanicus* (Fig. 2).

Seedling responses in the jar microcosm were observed only in above-ground measurements (Fig. 3), where seed treatment influenced seedling mass ($F = 12.6$; df = 1,37; $P < 0.001$) and height ($F = 33$; df = 1,37; $P < 0001$). Obvious below- and above-ground feeding by carabids on seedlings was not noted, whereas adult western corn rootworm above-ground feeding was readily observed; thus, feeding on the seedling was attributed to corn rootworm. This herbivory lowered final seedling mass and height of fungicide-only seedlings (67 mg, 9.3 cm) compared with those in the clothianidin treatment (162 mg, 17.0 cm). Root length and dry mass below the crown were not impacted significantly by the seed treatments ($F = 0.18$; df = 1,35; $P = 0.67$ for root length; $F = 0.25$; df = 1,37; $P = 0.62$ for dry mass). No effects on seedlings could be attributed to carabid species or the interaction of carabid species with seed treatment.

Discussion

Our goal was to develop bioassays and study the potential impact of *Bt* Cry toxins and seed treatments used in commercial agriculture on ground beetle biodiversity in field and sweet corn agroecosystems. Carabidae from our field plots comprised 37 species (T.W.L., unpublished data). A series of laboratory-feeding and defined-dose bioassays effectively delivered or exposed representative carabid species collected from these field plots to Cry toxins and seed treatments. We found that the neonicotinoid/fungicide seed treatments, and not Cry3Bb1, were a major mortality factor for the 16 ground beetle species tested. Corn pollen expressing 30–90 μg Cry3Bb1/g pollen based on immunoassay was fed to 16 carabid species. This coleopteran-selective *Bt* pollen was compared with a sweet corn pollen expressing the lepidopteran-targeting Cry1Ab/c toxins. No reduction in adult carabid survivorship was noted for either treatment. Beetles survived, on average, >123 d solely on pollen and water. We did not anticipate that ground beetles would survive for such a long period with a single plant food source in a small petri dish arena. These beetles were actively consuming pollen during the full course of the bioassay. We considered longevity beyond 30 d to indicate lack of treatment-dependent acute toxicity that we could measure in this bioassay. Parallel tests with non-Cry toxin isolines,

which would have required months of time to complete, were therefore not conducted. This study is the first to document the broad capability of a diverse range of adult carabid species, representing many feeding ecologies (seed predator, carnivore, omnivore; Larochelle and Larivière 2003), to survive long-term on pollen as a sole food source.

Other fitness factors potentially impacted by chronic ingestion of Cry3Bb1 such as time in immature life stages, growth rates, and fecundity were not tested here because of insufficient knowledge on successful full-generational rearing of the carbabid species used, although some are known to take >1 yr to develop as larvae. In addition, only very low levels of Cry 3Bb1 protein (<1% of that ingested by adult Carabidae in pollen-feeding bioassays) were found in our northeastern soil extracts from transgenic field plots (unpublished data) and in soils from Kansas (Ahmad et al. 2005). These results suggest that Cry3Bb1 corn rootworm toxins from transgenic corn tissues do not target the adult stage of these generally predaceous beetles, as noted for the coccinellid *Coleomegilla maculata* DeGeer fed pollen in the laboratory (Duan et al. 2002, Lundgren and Wiedenmann 2002) and in field experiments (Al-Deeb and Wilde 2003). Future experiments are needed to address the chronic effects of beetle-selective Cry toxins on larvae and other carabid life stages. Adult carabids may have low susceptibility to beetle-targeting Cry toxins because of tolerance conferred by a preoral proteolytic or foregut digestive strategy (Cheeseman and Gillott 1987) that relies on regurgitated proteases from a more toxin-susceptible, noncuticular mid-gut. Enhanced proteolytic degradation of ingested Cry toxins within the insect midgut is another mechanism by which insects can resist Bt toxins (Oppert 1999).

In contrast, systemic neonicotinoid seed treatments, including imidacloprid, thiamethoxam, and clothianidin at the doses used commercially, elicited nearly complete mortality among all 18 Carabidae species tested in petri dish seedling bioassays (Fig. 1; Table 1). The high acute toxicity for adult *D. v. virgifera* (>90% in 24 h) feeding on transgenic corn seedlings germinated on filter papers (Mullin, unpublished data) led us to examine seed treatments as a possible mortality factor for ground beetles. Western corn rootworm is the targeted pest species for the Cry3Bb1 toxin and the adult is a model pollen feeder for studying phytochemical-insect interactions (Mullin and Kim 2001). Similar carabid acute toxicity results from the seedling bioassays were observed in the jar microcosm bioassay comprising soil, 6-d corn seedlings, and corn rootworm prey, where clothianidin seed treatments killed adult *S. quadriceps* relative to a Maxim xl control (Fig. 2), although the smaller *H. pensylvanicus* was more tolerant. This latter bioassay better represents a field situation for the carabid where alternative foods, a soil medium for burrowing, and an actively respiring corn seedling are present. Industrial laboratory trials on nontarget arthropods to support EU registration of clothianidin also indicated its moderate to high toxicity for adult and particularly larval *Poecilus cupreus* L. in unspecified small-scale tests with treated corn and other seeds (Schmuck and Keppler 2003). Further development of our microcosm arena would provide opportunities to dilute the initial high dose of the seed treatment through weathering into the rhizosphere and uptake into a rapidly growing plant that should enhance degradation of this neurotoxicant and decrease exposure to the nontarget insect. Based on the high carabid mortality in the microcosm test, we predict that spring emerging adult carabid species such as *S. quadriceps*, which are predacious on corn rootworm (Larochelle and Larivière 2003) and on seeds as observed here, will be impacted by direct or indirect exposures to neonicotinoid seed treatments.

Carabids are among the most tolerant family of arthropods to pesticide treatments relative to their prey (Croft 1990), and thus survive toxic environments where many other beneficials fail. It is surprising that a large carabid species such as the 519-mg *S. quadriceps* (Table 1) is more susceptible to the neonicotinoid seed treatments than the 139-mg *H. pensylvanicus* (Fig. 2) or the 61-mg *P. chalcites* (Fig. 1). Generally, larger ground beetles are more tolerant to other classes of insecticides, including cyclodienes, organophosphates, carbamates, and pyrethroids, than smaller species (reviewed in Croft 1990). Kunkel et al. (2001) reported a greater toxicity of the carbamate bendiocarb than the neonicotinoid imidacloprid on adult *H. pensylvanicus* by contact or ingestion at turfgrass spray rates. This was primarily caused by beetles recovering from imidacloprid intoxication a few days after exposure, although exposed beetles under field conditions suffered high predation by ants before recovery. While *H. pensylvanicus* and *P. chalcites* recovered more from this neonicotinoid seed treatment than the other carabid species tested here, clothianidin and thiamethoxam treatments killed more rapidly (Table 3) in the case of these tolerant species, where 20% at most exhibited signs of recovery from paralysis only to succumb later. As suggested by Kunkel et al. (2001) for *H. pensylvanicus*, it is unlikely that carabids paralyzed for several days in the field would escape predation. Carabids are notable natural enemies of all life stages of corn rootworms (Kromp 1999), and they readily fed on adults in jar microcosm tests. Although field relevance of carabid predation on western corn rootworm adults may be limited (Kirk 1982), perhaps to times when corn rootworm adults are ovipositing, it was easier to score this predation in microcosm bioassays than it would be for the more cryptic larval stage.

The newly germinated corn seedlings from neonicotinoid-treated seeds resulted in a latency to feeding among ground beetles, whereas consumption of fungicide treatments was generally observed within 1 d (Table 3). Feeding inhibitory effects of neonicotinoids have been noted previously for other insects including Hymenoptera (Morandin and Winston 2003, Schmuck 2004). The potential use of the feeding deterrent/repellent effects of selected neonicotinoids on carabids to reduce some of the nontarget toxicities

Table 4. Representative EPA active corn seed treatments[a]

Registered product name	U.S. EPA reg. no.	Percent active ingredients	Manufacturer
Apron flowable Arpentia (fungicide)	2935-427	28.35 metalaxyl	Wilbur Ellis
Apron xl ws (fungicide)	100-797	45 mefenoxam	Syngenta
Cruiser xl (fungicide + insecticide)	100-1184	1.25 fludioxonil, 0.5 mefenoxam, 25 thiamethoxam	Syngenta
Dividend extreme fungicide	100-1141	7.73 difenoconazole, 1.87 mefenoxam	Syngenta
Flint fungicide	264-777	50 trifloxystrobin	Bayer Crop
Gaucho 600 flowable (insecticide)	7501-173	48.7 imidacloprid	Gustafson
Gaucho 75 st insecticide	7501-159	75 imidacloprid	Gustafson
Gustafson allegiance 50wp (fungicide)	7501-174	50 metalaxyl	Gustafson
Gustafson captan 400 (fungicide)	7501-26	38.3 captan	Gustafson
Gustafson vitaflo 280 flowable fungicide	7501-133	14.9 carboxin, 13.2 thiram	Gustafson
Imidacloprid vitavax metalaxyl seed trt	7501-198	14 carboxin, 25 imidacloprid, 1 metalaxyl	Gustafson
Maxim 4fs (fungicide)	100-758	40.3 fludioxonil	Syngenta
Maxim xl fungicide	100-916	21 fludioxonil, 8.4 mefenoxam	Syngenta
Poncho 600 (insecticide)	264-789	48 clothianidin	Bayer Crop
Vitavax - t fungicide (with thiram)	400-92	37.5 carboxin, 37.5 thiram	Crompton
Vitavax-3f fungicide	400-152	34 carboxin	Crompton

[a] Data assembled here is accessible from the Pesticide Action Network Pesticides Database, http://www.pesticideinfo.org/Index.html

needs further exploration. Some of the bioassays developed here may supplement other standardized testing procedures available in Europe through the Beneficial Arthropod Regulatory Testing (BART) group to assess toxicant impacts on carabid populations as a key ecotoxicity bioindicator group (Rombke and Heimbach 1996). Such tests are useful for regulatory evaluations that will identify acceptable pest control practices for agroecosystems so that biological control is not compromised.

Virtually all hybrid corn seed in the United States is treated with a fungicide or a combination of fungicide/insecticide (Table 4), many of which are broadly used on other crop seeds. Seed treatments are becoming an important part of the total U.S. pesticide use as evident from recent applications of neurotoxic neonicotinoids to most biotech corn (Table 4) to protect planted seeds and seedlings from many below- and above-ground insect pests. Seeds are treated at up to 1.34 (imidacloprid), 1.125 (thiamethoxam), and 1.25 (clothianidin) mg (AI) per kernel (Van Duyn 2003), which at a planting rate of 20,000 seeds per acre is equivalent to 22.5–26.8 g (AI)/acre for these new generation insecticides. These field rates are similar to that of higher generation pyrethroids and other modern insecticides used in corn (Smith et al. 2004). This is a dose of five parts per thousand per seed, although our studies were conducted with <3 parts per thousand (Table 2). Such a high dose further focused as a film on the seed surface represents perhaps the highest concentrated local dose of insecticide used in modern agriculture. That these insecticides are applied to seeds already dosed with two or three other fungicides allows for synergistic interactions to occur (Pedersen et al. 2003), which may change efficacy and selectivity of the insecticide (Schmuck et al. 2003).

While imidacloprid with a 10-yr and 120 country market history may pose a risk to honey bees if residual seeds are left above ground, extensive research has shown that its residues in the germinated seed, seedling, or soil are harmless to all tested nontarget Hymenoptera when the coated seed is drilled into the soil (Morandin and Winston 2003). Similar neonicotinoids including clothianidin and thiamethoxam and particularly later-generation analogs lacking a nitro-group substituent such as thiacloprid are slightly to much less toxic to honey bees (Iwasa et al. 2004). Nevertheless, accidental ingestion of seed treated for sowing has been a primary cause of human death associated with pesticides (Ferrer and Cabral 1995). Considerable mammalian and vertebrate toxicity, including analgesia and cytotoxicity, have been established for neonicotinoids (Tomizawa 2004). Systemic insecticides with relatively high persistence such as neonicotinoids require cautious use to negate ecotoxic effects on other beneficial animal species or pesticide handlers. It is essential that a robust regulatory pathway for implementing and monitoring seed treatments is maintained so that unwanted nontarget effects do not occur.

Transgenic cultivars combining insecticidal proteins with neonicotinoid seed treatments now dominate in biotech maize production. Seed treatments are an excellent strategy to minimize above-ground exposures and environmental loadings of pesticides, yet achieve excellent insect control. Our jar microcosm bioassay showed that clothianidin seed treatments rapidly killed 90% of corn rootworm in 1 d and greatly reduced its seedling consumption (Fig. 3), confirming the efficacy of this systemic neonicotinoid seed treatment for rootworm control. Our studies to assess the acute and chronic toxicities of Cry3Bb1 corn on adult Carabidae relevant to corn agroecosystems are consistent with other studies showing its ecosafety. However, there is a critical need to carefully define and optimize the use of seed treatments, particularly insecticides, both for pest management and for minimizing nontarget effects within transgenic and conventional agroecosystems.

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