**EFFECTS OF CRY3Aa δ -ENDOTOXIN ON *PODISUS MACULIVENTRIS*
(HEMIPTERA: PENTATOMIDAE) AND *COLEOMEGILLA MACULATA*
(COLEOPTERA: COCCINELLIDAE).**

by

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**A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirements for the degree of**

Master of Science

Department of Biology

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Thesis Supervisor

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ABSTRACT

In this study, I fed *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) and *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae) different concentrations of purified Cry3Aa δ -endotoxin, the insecticidal protein expressed by NewLeaf™ transgenic potatoes. The maximum dose was 50µg Cry3Aa/g food for *P. maculiventris* and 200 µg Cry3Aa/g food for *C. maculata*. I found no effect on either developmental rate or survivorship for either species, even at the highest doses, strongly indicating that Cry3Aa poses virtually no risk to these predators. Frass from *C. maculata* in the highest dose treatment contained enough active Cry3Aa protein to cause significant mortality in early instar *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Colorado potato beetle). Colorado potato beetle showed no significant mortality when exposed to frass from *P. maculiventris* in either the highest dose treatment or an additional treatment of 500 µg Cry3Aa/g food, although some mortality was observed when they ingested *P. maculiventris* carcasses from the 500 µg/g treatment. This suggests that *P. maculiventris* digests Cry3Aa, while *C. maculata* does not.

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INTRODUCTION

Insect-resistant crops arrived in the agricultural marketplace in 1995 when Ciba Seeds released Maximizer™ corn with “built-in” Knockout™ control for European corn borers (ECB). Several companies followed suit with similar corn products, and in 1996 Monsanto Company released Bollgard® transgenic cotton and NewLeaf™ transgenic potatoes. All of these plants can be grouped as “Bt” transgenic crops—that is, plants that have been genetically engineered to express toxins that are ordinarily only found in subspecies of the bacteria, *Bacillus thuringiensis*¹.

B. thuringiensis is a group of bacteria that produces insecticidal crystal proteins (ICP) during sporulation. Different subspecies produce different ICPs, each of which contains the precursors (or protoxins) for one or more δ -endotoxins—the active proteins that can be acutely toxic to specific target insects. This specificity was the basis for grouping and naming Bt toxins (also known as Cry proteins) for almost 90 years, until Crickmore *et al.* developed the current nomenclature based on amino acid sequences (1993).

Transgenic corn, cotton, and potatoes, for example, each contain a different gene for expressing a δ -endotoxin that targets a different pest. Each introduced gene is a synthetic construct that has been optimized for expression in plants by increasing the cytosine:guanine base-pair ratio; these modifications, however, do not alter the final protein, which has an amino acid sequence identical to that of the wild-type δ -endotoxin (Perlak *et al.*, 1993). Several commercially-important Cry proteins and the *B. thuringiensis* subspecies in which they are found are shown in Table 1.

¹ In this paper, *Bacillus thuringiensis* refers to the bacteria, while Bt refers to the bioinsecticide.

Table 1. Overview of some of the more widely used *Bacillus thuringiensis* toxins. (Crickmore *et al.*, 2002, and van Frankenhuyzen, K. and C. Nystrom, 2002)

Toxin	Bt subspecies	Target order	Target species for commercial applications	Commercial GM crops with toxin gene
Cry1Aa	<i>kurstaki</i> , <i>sotto</i> , <i>aizawai</i> , <i>entomocidus</i> , <i>dendrolimus</i>	Lepidoptera	diamondback moth, leafrollers	none
Cry1Ab	<i>berliner</i> , <i>kurstaki</i> , <i>aizawai</i>	Lepidoptera	European corn borer, corn earworm, spruce budworm, tent caterpillar, gypsy moth, diamondback moth, cabbage looper, tobacco budworm, and cabbage worm	corn
Cry1Ac	<i>kurstaki</i> , <i>kenyae</i>	Lepidoptera	cotton bollworm, tobacco budworm and pink bollworm, European corn borer	cotton, corn
Cry1Ca	<i>aizawai</i> , <i>entomocidus</i>	Diptera, Lepidoptera	mosquitoes	none
Cry2Aa	<i>kurstaki</i> , <i>sotto</i> , <i>kenyae</i>	Lepidoptera (Diptera?)	gypsy moth, tobacco budworm, European corn borer, mosquito	none
Cry3Aa	<i>san diego</i> , <i>tenebrionis</i> , <i>morrisoni</i>	Coleoptera	Colorado potato beetle	potato**
Cry4Aa	<i>israelensis</i>	Diptera	mosquitoes	none
Cry4Ba	<i>israelensis</i>	Diptera	mosquitoes	none
Cry9C	<i>tolworthi</i>	Lepidoptera	European corn borer	corn**

Insecticides containing cultures of *B. thuringiensis* subspecies and ICPs have been used as alternatives to chemical pesticides for decades. They degrade quickly in the presence of ultra-violet radiation (Pusztai *et al.*, 1991), thereby reducing the risks associated with environmental persistence. As well, they tend to be much more target-specific than synthetic insecticides because the Bt protein in the formulation cannot be processed into an active toxin or bound to an active site unless the environment inside the insect is suitable. Once an ICP has been ingested, it must dissolve in the insect's digestive fluids (requiring a specific pH), then be processed by specific enzymes into a truncated, active form that must bind to specific receptors before it can have any effect on the insect that consumed it (Oppert, 1999; Knowles, 1994; Gill, 1992; Li, 1991).

Elements of these environmental safeguards are lost when *B. thuringiensis* proteins are expressed in transgenic crop plants (e.g., see Jepson *et al.*, 1994; Schuler *et al.*, 1998; de Maagd *et al.*, 1999; Cannon, 2000; Hilbeck, 2001; and Obrycki *et al.*, 2001). Environmental persistence becomes a concern because the plants express the protein throughout the entire growing season, and possibly longer if residues are left in the soil (e.g. Stotzky, 2000). As well, the protein expressed by transgenic plants is several processing-steps closer to the active toxin than are the precursor forms applied in traditional Bt insecticides, possibly broadening the spectrum of activity (e.g. Hilbeck, 2001; Perlak, 1993).

Preserving healthy populations of beneficial, non-target insect species is a critical part of integrated pest management (IPM), which, in turn, is an essential strategy if farmers are to realize the economic and environmental benefits offered by transgenic crops. Predators and parasitoids can reduce the abundance of non-susceptible pest species

and stages, as well as help curb the rise of resistance in a population (Reed *et al.*, 2001; Riddick *et al.*, 2000; Arpaia *et al.*, 1997). It is, therefore, important to ensure that proteins expressed by transgenic crops do not accidentally intoxicate beneficial insects along with target pest species.

Naturally occurring predatory insects in potato fields include coccinellid and carabid beetles, tachinid flies, and predatory hemipterans, including members of the families Nabidae, Anthocoridae, Lygaeidae, and Pentatomidae. In this study, I examined the effects of chronic Cry3Aa exposure on nymphs of *Podisus maculiventris* Say (Hemiptera: Pentatomidae) and *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae). Both species are native with ranges that span large areas in the United States and Canada. Both are also reared commercially as biocontrol agents and are considered important for controlling *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Colorado potato beetle (CPB)) ((Boiteau, 1988, in Biever and Chauvin, 1992; Groden *et al.* 1990; Arpaia, 1997).

I chose to focus on a hemipteran and a coccinellid because members of these groups possess digestive enzymes similar to those of chrysomelid beetles (including CPB): that is, they are cysteine-based (Terra and Ferreira, 1994). Tachinid flies and carabid beetles, on the other hand, have trypsin-based digestive enzymes, and therefore are less likely to possess the proteinases required to cleave the Cry3Aa protein into its active form.

The null hypothesis for this study is that there will be no difference in the developmental rate and the survival rate between predators reared on diet containing Cry3Aa δ -endotoxin and those reared on untreated diet. The alternative hypothesis is that

predators reared on Cry3Aa will take longer to develop or more will die before reaching maturity when they are reared on Cry3Aa.

I also conducted a much smaller investigation into the fate of Cry3Aa after ingestion by a predator. In particular, I wanted to know whether *P. maculiventris* or *C. maculata* were able to digest the toxin, thereby eliminating it from the environment, or if it passed right through, creating another route of exposure for non-target insects and possibly accumulating in the soil.

Background

Mechanism of action and CPB response to toxicity

Cry3Aa is one of the proteins produced by *B thuringiensis* subspecies *tenebrionis*, and *san diego* and has been genetically engineered into Monsanto Company's NewLeaf™ potato line². It is toxic to insects from the families Tenebrionidae and Chrysomelidae, and is particularly effective against early-instar CPB, not only killing the insects but also quickly inhibiting feeding activity, thereby minimizing damage to foliage (Ferro and Gelernter, 1989).

Cry3Aa is typical of Bt toxins in that it damages the gut lining of insects (eg, Cannon, 1996; Gill *et al.*, 1992; Slaney *et al.*, 1992, Knowles, 1994). In susceptible insects like CPB, it recognizes specific receptors on the brush border membrane cells of the gut epithelium, binds, and penetrates the cell membrane. Aggregates form, creating pores that permit ions to rush into the cell, causing it to swell and eventually lyse. Within a matter of days (depending on the dose), sections of the midgut lining are destroyed and the insect dies.

² This line was discontinued in 2001 when several fast food chains, including MacDonald's, bowed to public pressure and refused to buy genetically modified potatoes (Wall Street Journal REF).

Proteolytic processing

NewLeaf™ potatoes express a 68 kDa protein that appears to be the same as the δ -endotoxin from *B. thuringiensis* subsp. *tenebrionis* (CFIA, 1996). Slaney *et al.* (1992) reported that this δ -endotoxin was “naturally truncated” and required no proteolytic processing to become active, based on their failure to find shorter, stable proteolytic fragments after treating it with CPB digestive juices. Later studies, however, have shown that digesting the protein with chrysomelid gut juices can produce smaller, stable fragments that are more soluble at chrysomelid gut pH (4.5-6.6) and more structurally suited to binding (Oppert, 1999; Carroll *et al.*, 1997).

Carroll *et al.* (1997) found that chymotrypsin from CPB midguts trimmed the δ -endotoxin to a 49 kDa protein that was soluble at pH 2.9 – 9.1 and bound readily to brush border membrane vesicles made from CPB midguts. This 49 kDa protein was not present in samples digested with yellow mealworm (*Tenebrio molitor*) gut extract; instead, a slightly larger (though still truncated) 55 kDa protein was found, closely associated with an 8-11 kDa fragment. This 55 kDa product was not soluble at the near-neutral pH of the mealworm midgut, even though mealworms are somewhat susceptible to Cry3Aa, suggesting that other factors may be involved. This raises the possibility that the final stages of processing and even the resulting activated toxin may differ between target species.

The degree to which the 68 kDa protein must be processed and the stability of the resulting products are important when considering non-target effects. If processing is required to activate a toxin, it is less likely to affect insects that do not possess the necessary proteases, or whose digestive tract is at the wrong pH. Current knowledge of insect digestive enzymes is rather sparse but, even so, it may be possible to identify

insects that may be at greater risk than others. *C. maculata* and *P. maculiventris*, for example, both have midguts at pH 5-6, which is in the same range as members of Chrysomelidae (Terra and Ferreira, 1994). As well, chrysomelids, coccinellids, and hemipterans all rely on cysteine proteinases as the predominant proteinases in their digestive systems—a physiological characteristic that is unique to Hemiptera and some members of Coleoptera (Terra and Ferreira, 1994).

Furthermore, if the final, activated toxin is stable, it might be passed on in its active form to non-target predators. This possibility has not been widely explored; however, Hilbeck *et al.* (1999) alluded to it in attempting to explain their findings that common green lacewings (*Chrysoperla carnea*) suffered higher mortality and greater delays in development when reared on Cry1Ab-fed prey compared to those fed Cry1Ab in artificial diet.

Effects of transgenic crop-fed prey on predators: previous studies

Given the number of insect species exposed to Bt toxins in the cause of pest control, there are few published studies assessing the toxicity of Bt toxins on non-target, predatory insect species (Tables 2 and 3). Of these, only 10 involve purified δ -endotoxins produced by transgenic crops (or the equivalent protein produced in *E. coli*), including findings from 2 groups that directly contradict each other.

In one study, Pilcher *et al.* (1997) found that ingesting pollen from transgenic corn plants (expressing Cry1Ab) had no effect on mortality of common green lacewings. Mortality rates were greater than 50% in both the treated and the control groups, however, possibly because the lacewings had to be coerced into eating the pollen by having their preferred food withheld for 24h periods at three points in their development. Even so, the authors concluded that lacewings were not susceptible to Cry1Ab.

Table 2. Studies investigating non-target effects of lepidopteran-specific Cry proteins on predaceous non-target species. L1=first instar; L2=second instar; L3=third instar; L4=fourth instar; A=adult; NS=not specified. Novo-biobit, Delfin, Dipel®, and Bactospeine are all commercial *B. thuringiensis* bioinsecticides based on *B. thuringiensis* subsp. *kurstaki*.

Species	Order	Family	Life stage	Source of cry proteins	Exposure route	Response	Effect	Environment	Reference
<i>Bembidion lampros</i>	Coleoptera	Carabidae	A	Dipel	sprayed host plants	predation efficiency, mortality	0	field	Obadofin & Finlayson 1977
<i>Coleomegilla maculata</i>	Coleoptera	Coccinellidae	L1-A	transgenic corn (Cry1Ab)	pollen	development time, mortality	0	lab	Pilcher <i>et al.</i> 1997
<i>C. maculata</i>	Coleoptera	Coccinellidae	L1-A	transgenic corn (Cry1Ab)	pollen/prey	abundance	0	field	Pilcher <i>et al.</i> 1997
<i>Hippodamia convergens</i>	Coleoptera	Coccinellidae	A	Cry2A purified from <i>E. coli</i>	artificial diet	mortality	0	lab	Sims, 1995
<i>H. convergens</i>	Coleoptera	Coccinellidae	A	Cry1Ac purified from <i>E. coli</i>	artificial diet	mortality	0	lab	
<i>Orius insidiosus</i>	Hemiptera	Anthocoridae	L1-A	transgenic corn (Cry1Ab)	pollen	development time, mortality	0	lab	Sims, 1995 Pilcher <i>et al.</i> 1997
<i>O. majusculus</i>	Hemiptera	Anthocoridae	L1-A	transgenic corn (Cry1Ab)	prey	development time, mortality	0	lab	Zwahlen <i>et al.</i> 2000
<i>O. insidiosus</i>	Hemiptera	Anthocoridae	L1-A	transgenic corn (Cry1Ab)	pollen/prey	abundance	0	field	Pilcher <i>et al.</i> 1997
<i>Geocoris punctipes</i>	Hemiptera	Lygaeidae	A	<i>B. t. kurstaki</i> : commercial preparation	prey (<i>Pseudoplusia includens</i>)	mortality	0	lab	Boyd & Boethel 1998
<i>Dicyphus tamaninii</i>	Hemiptera	Miridae	L3-L4	Novo-biobit® Delfin®	sprayed on foliage	mortality	0	lab	Castane <i>et al.</i> 1996
<i>Nabis capsiformis</i>	Hemiptera	Nabidae	A	<i>B. t. kurstaki</i> : commercial preparation	prey (<i>P. includens</i>)	mortality	0	lab	Boyd & Boethel 1998
<i>Podisus maculiventris</i>	Hemiptera	Pentatomidae	L3	<i>B. t. kurstaki</i> : commercial preparation	prey (<i>P. includens</i>)	mortality	0	lab	Boyd & Boethel 1998

Species	Order	Family	Life stage	Source of cry proteins	Exposure route	Response	Effect	Environment	Reference
<i>P. maculiventris</i>	Hemiptera	Pentatomidae	A	<i>B.t. kurstaki</i> : commercial preparation	prey (<i>P. includens</i>)	mortality	0	lab	Boyd & Boethel 1998
<i>P. maculiventris</i>	Hemiptera	Pentatomidae	A	Bactospeine	drinking water	mortality	0	lab	Mohaghegh <i>et al.</i> 2000
<i>P. maculiventris</i>	Hemiptera	Pentatomidae	L4	Bactospeine	drinking water	mortality	0	lab	Mohaghegh <i>et al.</i> 2000
<i>Podisus nigrispinus</i>	Hemiptera	Pentatomidae	L2-A	Dipel	prey (<i>B. mori</i>)	fecundity	-	lab	Nascimento <i>et al.</i> 1998
<i>P. nigrispinus</i>	Hemiptera	Pentatomidae	L2-A	Dipel	prey (<i>B. mori</i>)	presence of cry proteins in gut and feces	+	lab	Nascimento <i>et al.</i> 1998
<i>P. nigrispinus</i>	Hemiptera	Pentatomidae	L2-A	Dipel	prey (<i>Bombyx mori</i>)	presence of cry proteins in hemolymph mortality	0	lab	Nascimento <i>et al.</i> 1998
<i>Tenodera aridifolia sinensis</i>	Mantodea	Mantidae	L3-L4	<i>B.t. kurstaki</i> : commercial preparation	prey (<i>Trichoplusia ni</i>)	mortality	0	lab	Yousten 1973
<i>Chrysoperla carnea</i>	Neuroptera	Chrysopidae	nymphs	Cry1Ac purified from <i>E. coli</i>	mixed with <i>Sitotroga cerealella</i> eggs	mortality	0	lab	Sims, 1995
<i>C. carnea</i>	Neuroptera	Chrysopidae	nymphs	Cry2A purified from <i>E. coli</i>	mixed with <i>Sitotroga cerealella</i> eggs	mortality	0	lab	Sims, 1995
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1-A	transgenic corn (Cry1Ab)	pollen	development time, mortality	0	lab	Pilcher <i>et al.</i> 1997
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1-A	transgenic corn (Cry1Ab)	pollen/prey	abundance	0	field	Pilcher <i>et al.</i> 1997
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1-A	transgenic corn (Cry1Ab)	prey (<i>Ostrinia nubilalis</i>)	development time, mortality	+	lab	Hilbeck <i>et al.</i> 1998a

Species	Order	Family	Life stage	Source of cry proteins	Exposure route	Response	Effect	Environment	Reference
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1-A	transgenic corn (Cry1Ab)	prey (<i>Spodoptera littoralis</i>)	development time, mortality	+	lab	Hilbeck <i>et al.</i> 1998a
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1-A	Cry1Ab purified from <i>B.t. kurstaki</i>	artificial diet	development time, mortality	+	lab	Hilbeck <i>et al.</i> 1998b
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1-A	Cry1Ab purified from <i>B.t. kurstaki</i>	prey (<i>O. nubilalis</i>)	development time, mortality	+	lab	Hilbeck <i>et al.</i> 1999
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1-A	Cry1Ab purified from <i>B.t. kurstaki</i>	prey (<i>S. littoralis</i>)	development time, mortality	+	lab	Hilbeck <i>et al.</i> 1999
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1-A	Cry1Ab prototoxin purified from <i>B.t. kurstaki</i>	prey (<i>O. nubilalis</i>)	development time, mortality	+	lab	Hilbeck <i>et al.</i> 1999
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1-A	Cry1Ab prototoxin purified from <i>B.t. kurstaki</i>	prey (<i>S. littoralis</i>)	development time, mortality	+	lab	Hilbeck <i>et al.</i> 1999
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1-A	Cry2A purified from <i>B.t. kurstaki</i>	prey (<i>S. littoralis</i>)	development time, mortality	+	lab	Hilbeck <i>et al.</i> 1999
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1-A	Cry2A purified from <i>B.t. kurstaki</i>	prey (<i>O. nubilalis</i>)	development time, mortality	+	lab	Hilbeck <i>et al.</i> 1999
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1, L2	transgenic corn (Cry1Ab)	prey (<i>S. littoralis</i>)	prey preference	0	lab	Meier & Hilbeck 2001
<i>C. carnea</i>	Neuroptera	Chrysopidae	L3	transgenic corn (Cry1Ab)	prey (<i>S. littoralis</i>)	prey preference	-	lab	Meier & Hilbeck 2001
<i>C. carnea</i>	Neuroptera	Chrysopidae	L1, L2, L3	transgenic corn (Cry1Ab)	prey (<i>Rhopalosiphum padi</i>)	prey preference	0	lab	Meier & Hilbeck 2001

Table 3. Studies investigating non-target effects of coleopteran-specific Cry proteins on predaceous non-target species. N1=first instar; N2=second instar; N3=third instar; A=adult; NS=not specified. Novodor® and M-One® are commercial bioinsecticides based on *B. thuringiensis* subsp. *tenebrionis*.

Species	Order	Family	Life stage	Source of <i>cry</i> proteins	Exposure route	Response	Effect	Environment	Reference
spider spp	NS	NS	NS	transgenic potatoes (Cry3Aa)	not specified	abundance	+	field	Riddick <i>et al.</i> 2000
<i>Chauliognathus lugubris</i>	Coleoptera	Cantharidae	A	Novodor	sprayed eggs (Chrysophtharta bimaculata)	predation efficiency	0	lab	Beveridge & Elek 1999
<i>C. lugubris</i>	Coleoptera	Cantharidae	A	Novodor	spray	mortality	0	lab	Beveridge & Elek 1999
<i>Cicindela punctulata punctulata</i>	Coleoptera	Carabidae	NS	transgenic potatoes (Cry3Aa)	NS	abundance	0	field	Riddick <i>et al.</i> 2000
<i>Poecilus chalcites</i>	Coleoptera	Carabidae	NS	transgenic potatoes (Cry3Aa)	NS	abundance	0	field	Riddick <i>et al.</i> 2000
<i>Poecilus lucublandus lucublandus</i>	Coleoptera	Carabidae	NS	transgenic potatoes (Cry3Aa)	NS	abundance	0	field	Riddick <i>et al.</i> 2000
<i>Scarites quadricaps</i>	Coleoptera	Carabidae	NS	transgenic potatoes (Cry3Aa)	NS	abundance	0	field	Riddick <i>et al.</i> 2000
<i>Scarites subterraneus</i>	Coleoptera	Carabidae	NS	transgenic potatoes (Cry3Aa)	NS	abundance	0	field	Riddick <i>et al.</i> 2000
<i>Coccinella septempunctata</i>	Coleoptera	Coccinellidae	NS	transgenic potatoes (Cry3Aa)	NS	abundance	0	field	Riddick <i>et al.</i> 2000
<i>C. maculata</i>	Coleoptera	Coccinellidae	N2-A	transgenic potatoes (Cry3Aa)	prey (L. decemlineata)	development time, live weight, fecundity,	0	lab	Riddick & Barbosa 1998
<i>C. maculata</i>	Coleoptera	Coccinellidae	N1-A	M-One	treated pollen	development time	+	lab	Giroux <i>et al.</i> 1994
<i>C. maculata</i>	Coleoptera	Coccinellidae	N1-A	M-One	treated pollen	mortality	0	lab	Giroux <i>et al.</i> 1994

Species	Order	Family	Life stage	Source of cry proteins	Exposure route	Response	Effect	Environment	Reference
<i>C. maculata</i>	Coleoptera	Coccinellidae	N3	M-One	treated eggs (L. decemlineata)	predation efficiency	-	lab	Giroux <i>et al.</i> 1994
<i>Harmonia axyridis</i>	Coleoptera	Coccinellidae	NS	transgenic potatoes (Cry3Aa)	NS	abundance	0	field	Riddick <i>et al.</i> 2000
<i>Hippodamia convergens</i>	Coleoptera	Coccinellidae	N2-A	transgenic potatoes (Cry3Aa)	prey (Myzus persicae)N1-A	time between moults, pupal weight, fecundity, prey consumption, longevity	0	lab	Dogan <i>et al.</i> 1996
<i>H. convergens</i>	Coleoptera	Coccinellidae	NS	transgenic potatoes (Cry3Aa)	NS	abundance	0	field	Riddick <i>et al.</i> 2000
<i>O. insidious</i>	Hemiptera	Anthocoridae	NS	transgenic potatoes (Cry3Aa)	NS	abundance	Y1: + Y2: 0	field	Riddick <i>et al.</i> 2000
<i>G. punctipes</i>	Hemiptera	Lygaeidae	NS	transgenic potatoes (Cry3Aa)	NS	abundance	0	field	Riddick <i>et al.</i> 2000
<i>Nabis</i> spp	Hemiptera	Nabidae	NS	transgenic potatoes (Cry3Aa)	NS	abundance	0	field	Riddick <i>et al.</i> 2000

Another study, however, suggests that lacewings are susceptible to Cry1Ab. In 1998, Hilbeck *et al.* found that 62% of lacewings fed lepidopteran prey (ECB and *Spodoptera littoralis*) reared on Cry1Ab-transgenic corn foliage died before reaching adulthood, compared to 37% in the control group (fed lepidopteran prey reared on non-transgenic corn). In response to criticism from many sources, including the Select Committee on European Communities (1999), Hilbeck *et al.* conducted follow-up studies using prey reared on Cry1Ab +/- artificial diet (1999) and Cry1Ab +/- lacewing artificial diet (1998b). In both cases, lacewings exposed to Cry1Ab (in whatever form) experienced significantly higher mortality than those fed non-Cry1Ab diets.

Studies assessing non-target effects of Cry3Aa are especially scarce (Table 3). In 1994, Giroux *et al.* (1994) reared *C. maculata* on pollen and found that they developed significantly more slowly when the pollen had been treated with M-One™, a commercial formulation of *B. thuringiensis* subspecies *san diego* from Mycogen Corporation. Dogan *et al.* (1996) fed aphids reared on Cry3Aa potatoes to ladybird beetles (*Hippodamia convergens*) and looked for effects on aphid consumption, development times (for each larval stage plus pupation), fecundity, and longevity. They found no difference between beetles fed aphids from transgenic potato plants expressing Cry3Aa and those in the control (i.e., fed non-transgenic potato plants). However, aphids feed on phloem sap and therefore may not ingest Cry3Aa at all (Raps *et al.*, 2001).

In 1998, Riddick and Barbosa conducted several experiments to determine whether CPB larvae reared on transgenic foliage expressing Cry3Aa had an effect on the appetite, development and fecundity of *C. maculata*. They found no effects, although their study has been criticized by Hilbeck *et al.* (2000) for several reasons, including very

high mortality in all treatments, including the controls (>50%) and failure to study first-instar *C. maculata*. In general, susceptibility to Bt toxins decreases with age (Wierenga *et al.*, 1996). Only second instars and older were used in this study.

Routes of exposure

C. maculata and *P. maculiventris* are not foliage feeders; however, pollen is an important element of *C. maculata*'s diet (Pilcher *et al.*, 1997), while *P. maculiventris* are polyphagous and will occasionally feed on plant juices. Consequently, there is a chance that they will ingest Cry3Aa directly, through plant matter. If they visit damaged transgenic plants, they might also become contaminated by plant juices and then ingest them while grooming.

More likely, the route of greatest exposure for generalist predators such as *P. maculiventris* and *C. maculata* will be by consuming prey that has fed on transgenic foliage. Hilbeck's studies with lacewings and lepidopteran prey (1998a and 1999) would seem to suggest that the toxin can be ingested by either a susceptible or a non-susceptible insect and still retain some activity. Head *et al.* (2001) demonstrated definitively that this was the case, at least with *Rhopalosiphum padi* and three lepidopteran species, including *Ostrinia nubilalis* (European corn borer (ECB)). They fed insects either transgenic or non-transgenic corn foliage for seven days, or artificial diet containing different concentrations of Cry1Ab for three days, and then measured the level of Cry1Ab in the insects' tissues using enzyme-linked immunosorbent assays (ELISA). Cry1Ab was detected in all species (although not in all treatments, depending on the concentration in the diet), with the highest levels found in the least susceptible species (i.e., the ones that fed more). They confirmed the activity of the toxin through bioassays in which aphids

and ECB were fed Cry1Ab (in artificial diet), then were lyophilized, crushed, and incorporated into artificial diet that was fed to ECB. The toxin retained enough activity to negatively affect the ECB.

It does not seem impossible, then, that the protein could be ingested by an herbivorous insect and retain its potential to intoxicate a sensitive predator.

MATERIALS AND METHODS

Insects

All insects used in bioassays were obtained from lab colonies established for this study and maintained at 16:8 (L:D) photoperiod. Relative humidity and temperature were not controlled; temperature ranged from 21-29°C.

I established a colony of *Leptinotarsa decemlineata* (CPB) from eggs obtained from the permanent colony at the Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, London, Ontario. Adults and larvae not used in bioassays were maintained on several varieties of potato plants (depending on availability of tubers).

The *P. maculiventris* colony was started from nymphs purchased from Koppert Canada Ltd., Scarborough, Ontario. Adults and nymphs were reared as described in Mukerji and LeRoux (1964), on mealworm larvae and green beans.

For *C. maculata*, I obtained approximately 50 adults from François Lorenzetti, Département des Sciences Biologiques, Université du Québec à Montréal, and maintained them in an aquarium on ECB eggs, lyophilized beef, and wildflower pollen. When eggs were required, pairs of adults were isolated in 90mm Petri dishes with food, water, and a piece of wax paper on which to oviposit.

***Bacillus thuringiensis* proteins**

I used 1.0 µg/mL Cry3Aa δ -endotoxin stock solution supplied by Monsanto Company, St Louis, MO, for all bioassays except for the first four feeding trials with *P. maculiventris* and bioassays with CPB that were done to support those trials. For these, I used Cry3Aa δ -endotoxin that was made for me by Mohsin Zaidi (from the University of Ottawa) from transformed *Escherichia coli* JM103 (Wu and Dean, 1996), obtained from

the Bacillus Genetic Stock Center, Department of Biochemistry, Ohio State University, Columbus, Ohio.

To harvest the protein, Mohsin and I grew the *E. coli* in YEP broth with 1 µg/mL ampicillin for 24h at 37°C, then divided this culture between six flasks (each containing 1.5L of LB broth) and incubated, with continuous shaking, at 30°C for 48h. We purified the δ -endotoxin by the method described in Ge *et al.* (1991) with one modification: to lyse the cells, we resuspended the cell pellets in lysis buffer (50mM Tris, pH 8.0; 50mM EDTA; and 15% sucrose) with 1mg/mL lysozyme overnight and incubated overnight at 37°C with continuous, slow shaking.

We solubilized the protein crystals by incubating for 3h at 37°C in 50 mM Na₂CO₃ and 10 mM dithiothreitol (pH 10.0). We centrifuged at high speed for 15 minutes and collected the solubilized protein in the supernatant. Shortly after the final product was collected in August, 2001, the solubilized proteins were separated using sodium dodecyl sulfate - polyacrymide gel electrophoresis (SDS-PAGE) and the concentration of CryAa measured by absorption spectroscopy after staining with Coomassie Blue dye. The concentration of three samples from this stock solution was measured again, using the same technique, in April, 2002.

Both Cry3Aa proteins were stored at -20°C.

For clarity in this paper, when I used Cry3Aa δ -endotoxin from Monsanto, I identify it as “MON”; when it is from our “home-made” batch, I identify it as “UO.”

Bioassays to Test δ -endotoxin Activity

To assess the bioactivity of the Cry3Aa proteins (from both sources), I coated excised potato leaflets with solutions containing the protein or a control, allowed them to

air dry, and then exposed early instar CPB for 48-96h. Leaflets were kept fresh in improvised floral pics made from Eppendorf tubes stoppered with cotton batting. Mortality was assessed every 24h.

Details of the bioassays are presented in Table 4.

I quantified the amount of damage done to each leaflet by CPB feeding using a 0.4 cm grid. Some of the squares in the grid were blacked out in order to reduce the amount of counting required for these measurements, although in all cases the leaflet was visible through at least 65 squares (blacked-out squares were chosen randomly). By counting the number of grid squares that contained signs of feeding and dividing by the total number of squares that touched the leaflet, I derived a value for the percentage of the leaflet that was damaged. In cases where a square appeared to contain no leaf at all but it was clear that the area had been completely consumed, the square was counted as showing feeding damage.

Feeding trials

P. maculiventris

I reared *P. maculiventris* from the first instar to adulthood on artificial diet dosed with Cry3Aa in order to identify effects on survivorship and developmental rate. The artificial diet was similar to that described by De Clerq *et al.* (1992), modified so that I could prepare small amounts of fresh diet as it was required. I made a dry meat mixture of lyophilized beef liver and medium ground beef (50:50 (fw:fw)), 1% sucrose, and 0.05% ascorbic acid, which was stored at 4°C. New diet was made every 48h by adding 1.25mL of hen's egg yolk mixed with distilled water (1:12 (v:v)) and 250 µL of toxin and/or buffer for every 1g of dry meat mixture. Food was presented to *P. maculiventris*

Table 4. Details of bioassays to assess Cry3Aa protein bioactivity during the experimental period.

Start date of test	Source of Cry3Aa δ -endotoxin	Treatments Concentration* ($\mu\text{g/mL}$) Control	Duration (h)	No. trials/ treatment	No. CPB/ leaflet	Comments
22/12/2001	UO	200 alkalic buffer	48	1	10	Treatment solutions kept at room temperature for 48h before the start of the test
23/03/2002	UO	200 alkalic buffer	96	3	10	leaves replaced after 48h
22/04/2002	UO1* UO2 MON	2, 20, 200 2, 20, 200 100, 50, 10, 5, 1 alkalic buffer	96	2	4-5	leaves replaced after 48h
11/06/2002	MON	62.5 distilled water	72	1	5	
14/06/2002	MON	62.5 distilled water	90	1	7	
02/07/2002	MON	50 distilled water	48	1	4 (treated) 3 (control)	

* Calculated concentration, based on concentrations originally measured with SDS-PAGE (UO: 2.0 $\mu\text{g/mL}$) or reported from source (MON: 1.0 $\mu\text{g/mL}$).

**UO1 and UO2 refer to samples of the UO protein that were stored separately in the freezer.

packaged in Parafilm. This allowed the insects to feed by piercing the Parafilm with their rostrum, much as they would pierce the cuticle of their prey

I repeated the feeding tests seven times: four times with UO δ -endotoxin, and three times with MON δ -endotoxin (Table 5). Sample sizes differed between trials due to the availability of neonate nymphs. In all trials, first instar *P. maculiventris* were housed individually in 55mm Petri dishes with artificial diet, a cotton ball soaked with distilled water, and a crumpled piece of unbleached paper towel. Fourth instars were moved to plastic jars with tight-fitting ventilated lids to complete their development. Insects were fed *ad libitum* and given water and fresh food at approximately the same time every day.

I recorded deaths and the developmental stage of each surviving individual approximately every 24h.

Concentration of Cry3Aa δ -endotoxin in the artificial diet

I conducted the first two feeding trials (Trials 1 and 2) from late November to late December, 2001, and the second two (Trials 3 and 4) from mid-January to mid-February. I dosed the diet in all of these trials with UO Cry3Aa δ -endotoxin, diluted 1:10, 1:100, and 1:1000 with alkalic buffer. The results of a short bioassay conducted 22-24 December, 2001, indicated that the activity of the toxin was within the expected range (see Table 9, Results section). However, when I conducted a second bioassay in late March (in conjunction with later feeding assays that were not included in this paper), I found that the activity had decreased somewhat (see Table 9, Results section).

Table 5. Start dates, sample sizes, treatments, and duration of *P. maculiventris* feeding trials.

Trial no.	Start date	Treatments: Control µg Cry3Aa/g food	Source of Cry3Aa δ- endotoxin	Nymphs/ treatment	Length of test (d)
1	27/11/2001	alkalic buffer 0.5, 5, 50	UO	5	30
2	29/11/2001	alkalic buffer 0.5, 5, 50	UO	5	30
3	09/01/2002	alkalic buffer 0.5, 5, 50	UO	10	35
4	15/01/2002	distilled water 50	UO	10	35
5	12/06/2002	distilled water 50	MON	17	35
6	13/06/2002	distilled water 50	MON	16	35
7	14/06/2002	distilled water 50	MON	18	35

As a result, I cannot state with confidence the exact concentration of Cry3Aa δ -endotoxin in the diet for Trials 1-4; however, comparing the relative feeding damage of the 1:10 dilution of UO1 and various dilutions of the MON Cry3Aa in the CPB bioassays suggests that the highest dose was likely at least 50 $\mu\text{g/g}$ food (Tables 9-13, Results section). The rationale behind this decision is spelled out in more detail below.

The lower-dose treatments were prepared by serial dilution of the stock UO toxin with alkalic buffer (1:10 and 1:100) and, therefore, were considered as 5 and 0.5 μg Cry3Aa/g food. The control contained only alkalic buffer.

Trials using the UO δ -endotoxin were completed 2.5 months before the problems were recognized; therefore, in order to verify our first results, I repeated the highest-dose treatment using 50 μg MON Cry3Aa δ -endotoxin/g artificial diet (Table 5). I did not dilute the protein stock before adding it to the artificial diet, so I used distilled water instead of alkalic buffer in the control diet for these trials.

Justification for assigning a value of 50 $\mu\text{g/g}$ to the highest dose treatment in Feeding Trials 1-4 for *P. maculiventris*

I designated the highest-dose treatment as 50 μg Cry3Aa/g food in Trials 1-4 on the basis of the feeding damage measured in the first two bioassays. I did not consider survival rates because the first assay ran for only 48h, which was not long enough to properly assess effects on survival (Robertson *et al.*, 1995).

I added 250 μL of undiluted UO Cry3Aa stock per gram of food in the highest dose treatment for Trials 1-4 with *P. maculiventris*. Therefore, the minimum stock concentration required for a dose of 50 μg Cry3Aa /g food is 200 $\mu\text{g/mL}$.

A 1:10 dilution of UO Cry3Aa prevented feeding almost completely in the

bioassays started on 22 December, 2001 (Table 9)—an effect corresponding to between 50 and 100 $\mu\text{g/mL}$ of the Monsanto toxin. As this bioassay was conducted towards the end of Trials 1 and 2, this suggests that the stock solution, which was 10X higher, was at least 500 $\mu\text{g/mL}$.

Trials 3 and 4 started on January 9 and 16, 2002 (respectively), less than one month after the first bioassay, and finished in early February, 2002, 4-6 weeks before the second bioassay (March 23, 2002). In the second assay, larvae fed more extensively on treated leaves than they had in December, but they were still clearly affected by the toxin. Feeding damage in this assay was 14.0-16.6% (95% confidence limits), which is almost exactly the level of damage measured for 50 $\mu\text{g/mL}$ of MON Cry3Aa (the 1:200 dilution), and almost half that for 10 $\mu\text{g/mL}$ MON Cry3Aa (Table 11). Again, this value was for a 1:10 dilution; consequently, the concentration of stock was likely still higher than 200 $\mu\text{g/mL}$.

C. maculata

I reared *C. maculata* from first instar to adulthood on artificial diet dosed with MON Cry3A δ -endotoxin at three concentrations: 200 $\mu\text{g/g}$ food, 20 $\mu\text{g/g}$ food, and 2 $\mu\text{g/g}$ food. I added straight protein stock solution to the artificial diet in the highest dose treatment, and prepared lower-dose treatments using the same volume of 1:10 and 1:100 serial dilutions (stock:distilled water). The control treatment contained distilled water.

C. maculata artificial diet contained 37% dried meat mixture (the same as used for the *P. maculiventris*), 37% wildflower pollen (from Kardish Food), and 26% brewer's yeast, suspended in 30mL 1.5% agar, prepared weekly and stored at 4°C. In order to

reduce the risk of the toxin degrading during the week, I dosed small amounts of diet every 2 days by melting the agar in the microwave, and adding the desired amount of Cry3Aa. Insects were fed *ad libidum* and food was replaced daily.

I ran the *C. maculata* feeding trials four times, using as many individuals as I could for each trial, depending on the availability of neonates (Table 6). For each trial, *C. maculata* were housed individually (within 24h of hatching) in shallow 50mm Petri dishes with tight-fitting lids. I recorded deaths and the developmental stage of each surviving individual approximately every 24h for 30d, or until all individuals in the trial had either died or reached adulthood.

Bioassays to Test for Toxin in Predators and Frass

I tested for the presence of Cry3Aa δ -endotoxin in frass from *P. maculiventris* and *C. maculata* by mixing the frass with distilled water, painting it on potato leaflets, and exposing early instar CPB. I performed a similar experiment using crushed carcasses of predators mixed with distilled water and painted on potato leaflets to determine whether an effective amount of toxin remained in the predator after ingestion. The details of each of these trials are shown in Tables 7 and 8.

I used feeding inhibition as the main indicator of the presence or absence of Cry3Aa in a treatment, as indicated by the level of feeding damage, which I measured as described earlier. Mortality was also recorded at the end of each test.

Because of the difficulty in obtaining sufficient frass for the bioassays and the irregular availability of CPB larvae, none of the bioassays were replicated exactly, although the *C. maculata* trials were considered close enough to compare using the Student's t-test.

Table 6. Start dates, sample sizes, and duration of *C. maculata* feeding trials.

Trial no.	Start date	Nymphs/treatment
1	13/04/2002	10
2	21/04/2002	8
3	22/04/2002	10
4	28/04/2002	14

Table 7. Specifications for bioassays to test for presence of active Cry3Aa in crushed predators.

Number of CPB larvae exposed/ leaflet	Predator species	[Cry3Aa] fed to predator ($\mu\text{g/g}$)	Size/ stage of predator	Volume dH ₂ O (mL)	duration (h)
4	<i>C. maculata</i>	200	5 adults	0.5	72
6	<i>C. maculata</i>	200	10 adults	0.5	72
4	<i>P. maculiventris</i>	50	4 L3/L4	0.5	96
5	<i>P. maculiventris</i>	500	5 L5	1.0	96

Table 8. Specifications for bioassays to test for presence of active Cry3Aa in predators' frass. A "dish" refers to one of the Petri dishes used to house the insects, and contains 3 days' worth of frass.

Number of CPB larvae exposed/ leaflet	Predator species	[Cry3Aa] fed to predator ($\mu\text{g/g}$)	Amount of frass	Volume dH ₂ O (mL)	duration (h)
4	<i>C. maculata</i>	200	6 dishes	1.0	96
5	<i>C. maculata</i>	200	5 dishes	1.0	72
6	<i>C. maculata</i>	200	4 dishes	1.0	72
4	<i>P. maculiventris</i>	50	4 dishes	0.7	96
5	<i>P. maculiventris</i>	500	7 dishes	1.5	96

As well, while I always intended to run each assay for 96h, I had to cut some of them short when the leaflets were either entirely consumed or became inedible before the scheduled end of the test.

Statistical Analysis

I used JMP statistical software (SAS Institute, 1995) to perform all statistical analyses.

For the bioassay that began on March 23, I used Student's t-test to compare the effects of treated and control potato leaflets on CPB survivorship and feeding activity. I also used the t-test to compare feeding activity and mortality among CPB feeding on leaflets painted with *C. maculata* frass or carcasses.

I analyzed the effect of treatment and trial on developmental times using two-way ANOVAs. When I found a significant effect, I used the Tukey-Kramer test to separate treatment means via all pairwise comparisons.

I compared survivorship at different doses and in different trials using separate Kaplan-Meier survival analyses. I identified significant differences using a non-parametric generalized Wilcoxon test to estimate Chi-square values. I chose the Wilcoxon test because it is more sensitive to differences in survivorship early in the study period, which corresponds to earlier instars when insects tend to be more susceptible to *B. thuringiensis* δ -endotoxins. Data for individuals that were still alive at the end of a test but had failed to reach adulthood were not censored because it was assumed that they would never reach the next instar.

RESULTS

Bioassays to Test Purified δ -endotoxin Activity

The results of the first of these bioassays (started on December 22, 2001) confirmed that the Cry3Aa δ -endotoxin in the UO protein stock solution was biologically active. Only 70% of the early instar CPB exposed to the 1:10 dilution was still alive after 48h, compared to 100% of CPB in the control treatment. Surviving larvae on the treated leaflets were approximately half the size of those on the control. As well, only 2% of the leaflet coated with the 1:10 dilution showed signs of feeding, compared to 53% feeding damage to the control leaflet. I considered these results—particularly the degree of feeding inhibition—as evidence that the concentration of the UO protein was quite high.

I tested the UO Cry3Aa δ -endotoxin again starting on March 23. I was able to use only 2 replicates from each treatment to measure survivorship because of an escape that occurred during the last 24h of the test. Even so, I found that significantly fewer CPB survived after 96h on the treated leaflet than on the control ($df = 2$; $t_{\text{stat}} = 8.23$; $P < 0.01$), and that feeding damage after 48h was significantly less on the treated leaflet than on the control ($df = 4$; $F = 5.62$, $P < 0.01$). However, the survival rate for both treatments was identical 48h into the test, and the UO toxin did not seem to be inhibiting feeding as completely as it had in the earlier bioassay (Table 9 and personal observation). This led us to suspect that the UO Cry3Aa had degraded somewhat during the intervening 3 months.

Table 9. Results of two bioassays conducted to confirm the activity of UO Cry3Aa δ -endotoxin. Results from December 22, 2001 are from one trial only; those from March 23, 2002 are the mean (SE) from two trials.

Start date of bioassay Dependent variable (after 48h*)	control	200 $\mu\text{g/mL}$
December 22, 2001		
% survival	100	70
% feeding damage	53	2
March 23, 2002		
% survival	96.7 (2.4)	96.7 (2.4)
% feeding damage	55.7 (4.9)	15.3 (1.3)

*The second bioassay ran for 96h, but survivorship was measured and recorded every 24h.

I confirmed our suspicions after Mohsin measured three samples of the UO protein stock solution (using SDS-PAGE) and found that the concentration of two of them (UO1 and UO3) had diminished by at least half—from 2.0 mg/mL to 1.0 mg/mL—while the third sample (UO2) was almost 50 times less concentrated than we had believed (0.04 mg/mL). When I compared the survival rates from bioassays using two of these samples (UO1 and UO2) with those from bioassays of serial dilutions of the MON δ -endotoxin stock, I found that the activity of a 1:10 dilution of UO1—which should have contained 200 μ g Cry3Aa/mL according to our original measurements, and 100 μ g/mL Cry3Aa/mL according to our new measurements—compared more closely to the 1 μ g/mL of the MON Cry3Aa (Table 10). The activity of the UO2 Cry3Aa was even lower.

Feeding damage was also unexpectedly extensive when CPB larvae were exposed to leaflets treated with UO2 or UO3 Cry3Aa, strongly suggesting that there was something wrong with the UO protein stock solution (Table 11).

The feeding damage measured in the 1:10 dilutions of UO1 falls somewhere between the 1:1000 and 1:200 dilutions of MON Cry3Aa samples, which correspond to concentrations of between 1 and 5 μ g/mL (Table 11), much lower than 100 μ g/mL that was expected.

In response to these findings, I stopped using the UO Cry3Aa δ -endotoxin stock and switched to the supply from Monsanto for *P. maculiventris* Trials 5-7, all *C. maculata* trials, and all bioassays testing for Cry3Aa in predators' bodies or frass.

In all three of the bioassays conducted to support the feeding trials using MON protein, all of the CPB exposed to MON toxin leaves died before the end of the 96h test, while those on the controls thrived (Table 12). I did not quantify feeding damage in these

trials, but I saw very little evidence of feeding, especially after the first 24h. These bioassays confirmed that the MON protein stock retained its activity at least until all of the feeding trials with the predators were complete.

Table 10. Survival rates of CPB exposed to potato leaflets painted with one of two samples of UO toxin (stock concentration uncertain) or MON toxin (stock concentration = 1.0 µg/mL), at different dilutions.

Dilution	% Survival (SE)		
	MON	UO1	UO2
control	100.0 (0.0)		
1:1000	50.0 (10.0)	90.0 (10.0)	90.0 (10.0)
1:200	46.5 (13.5)		
1:100	30.0 (10.0)	60.0 (0.0)	70.0 (10.0)
1:20	0.0 (0.0)		
1:10	0.0 (0.0)	50.0 (10.0)	71.5 (11.5)

Table 11. Feeding damage by CPB on potato leaflets painted with one of two samples of UO toxin (stock concentration uncertain) or MON toxin (stock concentration = 1.0 µg/mL), at different dilutions.

Dilution	MON	% Feeding damage (SE)	
		UO1	UO2
control	100.0 (0.0)		
1:1000	71.0 (0.5)	78.0 (7.0)	77.0 (17.5)
1:200	26.0 (0.0)		
1:100	32.0 (18.0)	50.5 (18.5)	76.0 (6.0)
1:20	17.0 (1.0)		
1:10	0.0 (0.0)	38.4 (6.0)	72.5 (15.5)

Table 12. Survivorship of CPB exposed to potato leaflets painted with MON Cry3Aa or distilled water (control), in three separate 96-hour bioassays.

Start date of bioassay	% survivorship	
	control	Cry3Aa+
June 11, 2002	0	100
June 14, 2002	0	100
July 2, 2002	0	100

Effects of long-term exposure to Cry3Aa protein on development of *P. maculiventris*

Because *P. maculiventris* do not feed during the first instar (Mukerji and LeRoux, 1964), I measured developmental time beginning from the second instar. Individuals that died before reaching the second instar were not counted. Two individuals escaped during the study and were not counted.

Trials 1-4

Ingesting Cry3Aa δ -endotoxin had no significant effect on developmental time from second instar to adult (Table 13, Figure 1.).

Trial had a significant effect on developmental time (Table 13 and Figure 2). Tukey-Kramer means comparisons revealed that individuals in Trials 1 and 2 developing more quickly than those in Trials 3 and 4 ($\alpha = 0.05$). There was no significant interaction between Trial and Treatment (Table 13).

Ingesting UO Cry3Aa had no significant effect on the length of any instar for *P. maculiventris* in Trials 1-4 (Table 14). Trial had a significant effect on the length of every instar (Table 14), but there was no significant interaction between Trial and Treatment (Table 14).

Table 13. Two-way ANOVA testing the effect of the independent variables Trial and Treatment (UO Cry3Aa concentration) on *P. maculiventris* developmental time from second instar to adult (for Trials 1-4).

Independent variable	df	F	P
Treatment	3	0.49	0.69
Trial	3	14.21	<0.01
Treatment x trial	9	0.85	0.57

Figure 1. Developmental time (mean $\pm$ SE) from second instar to adult for *P. maculiventris* reared on artificial diet containing different concentrations of UO Cry3Aa or alkalic buffer alone (control) (Trials 1-4).

Figure 2. Developmental time (mean $\pm$ SE) from second instar to adult for *P. maculiventris* in each of the first four trials.

Table 14. Two-way ANOVAs testing the effect of the independent variables Trial and Treatment (UO Cry3Aa concentration) on the length (in days) of each instar for *P. maculiventris* (Trials 1-4). n= the total number of individuals from all four trials that completed the instar.

Instar	n	Treatment			Trial			Trial x Treatment		
		df	F	P	df	F	P	df	F	P
N2-N3	110	3	0.45	0.71	3	3.87	0.01	9	1.20	0.31
N3-N4	106	3	0.05	0.98	3	11.08	<0.01	9	1.06	0.40
N4-N5	101	3	0.48	0.70	3	6.41	<0.01	9	1.07	0.39
N5-A	96	3	0.25	0.86	3	12.31	<0.01	9	1.08	0.38

*N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; A = adult

Trials 5-7

Ingesting MON Cry3Aa δ -endotoxin had no significant effect on the developmental time from second instar to adult for *P. maculiventris* in Trials 5-7 (Table 15 and Figure 3).

Trial also had no significant effect on developmental time, nor was there any significant interaction between Trial and Treatment (Table 15 and Figure 4).

Ingesting MON Cry3Aa had no significant effect on the length of any instar for *P. maculiventris* in Trials 5-7 (Table 16). Trial had a significant effect on the length of the third instar and fifth instars (Table 15), although overall development time (N2-A) was not significantly affected (Table 16). There was no significant interaction between Trial and Treatment (Table 16) for any instar.

Table 15. Two-way ANOVA testing the effect of the independent variables Trial and Treatment (MON Cry3Aa concentration) on *P. maculiventris* developmental time from second instar to adult (for Trials 5-7).

Independent variable	df	F	P
Treatment	2	1.37	0.26
Trial	1	13.49	0.07
Treatment x trial	2	0.63	0.54

Figure 3. Developmental time (mean $\pm$ SE) from second instar to adult for *P. maculiventris* reared on artificial diet containing different concentrations of MON Cry3Aa or distilled water (control) (Trials 5-7).

Figure 4. Developmental time (mean $\pm$ SE) from second instar to adult for *Podisus maculiventris* in Trials 5, 6, and 7.

Table 16. Two-way ANOVAs testing the effect of the independent variables Trial and Treatment (MON Cry3Aa concentration) on the length (in days) of each instar for *P. maculiventris* in Trials 5-7

Instar*	Treatment			Trial			Treatment x Trial			
	n	df	F	P	df	F	P	df	F	P
N2-N3	94	1	0.96	0.33	2	1.35	0.26	2	0.40	0.67
N3-N4	90	1	0.20	0.66	2	3.40	0.04	2	0.45	0.64
N4-N5	87	1	2.86	0.09	2	2.75	0.07	2	0.40	0.67
N5-A	81	1	2.82	0.09	2	3.67	0.03	2	0.63	0.54

*N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; A = adult

Effects of long-term exposure to Cry3Aa protein on survivorship of *P. maculiventris*

Trials 1-4

There was no significant difference in survivorship between *P. maculiventris* fed UO Cry3Aa δ -endotoxin and those in the control group (Figure 5) ($df = 3$, Chi-square = 0.31, $P = 0.96$) for Trials 1-4, nor was there a significant difference in survivorship between the trials (Figure 6) ($df = 3$, Chi-square = 4.79, $P = 0.19$).

Neither Treatment nor Trial had a significant effect on the survival rate at the end of each instar for *P. maculiventris* in Trials 1-4 (Table 17). There was no significant Trial x Treatment interaction (Table 17).

Mean survivorship in the control treatment was $88.89\% \pm 13.47$.

Figure 5. Kaplan-Meier survival analysis of *P. maculiventris* fed artificial diet containing different amounts of UO Cry3Aa or buffer alone (control), at the end of each instar, for Trials 1-4.

Figure 6. Kaplan-Meier survival analysis of *P. maculiventris* at the end of each instar, by trial, for Trials 1-4.

Table 17 Two-way ANOVAs testing the effect of the independent variables Trial and Treatment (UO Cry3Aa concentration) on the survivorship at each instar for *P. maculiventris* in Trials 1-4.

Instar	Treatment			Trial			Trial x Treatment			
	n	df	F	P	df	F	P	df	F	P
N2-N3	16	1	0.47	0.51	3	0.28	0.84	3	3.37	0.07
N2-N4	16	1	0.04	0.86	3	2.19	0.17	3	0.82	0.52
N2-N5	16	1	0.15	0.71	3	1.32	0.33	3	0.62	0.62
N2-A	16	1	0.10	0.75	3	0.77	0.54	3	1.65	0.25

* N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; A = adult

* N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; A = adult

Trials 5-7

Ingesting Cry3Aa δ -endotoxin had no significant effect on *P. maculiventris* in Trials 5-7 (Figure 7) ($df = 1$, Chi-square = 2.24, $P = 0.13$).

Trial had a significant effect on survivorship ($df = 1$, Chi-square = 7.87, $P = 0.02$) (Figure 8).

Neither Treatment nor Trial had a significant effect on the survival rate at the end of each instar for *P. maculiventris* in Trials 5-7 (Table 18). There was no significant Trial x Treatment interaction (Table 18).

Mean survivorship in the control treatment was $86.13\% \pm 11.06$

Figure 7. Kaplan-Meier survival analysis of *P. maculiventris* fed artificial diet containing either distilled water (control) or 50 $\mu\text{g/g}$ MON Cry3Aa, at the end of each instar, for Trials 5-7.

Figure 8. Kaplan-Meier survival analysis of *P. maculiventris* at the end of each instar, by trial, for Trials 5-7.

Table 18. Two-way ANOVAs testing the effect of the independent variables Treatment (UO Cry3Aa concentration) and Trial on the survivorship at each instar for *P. maculiventris* in Trials 5-7.

Instar	Treatment			Trial			Trial x Treatment		
	n	df	F	P	df	F	df	F	P
N2-N3	6	1	3.00	0.23	1	2.00	1	3.00	0.23
N2-N4	6	1	1.77	0.3	1	0.51	1	1.77	0.3
N2-N5	6	1	0.49	0.56	1	0.75	1	0.49	0.561
N2-A	6	1	0.18	0.91	1	0.33	1	0.16	0.73

* N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; A = adult

Effects of long-term exposure to Cry3Aa protein on development of *C. maculata*

One *C. maculata* escaped and I killed two accidentally. None of these individuals were counted.

Ingesting MON Cry3Aa δ -endotoxin had no significant effect on the time required for *C. maculata* to develop from neonate to adult (Table 19 and Figure 9).

Trial had a significant effect on developmental time (Table 19 and Figure 10). When I compared all pairs using the Tukey-Kramer test, I found that insects in Trial 4 took significantly longer to develop than those in Trials 1-3 ($\alpha = 0.05$).

Ingesting MON Cry3Aa had no significant effect on the mean time required for *C. maculata* to complete any instar (Table 20). Trial had a significant effect on the length of every instar except the second (Table 20). There was no significant interaction between Trial and Treatment (Table 20) for any instar.

Effects of long-term exposure to Cry3Aa protein on survivorship of *C. maculata*

There was no significant difference in survivorship from neonate to adult when I compared *C. maculata* that had consumed Cry3Aa δ -endotoxin (at any concentration) with those that had not ($df = 3$, Chi-square = 1.80, $P = 0.62$) (Figure 11).

Trial had a significant effect on survivorship ($df = 3$, Chi-square = 10.44, $P = 0.02$), with the survival rate being significantly lower in Trial 2 (Figure 12).

Neither Treatment nor Trial had a significant effect on the survival rate of *C. maculata* at the end of each instar for (Table 21). There was no significant Trial x Treatment interaction (Table 21).

Mean survivorship in the control treatment was $66.93\% \pm 4.12$.

Table 19. Two-way ANOVA testing the effect of the independent variables Trial and Treatment (MON Cry3Aa concentration) on *C. maculata* developmental time from neonate to adult.

Independent variable	df	F	P
Treatment	3	0.17	0.69
Trial	3	23.50	<0.01
Treatment x trial	9	2.59	0.06

Figure 9. Developmental time (mean $\pm$ SE) from neonate to adult for *C. maculata* reared on artificial diet containing different concentrations of MON Cry3Aa or distilled water (control).

Figure 10. Mean developmental time ($\pm$ SE) from neonate to adult for *C. maculata* in each trial.

Table 20. Two-way ANOVAs testing the effect of the independent variables Trial and Treatment (MON Cry3Aa concentration) on the length (in days) of each instar for *C. maculata*.

Instar	Treatment			Trial			Treatment x Trial		
	n	df	F	P	df	F	df	F	P
N1-N2	136	3	0.24	0.87	3	20.23	9	1.28	0.26
N2-N3	123	3	1.40	0.25	3	0.78	9	0.79	0.62
N3-N4	120	3	3.65	0.29	3	10.05	9	0.52	0.85
N4-P	111	3	0.09	0.97	3	6.94	9	0.87	0.57
P-A	109	3	0.96	0.42	3	7.06	9	0.72	0.10

*N2 = second instar; N3 = third instar; N4 = fourth instar; P = pupa; A = adult

Figure 11. Kaplan-Meier survival analysis of *C. maculata* fed artificial diet containing different concentrations of Cry3Aa or distilled water (control) or 50 μ g/g Cry3Aa, at the end of each instar, for Trials 1-4.

Figure 12. Kaplan-Meier survival analysis of *C. maculata* at the end of each instar, by trial, for Trials 1-4.

Table 21. Two-way ANOVAs testing the effect of the independent variables Trial and Treatment (UO Cry3Aa concentration) on the survivorship at each instar for *C. maculata*.

Instar	Treatment			Trial			Trial x Treatment			
	n	df	F	P	df	F	P	df	F	P
N1-N2	16	1	1.11	0.31	1	1.10	0.32	1	0.22	0.64
N2-N3	16	1	1.15	0.70	1	1.14	0.30	1	0.11	0.74
N3-N4	16	1	0.14	0.72	1	0.74	0.41	1	0.07	0.79
N4-P	16	1	0.16	0.64	1	0.05	0.83	1	0.01	0.91
P-A	16	1	0.09	0.77	1	0.003	0.99	1	0.05	0.82

*N1 = first instar; N2 = second instar; N3 = third instar; N4 = fourth instar; P = pupa; A = adult.

Bioassays to Test for Toxin in Predator Frass and Carcasses

For the sake of convenience, I will identify carcasses and frass from predators that were fed MON Cry3Aa as “Cry3Aa+”. In this case, “+” indicates that the predator was exposed to the protein, not that it was necessarily present in the carcass or the frass.

Feeding damage was at least 75% in all of the control treatments (Tables 22 and 23), indicating that the route of exposure in this experiment was not in itself a major feeding deterrent. The CPB larvae in these trials did not appear to be put-off by the presence of crushed insects or frass on their food.

***P. maculiventris* frass and carcasses**

Fewer CPB survived after ingesting Cry3Aa+ *P. maculiventris* from the 500 µg/g group than from either the 50 µg/g group or the corresponding controls (Table 22). Similarly, feeding damage was also much lower in this treatment (Table 22).

Survival was high in all treatments when CPB ingested *P. maculiventris* frass, regardless of the predators’ diets (Table 22). Feeding damage was also very extensive in all treatments (Table 22).

***C. maculata* frass and carcasses**

Ingesting Cry3Aa+ *C. maculata* had no significant effect on CPB survivorship or feeding damage (Table 23). Ingesting frass from Cry3Aa+ *C. maculata*, on the other hand, significantly reduced both survivorship and feeding damage (Table 23).

Table 22. Survival rates and relative feeding damage caused by CPB larvae after ingesting frass or carcasses from *P. maculiventris*. Cry3Aa+ *P. maculiventris* were fed artificial diet containing MON Cry3Aa at the dose specified in parentheses; those in the control treatments received artificial diet dosed with distilled water.

Medium	% Survival	% Feeding Damage
Carcasses		
control	100	100
Cry3Aa+ (50 µg/g food)	100	100
control	100	100
Cry3A+ (500 µg/g food)	40	5
Frass		
control	75	100
Cry3Aa+ (50 µg/g food)	100	100
control	80	96
Cry3A+ (500 µg/g food)	80	100

Table 23. Results of t-tests comparing a) the survival rates of CPB larvae, and b) the relative amount of feeding damage they caused after ingesting *C. maculata* frass or carcasses from different treatments. Cry3Aa+ *C. maculata* were fed artificial diet containing 200 µg MON Cry3Aa/g food; those in the control treatments received artificial diet dosed with distilled water.

Independent variable	% Survival				% Feeding Damage			
	mean (SE)	df	t _{stat}	P	mean (SE)	df	t _{stat}	P
Treatment								
Carcasses								
control	100	control and treatment results were identical			76.5 (1.1)	4	-1.4	0.15
Cry3Aa+	100				80 (1.4)			
Frass								
control	91.7 (7.2)	4	5.09	<0.01	94.7 (4.6)	4	12.4	<0.01
Cry3Aa+	19.0 (8.2)	2			13.3 (2.7)			

DISCUSSION

Bioassays to test purified Cry3Aa δ -endotoxin

Ferro and Gelernter (1989) reported that the feeding activity of second-instar CPB decreased dramatically after they had ingested of *B. thuringiensis* var *san diego*. In particular, they noted that, at higher doses, CPB stopped feeding within the first 24h, even though they tended not to die until close to the end of the 96-hour test (the researchers did not identify the concentrations at which this behaviour occurred).

During the course of this experiment, I also noticed that feeding inhibition was very apparent early in a bioassay. Only 24 hours into a 96-hour bioassay, it was clear from the insects' feeding behaviour which treatments contained higher concentrations of Cry3Aa. I used this characteristic as the main indicator of Cry3Aa presence and activity throughout this study, particularly when trying to assess the activity of the UO toxin used in the first assays. Given the very small sample sizes in our experiments, I had much more confidence in the sight of an uneaten leaf than I did in survival statistics that could be heavily skewed by a single, chance death.

This being said, assays can only properly support an experiment if they are done regularly. In hindsight, performing bioassays more frequently, and testing a greater range of concentrations each time (i.e., more serial dilutions), would have allowed us to predict the concentration of the stock solution more accurately, and to be more aware of any decrease in toxin activity over time.

Effects of long-term exposure to Cry3Aa protein on predator development and survivorship

NewLeaf™ potato plants express Cry3Aa δ -endotoxin at between 14.4 and 59.9 $\mu\text{g/g}$ fresh weight in the leaves (CFIA, 1996; CFIA, 1997). Neither *P. maculiventris* nor *C. maculata* appear to be susceptible to the toxin at doses within that range (*P. maculiventris*) (Tables 13-18) or higher (*C. maculata*) (Tables 19-21). Developmental rate was not significantly delayed, nor was survivorship significantly reduced in treatments where individuals were reared on artificial diet containing Cry3Aa. This strongly indicates that ingesting Cry3Aa δ -endotoxin is not harmful to these predators.

Our findings with respect to *C. maculata* are in-line with those of Riddick and Barbosa (1998), who found that nymphs reared on CPB that had eaten transgenic potato foliage did not differ significantly from those in the control treatment with respect to developmental time, live weight, or fecundity (Table 3).

Our findings do conflict with Giroux *et al.* (1994), who found that nymphs fed pollen that had been treated with a field-strength concentration of M-One (a *B. thuringiensis* subsp. *tenebrionis*-based bioinsecticide) took almost one week longer to develop from neonate to adult than those in the control group (Table 3). As M-One is a spore/crystal preparation, it is quite possible that this effect was caused toxic proteins in the spores other than Cry3Aa, such as cytotoxins, which are much less specific than δ -endotoxins.

There have not been any previous investigations of Cry3Aa on *P. maculiventris* (Table 3).

Trial had a significant effect on developmental time for *P. maculiventris* in Trials 1-4 and for *C. maculata*, but not for *P. maculiventris* in Trials 5-7 (Tables 13-18). This

can probably be attributed to differences in the uncontrolled environmental conditions, including periodic jumps or drops in room temperature during the experiments. The fact that there is no significant difference in developmental times between Trials 5, 6, and 7 supports this claim, as these were the only trials to start on consecutive days (Tables 5, 15 and 16). In the other feeding assays, trials were sometimes held weeks apart.

It is possible that my inability to detect an effect in the treated groups is because the sample sizes were too small. Power analysis would have helped remove any doubts in this area, and will be included in any future reports of my findings. This being said, over the course of this study, hundreds of individuals from each species were reared to different stages but not included in the analysis (for various reasons), and nothing was observed to indicate that Cry3Aa was harmful. Moreover, most effects, although small and nonsignificant, were in the wrong direction (better performance when fed Cry3Aa-laced diet).

Direct toxicity lab studies such as these cannot replicate the complex conditions in the field; however, they do represent a logical first step by providing information necessary to quickly identify significant hazards that require further investigation (Poppy, 2000).

The tests performed for this paper cannot rule out the possibility that one of the truncated forms of Cry3Aa found by Carroll *et al.* (1997) could be more toxic to *C. maculata* or *P. maculiventris* than the 68 kDa form used in these analyses. For example, it may be that the protein is cleaved in the prey species' gut into a form that is more soluble in the predator's gut. However, as mentioned, Riddick and Barbosa (1998) found nothing to suggest that this was the case, at least for *C. maculata* feeding on CPB.

Given these findings, it is likely that both *C. maculata* and *P. maculiventris* could safely play a role in a Bt-potato IPM strategy. As generalists, both species would be capable of dealing with pests that are not susceptible to Cry3Aa such as ECB, leafhoppers, aphids, and resistant CPB larvae. Arpaia *et al.* (1997) found that the predation efficiency of *C. maculata* feeding on CPB eggs was inversely density-dependent—that is, *C. maculata* adults ate a greater proportion of eggs in each egg mass when there were fewer egg masses available, in closed-cage experiments. They then developed a model and demonstrated that augmentative field releases of *C. maculata* could help delay the rise of Cry3Aa resistance in CPB populations in transgenic fields. As well, Biever and Chauvin (1992) found that third-instar *P. maculiventris* attacked CPB larvae at a rate that prevented more than half of 1800+ larvae (released in a closed-cage) from reaching adulthood.

Bioassays to Test for Toxin in Predators and Frass

CPB larvae consumed potato leaves coated with the crushed carcasses of adult *C. maculata* that had been reared to pupation on Cry3Aa and experienced no ill effects. This indicates that very little toxin remains in the insect after pupation. This experiment should be repeated using pre-pupal *C. maculata* as there is currently no way of knowing whether the toxin was degraded by enzymes present only during metamorphosis and, therefore, would otherwise be present in an effective dose in the larva.

Mortality and feeding rates in the *C. maculata* frass treatments, on the other hand, suggest that Cry3Aa may pass through the *C. maculata* digestive system intact. SDS-PAGE analysis could be used in the future to identify the size of the protein after it has passed through the *C. maculata* digestive system in order to see if any proteolysis has occurred.

The deposition of Cry3Aa in the soil via *C. maculata* frass could conceivably lead to a build up of Cry3Aa in the soil in transgenic fields, thereby exposing soil-dwelling organisms to higher-than-expected levels of toxin. Stotzky (2000) and Saxena *et al.* (1999) have shown that Cry1Ab can persist for 234 days when bound to clay particles. However, the amount of Cry3Aa introduced to the soil via *C. maculata* frass would likely be very small compared to that introduced by potato root exudates and plant residues, and thus contribute little to the total concentration.

P. maculiventris carcasses from the control and 50 µg/g treatments had no visible effect on the health of CPB ingesting them, while larvae fed crushed *P. maculiventris* carcasses from the 500 µg/g treatment fed considerably less than those fed carcasses from the control. This implies that the *P. maculiventris* had consumed and retained a dose of toxin large enough to affect CPB, although it is unclear whether the toxin became concentrated within the *P. maculiventris* or if the concentration in the food was simply high enough that very small amounts (such as that residing in the *P. maculiventris*' digestive tracts during normal digestion) were able to cause an effect.

The results of the bioassay involving *P. maculiventris* frass were more clearly an indication of a physiological process inside the insect—one that appears to be absent in *C. maculata*. All of the CPB in the treatments involving *P. maculiventris* frass thrived and ate well, even when *P. maculiventris* had been fed diet containing 500 µg/g toxin (Table 22). When compared to survival rates of less than 20% in CPB fed frass from *C. maculata* exposed to toxin 2.5 times less concentrated (Table 23), this result suggests that *P. maculiventris* digests Cry3Aa much more completely than *C. maculata*—to the point where the toxin is no longer in a biologically active form.

P. maculiventris digest much of their food extra-orally; that is, they pierce their prey with their rostrum, inject powerful enzymes under the exoskeleton, and then suck back the liquefied innards (Cohen, 1995). The fact that heavily-dosed *P. maculiventris* carcasses are toxic to CPB (and, thus, must contain some intact Cry3Aa) demonstrates that the enzymes injected into the prey do not completely degrade the Bt toxin. On the other hand, *P. maculiventris* frass had no effect on CPB, indicating that it is the enzymes in the *P. maculiventris* gut that break Cry3Aa into non-toxic polypeptides before it is eliminated.

CONCLUSION

Risk is defined by the World Health Organization as follows:

$$\text{Risk} = \text{Hazard} \times \text{Exposure}.$$

This study measured the hazard component of the equation by assessing the direct toxicity of Cry3Aa δ -endotoxin towards *P. maculiventris* and *C. maculata*. There was no significant effect on survivorship or developmental rate in either case, indicating that Cry3Aa poses a very small hazard—and, therefore, a very small risk—to these predators.

P. maculiventris could completely digest large doses of Cry3Aa, while *C. maculata* could not. However, it seems unlikely that a lady beetle would consume such a large dose of Cry3Aa under field conditions, so there seems little danger of unexpectedly high loads of Cry3Aa accumulating in the environment as a result of lady beetle frass.

Based on the results of this study, Cry3Aa can be considered compatible with IPM programs involving *P. maculiventris* and *C. maculata*.

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