INTERACTIONS OF ARTHROPOD PREDATORS AND CRY1AC-TRANSGENIC COTTON

by

JORGE BRAZ TORRES

(Under the Direction of John R. Ruberson)

ABSTRACT

Interactions of predators and Bt-cotton plants (containing a gene from *Bacillus thuringiensis* that expresses Cry1Ac toxin) were investigated in the field, greenhouse, and laboratory. Abundance of predatory arthropods was monitored from 2002 to 2004 in three pairs of adjacent Bt and non-Bt fields (5 to 15 ha each). Analysis of predator abundance and dynamics showed variation among sampling dates and among seasons for some specific taxa collected through whole plant and drop cloth sampling in favor of either cotton. However, when averaged over three years, differences were nearly all eliminated. Of 65 ground-dwelling arthropods collected in pitfall traps, no differences were found between cotton types for abundance, diversity, and species richness.

Field-collected materials (plant-herbivore-predator) were assayed for Cry1Ac toxin using ELISA. Bt-cotton and lepidopteran larvae were positive on all sampling dates, while among seven predator species only *Podisus maculiventris* and *Chrysoperla rufilabris* were positive (on one and two sampling dates, respectively) concurrent with high abundance of lepidopteran larvae in the fields. Ingestion of Cry1Ac toxin by four common predatory heteropterans (*Geocoris punctipes*, *Nabis roseipennis*, *Orius insidiosus*, and *P. maculiventris*) was studied using prey fed Bt-cotton in a greenhouse or dilutions of purified Cry1Ac in the laboratory (*Geocoris punctipes*).

Predatory heteropterans were unable to pick up toxin directly from the plant, despite plant feeding behavior, but may acquire Cry1Ac from prey fed Bt-cotton. The amount of prey consumed by small predatory heteropterans (*Orius, Geocoris,* and *Nabis*) seems to limit ingestion of Cry1Ac below detectable levels in their bodies. *G. punctipes* was able to pick up toxin from Cry1Ac purified dilutions in water at detectable levels but from concentrations higher than levels detected in cotton plants and greater than conveyed by prey fed Bt-cotton. Most of the ingested Cry1Ac, however, was excreted and was not detected in the predators' bodies or feces more than 48 to 72h after feeding.

Prey- and/or plant-mediated effects on the omnivorous predator *G. punctipes* were studied in the field. Predators were exposed to a combination of prey with and without toxin, and Bt and non-Bt plants from egg hatch until death. The results showed no effect of prey fed-Bt or direct effect of Bt-cotton plants on life history parameters of the predator.

INDEX WORDS: Insecta, *Bacillus thuringiensis*, transgenic cotton, biological control, tritrophic interactions, predatory heteropteran.

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DEDICATION

This dissertation is dedicated to my large and lovely family that always gave me support. To my wife Christian for her constant companionship and balance given to our relationship. To all professors along my academic life and especially to my mother and father who with their simplicity showed me the value of education.

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CHAPTER 1

INTRODUCTION

Cotton is one of many agriculture commodities that does not need many words to justify its importance not only in the US, but also for many other countries around the world. The economic impact of cotton and the importance of pest management for cotton production is reflected in the recent investments made by biotechnology companies to develop transgenic cotton resistant to insect pests. For instance, the releases of Bollgard®, Bollgard II®, Widestrike™ and VipCot™ varieties all sharing the unique goal of managing nearly all lepidopteran larvae, a group of key pests in cotton fields everywhere. The broad acceptance by growers and great potential for Bt-cotton cultivation throughout cotton growing regions fuel many ecological questions from agronomists, ecologists, and especially from entomologists about potential negative impactS of the technology on nontarget organisms. This subject evolved into a major issue for adopting transgenic plants during this past decade, not only as a matter for scientific discussion but also for public debate.

Transgenic cotton plants have been produced containing genes that express Cry toxins (Cry = crystal) from the bacterium *Bacillus thuringiensis* (Bt). Bt-cotton expressing the Cry1Ac toxin has been cultivated on a large scale in the US and other countries such as Australia, Argentina, China, India, Indonesia, Mexico, and South Africa (Fitt, 2000; Tianzhen and Canming, 2000; Yousouf et al., 2001; Perlak et al., 2001; Edge et al., 2001; Qaim and De Janvry, 2003; Toenniessen et al., 2003; Wu and Guo, 2005). Other varieties containing genes for expression of Cry2Ab, Cry1F and VIP (VIP stands for Vegetative Insecticidal Proteins) are coming to the

market. The broad use of Bt-cotton is projected for many more countries, but the authorization to plant transgenic cottons is awaiting studies of nontarget effects, among others factors. Many questions have been generated and debated about the impact of transgenic plants on beneficial or nontarget organisms. Results have been published covering different aspects of transgenic Bt-crops; however, few studies have properly addressed interactions of Bt-crops and natural enemies, excluding those in the literature review (CHAPTER 2). From these results, some important concerns related to Bt-plants and natural enemies can be addressed. The herbivores targeted by Bt-toxins are eliminated or become less suitable prey or hosts for natural enemies. Prey/host absence has obvious adverse effects on predators and parasitoids. In addition, unhealthy prey/hosts may be more readily attacked by predators and parasitoids causing a cumulative sublethal effect of Bt toxin on natural enemy population increase. On the other hand, reduction of insecticide use in Bt crops tends to enhance natural enemy populations compared to insecticide-managed non-Bt fields. Most laboratory and field experiments have indicated that Bt-plants have no direct effect on insect predators. This outcome, however, may change when indirect effects are evaluated. Some laboratory studies reported indirect effects of Bt-plants on arthropod predators and parasitoids through unhealthy prey and hosts (Hilbeck et al., 1999; Schuler et al., 1999; Dutton et al., 2002; Baur and Boethel, 2003; Ren et al., 2004). The results suggest at first glimpse that the availability of prey/host fed Bt-cotton can inflict cumulative effects, which would bring about changes in the population dynamics of predators and parasitoids in the field. The results, however, have been criticized because the studied predator or parasitoid had only the target pest as a prey or host under laboratory conditions (Crawley, 1999; Lövei and Arpaia, 2005). In nature, this situation is quite different, considering the broad spectrum of prey and hosts not targeted by

Bt toxins that can be exploited by predators and parasitoids, especially in the cotton agroecosystem.

Although large-scale and long-term studies of insect communities in commercial fields yield the most realistic and valid results (Marvier, 2002; O'Callaghan et al., 2005), data from such trials are often difficult to interpret due to irregular and variable insect population densities, limited replication, and unpredictable environmental conditions depicted in most published results (CHAPTER 2). Moreover, long-term investigations of arthropod populations in agricultural fields have limitations no matter what scale (O'Callaghan et al., 2005). Therefore, a sequence of intermediate scale studies between laboratory, small-scale greenhouse and field bioassays and large-scale field experiments has been recommended (Firbank et al., 1999; Schuler et al., 2001). However, one must be aware that many of these studies do not necessarily provide realistic predictions about long-term population dynamics at larger spatial scales (Crawley, 1999). Generalist natural enemies will find in each patch a different ecological community with a different array of prey. For these reasons, Poppy (2000) suggested that comparisons of data should be considered only cautiously because experiments are conducted at different scales, at different times, and at different locations.

This dissertation presents results of a series of studies from laboratory, greenhouse and commercial field surveys investigating interactions of transgenic Bt-cotton (Bollgard®), expressing the Bt toxin Cry1Ac, with predators important for cotton pest management. These studies focused on the following questions:

a) Are the communities and population dynamics of canopy- and ground-dwelling predatory arthropods different between Bt and non-Bt commercial cotton fields?

b) Does the toxin Cry1Ac expressed in the cotton plants move through trophic levels: Bt-cotton plants to herbivorous prey to insect predators?

c) Are predatory heteropterans, which exhibit plant-feeding behavior, able to ingest Cry1Ac toxin from Bt-cotton plants or prey fed Bt-cotton?

d) Is the omnivorous predatory heteropteran *Geocoris punctipes* affected by the Bt-toxin Cry1Ac through prey fed-Bt cotton or plant feeding behavior?

e) Do Bt cotton plants influence the oviposition pattern of the omnivorous predator *Geocoris punctipes* and of its prey, heliothine moths?

The dissertation contains eight chapters with the first two containing a brief introduction and literature review of the interactions of Bt transgenic plants and natural enemies, and some details on the life history of the predator *G. punctipes*, which was partially the focus of this study. In the next five chapters are presented the results addressing the questions above, ending with the last chapter summarizing the outcomes and addressing final considerations about the interaction of Bt-cotton and natural enemies found in cotton fields.

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CHAPTER 2

LITERATURE REVIEW

Cotton in the United States is grown in 17 different states grouped into 4 major production regions - Far West, Southwest, Midsouth, and Southeast. The Southwest and Southeast regions comprise the highest acreage harvested, with Texas and Georgia ranked first and second in planted area, respectively. In the US and throughout the world, cotton ecosystems contain a wide diversity of arthropod pests, predators and parasitoids, which are influenced by cotton varieties, uncultivated plants and weather interactions. Among the arthropod pests of cotton, largely polyphagous species such as heliothines, aphids, spider mites and whiteflies are found in the production regions in the US. Despite advanced technologies applied to pest management in cotton, no single control method has provided complete control of the pest complex. Special emphasis has been placed on boll weevil, bollworms and plant bugs complexes. The two former groups have been well managed through eradication practice and adoption of Bt cotton, respectively, but plant and stink bugs continue to require intensive management. Although Bt cotton effectively controls bollworms, there is lack of efficacy against sucking pests commonly present in the cotton ecosystem. Hence, the complex of bug pest has been elevated to key pest status in many states following boll weevil eradication and adoption of Bt cotton.

Engineered or transgenic plants have received special attention from biotechnologists and entomologists who have a special interest in obtaining plants resistant to arthropod pests from breeding programs. The greatest research effort in developing pest-resistant transgenic crops has gone into incorporating genes expressing δ-endotoxins of *Bacillus thuringiensis* (Bt) in plants. Bt

δ-endotoxin genes have been inserted into at least 17 cultivated plant species to control species of two major groups of agricultural pests (Lepidoptera and Coleoptera). A complete list of Bt genes and other sources for transgenic plants available or in development is reported in Sharma et al. (2004) and O'Callaghan et al. (2005). The Bt δ-endotoxins constitute a family of related proteins for which over 295 Cry genes have been described (Crickmore et al., 2004). Different toxins have different specificities for different orders of insects, mainly for lepidopteran larvae (Cry1A, 1B, 1C, 1D, 1E, 1F, 1L, 1J, 2A, 9A, 9B, 15A), coleopteran larvae (Cry1B, 1L, 3A, 3B, 3C, 7A, 8A, 8B, 8C, 14A), dipteran larvae (Cry4A, 4B, 10A, 11A, 2A), and nematodes (Cry5A, 6A, 6B, 12A, 13A). However, susceptibility to Cry proteins of different species within an order can differ enormously (Slaney et al., 1992; Glare and O'Callaghan, 2000).

Transgenic plants have been produced using sources of resistance genes other than Bt. For instance, α-amylases, protein inhibitors (PI) such as CpTI inhibitors of trypsin against *Heliothis virescens* (Fabr.) in tobacco, and Pot PI inhibitor of proteinases against *Helicoverpa armigera* (Hübner) have been used. Anti-aphid mannose-specific lectin GNA derived from *Galanthus nivalis,* and other lectin compounds and other PI have been used in plant transformation affecting larval development and survival of lepidopteran pests of crops other than cotton (Ryan, 1990; Oppert, 2001; Sharma et al., 2004; O'Callaghan et al., 2005). In cotton, transgenic varieties have been produced with Cry2Ab, Cry1F, Cry1A+CpTi (=Cowpea Trypsin inhibitor), VIP (=Vegetative Insecticidal Proteins), Arrowhead PI and pea lectin in different countries (Estruch et al., 1996; Wang et al., 1999; Tianzhen and Canming, 2000; Huang et al., 2001; Cui et al., 2002; Adamczyk and Gore, 2004).

Genetically engineered cotton containing Bt-genes expressing Cry1Ac toxin protect cotton against the bollworm complex (*Helicoverpa*, *Heliothis* and *Pectinophora*). Recently other cotton

varieties have been developed with genes of Cry2Ab, Cry1F, and VIP toxins offering extended control to other lepidopteran larvae such as loopers and armyworms, only partially susceptible to Cry1Ac (Estruch et al., 1996; Adamczyk and Gore, 2004). In addition, simultaneous expression of a combination of different toxins (called "gene stacking" or "pyramiding") is considered an important tool in resistance management (Roush, 1997; Greenplate et al., 2003), especially for pest species showing a physiological potential to respond evolutionarily to different toxins simultaneously (Jurat-Fuentes et al., 2003).

Although cotton plants expressing Bt Cry1Ac toxin have produced impressive results against the bollworm complex, as reported by Perlak et al. (2001), Bt-cotton failed to control *Helicoverpa armigera* (Hübner) in Australia, and *Helicoverpa zea* on at least 20,000 acres in Texas (Hilder and Boulter, 1999). Moreover, 4 out of 5 pests with strains able to survive diet containing Cry toxins are cotton pests (i.e., tobacco budworm, pink bollworm, old-world bollworm and cabbage looper; the fifth one being diamondback moth, a pest of crucifers) (Gould et al., 1997; Liu et al., 2001; Fengxia et al., 2003; Janmaat and Myers, 2003). Many reasons have been used to justify variation in susceptibility to Bt such as environmental influences, inadequate expression levels, local resistance, high population pressure, and genetic and physiological adaptation of species coping continuously with Bt-toxins (Gould, 1998; Liu et al., 2001; Tabashnik et al., 2000; Carrière and Tabashnik, 2001; Gahan et al., 2001; Perlak et al. 2001). Therefore, multiple integrated, rather than single tractic pest management practices are recommended to avoid any further adverse outcome.

The clear need for integrated management of cotton pests can be visualized from results in China where cotton hosts a complex of pests. Jing-Yuan et al. (1999) reported that from 1994 to 1998 cotton varieties expressing Bt toxins in China controlled the old-world bollworm, *H.*

armigera, and that the degree of cotton resistance fluctuated in time and space. Use of Bt cotton coupled with other management practices reduced the status of bollworms as key pests in Bt cotton, but red spider mites, aphids, and thrips then became the main pests. Thus, important pests continue attacking Bt cotton varieties, necessitating additional spraying for their control. For these reasons and the likely event that Bt-targeted insects become resistant, cultural control through cropping practices and preservation of natural enemies continue to be important tactics for success in cotton pest management.

From intensive use of insecticides to integrated, multilateral pest control, cotton pest management in the US has evolved to a low insecticide requirement compared to periods preceding boll weevil eradication (Haney et al., 1996; Meyer and Smith, 1999) and the availability of Bt-cotton (Luttrell and Herzog, 1994; Betz et al., 2000). The reduction of insecticide usage by adopting these two foundational pest management practices in US cotton fields opened a window of opportunity to enhance the role of biological control agents occurring naturally in cotton fields. Conservation of biological control agents is an option to ensure that existing technologies are not as easily lost in the short term. It is generally agreed in most recent reviews of cotton pest management in the US that future cotton pest management programs will rely much less on chemical insecticides and more on plant resistance and biological control. Relative to this point, it has been suggested that the reduction in insecticide associated with transgenic varieties should increase the abundance of beneficial insects and improve the natural control of many pests (Hilder and Boulter, 1999). However, other pests not controlled by Bt-cotton may assume greater importance and insecticides would be used as much as before, especially if there are negative effects of transgenic plants on natural enemies. Furthermore, Hagerty et al. (2005) found that the bollworm, *H. zea*, exceeded the economic threshold twice

when natural enemies were disrupted in Bt-cotton fields, reinforcing the need to conserve natural enemies.

Tritrophic interactions in Bt-cotton fields. In a cotton pest management program, practices that conserve natural control caused by insect pathogens, predators, and parasitoids are a promising approach to pest control, but according to Luttrell et al. (1994) and Bradley (1996) insecticides are still the primary control method used worldwide in cotton production systems. It is somewhat justified because the reduction in cotton yield, in different regions where cotton is cultivated, are estimated to be as high as 84% in the absence of pesticide use (Oerke et al., 1994). For instance, the loss due to insect damage was estimated to be 2.26% for Georgia cotton in 2004 despite adopting all current pest management practices, and accounted for a total cost to growers of $90,569,000 (including yield loss by insect damage and control costs) (Williams, 2005).

The overall adoption of Bt cotton in the US accounted for 58% of acreage cultivated, and nearly 70% of 1.3 millions acres cultivated in Georgia during the season 2004 (Williams, 2005). Pest management in cotton fields was expected to change with adoption of Bt cotton (Perlak et al., 1990; Luttrell and Herzog, 1994). Despite the benefits of Bt cotton, controversy has surrounded the broad-scale use of transgenic cotton resistant to pests because of potential risks of negative impact on the natural enemies of herbivores and on other nontarget organisms, the possibility of gene flow to wild relatives, and the rapid evolution of resistance in pests (Hail, 2000).

Being cultivated under a number of different cropping systems, cotton is exposed to losses from a diverse complex including Bt-targeted and nontargeted pests such as aphids, whiteflies, the bug complex, mites and others. In addition, effectiveness of the insect-resistant traits is limited. Many of those pests are not susceptible to any known Bt strains and some amount of conventional

insecticides will still be used for their control (Meyer and Smith, 1999). Therefore, the rapid pace at which research is proceeding in this field naturally calls for an integrated approach to problems concerning insects and host plant resistance. In the search for alternative strategies and natural enemy conservation, the combined efforts of entomologists, ecologists, and recently biotechnologists have focused on the role of tritrophic interactions in the biocontrol of insects. In cotton fields of Georgia, there are many possible interactions between herbivores and natural enemies, opportunities for natural enemies to interact with Bt toxins (Fig. 2.1). For natural enemies that feed directly on plants (e.g., predatory heteropterans) or use plant products such as pollen and nectar (e.g., lady beetles and parasitoids), direct interactions with Bt toxins may occur. In addition, prey-mediated indirect interactions may occur when prey consume cotton containing Cry toxins. The potential for negative interactions are most likely for natural enemies attacking prey conveying Bt-toxins. Almost all arthropod predators found in cotton fields are generalists and use a mixed diet of Bt(–) and Bt(+) prey species when available, and plant products (sap, nectar, pollen, etc.). Negative interactions that may occur via direct toxicity of Bt toxin or through prey-mediated effects might be diluted and not detected through population dynamics in the field or in the laboratory as reported (results in Table 2.1, and CHAPTERS of this dissertation).

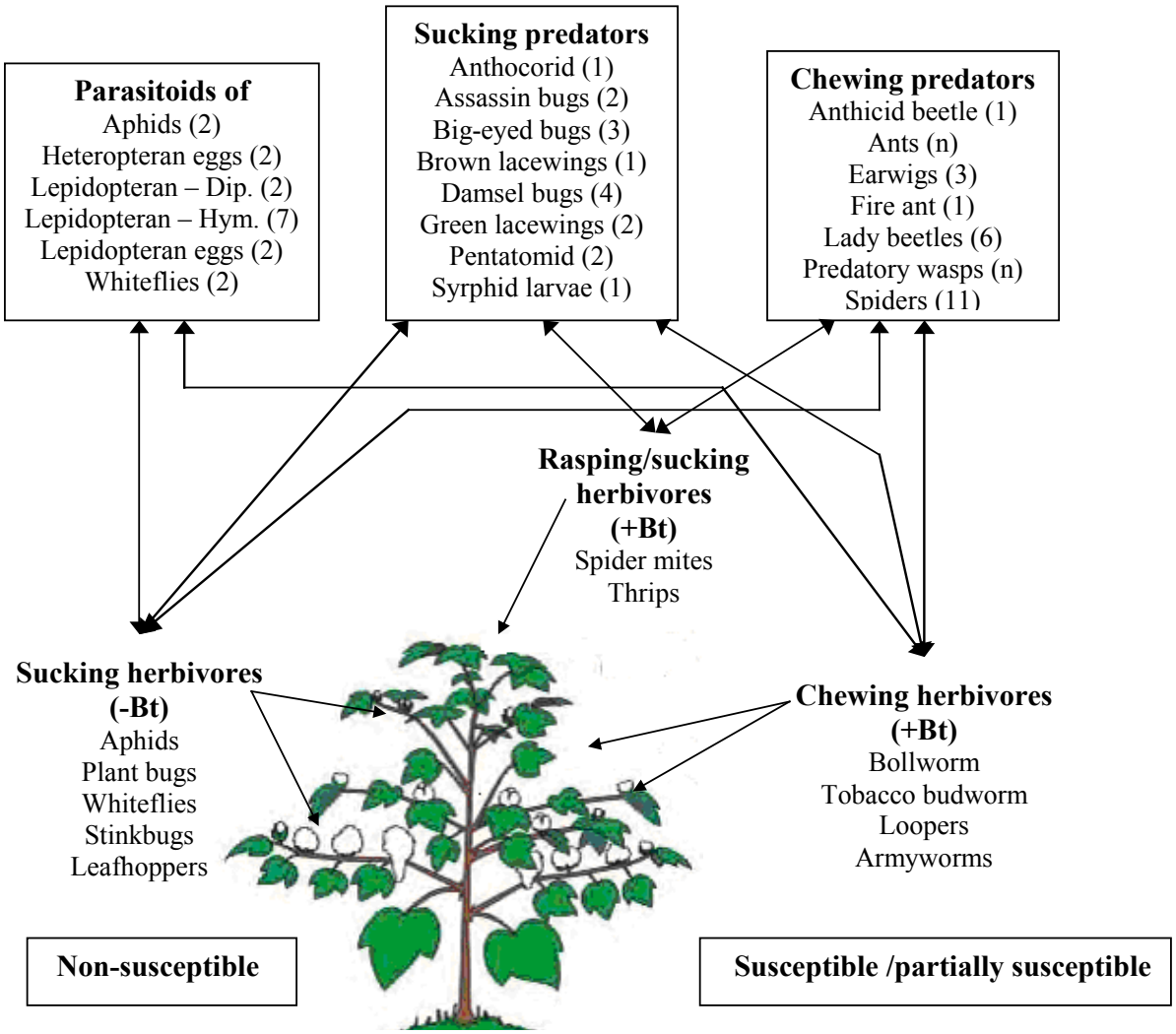

Fig. 2.1. Examples of major likely trophic interactions on the plant canopy in cotton fields cultivated in the Georgia Coastal Plain. Numbers between parentheses stand for number of common identified species and "n" for many unidentified species (see CHAPTERS 3 and 4 for more details on common arthropod predators).

The specificity of Bt is such that it was expected to have no direct effects on predator populations, although indirect effects due to sick or suboptimal prey would be expected. Brief synopses of the most significant findings of published results concerning the interaction between Bt crops and natural enemies are presented in Table 2.1. From 73 research results, an adverse

effect of Bt toxins or Bt-reared prey on arthropod predators is reported in three laboratory studies (Hilbeck et al., 1998a and b; Ponsard et al., 2002) and in one field survey (Sun et al., 2003). In the laboratory, larvae of the green lacewing *Chrysoperla carnea* (Stephens) and the predatory heteropterans *Geocoris punctipes* (Say) and *Orius tristicolor* White were negatively affected when fed diet containing Cry1Ab or prey fed Bt cotton. Later studies determined that the effect on *C. carnea* was a result of suboptimal prey quality and not toxicity of Bt-toxin (Dutton et al., 2002; Romeis et al., 2004). For *G. punctipes* and *O. tristicolor*, lower survival was found in one and two trials out of four trials, respectively, when field-collected predators were confined in the laboratory with *Spodoptera exigua* larvae reared on Bt cotton. The authors recognized problems with the caging method; especially prey size and moisture offered to the predators during the experiment, which are factors that can cause variation in survival of these predators.

In 14 field survey studies in Bt and non-Bt cotton fields in the US (7), China (6) and Australia (1), comprising 77 predator and 2 parasitoid species (Table 2.1), it was reported that 8 and 5 taxa had greater and lower densities, respectively, in Bt-cotton fields. The remaining 66 taxa were similarly abundant in Bt and non-Bt cotton fields. These results were generated under various environmental conditions and experimental designs that do not allow comparisons among them. In reports with greater populations in Bt cotton, insecticide use in non-Bt cotton usually is offered as the explanation, but no explanation is offered for the opposite results.

Field results although not distinguishing between population-level, prey-mediated or direct Bt-toxin effects, have varied from no effect, to increase and decrease of specific predator taxa in relation to Bt crops (Table 2.1). The lack of consistency has resulted from a variety of confounding sources, such as small plots, number of years surveyed, and number of samples taken during the crop season. From 14 field surveys comparing predator abundance between Bt and

non-Bt cotton fields, 78.6% (11 out of 14) of the results were generated in small field plots (e.g., 8-10 rows per 10-30 m in length or 100-200 m^2). Studies were done during one (50%), two (21.4%), or three seasons (28.6%); few studies were of sufficient duration to account for environmental variability among years. In addition, 38.5% of the studies conducted only one to three samples during the entire season, restricting statistical power to detect significant interactions (variation in toxin expression in the plants; herbivore and predator dynamics) occurring during the season. Therefore, small experimental fields, few years, and one or few sampling dates within the crop season provide weak control of environmental variability, and interactions of plant and natural enemy phenologies, issues often recognized by the authors themselves. Furthermore, when using standard grower agronomic practices, adoption of insecticides to control pests not targeted by Bt cotton adds another variable to population dynamics (e.g., CHAPTER 3).

Parasitism of pests susceptible to Bt toxins typically failed because of the host death before the parasitoid could complete development, or parasitoid development was delayed by low host quality (Table 2.1). Parasitism of pests fed Bt plants or diet containing Bt toxins, but only partially susceptible to Bt, allowed parasitoids to successfully complete development. However, in most cases sublethal effects, such as delayed larval development, lower pupal and adult weight, were noted (Bernal et al., 2002; Baur and Boethel, 2003; Lu et al., 2004; Ren et al., 2004; Meissle et al., 2004; Liu et al., 2005). However, this type of outcome has also been detected for parasitoids from Hymenoptera and Diptera reared on hosts treated with commercial Bt formulations, where hosts typically die before parasitoid development is complete (Blumberg et al., 1997; Atwood et al., 1999; Erb et al., 2001).

Table 2.1. Summarized experimental procedures and relevant findings of studies of interactions between Bt transgenic plants and natural enemies.

Toxin/Crop/ Locale/Guild	Experimental Procedures – Natural enemies	Relevant findings	Ref.
Cry1Ac Cotton Field PR[1]	Sweep-net samples of *Geocoris punctipes*, *Nabis* spp., *Collops vittatus*, *Orius tristicolor*, *Hippodamia convergens*, *Chrysoperla carnea*, and *Lygus hesperus* were taken during the season 1994 in field plots of 4 rows of 9.1m long.	There were no significant differences in densities of any of the collected predators between unsprayed non-Bt cotton and Bt cotton plots.	1
Cry1Ac Cotton Field PR	Field plots (4 rows wide and 9.1m long) of Bt and non-Bt cotton were surveyed weekly for *G. punctipes*, *C. vittatus*, *C. carnea*, *H. convergens*, *Nabis* spp., and *O. tristicolor* using 40 sweeps per plot during 1990.	Overall low densities of predators are reported in both cottons, except on one date when fewer *G. punctipes* were found on Bt-cotton (3.17) than on non-Bt cotton (11.5 individuals).	2
Cry1Ac Cotton Field PR	*Geocoris* sp., *Nabis* sp., *Orius* sp., coccinellids, lacewings, and syrphid larvae were surveyed using sweep-net and visual whole-plant inspection in Bt-cotton, and non-Bt cotton treated and untreated plots of 2 ha each during seasons 1992-1994.	Pooled predators counts from whole-plant samples of untreated non-Bt cotton were higher than Bt-cotton and treated non-Bt cotton, but not for sweep-net data. No differences between Bt-cotton and treated non-Bt cotton were observed.	3
Cry1Ac Laboratory PA[2]	Bt toxin was mixed at a concentration of 20 μg/ml in diet (25% honey solution and offered to parasitoid *Nasonia vitripennis* adults; 47% honey solution and offered to *H. convergens* adults). Toxin was diluted in water and after sprayed on lepidopteran eggs, then offered to *C. carnea* larvae.	Although using concentration higher than 100 times that of toxin found in nectar and pollen of Bt cotton in the field, all natural enemies survived similarly on both diets with and without toxin.	4
Cry1Ac Cotton Field PR	*Orius tristicolor*, *G. punctipes*, *Nabis* spp., lacewings, *Collops* spp., *Olla v-nigrum*, *H. convergens*, *Coccinella septempuncata*, spiders, and *Notoxus* spp. were surveyed in Bt and non-Bt cotton fields from 12 to 32 ha. Samples consisted of 15 randomly selected sites per field using sweep net (beat-net technique), during two dates in 1999.	No significant difference was observed in total number of predators between Bt and non-Bt cotton fields, except for total pooled ladybeetles species and *G. punctipes* that were more abundant in non-Bt and Bt-cotton fields, respectively.	5
Cry1Ac Cotton Field PR	Ladybeetles, big-eyed bugs, spiders and minute pirate bugs were surveyed from Bt and non-Bt cotton fields under intensive insecticide use to control tarnished plant bug. Samples consisted of 4-drop cloths or 100 sweep samples per field from 8 to 12 ha from 1995 to 1997.	Under tarnished plant bug management, there were no differences in predatory arthropods between Bt and non-Bt cotton. However, comparing both untreated cottons, lady beetles and ants showed higher seasonal averages in Bt-cotton fields.	6
Cry1Ac Cotton Field PR	Big-eyed bugs, ants, and spiders were surveyed in Bt cotton and non-Bt cotton plots of 36-40 rows x 35m long under insecticide use practices. Drop cloth sampling was used on 7 dates during 1999.	Untreated non-Bt cotton and Bt-cotton fields had similar abundance of geocorids, ants and spiders and greater than any insecticide practice adopted in regular cotton fields.	7

Table 2.1. Continued.

Cry1Ac Cotton Field PR	Minute pirate bug, green lacewing and big-eyed bugs adults were surveyed in Bt and non-Bt cotton fields of 4-8 rows by 10-12 m long. Samples consisted of 50 net sweeps per plot taken weekly during cotton growing season 1999 (Bollgard) and 2000 (Bollgard II).	Seasonal averages for Bollgard trial were similar for minute pirate bug and green lacewing adults, but big-eyed bugs were more abundant in Bt cotton compared to non-Bt cotton. Populations of green lacewing adults were lower in Bollgard II and in regular cotton compared to Bollgard cotton.	8
Cry1Ac Laboratory PR	Field-collected predators *G. punctipes*, *O. tristicolor*, *Zelus renardii* and *Nabis* sp. adults were reared on *S. exigua* larvae fed Bt cotton of different ages according to predator preferences.	No effect on *Nabis* was observed, while longevity of *G. punctipes* and *O. tristicolor* was reduced in one and two out of four trials, respectively; *Z. renardii* lived longer in one out of three trials when fed prey fed Bt cotton.	9
Cry1Ac Laboratory PA	Parasitism of Bt-reared *Pseudoplusia includens* by *Copidosoma floridanum* and *Cotesia margiriventris* was studied using plastic containers in the laboratory.	Development of both parasitoids was delayed, with lower longevity and fecundity from host fed Bt-cotton compared to non-Bt cotton. However, the results were similar to those yielded with hosts fed conventional resistant soybean.	10
Cry1Ac Cotton Field PR-PA	Visual and sweep-net counts of arthropods in experimental plots, under local standard crop management, of Bt and non-Bt cotton of ≈ 0.4 ha were carried out from 15 May to 10 September 1999-2001.	Densities of natural enemies (16 taxa) in Bt-cotton fields were similar to non-Bt cotton over three years and greater than non-Bt treated fields. Diverisity indices showed decresased natural enemy diversity in Bt-cotton compared to non-Bt cotton in 2001.	11
Cry2A Diet PA-PR	Cry2A toxin dispensed in diets at rate of 50 ppm was tested. Toxin solution was sprayed on *Sitotroga cerealella* eggs and offered to *C. carnea* larvae, or diluted (50%) in honey and water solution and offered to *Macrocentrus ancylivorus*, *Meteorus pulchricornis* and *N. vitripennis* adults and in 50% sucrose water solution and offered to *H. convergens* adults.	Survival of *C. carnea* up to 50% of larva pupation in control treatement (free of Bt-toxin) and of the parasitoids and *H. convergens* adults until 20% of death in the controls were similar between individuals unfed and fed Cry2A toxin.	12
Cry1Ac Cotton Field PR	Survey of pests and natural enemies was carried out every 5 days during 2000-2001 seasons in fields of 100 m^2 of Bt-cotton and non-Bt cotton under standard local pest management.	Unsprayed Bt-cotton had higher densities of non-target pests and natural enemies. *Geocoris pallidipennis* and 2 spider and 2 coccinellid species were more abundant in Bt-cotton fields compared to untreated non-Bt cotton.	13
Cry1Ac Cotton Field PR	Arthropod predators (Araneida, *Chrysopa sinica*, *Propylaea japonica*, *Orius minutus*) were surveyed in 9 dates across season 2002 in fields of 100 m^2 and evaluating 100 plants per date.	Lower densities of *C. sinica* (eggs and larvae), *P. japonica* and *O. minutus* were found in Bt-cotton fields on one sampling date, and Araneida on several mid-late season dates, but with no difference for seasonal averages.	14

Table 2.1. Continued.

Cry1A Cotton Field PR	Sixteen samples of 100 plants were taken during 1999 and 2000 in Bt and non-Bt plots of 0.03ha each. Plants were inspected in the field for predators. Fields were managed according to local standard practices including insecticide applications.	No differences were found for 3 coccinellids, 4 lacewings, 2 spiders and *Orius similes* between Bt and non-Bt untreated plots. Insecticide treated non-Bt plots had lower predator densities than Bt and non-Bt untreated plots.	15
Cry1Ac Cotton Field PR-PA	Nontarget insects (chewing, sucking and rasping-sucking and natural enemies) were surveyed in fields of 32-40 rows of 183 m long planted under row mixture of Bt-cotton. One sample was taken in 2001 and 2 samples in 2002 of 10 plants per plot. Some of the fields were treated with insecticides to control *Lygus* and whiteflies.	Results were variable with all arthropods sampled and natural enemies separately being more abundant on non-Bt cotton and in-field row mixture fields compared to Bt cotton. Abundance, diversity and species richness for family levels were estimated with no clear pattern due to limited data.	16
Cry1Ac Laboratory PR	*Helicoverpa armigera* larvae, *Aphis gossypii* and the ladybeetle, *P. japonica*, were collected from Bt-cotton plots of 0.2 ha each and tested to Cry1Ac toxin. Life history of *P. japonica* was studied in the laboratory where they were offered field-collected *A. gossypii* from Bt and non-Bt cotton plots.	Bt toxin rated per g of fresh weight detected in field-collected material was: *H. armigera* (15 ng), *A. gossypii* (2.5 ng), *P. japonica* larvae (10 ng) and adults (20 ng). *P. japonica* fed cotton aphids from Bt or non-Bt cotton produced similar life history parameters.	17
Cry1Ac Cotton Field PR	The spiders, *Erigone* and *Thomisus*, and the ladybeetle *P. japonica* were surveyed in Bt and non-Bt cotton fields of 0.4 h insecticide treated and untreated through visual counts on six plants once every five days from 1999 to 2001.	Bt and non-Bt cotton received similar numbers of pesticide applications due to infestations of non-target pests of Bt cotton. Bt cotton did not affect populations of the ladybeetle, but reduced seasonal average of spiders.	18
Cry1Ac Laboratory PA	*Helicoverpa armigera* larvae were reared on diet containing 0.5, 1, 2, 4 and 8 μg g^{-1} of Cry1Ac toxin and used as hosts for the larval parasitoid *Microplitis demolitor*. Longevity and offspring development of the parasitoid adults fed 125, 250 and 500 μg Cry1Ac ml^{-1} diluted in 10% honey solution were investigated. Parasitoid adults were positive for Cry1Ac when fed toxin dilutions.	*H. armigera* fed Cry1Ac diet delayed development and prolonged host acceptability. Host fed Bt diet negatively affected parasitoid larval development, pupal and adult weight, and female longevity. Parasitoid adults fed directly on Cry1Ac solution were not affected in their longevity or life histories of their offspring.	19
Cry1Ac+2Ab Cotton Field PR	Bt (Bollgard™ and Bollgard™ II) and non-Bt cotton plots (18 rows x 32 m long) were monitored 3x in 2000, 5x in 2001 and 7x in 2002 for predator (heteropteran, fire ants, coccinellids) and bollworm populations. Fields were treated with broad-spectrum insecticide to measure contributions of predators to pest control on both cottons.	There was no difference for any predatory group between Bt and non-Bt cottons. Bollworm exceeded economic threshold twice in Bollgard cotton (Cry1Ac) fields under predator disruption. Bollgard II (Cry1Ac + 2Ab) provided better control of bollworms than Bollgard (Cry1Ac).	20
Cry1A+CpTI Laboratory PA	Larvae of *Helicoverpa armigera* fed diet containing powder of stacked Bt-cotton leaves expressing Cry1A and CpTI toxins were used as hosts for *Microplitis mediator* and *Campoletis chlorideae*.	Both parasitoids had slower larval development, cocooning rate and weight on hosts fed mixed diet. Hosts had lower total hemolymph protein content when fed mixed diet, a possible cause of CpTI toxin and not from Cry1A toxin.	21

Table 2.1. Continued.

Cry1Ac Laboratory PA	*Myzus persicae* maintained on Bt oilseed rape were exposed to parasitism by *Diaretiella rapae* in cages in controlled conditions.	Aphids fed Bt oilseed rape had no effect on development, parasitim rate and sex ratio of the parasitoid *Diaretiella rapae*.	22
Cry1Ac Laboratory PA	Host-finding behavior and parasitism success of *Cotesia plutellae* parasitizing Bt-oilseed rape reared *Plutella xylostella* was studied using wind tunnel and cages.	No difference on host finding behavior for Bt-resistant larvae, but no parasitoid development was observed on susceptible hosts due to death of the host.	23
Cry1Ac Laboratory PA	Parasitism and development of the parasitoid *C. plutellae* parasitizing Bt-resistant and susceptible strains of *P. xylostella* was studied using Petri dish arenas.	Bt-resistant host promoted similar developmental time, survival and parasitism rate comparable to host fed non-Bt oilseed rape; while parasitism on Bt-susceptible pest strain was not successful due to death of the host.	24
Cry1Ac Rape Field PR	Ground-dwelling arthropods were monitored using pitfall traps every 14 days during 2003 in three plots of 200 m^2 of Bt oilseed rape.	Total numbers of Carabidae, Staphylinidae, and Araneae collected were not different between Bt and non-Bt oilseed rape fields.	25
Cry1Ab Laboratory PA	Parasitism of *Heliothis virescens* fed Bt tobacco plants by *Campoletis sonorensis* were evaluated in cages in the laboratory.	Parasitism observed for 1-4 h was lower on transgenic plants with susceptible larvae.	26
Cry1Ac Tobacco Field PR	Bt tobacco was cultivated in plots of 1 row of 10 plants. During 1st and 4th weeks after pest infestations, 3 plants per plot were cut, placed in plastic bags and examined.	Predatory *Nabis* sp., and aphids and chrysomelid beetles were similar between Bt and non-Bt tobacco plots.	27
Cry1Ac Tobacco Field PA	Bt tobacco cultivated in plots of 60-70 plants each was artificially infested with *H. virescens.* Plants were censused on 3 to 7 dates during 1989 and 1990 in three locations. Larval parasitism by *C. sonorensis* and *Cardiochiles nigriceps* was evaluated from field-collected larvae.	From 25 sampling dates on three different locations, parasitism rate of *H. virescens* by *C. sonorensis* was greater on larvae collected from Bt-tobacco plots on three dates from one location, and lower parastism by *C. nigricipes* on one date.	28
Cry1Ab Laboratory PR	Artificial diet containing purified Cry1Ab protoxin at 100 μg/ml of diet was offered to *C. carnea* larvae.	Higher mortality for larvae fed toxin-impregnated artificial diet but no effect on developmental time was reported.	29
Cry1Ab Corn Field PR	Aphids fed Bt-corn event 176, plus corn pollen, were offered to immatures of the predators *Coleomegilla maculata*, *C. carnea* and *Orius insidiosus* in plastic containers.	There was no effect of aphids fed Bt corn and pollen on developmental time and survival of immature predators.	30
Cry1Ab Corn Field PR	*Nabis* spp., *C. maculata*, and *H. convergens* adults, *O. insidiosus* and lacewing immatures and adults were surveyed in Bt corn plants cultivated in plots of 4 rows by 7.6 m long (1994-1995).	No effect on predator densities was observed between Bt and non-Bt corn fields before shedding, during shedding, and after pollen shed.	31
Cry1Ab Laboratory PR	Development and reproduction of *Rhopalosiphum padi* feeding on Bt corn was monitored and aphids were later offered as prey to *C. carnea* larvae in glass containers.	Development and reproduction of aphids reared on Bt cotton were not affected, nor were development and survival of lacewing preying on aphids fed Bt-corn.	32

Table 2.1. Continued.

Cry1Ab Corn Field PA	Parasitism rate of *Ostrinia nubilalis* by *Eriborus terebrans* and *Macrocentrus grandii* and egg predation by *C. maculata*, *O. insidiosus* and lacewing larvae were evaluated in experimental plots of 64 x 62 m with three sampling dates in 1994.	Bt corn does not show antixenosis to *O. nubilalis* oviposition. No effect on parasitism, predation, or on predator densities was recorded.	33
Cry1Ab Laboratory PR	*Spodoptera littoralis* and *O. nubilalis* 1-d-old fed Bt corn were offered to *C. carnea* larvae using 150-ml plastic bottles.	Lower survival and development of *C. carnea* larvae fed *O. nubilalis* reared on Bt corn is reported, but was unaffected by *S. littoralis* reared Bt corn as prey.	34
Cry1Ab Corn Field PR	Field survey of Carabidae (pitfall traps) and aerial fauna (Malaise traps) were conducted during 12 sampling weeks in Bt-corn fields of 2.5 ha during seasons 1997-1998.	No difference was found on abundance and diversity between conventional and transgenic Bt-corn for sampled communities.	35 36
Cry1Ab Laboratory PR	Thrips (*Anaphothrips obscurus*) fed Bt corn were offered to *Orius majusculus* nymphs in specially-designed plate of 54 hole-cages.	Thrips fed Bt corn had no effect on the development and survival of the predator *O. majusculus.*	37
Cry1Ab Corn Field PA	Parasitism rate of *O. nubilalis* larvae by *M. grandii* and *E. terebrans* is reported from collections made in Bt corn fields from 13 different locations across six states (US).	Significant lower rates of parasitism were observed on larvae collected from Bt-corn fields compared to regular corn.	38
Cry1Ab Laboratory PR	Prey preference by *C. carnea* for *S. littoralis* larvae fed non-Bt and Bt-corn was tested.	Lacewing larvae preferred *S. littoralis* larvae fed non-Bt corn to Bt-fed larvae.	39
Cry1Ab Corn Field PR	Visual surveys (1998-99) of *C. carnea*, *Nabis americoferus* and 6 species of coccinellids were conducted over 6 dates on 3 consecutive Bt and non-Bt corn plants in plots of 30 rows x 24.4 m long.	No differences in the densities of beneficial insect populations were reported between Bt and non-Bt sweet corn.	40
Cry9C Laboratory PA	Development of the ectoparasitoid *Parallorphagas pyralophagus* on stemborer *Eoreuma loftini* fed Bt corn was studied in the laboratory.	Host-mediated effect is reported, with greater larval mortality and delayed parasitoid development for those reared on hosts fed Bt corn.	41
Cry1Ab Laboratory PR	Brown planthopper, *Nilaparvata lugens*, reared on different Bt-rice lines was offered as prey to nymphs of the mirid predator *Cyrtorhinus lividipennis* using 10-ml glass tubes in the laboratory.	Nymphal development, survival and adult predator weight were not affected when reared on prey conveying Cry1Ab toxin, which was detected in honeydew excreted by the prey.	42
Cry1Ab Corn Greenhouse PR	*Tetranychus urticae*, *R. padi*, and *S. littoralis* fed Bt corn were offered as prey to *C. larvae* using Plexiglas cages of 52.6 x 13.9 x 1.9 cm attached to the plant leaves.	No effect was observed on *C. carnea* larvae fed *T. urticae* and *R. padi*, but negative effect was observed for predator larvae fed *S. littoralis* reared on Bt corn. Both *T. urticae* and *S. littoralis* tested positive for Cry1Ab.	43
Cry1Ab Corn Field PR	Predators of Araneida, Nabidae, Lygaeidae Anthocoridae and Coccinellidae were collected using D-Vac suction in plots of 8 rows by 6.1m long of Bt and non-Bt sweet corn during two dates in 2000.	Similar densities of predators were observed in both corn genotypes from samples taken on early- and late-planted fields.	44

Table 2.1. Continued.

Cry1Ab Corn Field PA-PR	Parasitism rate of *O. nubilalis*, and parasitoids and predators of aphids in Bt corn were evaluated during 1998 by sampling 100 to 561 stalks or ears in plots of ~200 m^2.	Lower parasitism of *O. nubilalis* in Bt-corn fields by tachnid flies is reported, without excluding the effect of host abundance. Predators and parasitoids of aphids had similar densities between Bt and non-Bt fields.	45
Cry1Ab Corn Field PR	Plots of 7 rows wide and 6.1 m long of Bt and non-Bt sweet corn treated with insecticides were surveyed for *Harmonia axyridis*, *C. maculata* and *O. insidiosus* during 2000 and 2001 by inspecting 10 consecutive plants.	Predators were not affected in Bt sweet corn and higher densities were observed in untreated fields than in fields treated with lambda-cyhalothrin to control *O. nubilalis*.	46
Cry1Ab Corn Field PR-PA	Adult of natural enemies were surveyed in 6 paired Bt and non-Bt corn fields from 5.2 to 16ha each using 2 yellow stick trap per field. Trpas were attached to corn stalk near the ear zone and replaced weekly.	Seasonal means of 4 coccinellids, spiders, parasitoid wasps, syrphid flies, green lacewings, brown lacewings and *Orius* were similar between Bt and non-Bt corn fields.	47
Cry1Ab Corn Field PR	Arthropods were surveyed in fields of Bt and non-Bt corn (1.18-1.69 ha) treated with Bt commercial formulations or insecticides to control *O. nubilalis* during 1998. Sampling was made with pitfall traps (8 dates), sticky traps (10 traps per field during 6 dates); and beating-funnel method (50 to 100 plants per date).	No effects of Bt-corn on ground-dwelling and nontarget canopy-dwelling arthropods were reported through principal response curve analysis relative to non-Bt corn treated with Bt formulations and untreated, but with higher abundance than cyhalothrin treated non-Bt cotton.	48
Cry1Ab Laboratory PA	Effect of *S. littoralis* reared on a mixture of leaf and stem parts of Bt corn (MEB307Bt) and used as a host to *C. sonorensis* was studied in the laboratory. Development of parasitoid larvae, cocoon weight and sex ratio, Bt-toxin concentration in host and parasitoid cocoon was determined.	Only development from parasitism to pupation was prolonged when using hosts fed Bt corn. Nearly 40% of original Cry1Ab in the plant (1597 ng/g fresh weight) was found in the host (645 ng/g), but only 7% (110 ng/g) was detected in the parasitoid cocoon (and 17% from the host).	49
Cry1Ab Laboratory PR	Ten newly-hatched nymphs of *O. majusculus* were fed *Ephestia kuehniella* eggs and supplied with non-Bt or Bt-corn (Event 176) leaves or pollen in the laboratory using individual cages (53x32 mm).	All biological parameters (developmental time, nymph mortality, sex ratio, size and weight of teneral adults) did not differ between treatments.	50
Cry1Ab Corn Field PR	Aphid predators were surveyed on whole-harvested plants (3 plants, 4 locations with 5 sampling dates in 2000) or visual counts (3 plants per plot on 5 to 10 sampling dates in 2001). In addition, pitfall traps were used to survey carabids and spiders during 4 weeks (2000-2003). All samples were conducted in fields from 3 to 11 ha.	Using ordination analysis, the variance for aphids and ground predators (carabids and spiders) explained between Bt genotypes was only 2.7 and 2.1%; hence, no significant effect is reported, while year and field and sampling dates had signicant effects on predator population densities.	51

Table 2.1. Continued.

Cry1Ab Corn Field PR	Yellow sticky trap (1 per plot replaced every week) and whole plant counts (3 pls per plot during five sampling dates) were used to survey predatory Anthocoridae, Miridae, Syrphidae, Staphylinidae, Chrysopidae and 5 species of coccinellids in Bt corn (cv. Compa CB) in 10 plots of 64 m^2 each during 2002.	Predator populations were similar in Bt and non-Bt corn through whole plant counts. From yellow stick trap, only the coccinellid *P. quatuordecimpunctata* had two-fold lower densities in Bt-corn.	52
Cry1Ab Laboratory PR	Sucrose diet containing 0.1% Bt toxin (10,000x higher than the amount ingested through Bt-reared prey lepidopteran larvae) was offered at 0.5µl to *C. carnea* larvae in Petri dishes.	No effect of Bt-toxin was found on *C. carnea* larval development, weight gain and prey consumption.	53
Cry1Ab Corn Field PR	Visual sampling of generalist predators was conducted weekly over 13 weeks using 5 adjacent plants in 5 points per field (4 fields) of Bt and non-Bt corn of 125 m^2 plots during 2001 and 2002.	*Scymnus levaillanti*, *Stethorus gilvifrons*, *Nabis* spp. and *Orius* spp. exhibited similar populations between cotton types, except *C. carnea* that had higher abundance in the Bt-corn fields in 2002.	54
Cry1Ab Corn Field PR	Spider communities were surveyed using 10 pitfall traps per field of Bt and non-Bt corn (from 7 to 29 ha each) from 2000 to 2002, using three different locations, but one field and one location each year.	No negative effect of Bt-corn was observed on spider communities. Canonical correspondence analysis showed that other factors such as field characteristics and seasonal environmental changes were more important for community composition than planting Bt corn.	55
Cry1Ab Corn Field PR	*Orius* sp. and lacewing eggs were surveyed on 10 plants in each of 5 non-Bt and Bt-corn fields of 0.5 ha each in 2002 (4 sampling dates) and in 2003 (5 sampling dates). Carabids, staphylinids and spiders were collected using 4 pitfall traps/field 6 times in 2002 and 5 traps/field 5 times in 2003.	Abundance of the predatory bug *Orius* sp., and of lacewing eggs were similar between non-Bt and Bt-corn fields. In addition, diversity and abundance of carabids, spiders, and staphylinids were also similar between non-Bt and Bt-corn fields.	56
Cry1Ab Corn Field PA	Viability of the tachinid parasitoid, *Lydella thompsoni*, parasitizing *O. nubilalis* from non-Bt and Bt-corn fields was determined. Fifth instars of second-generation *O. nubilalis* (n=300 per field) were collected in 9 different locations (9 fields) in northern Italy during 1999 and 2000.	Diapause of *O. nubilalis* and parasitoid larvae was broken in the laboratory. Three out of 9 locations showed lower parasitism rate of *O. nubilalis* from Bt-corn fields, but parasitoid development and adult longevity fed 10% honey solution were similar on both corn types.	57
Cry1Ab+2A Laboratory PR	Larvae of *S. littoralis* fed artificial diet containing 25, 50, and 100 µg g^{-1} of Cry1Ab and 100 µg g^{-1} of Cry2A toxins were offered as prey to *C. carnea* larvae in 150-ml plastic containers.	Higher mortality of predator larvae fed on prey fed diet containing toxin is reported; but no effect on developmental time.	58
Cry1Ab Rice Laboratory PR	Functional response of wolf spider *Pirata subpiraticus* was studied in laboratory using rice leafroller, *Cnaphalocrocis medinali*, and brown planthopper, *Nilaparvata lugens* fed Bt rice.	Predation rate showed a type II functional response on both prey items, and there were no differences in prey killed and handling time for prey from Bt or non-Bt rice.	59

Table 2.1. Continued.

Cry1Ab Rice Laboratory PA	Parasitism of *Chilo suppressalis* by *Apanteles chilonis* was studied in the laboratory using 3rd, 4th, and 5th instar larvae fed Bt rice during all development.	Parasitism rate and coccon formation were significantly lower on hosts fed Bt rice, but no differences were observed for larval development, cocoon weight, emergence rate or sex ratio.	60
Cry1Ab+1Ac Rice Field PA-PR	Arthropod communities were surveyed in rice paddies of 300 to 500m^2 cultivated with two transgenic and one non-transgenic variety during 2000 and 2001.	There were no differences in abundance of 26 families of arthropod predators, 14 familes of parasitoids, and community diversity and abundance indices and temporal dynamics between Bt and non-Bt rice fields.	61
Cry3Bb Laboratory PR	Lyophilized fruit fly eggs mixed with Bt-pollen at rate of 50% mixed diet was offered to *C. maculata* larvae in Petri dishes.	Diet containing Bt-pollen had no negative effect on development, survival and adult reproduction of *C. maculata.*	62
Cry3Bb Laboratory PR	Aphid plus Bt-corn pollen in proportion from 0 to 100% mixture was offered to *C. maculata* larvae in cups.	There were no effects on larval development, survival, adult weight or adult reproductive output of *C. maculata* fed with different proportions of Bt corn.	63
Cry3Bb Corn Field PR	Three to four visual inspections of 15-20 randomly-selected plants during 2000-2001 were conducted to survey *O. insidiosus*, *H. convergens*, *C. maculata* and *Scymnus* sp. in plots of 4 rows of 30.48 m in length.	No significant differences in numbers of either immature or adult predators was found between Bt corn and non-Bt isoline.	64
Cry3A Potato Laboratory PR	Bt or non-Bt potato leaflets infested with *Myzus persicae* were offered to *H. convergens* larvae and adults.	No effect of aphid fed Bt potato was observed on predation rate, developmental time survival of predator larvae, pupal weight, adult reproduction, or longevity.	65
Cry3A Potato Field PR	Abundance of *Lebia grandis* and *C. maculata*, predators of *L. decemlineata*, was estimated weekly from mid-May to late July using sweep-net and visual counts in experimental plots of 24 rows x 23 m long of 0, 50, 70 and 100% of Bt-potato seed mixture in 1994 and 1995.	*Lebia grandis* exhibited lower densities in mixed seedings and 100% Bt-potato fields compared to non-Bt fields, a possible effect of low prey density in Bt-fields inducing movement of an active predator among small plots, while *C. maculata* showed similar densities across all treatments.	66
Cry3A Laboratory PR	Neonate *Leptinotarsa decemlineata* fed Bt-potato foliage were offered as prey to *C. maculata*.	No difference in proportion of prey consumed, developmental time, survival or pupal and adult weights of *C. maculata* is reported.	67
Cry3A Laboratory PR	Quality of *L. decemlineata* neonate larvae fed Bt potato and non-Bt potato as prey for *L. grandis* was investigated using Petri dishes in the laboratory.	Prey fed Bt potato showed no adverse effect on predator acceptance but caused lower total consumption by predators since prey fed Bt-potato weighed less.	68
Cry3A Laboratory PR	Longevity of field-collected *Nabis* sp., *O. tristicolor* and *L. hesperus* nymphs and adults, and adults of *Geocoris* sp. were evaluated caging bugs on Bt-potato leaf discs deprived of prey.	No significant differences in predators' longevity caged with Bt or non-Bt potatoes foliage were observed.	69

Table 2.1. Continued.

Cry3A Potato Field PR	Abundance of arthropod predators in the potato ecosystem (*O. insidiosus*, *G. punctipes*, *Nabis* spp., *C. septempunctata*, *H. axyridis*, *H. convergens*, *Cicindela punctulata*, *Poecilus* spp., *Scarites* spp., ants and spiders) were surveyed weekly during 1994 and 1995 from mid May-June to late July-August, respectively, using sweep nets and pitfall traps in experimental plots of 24 rows x 23 m long of Bt and non-Bt potato.	Among 3 predatory heteropterans, only *O. insidiosus* had greater abundance in Bt potato during 1994, and spiders in 1995. Other predators were similarly abundant in Bt and non-Bt potato fields.	70
Cry3Aa Potato Field PR	Visual and drop cloth survey of arthropod predators (big-eyed bugs, damsel bugs, minute-pirate bugs, lady beetles, brown lacewings, syrphid flies, assassin bugs and spiders) were conducted in untreated and treated Bt and non-Bt potato fields of 1.2 ha each during 1992 and 1993 from mid June to late August.	Predator abundances were similar in Bt-potato fields compared to regular untreated potato fields, and both had greater abundances than insecticide treated non-Bt fields.	71
Cry3A Potato Field PR	Epigeal arthropods (Collembola, Carabidae, Staphylinidae and Araneae) were surveyed using pitfall traps during 1992 and 1993 from mid June to late August in Bt-potato and non-Bt fields of 1.2 ha each under conventional pest management practices.	Major ground-dwelling arthropod predators and Collembola exhibited dynamics and densities in Bt-potato fields comparable to non-Bt untreated fields and higher densities than conventionally-managed fields.	72
Cry toxins Laboratory PR	Larvae of *O. nubilalis* were fed diet mixed with Bt formulations containing the toxins Cry1Aa, 1Ab, 1Ac and 2B and offered to *O. insidiosus* nymphs and adults in plastic cages of 30 x 30 cm.	Developmental time, body weight and length, and adult longevity were similar for predators given prey fed Bt mixed diet or prey fed non-Bt mixed diet.	73

[1]PR = predator; [2]PA = parasitoid. 1. Flint et al., 1994; 2. Wilson et al., 1992; 3. Luttrell et al., 1995; 4. Sims, 1995; 5. Armstrong et al., 2000; 6. Stewart et al., 1998; 7. Hagerty et al., 2000; 8. Goodell et al., 2001; 9. Ponsard et al., 2002; 10. Baur and Boethel, 2003; 11. Men et al., 2003; 12. Sims, 1997; 13. Deng et al., 2003; 14. Sun et al., 2003; 15. Wu and Guo, 2003; 16. Sisterson et al., 2004; 17. Zhang et al., 2004; 18. Men et al., 2004; 19. Liu et al., 2005; 20. Hagerty et al., 2005; 21. Ren et al., 2004; 22. Schuler et al., 2001; 23. Schuler et al., 2003; 24. Schuler et al., 2004; 25. Burgio et al., 2004; 26. Johnson et al., 1997; 27. Hoffman et al., 1992; 28. Johnson and Gould, 1992; 29. Hilbeck et al., 1998a; 30-31. Pilcher et al., 1997; 32. Lozzia et al., 1998; 33. Orr and Landis, 1997; 34. Hilbeck et al., 1998b; 35. Lozzia, 1999; 36. Manachini, 2000; 37. Zwahlen et al., 2000; 38. Siegfried et al., 2001; 39. Meier and Hilbeck, 2001; 40. Wold et al., 2001; 41. Bernal et al., 2002; Bernal et al., 2002; 43. Dutton et al., 2002; 44. Hassell and Shepard, 2002; 45. Bourguet et al., 2002; 46. Musser and Shelton, 2003; 47. Jasinski et al., 2003; 48. Candolfi et al. 2004; 49. Meissle et al., 2004; 50. Pons et al., 2004; 51. Freier et al., 2004; 52. Delrio et al., 2004; 53. Romeis et al., 2004; 54. Güllü et al., 2004; 55. Volkmar et al., 2004; 56. Sehnal et al., 2004; 57. Manachini and Lozzia, 2004; 58. Hilbeck et al., 1999; 59. Liu et al., 2003a; 60. Jiang et al., 2004; 61. Liu et al., 2003b; 62. Duan et al., 2002; 63. Lungren and Wiedenmann, 2002; 64. Al-Deeb and Wilde, 2003; 65. Dogan et al., 1996; 66. Riddick et al., 1998; 67. Riddick and Barbosa, 1998; 68. Riddick and Barbosa, 2000; 69. Armer et al., 2000; 70. Riddick et al., 2000; 71. Reed et al., 2001; 72. Duan et al., 2004; 73. Al-Deeb et al., 2001.

The delayed development of parasitoid larvae indicates a sublethal effect of host suitability because the availability of a Bt-resistant host fed Bt plants causes no such effect on parasitoid development, although the parasitoid is in contact with Bt-toxins in the host body (Schuler et al., 2004). Additionally, direct ingestion of toxin by parasitoid adults does not affect their survival (Sims, 1995; 1997; Liu et al., 2005). Therefore, no direct adverse impact of Bt toxins on parasitoid larvae have been found. Reduction in host quality (smaller size, and possible lower food quality from sickened hosts) seems to be the cause of sublethal effects. Parasitoids that are specific to hosts targeted by Bt toxins, such as the bollworm complex, are at risk for population reductions due to total failure of parasitism (host dying before parasitoid development) or sublethal effects resulting from poor host quality (Johnson et al., 1997; Yang et al., 2001; Schuler et al., 2001; Lu et al., 2004). However, these results parallel the outcome of hosts treated with insecticides (including commercial formulations of Bt; Blumberg et al., 1997) or reared on conventional resistant varieties (Baur and Boethel, 2003) that kill the host during parasitoid development, or reduce host quality. Host populations in Bt-cotton fields are reduced in similar ways as is the case with insecticide applications, but the effect on populations of common bollworm parasitoids on a large scale might be reduced because bollworm populations can be available in weeds, neighboring crop fields, or in the mandatory non-Bt refuges.

Results by Hilbeck et al. (1998a; 1998b) suggest that there may be a reduction in fitness of predatory chrysopid larvae directly attributable to preying on Bt-reared caterpillars. Likewise, the parasitoid *Cotesia plutellae* Kurdjumov does not emerge from Bt-susceptible *Plutella xylostela* (L.) larvae fed Bt-oilseed rape because the host dies before parasitoid development can be completed (Schuler et al., 1999). However, other studies focused on other aspects of the interactions between Bt plants and predators have found no detrimental effects in the laboratory

and field (results and references in Table 2.1). Tritrophic interactions have not been clarified completely due the complexity of multiple possible interactions, such as that seen in cotton fields (Fig. 2.1). The primary view is that effects of Bt-transgenic plants are species-specific for pests and predators, and it seems that the effects depend on the degree of linkage in the predator-prey and Bt-plant interactions. Cry1Ab toxin in plant sap from transgenic corn plants was not detected or only in minute amounts (Raps et al., 2001), but toxin was readily detectable when plant cells were damaged. These authors also reported inconsistent detection of Cry1Ab toxin in the body of aphid *Rhopalosiphum padi* L. Head et al. (2001) reported that no Bt toxin was detected in *Rhopalosiphum maidis* (Fitch) fed Bt-corn, but when the aphid fed on diet containing 20 and 200 ppm of pure Cry1Ab, the toxin was detected in their bodies and was active against European corn borer. Broad generalist plant feeders, such as thrips and mites, also have been found to contain detectable levels of Bt toxin acquired from their Bt-host plants (Dutton et al., 2002 and 2004). Therefore, these studies indicate that Bt-toxins can be conveyed to natural enemies through nonsusceptible pests used as prey or hosts (results in CHAPTER 6).

Bt toxins only affect insects after ingestion, suggesting that feeding on toxified prey and plant sap containing debris from mechanical cell damage may link Bt toxins and predators in the third trophic level (Fig. 2.1). Feeding behavior of predators and parasitoids may play an important role in possible nontarget effects of Bt toxins expressed in transgenic plants. Therefore, predatory heteropterans or those predators such as lacewings and ladybeetles that use plant products (nectar, pollen, etc.) as supplementary food may come in contact (ingestion) with Bt toxins and may suffer direct effects if they are susceptible. Moreover, important predatory heteropterans, such as the spined soldier bug, *Podisus maculiventris* (Say) and big-eyed bug, *G. punctipes*, produce salivary secretions with the enzyme amylase (Stamopoulus et al., 1993; Zeng and Cohen, 2000). These

findings suggest that the big-eyed bug and spined soldier bug are able to use starch granules from plant tissues, rather than using plant only as source of water. Therefore, part of this dissertation will examine field dynamics and greenhouse studies of tritrophic interactions of four major predatory heteropterans found in cotton fields, and detailed laboratory studies of Cry1Ac toxin ingestion by *G. punctipes*.

Big-eyed bug Geocoris punctipes. The big-eyed bug, *G. punctipes*, is one of the best-documented geocorids in the United States cotton agroecosystem. Data on the ecology of this predator in many agricultural systems, such as cotton, corn, tomato and soybeans, are still limited. Our poor understanding of the impact of *G. punctipes* and other arthropod predators on pest populations limits our ability to use these predators effectively in integrated pest management. There are numerous difficulties in obtaining information about predator ecology and activity under field conditions, especially for generalists (Naranjo and Hagler, 1998, Symondson et al., 2002). Such difficulties are increased by factors such as small body size, diurnal and nocturnal activities, cryptic coloration, limited knowledge of behavior, and extra-oral digestion leaving little or no evidence of the predators' actions. Evaluating predator population dynamics relative to prey populations and plant phenology, although an indirect evaluation, is an option for estimating the role of *G. punctipes* in the cotton ecosystem.

Feeding Behavior of Geocoris punctipes. *Geocoris punctipes* feeds on a wide variety of small live prey or much larger dead prey. They have been observed feeding on many species of Hemiptera, Coleoptera, Lepidoptera, small Diptera, Hymenoptera (including ants), Thysanoptera, Collembola and Acarina (Crocker and Whitcomb, 1980; Readio and Sweet, 1982). In studies focusing on important pests of cotton, *G. punctipes* has been observed preying upon plant bugs (e.g. *Lygus* bugs) (Champlin and Sholdt, 1966; Leigh and Gonzalez, 1976), leafhoppers (Staten,

1970; Breene et al., 1989; Medal et al., 1995; and 1997), mites (Staten, 1970; Wilson et al., 1991), thrips and cotton leaf perforator (Staten, 1970), pink bollworm (Orphanides et al., 1971; Hagler and Naranjo, 1994), fall armyworm (Naranjo, 1987; Knutson and Ruberson, 1997), cotton leafworm (Gravena and Sterling, 1983), cotton bollworm and tobacco budworm (Lingren et al., 1968; Ables et al., 1978), beet armyworm (Ruberson et al., 1994), whiteflies (Watve and Clower, 1976; Hagler and Naranjo, 1994), aphids (Ables et al., 1978; Tamaki et al., 1981), soybean looper (Richman et al., 1980), and cabbage looper (Ehler, 1977).

Despite the broad range of prey, *G. punctipes* also use plants as supplementary food; therefore, they are labeled as an omnivorous or zoophytophagous predator (Stoner, 1970; Coll, 1998). Besides animal prey, *G. punctipes* feeds on seeds, leaves and pods of many plants (Stoner, 1970; Crocker and Whitcomb, 1980; Naranjo and Stimac, 1985; Thead et al., 1985; Eubanks and Denno, 1999; 2000a; 2000b). Cohen and Debolt (1983) stated that a source of water or plant sap is essential for survival of *G. punctipes* fed on *Lygus* eggs. In addition, studies have shown beneficial effects of plant feeding for this predator. *Geocoris punctipes* kept on prey and suitable host plants exhibited shorter developmental times for certain instars, greater nymphal survival, and increased weight of newly-emerged adults (Stoner, 1970; Naranjo and Stimac, 1985).

Many suggest that feeding at more than one trophic level furnishes omnivorous predators with complementary resources that allow them to survive periods when resources at one trophic level are not available or are of low quality (Eubanks and Denno, 1999). These authors also reported that *G. punctipes* populations seem to be intimately associated with variation in their host plants. Thus, more suitable host plants may contribute to larger predator populations in field crops.

Analyses have shown that the benefits of phytophagy are species-specific and dependent on predator age and the quality of the prey, plant types, and their structures (Coll, 1998; Eubanks and Denno, 2000a; 2000b). Yokoyama (1978) suggested that certain predatory arthropods sustain themselves by feeding on plants when prey are scarce and that nectar production is responsible for maintenance of predaceous species on nectaried cotton. This hypothesis was investigated by Thead et al. (1985), who found that *G. punctipes* ingested more material on nectaried plants than nectariless ones using radioactive labeled plants. *Geocoris punctipes* fed more on nectaried plants (12.08%) than on nectariless cotton either with prey or without prey (0.35 to 3.34%). Thus, these data prove that prey scarcity or in low quality does induce more plant feeding by *G. punctipes*, but the type of plant also mediates it.

The host plant of the prey also may play an important role for predators (Price, 1986). Plant species have been demonstrated to influence predator development, as well as predatory activity such as aphid predation by *Geocoris pallens* Stål, *Geocoris bullatus* (Say) (Tamaki and Weeks, 1972; Tamaki et al., 1981) and *G. punctipes* (Naranjo and Stimac, 1987). Plants influence predators in a number of ways. Plants may provide moisture and nutrients to predators when prey are scarce, and contribute important supplemental nutrients to a largely carnivorous diet or can have a negative impact when plants exhibit traits resistant to herbivorous prey (Rogers and Sulivan, 1986; Barbour et al., 1993).

Could Bt-cotton affect Geocoris punctipes*?* Feeding on more than one trophic level is a common phenomenon in ecological communities. In arthropod food webs, many predators are omnivores and they may not restrict their diets to herbivore species, but feed also on plants, other predators and even on conspecifics (Sabelis, 1992). Therefore, we can state that Bt-cotton plants

interact with *G. punctipes* population in many ways and that additional work is warranted to understand possible interactions.

Biological control typically is an intimate web involving plant, herbivorous pests, and natural enemies, resulting in tritrophic or sometimes tetratrophic interactions. Studies involving tritrophic interactions have been carried out with several species of insects, and the response of each species to individual plant compounds may differ because several natural products are involved as mediators in insect-plant and herbivore-natural enemy interactions, including both volatiles and nonvolatile compounds (Price, 1986; Vet and Dicke, 1992). The diversity of secondary plant compounds is astounding and more than 20,000 compounds have been isolated and characterized as having effects on interactions among trophic levels (Sing and Bakthavatsalam, 1996). In addition, the insertion of Bt genes in cotton plants to express Cry1Ac toxin has resulted in modifications in cotton plant physiology beyond expression of the toxin itself (Jallow et al., 1999; Zhang et al., 1999; Ding et al., 2001).

Plants also may exert direct effects on predators and their efficiency. Results show that plant food is essential for nymphal development and reproduction of *G. bullatus*, *G. pallens*, and *G. punctipes* on a poor diet such as pea aphids (Tamaki and Weeks, 1972; Eubanks and Denno, 1999). However, supplemental plant feeding is less important when these predators are offered any of various lepidopteran eggs (Cohen and Debolt, 1983; Eubanks and Denno, 2000a; 2000b). Corn earworm eggs were nutritionally superior to pea aphids as prey for *G. punctipes* (Eubanks and Denno, 2000a). This predator survived four times as long when fed corn earworm eggs than when fed pea aphids, although in choice tests *G. punctipes* attacked more pea aphids than corn earworm eggs (Eubanks and Denno, 1999). Instead of prey quality, prey mobility seems to govern

prey choice. *Geocoris punctipes* fed on corn earworm eggs about five times less frequently when pea aphids were present.

Despite the possible direct interactions of *G. punctipes* feeding on Bt-cotton, this predator may be primarily affected by prey quality. Prey fed on Bt cotton and escaping death might be not suitable for predator development. Herbert and Harper (1986) found that *H. zea* larvae treated with Bt beta-exotoxin reduced survival of 4th-instar *G. punctipes* when predators were maintained on this prey over time, but no toxicity was observed for adults. Additionally, development of partially-susceptible lepidopteran larvae fed Bt cotton is prolonged (Stewart et al., 2001). Therefore, predation on intoxicated larvae with Bt toxin can increase; especially if intoxicated prey remain small for prolonged periods, appropriate to more extended *G. punctipes* attack, or if prey are in a moribund, defenseless state available for predators. Moreover, insect larvae that are not or are only partially susceptible to Bt toxin may acquire the toxin and convey it to predators via ingestion (Head et al., 2001; Dutton et al., 2002; Howald et al., 2003). For example, Ponsard et al. (2002) reported decreased longevity *G. punctipes* adults when fed partially-susceptible *S. exigua* larvae fed Bt cotton. Moreover, predators such as *G. punctipes* may acquire Bt toxin directly by sucking fluids from the plants. On the other hand, *G. punctipes* may not be affected. Armer et al. (2000) found similar survival for males and females of *Geocoris* sp. and other plant feeding predators (*O. tristicolor*, and the facultative predator *Lygus hesperus* Knight), when they fed upon transgenic CryIIIA Russet Burbank potato and nontransgenic potato leaves, and were deprived of prey. In addition, for two predators found in cotton ecosystems, *C. carnea* and *Hippodamia convergens* Guerin-Meneville, that feed on Bt-cotton pollen and nectar, Sims (1995) found no direct toxicity of Cry1Ac toxin expressed in the tissues of transgenic cotton.

Bt-cotton may affects *G. punctipes* population through prey scarcity in the cotton agroecosystem resulting in more direct feeding on Bt-cotton plants and increased intraguild predation (i.e., feeding on conspecific or on other natural enemy). Reduction of prey diversity in a multi-pest ecosystem like cotton may stimulate intraguild predation by a generalist predator such as *Geocoris*. In addition, prey scarcity induces predators to increase foraging activity, which can expose them to predation and parasitism by other natural enemies, and increase exposure to insecticide residues. The worry is that although each situation can be examined alone, in all likelihood they act together and may be difficult to adequately reconstruct as a whole.

Being omnivorous, *G. punctipes* may prey upon a wide variety of pests, but it also feeds on plants by sucking sap. The probability of *G. punctipes* finding and interacting with prey fed Bt-cotton plants and feeding on plant may be influenced by decisions made during their long-range searching behavior, thus it is important to know the developmental and reproductive responses of *G. punctipes* to Bt cotton (CHAPTER 7).

Chemical properties of plants, such as nutritional quality, semiochemical profile, and allelochemcial content, may greatly influence insect predators (Schuster and Calderon, 1986; Singh and Bakthavastsalam, 1996). Plants may affect *G. punctipes* in different ways. Indirect effects of plants on predatory bugs through the presence of allelochemicals in prey have been studied. *Geocoris punctipes* nymphs developed slower and suffered higher mortality when fed velvetbean caterpillars reared on a resistant soybean genotype compared to susceptible one (Rogers and Sulivan, 1986). Likewise, Barbour et al. (1993) demonstrated that *G. punctipes* consumed fewer *H. zea* eggs and had lower nymphal development in wild tomatoes due to adverse effects of trichome exudates.

Analyses of *G. punctipes* feeding indicate that sucking plant sap is a common behavior in nature with or without prey. Hence, the host plant has great importance for tritrophic interactions involving big-eyed bugs. Phytophagy adaptations in predators allow them to maintain populations under variable prey conditions, enhancing their success in annual and perennial crops. This fact suggests that *G. punctipes* is able to survive in situations of prey shortage, especially early in the season as the cotton agroecosystem undergoes pest colonization. In addition, by feeding either on cultivated or on indigenous plants, *G. punctipes* can survive in crop borders. For this reason, it is believed that *G. punctipes* can function between generations of its prey. Therefore, the knowledge generated on ecology of this bug in the transgenic Bt-cotton agroecosystem will provide a foundation for the manipulation and adoption of conservation tactics for this predator.

Direct effects by feeding on cultivated plants and weeds, and their use as refugia can be considered an advantage for *G. punctipes* in IPM. Naranjo and Stimac (1987) studied the effects on development and predation of *G. punctipes* of 11 weed species common to soybean fields in Florida. The best predator performance was observed on knotweed. Knotweed associated with radish supported higher *Geocoris* spp. populations than other weed species (Bugg et al., 1987). High densities of *G. punctipes* also were found on cantaloupe planted with cover crops of vetch or subterranean clover, but not rye (Bugg et al., 1991). Also, *Ammi visnaga* (L.), a Eurasian summer annual weed, harbors large populations of *Geocoris* spp., which may reduce pest populations on adjacent crop plants (Bugg and Wilson, 1989). Coll (1998) reviewed plant feeding by heteropteran predators and reported that 7 out of 10 intercropping systems did not show effects on populations of *Geocoris*; 2 out of 10 intercroping systems (corn-bean-tomato, and crimson clover-ryegrass) produced lower populations and only 1 (pepper and knotweed) harbored higher populations of *G. punctipes*, supporting the results of Naranjo and Stimac (1987).

In general, reduced predator densities in Bt-resistant plants have been shown to be equal to, or lower or greater than reductions in pest densities (Table 2.1). However, it remains difficult to establish whether plant resistance reduces predator populations by absence, low quality or low diversity of prey, plant morphological structures and allelochemical content, or any combination of these factors.

Purpose of this study. It is recommended that risk assessments of transgenic crops on nontarget organisms be conducted in grower fields, incorporate natural plant and arthropod phenology, and be of sufficient duration to account for environmental variability. Time constraints to provide quick answers to society drove the experiments in this past decade -- the majority could not include the above recommendations (Table 2.1); hence, some controversial results were produced. The present study took a large-scale, long-term tritrophic approach to investigate interactions of Bt cotton and arthropod predators, considering multiple interactions of Bt-cotton plants, herbivores, and arthropod predators that might occur in cotton fields (Fig. 2.1).

Bt cotton is already deployed and widely cultivated in the US. Therefore, our study started in the field to detect possible differences in abundance, dynamics, and diversity of arthropod predators between Bt and non-Bt cotton fields. To accomplish this objective, multiple sampling methods were used to deal with predominant predator behaviors (epigeal, free living and resident plant canopy communities), numerous samples were taken during each growing season to deal with plant-herbivore-predator phenology, and the study was conducted over multiple seasons to address environmental variability. Although field surveys provide a big picture of the system, significant results typically lack mechanistic explanations. Thus, field cage, greenhouse, and laboratory experiments were conducted to generate mechanistic explanations to understand the large-scale results.

To provide details about the risk of Cry1Ac toxin in Bt-cotton moving in the food web and reaching predators in the third trophic level, toxin was measured in plants, herbivores, and predators collected throughout the growing season. To clarify how the toxin reached the third trophic level, greenhouse experiments were conducted using lepidopteran larvae fed Bt cotton and predatory heteropterans. To address the concern that modified Bt cotton may directly affect omnivorous predators common in cotton fields, and to assess risks of Cry1Ac toxin expressed in Bt-cotton reaching the third trophic level through herbivorous prey, experiments using field cages were conducted to evaluate Bt cotton as a host plant and to examine prey-mediated effects of Bt cotton. Additionally, the fate of Cry1Ac toxin in the body of a predator (*G. punctipes*) was investigated in the laboratory.

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CHAPTER 3

CANOPY- AND GROUND-DWELLING PREDATOR ARTHROPODS IN BT AND NON-BT COTTON FIELDS: PATTERNS AND MECHANISMS [1]

[1]Torres, J.B. and J.R. Ruberson. Submitted to *Environmental Entomology*. 3/22/05.

ABSTRACT The abundance of canopy- and ground-dwelling predators was monitored in three pairs of commercial Bt- and non-Bt cotton fields (5 to 15 ha) during three successive seasons using three sampling methods: bagged whole plants, drop cloth samples, and pitfall traps. Drop cloth and pitfall samples were taken weekly and whole plant samples at 10-d intervals throughout each growing season. Insecticides were applied to manage pests when economic thresholds were exceeded in both cotton types. Of 1518 possible date-by-date orthogonal contrasts used to evaluate abundance of immature and adult predators, univariate analysis of variance generated 25 contrasts in favor of non-Bt and 15 contrasts in favor of Bt-cotton. When data from all three seasons were pooled, only 11 contrasts out of 228 were significant, with one in favor of Bt (*Nabis* spp.) and 10 in favor of non-Bt cotton [*Hippodamia convergens* Guérin-Méneville and *Geocoris uliginosus* (Say)]. *H. convergenes* was responsible for eight out of 10 contrasts in favor of non-Bt cotton as a result of competition relief caused by insecticide use in non-Bt cotton fields. Analyses of predator community dynamics using principal response curves showed that the abundance of ground-dwelling predators was not affected by cotton type, while abundance of canopy predators varied across seasons with no particular trend for either cotton type. The abundance of predators in the cotton fields for 3 yrs with standard grower practices failed to demonstrate any negative impact of Bt-cotton on predator populations.

KEY WORDS transgenic plants, nontarget impact, predators, Cry1Ac, ladybeetle competition

INTRODUCTION

Transgenic cotton varieties expressing the Cry1Ac protein from Bt (Cry = crystal proteins and Bt = *Bacillus thuringiensis* Berliner) has become an important tool for managing lepidopteran larvae in cotton in the US. For example, nearly 80% of the 522,450 hectares of cotton cultivated in Georgia during 2004 was Bt-transgenic (Williams 2004). Insecticide use in Georgia cotton declined approximately 50% following boll weevil eradication (Haney et al. 1996), and was again reduced by approximately 50% with adoption of Bt-cotton in 1996, averaging fewer than three foliar applications per season to manage arthropod pests in cotton from 1995 to 2003 (data from Cotton Insect Losses Estimate, www.msstate.edu/Entomology/ctnloss). This trend of reduced insecticide use following adoption of Bt-transgenic cotton has been repeated across the southeastern US (Betz et al. 2000) and elsewhere (Fitt 2000, Pray et al. 2002). However, currently-available transgenic Bt-cotton varieties are not effective against sucking pests, and are marginally to moderately effective against certain lepidopterans; thus, Bt-transgenic cottons require continued use of pesticides and other tactics for effective pest management (Hilder and Boulter 1999). It is generally assumed that the shortage of lepidopteran larvae in Bt-cotton fields relative to non-Bt-cotton will adversely affect specific communities of parasitoids and predators. On the other hand, reduced insecticide use in Bt-cotton has the capacity to contribute to increased generalist predator abundance in Bt-cotton (Luttrell et al. 1995, Armstrong et al. 2000, Men et al. 2003, Wu and Guo 2003, Hagerty et al. 2005). Thus, it is expected that conservation of generalist natural enemies will translate into increased predation and parasitism of pests not effectively controlled by Bt-toxins. This result has been observed in the form of reduced aphid and caterpillar outbreaks in Bt-cotton (Wu and Guo 2003, Hagerty et al. 2005) and in Bt-potatoes (Reed et al. 2001), where insecticide applications to manage heliothines and Colorado potato

beetle, respectively, were eliminated. Changes in broad-spectrum insecticide use and spray frequency have yielded short-term benefits during the cropping season to generalist natural enemies (Reed et al. 2001, Wu and Guo 2003, Hagerty et al. 2005), but only long-term monitoring can demonstrate any real benefits of Bt-crops to natural enemy populations (Schuler et al. 2001, Obrycki et al. 2004).

Predation in the cotton ecosystem is accomplished primarily by generalist predators (Whitcomb and Bell 1964). Predators might be exposed to the Bt-toxin through feeding on lepidopteran larvae partially susceptible to Cry1Ac toxin, which experience some survival and developmental delay (Perlak et al. 2001, Stewart et al. 2001), or by feeding on other herbivores, such as two-spotted spider mites and thrips, that may pick up Bt-toxin from plants (Dutton et al. 2002 and 2004). Most studies to date have addressed species-specific interactions under laboratory conditions, while field studies have focused on experimental plots and other limited evaluations. This work evaluated the dynamics of major predatory groups in commercial Bt and non-Bt cotton fields throughout the growing seasons of three consecutive years (across multiple generations for most taxa) using different sampling methods to include the most common predator taxa.

Several authors have reviewed studies of the effects of Bt crops on natural enemies, in efforts to discern patterns (Groot and Dicke 2002, Obrycki et al. 2004, O'Callaghan et al. 2005, Lövei and Arpaia 2005). Most of the reviewed studies found no effects, but a few cases exhibited significant adverse or positive effects of the Bt-transgenic crop. All of the reviewers acknowledge the need for long-term, large-scale field experiments to obtain conclusive results. Crawley (1999) suggested that studies on nontarget species should cover entire life cycles and multiple generations under field conditions, and observed that short-term population studies are not ideal

for developing reasonable predictions about long-term population dynamics. Thus, several suggestions have been made to improve experimental risk assessment of Bt-transgenic plants (Marvier 2002, Dutton et al. 2003). One of the criticisms is that most experiments do not consider the real exposure of the toxins to organisms in nature. Experiments typically focus on one particular stage of the insect and plant, one generation of the test organism, or samples are taken only a few times during the entire crop season. These limited studies increase the risk of Type I (incorrectly observing effects that actually do not occur in the field) and Type II errors (incorrectly missing effects that do occur) relative to long-term, large-scale studies. Therefore, in this study we investigated the season-long predator community dynamics in adjacent commercial Bt and non-Bt cotton fields over 3 years. Sampling was conducted using pitfall traps, drop cloths, and whole plant observations throughout the cotton season to ascertain long-term integration of Bt-cotton for pest management on the arthropod predator community.

MATERIAL AND METHODS

Experimental designs and sampling procedures were similar for all three field pairs (each pair being considered one replication), each pair consisting of adjacent commercial Bt and non-Bt cotton fields (treatments) sampled weekly throughout the cotton season from the 1st- or 2nd week of June until the 4th week of August or 1st week of September. The cotton fields were located in Tift County, GA (Table 3.1). The crops were managed using standard agronomic practices determined by the growers and insecticide applications were in response to scouting data that determined spray timing, active ingredient, and rate (GA Pest Management Handbook, 2004). All fields received preventative in-furrow insecticide treatments to control early-season thrips all three years (Table 3.2). Fields in 2002 were planted with the Bt-cotton variety DPL 458 and non-

Bt cotton variety DPL 491. In 2003 and 2004 Bt fields were planted with DPL 555 and the non-Bt with DPL 493.

Study Site Descriptions. Detailed information on study sites and the management practices used at each location are presented in Tables 3.1 and 3.2. The fields at the Old House and Chula locations were completely surrounded by hardwoods/conifers, and the Ty Ty, Frazier, and Marchant locations were partially surrounded by hardwoods/conifers, and the remaining edges were surrounded by pasture and paved roads.

The non-Bt field at Chula was treated with insecticides twice within 15 days in July 2003 due to rainfall shortly after the first treatment. Heliothine larval populations exceeded economic thresholds in all non-Bt cotton fields in all years, requiring insecticide treatments (Table 3.2). In addition, Bt- and non-Bt fields were treated as needed for stink bugs (Table 3.2).

Toxin Expression. The amount of Cry1Ac present in the uppermost fully expanded cotton leaves was determined during 2003 and 2004, using leaves collected weekly from the field, in antibody-coated 96-well PathoScreen® plates for Bt-Cry1Ac/Cry1Ab enzyme-linked immunosorbent assay (ELISA) kit (Agdia® Inc., Elkhart, IN) following manufacturer instructions. Standards at concentrations from 0.158 to 20 ng/ml were used to calibrate the optical density curve that was used to derive estimates of the amount of toxin expressed in the plants (μg Cry1Ac g^{-1} fresh tissue).

Whole Plant Sampling. This procedure focused on predator life stages that are primarily sedentary on plants. Plants from each field were harvested at approximately 10-d intervals for evaluation (hereafter "sampling dates"). Transparent plastic bags 110 cm wide by 125 cm long (Stone Container Corporation, Mansfield, OH) were used to bag the plants. The bottoms of the bags were cut off and bags were tied around the base of the cotton plant and pressed on the

ground around the plant. Five to ten days following placement, the plastic bags were pulled quickly over the plant, tied at the top and the cotton plant was cut off at ground level and transferred to the laboratory for examination. Plants were inspected within 24 h of collection using a 10x magnification light. The total number of plants evaluated per field on each sampling date varied from 20 to 30 depending on the number of plants bagged. The experimental fields were seeded using a hill-drop system that placed 2-3 seeds in each spot. Thus, multiple plants were often bagged together, so that a total of 414 and 424, 581 and 607, and 516 and 517 Bt and non-Bt cotton plants, respectively, were collected and evaluated during 2002, 2003, and 2004.

Drop Cloth Sampling. Predatory insects and spiders were sampled weekly (hereafter "sampling weeks") with a standard 100-cm long white canvas cloth. The cloth was spread on the ground between two rows of cotton and the plants on the rows adjacent to the cloth were vigorously shaken over the cloth. Predators dropping on the cloth were immediately counted and sorted as either immatures or adults, and identified to species for those considered of major importance. This sorting procedure was based on previous scouting records for Georgia cotton and on identification provided in Knutson and Ruberson (1996). A total of 40 samples was taken along a transect from border to border of each of the cotton fields on each sampling date for 12 weeks per season.

Pitfall Traps. A modified pitfall trap was used to sample ground-dwelling insect predators. The traps themselves were 500-ml plastic cups, 18 cm deep. Two holes of approximately 2 cm in diameter were made 3-5 cm above the cup bottom. The holes were covered with nylon mesh to permit drainage of excess water from irrigation or rain events. Capture fluid in the traps consisted of water mixed with Tween 20 (Sigma®, St. Louis, MO) at 0.2% to break surface water tension, and 4-5 pellets per cup of water softener salt (Diamond Crystal®, Cargil Co., Minneapolis, MN) as

a preservative. The cup-traps were inserted into larger and deeper plastic cups (without bottoms to permit drainage) installed previously in field transects. A total of 20 traps were set up from border to border, in 10 equally-spaced sites. The sampling stations were set up immediately after seeding. Two traps were installed at each station, in the cotton rows and separated from one another by five rows. Trap contents were collected weekly (hereafter "sampling weeks") by replacing the insert cups and using the same base throughout the season. The data presented will focus on representative predator groups or species for the locale (e.g., ground beetles, tiger beetles, earwigs, rove beetles, big-eyed bugs, damsel bugs and spiders). Specimens collected in pitfall traps were washed and sorted to family. Spiders, ground beetles, earwigs, and tiger beetles were further identified to species using available keys (Kaston 1978, and Breene et al. 1993, for spiders; Lindroth 1961-1969 and Ciegler 2000, for ground beetles; Hoffman 1987, for earwigs, and Knisley and Schultz 1997, for tiger beetles). All material was stored in scintillation vials (Fisher Scientific, Pittsburgh, PA) containing 70% ethyl alcohol. Vials containing the weekly samples are deposited at the Biological Control Laboratory (UGA-CPES), in Tifton, GA, and voucher specimens are deposited at the University of Georgia Collection of Arthropods (UGCA), Athens, GA.

Data Analysis. Prior to analyses, data from whole plant evaluations, drop cloth samples and pitfall traps were transformed into standardized units. The number of individuals counted in whole plant evaluations (predator eggs or egg masses, nymphs or larvae, or adults) was averaged per plant to adjust for variation in the number of plants evaluated for each of three fields. The same procedure was adopted for pitfall trap counts by averaging the number of predator adults per pitfall trap per field on each sampling week, based on the number of traps recovered per field (discarding traps lost to flooding or other events). The number of predators collected in drop

cloth samples was summed for 40 samples per field on each sampling week for each taxon. All count data were square root (x + 0.5) or log (x + 1) transformed, when appropriate, prior to univariate analyses, but untransformed means are presented. Analyses of species abundance for the three-year average (sampling data and years as two repeated factors) considered only species that occurred in all three years to produce a balanced design. The results were submitted to one-way or two-factor repeated measures analysis of variance (ANOVA) with repeated measures on sampling dates or weeks within season, and sampling date or week and year as the two factors, respectively, and with field as a blocking factor because predator sampling was carried out in the same fields over the season (drop cloth - 12 sampling weeks, pitfall traps – 11 sampling weeks, and whole plant sample – 7 sampling dates in 2002 and 8 in 2003 and 2004). These analyses were carried out using PROC MIXED in SAS (SAS Institute 1999-2001), adapting the PROFILE statement as suggested by Littell et al. (1996). Orthogonal contrasts were used to test the null hypothesis that on each sampling date or week the mean abundance for each taxon did not differ significantly between Bt and non-Bt cottons. Additionally, retrospective power analysis of the probability of accurately detecting a 50% decrease or increase in species abundance between Bt and non-Bt cotton across years and locations was performed (after Perry et al. 2003), using parameters originating from fields and years as fixed effects from repeated measures ANOVA. Perry et al. (2003) stated that 50% difference in species abundance between treatments is ecologically significant and reasonable for detection in field studies.

The changes in predatory community dynamics in Bt-cotton compared to non-Bt cotton (as the control) throughout the cotton season were investigated using principal response curve (PRC) analysis. PRC analysis is a multivariate technique derived from redundancy analysis (RDA) that focuses on the proportion of the variance explained by the variables of interest, in this case

predator species or group abundance sampled on Bt-cotton and sampling dates or weeks over the cotton season. PRC models the treatment community response pattern ($T_{dtk} = b_k\ c_{dt}$) for each species as a multiple of species abundance weight (b_k) and the canonical coefficients (c_{dt}) of the partial RDA. These parameters were generated using the software CANOCO 4.5 for Windows (Lepš and Šmilauer 2003) through RDA least-squares estimates. By plotting values of c_{dt} for the treatment over sampling time, a PRC diagram is generated that depicts the dynamic changes in the community composition. For each set of analyses, the null hypothesis that the PRC does not explain significant treatment variance was tested using an *F*-type test obtained by permutating whole-time series in the partial RDA from which that PRC was obtained (Lepš & Šmilauer 2003). The deviation of principal response c_{dt} for Bt-cotton from the control (non-Bt, y=0 line) on each sampling date was tested using the Monte-Carlo method (999 permutations) performed with CANOCO 4.5. Abundance values (predators per plant, per pitfall trap or per 40 drop cloths) were log-transformed to reduce the effect of weights inflated due to highly abundant species. The reported data focused on predator communities, thus phytophagous ground beetles were omitted from analyses. The functional group for collected species of ground beetles was determined using information in Larochelle and Lariviere (2003).

RESULTS

The levels of Cry1Ac expression did not differ substantially within or between the 2003 and 2004 seasons. Cry1Ac was detected in all Bt-cotton fields and sample dates in 2003 and 2004, and ranged from 0.20 to 0.39, and 0.20 to 0.29 μg Cry1Ac g^{-1} fresh plant tissue, respectively. Thus, at least in these two years, we would not expect results to be significantly affected by toxin levels in the plants.

Canopy-Dwelling Predator Community. Representatives of 21 and 18 predator taxa were collected in Bt and non-Bt cotton fields in drop cloth and whole plant sampling, respectively (Table 3.3 and 3.4). The species composition was typical of predator communities in cotton in the region (Knutson and Ruberson 1996), with only two uncommon predatory taxa, *Geocoris floridanus* Blatchley and *Stiretrus anchorago* (F.) found. The big-eyed bug *G. floridanus* was collected only in 2003 and 2004, and the pentatomid *S. anchorago* in 2002 and in one sample in non-Bt cotton in 2003 (Table 3.3). The occurrence of other predators was consistent over the seasons but some species within groups were more abundant than others. *Geocoris punctipes* (Say) and *Orius insidiosus* (Say) were more abundant among predatory heteropterans during all years, including all damsel bug species pooled together: *Nabis roseipennis* Reuter, *Nabis americoferus* (Carayon), *Tropiconabis capsiformis* (Germar), and *Nabis alternatus* Parshley (Table 3.3). Data on damsel bugs are presented for all species and life stages pooled, because early nymphal stages of *Nabis* and *Tropiconabis* are very difficult to sort accurately to species when sampling in the field. Due to increased abundance of damsel bugs in 2004, adults were sorted to species level. The species contribution to the total adult damsel bug populations in Bt and non-Bt cotton in 2004 was *N. roseipennis* (68.4 and 59.7%), *T. capsiformis* (25.3 and 37.5%), *N. americoferus* (2.7 and 1.4%), and *N. alternatus* (3.6 and 1.4%). Among predatory heteropterans occurring all three years, the pentatomid *Podisus maculiventris* (Say) and the reduviids *Zelus* spp. and *Sinea* spp. had very low densities. However, all taxa collected in drop cloths were similarly abundant in Bt and non-Bt cotton fields when data for all years are pooled and analyzed with date-by-date contrasts (Table 3.5). Similar abundance patterns of predatory heteropterans were found in whole plant samples, except that *Geocoris uliginosus* (Say) was more abundant in non-Bt cotton fields and *Nabis* spp. were more abundant in Bt-cotton fields on 1 out

of 8 sample dates (Table 3.3). All other predator taxa in drop cloth and whole plant samples were similarly abundant in Bt and non-Bt cotton for all three seasons pooled (Table 3.5).

Six species of coccinellids were present in all years, with *Scymnus* sp. (nr. *loewii* Mulsant) being most abundant, followed by *Harmonia axyridis* (Pallas) (Table 3.3). The densities of other species were comparable across years, except for *Diomus* spp., which occurred at relatively low densities in 2002, but were more abundant during 2003 and 2004. Repeated measures ANOVA's indicate that *Coleomegilla maculata* (DeGeer) was more abundant in Bt-cotton fields in 2003 and 2004 on 5 successive weeks in mid- to late season. Also, *Scymnus* sp. and *H. axyridis* had higher densities in Bt-cotton on 2 and 5 successive weeks during 2002 and 2004, respectively. However, the opposite pattern was found for *Hippodamia convergens* Guérin-Méneville, with higher abundance in non-Bt cotton fields for 2 mid-season sampling weeks during 2002 and 4 mid- to late-season sampling weeks during 2004 (Table 3.3). The same pattern of higher densities of *H. convergens* and lower densities of *H. axyridis* in non-Bt cotton fields was observed in the whole plant sample data during 2004, and higher numbers of coccinellid egg masses and *Diomus* spp. were observed in non-Bt cotton fields during 2004 (Table 3.3). Nonetheless, pooled data for three successive seasons of coccinellids produced only two significant differences in favor of non-Bt cotton during 2004, higher densities of *H. convergens* and, correspondingly, coccinellid egg masses on whole plant samples (Table 3.5).

Repeated measures ANOVA's revealed no differences in abundance of green lacewing, *Chrysoperla rufilabris* (Burmeister), or brown lacewing, *Micromus* sp., larvae across years and for all three years pooled for either drop cloth or whole plant samples; except higher numbers of brown lacewing eggs observed in sampling dates 5 and 7 in Bt-cotton during 2003 (Table 3.3). However, no difference was detected in any sampling dates during 2002 or 2004, which resulted

in no significant differences in brown lacewing eggs or larvae when data from all 3 years were pooled (Table 3.5).

Spiders and red imported fire ants, *Solenopsis invicta* Buren, occurred at relatively higher densities than the other predator species individually, while the earwig *Doru taeniatum* (Dohm), the hooded beetle *Notoxus monodon* (F.), and syrphid larvae occurred at low densities. However, all of these predators showed similar densities in both cotton types (Tables 3.3, 3.4, and 3.5).

Predator community responses to Bt-cotton over the season can be portrayed using principal response curves (PRC). PRC diagrams show no significant changes in species abundance over time in drop cloth samples in 2002 ($P = 0.604$) and whole plant samples in 2002 ($P = 0.846$) and 2004 ($P = 0.906$). However, PRC diagrams showed consistent variability in canopy predator communities throughout 2003 and 2004 for drop cloth (Fig. 3.1) and whole plant samples during 2003 (Fig. 3.2). Of the total variance in species abundance in drop cloth samples during 2003 and 2004, 52.1% and 56.8% is explained by sampling weeks and 9.2 and 10.1% is explained by treatment (Bt-cotton), respectively. The variance exhibited in the first PRC diagrams was highly significant in 2003 ($F = 7.29$, $P = 0.001$) and 2004 ($F = 8.64$, $P = 0.002$), indicating that 63.7% and 63.9% of the variation in the community was due to interactions between sample weeks and cotton type (Fig. 3.1). The second PRC axis explained an additional 14.3 and 12% of the variance compared to the first PRC for the 2003 and 2004 seasons, but was not significant ($P > 0.05$). The Monte Carlo permutation test detected significantly higher species abundance on Bt-cotton in the last week of July and the first and last weeks of August in 2003, and lower abundance in the last two sampling weeks of July and the first week of August 2004 (Fig. 3.1).

The PRC diagram for whole plant samples was significant for 2003 ($F = 5.43$, $P = 0.026$). The first PRC axis explained that 60.9% of the variance in the community was due to sampling

date, whereas 15.1% can be attributed to Bt-cotton and sampling date interactions. Although the second PRC axis explained an additional 9.8% of variance, it was not significant ($P > 0.05$). Based on a Monte Carlo test run date by date, Bt-cotton had a significant influence on the species abundance in August 2003, with lower abundance in the samples obtained on 14 and 24 August, and greater abundance on 3 August (Fig. 3.2).

The contribution of each species to the community changes (response, c_{dt}) depicted by PRC diagrams can be also evaluated by considering each species' statistical weight (b_k), shown on the right side of each diagram (Fig.3.1 to 3.3). Species with positive weight values higher than +0.5 are most likely to follow the abundance changes displayed in the diagram, while negative values lower than -0.5 indicate species that trend in a direction opposite that depicted in the diagram. Values ranging between –0.5 to +0.5 do not contribute strongly to the overall community response (Van den Brink and Ter Braak, 1999). Thus, only taxa with significant relative contributions are detailed on the right side of the diagram, but the total number of taxa with species weights between –0.5 and +0.5 is also provided. Although their abundance did not differ significantly between cotton types in 2002, coccinellid egg masses and *O. insidiosus* contributed most to the deviation from the control. The species weights for the 2003 drop cloth samples suggest that higher densities of *S. invicta*, *C. maculata*, *Nabis* spp., *H. axyridis, Micromus* sp., *Coccinella septempunctata* L. and spiders occurred in Bt-cotton on sampling weeks 27 July, and 3 and 29 August (Fig. 3.1), while during 2004, *H. convergens*, *G. uliginosus*, *Scymnus* sp., *Diomus* sp., *S. invicta*, *C. rufilabris* and *O. insidiosus* were less abundant in Bt-cotton, which is consistent with results of the univariate repeated measures ANOVA for those species (Table 3.3). However, the PRC analysis indicated that other species than those with significantly different seasonal means, as determined by univariate analyses, were also important contributors to the community changes

(Figs. 1-3). This is the cumulative result of small differences in densities for these species across sampling weeks being captured by the permutation in the PRC analyses. For example, in 2002 the mean seasonal abundance of *C. septempunctata* was similar between Bt and non-Bt cotton (Table 3.3), but small variations in its abundance throughout the season became important to changes in overall predator abundance in the PRC analysis (Fig. 3.1).

Ground-Dwelling Predator Community. Twelve taxa representing the epigeal predator communities in cotton fields were individually submitted to univariate repeated measures ANOVA, and only three seasonal contrasts out of 36 were significant (Table 3.6). Higher abundance of *G. punctipes* in non-Bt cotton was found during sampling weeks 4 and 11 July in 2004. Also, analyses revealed higher numbers of ground beetles in the sampling week of 15 August 2003 in non-Bt cotton (Table 3.6). However, all taxa had similar densities in Bt and non-Bt cottons when data for all 3 years are considered (Table 3.5). *Megacephala carolina* L. and *Labidura riparia* (Pallas) were consistently the first and second most abundant taxa caught in the pitfall traps, except in 2003 when the number of spiders (all species pooled) was greater than *L. riparia*.

Among the ground-dwelling communities, the family Carabidae was the most diverse taxon, followed by spiders (11 species). Of the 44 carabid species collected in pitfall traps, predatory feeding behavior is reported for 21 (Larochelle and Lariviere 2003). Only predatory carabid species were submitted to PRC analyses of the epigeal community, along with spiders, tiger beetles, earwigs, big-eyed bugs, and damsel bugs. *Harpalus pennsylvanicus* DeGeer (31.6%), *Harpalus caliginosus* (Fabr.) (15.6%) and *Stenolophorus ochropezus* (Say) (11.3%) were the most abundant species in 2002; *Calosoma sayi* Dejean (43%), *S. ochropezus* (16.3%) and *H. pennsylvanicus* (12.8%) in 2003; and *C. sayi* (39.2%), *H. caliginosus* (10.4%) and *H.*

pennsylvanicus (8.9%) in 2004. A detailed description of dynamics, abundance, and diversity of ground-dwelling arthropods including phytophagous carabids is found in CHAPTER 4.

PRC analyses, in agreement with the univariate analyses (Table 3.5), showed no statistically significant impact of Bt-cotton compared to non-Bt cotton (standard reference) on the abundance of predatory ground-dwelling species (Fig. 3.3). In general, the variance of ground-dwelling predator communities over the seasons within each year depicted in the PRC diagram was driven by the most abundant predatory species, such as earwigs, *L. riparia*, the tiger beetles *M. carolina* and *Cicindela punctulata* Oliver, the ground beetles *C. sayi*, *H. pennsylvanicus*, and *S. ochropezus*, and the spiders *Pardosa pauxilla* Montgomery and *Oxyopes salticus* Hentz, that exhibited the highest statistical weights (Fig. 3.3).

Power of Statistical Test. The retrospective power analysis showed that all tests performed on data pooled for the 3 yrs yielded power to detect a 50% difference (increase or decrease) between Bt and non-Bt cotton for species abundance greater than 80% or approximating 100% (Table 3.5). Power greater than 80% is usually recognized as adequate for detecting a specified difference (Murphy and Myors 1998). Because the major interest was to evaluate the long-term effect of deploying Bt-cotton on predatory community, results from individual years were not tested.

DISCUSSION

A number of studies have investigated arthropod communities in Bt and non-Bt cotton (Luttrell et al. 1995, Armstrong et al. 2000, Hagerty et al. 2000, Men et al. 2003, Wu and Guo 2003, Sisterson et al. 2004, Hagerty et al. 2005), but the results reported here are among the first dealing with changes in plant canopy and ground dwelling predator communities over multiple cotton seasons and multiple predator generations under standard grower management practices.

Before discussing these results, some limitations of the study should be considered, such as the lack of Bt and non-Bt cotton fields without insecticide applications in our design. Using Bt and non-Bt fields, both unsprayed, would provide a means of separating insecticide effects from those attributable to plant type. Such studies have been done in experimental plots (above references). But most of those studies concluded that field experiments using grower practices are more relevant to commercial production. Results from unsprayed fields cannot be extrapolated to most commercial fields, since insecticide use is needed to assure cotton growers a profitable yield in most years in the southeastern United States. For this reason, the addition of non-Bt cotton fields with no insecticide use is not a realistic treatment for comparison in our region. Another limitation of this study is the absence of data on aerial predators, such as syrphid and dolichopodid flies, predatory wasps, dragonflies, etc. For syrphid flies, the larvae (which are plant residents and sampled) provide more pertinent information than adults, which forage large areas for food and are not predatory. Predatory wasps usually nest mainly in field borders, and dragonflies are more abundant in fields close to standing water; therefore, these predators are largely associated with adjacent landscape, making it difficult to isolate direct effects of the Bt-cotton from the adjacent landscape. Therefore, only those predator communities most directly related to the cotton ecosystem and directly exposed to grower practices were selected for consideration in this study.

We would anticipate higher abundance of natural enemies in Bt-cotton because use of broad-spectrum insecticides in cotton reduces the abundance and diversity of predatory species (Bartlett 1968, Wu and Guo 2003, Naranjo et al. 2004, Hagerty et al. 2005). This may have been the reason for higher species abundance in Bt-cotton during 2003 and 2004, as depicted by the PRC diagram (Fig. 3.2). The community was similar for both cotton types until mid-late July,

when two applications of lambda-cyhalothrin were made in non-Bt cotton at the Chula (C) farm (Table 3.2). However, the opposite pattern occurred in 2004, with higher species abundance in non-Bt cotton after insecticide applications (Fig. 3.2). However, only two species appear to account for the community changes displayed in the 2004 PRC diagram. These species were *H. convergens* (the highest positive species weight in the PRC, signifying the lowest population in Bt-cotton according to the diagram) and *H. axyridis* (with the lowest species weight, signifying higher densities in Bt-cotton). The results agree with results of univariate analyses (Table 3.3), and indicate a species shift after insecticide application. This observation agrees with the suggestion of Luttrell et al. (1995) that changes in the predatory arthropod communities in a Bt-cotton ecosystem are probably more related to changes in insecticide use patterns than the presence of toxins in the plants, and the species shift is typical of changes observed in predator communities in cotton after insecticide application (Naranjo et al. 2003 and 2004, Men et al. 2003).

The highest species weight values indicate the strength of the respective species' dynamics in shaping the pattern displayed in the PRC diagram. Different species were major contributors in the various years, and yielded different abundances between Bt and non-Bt cotton among dates for predators in the cotton canopy (Tables 3.3 and 3.4). These results suggest that the predator community in the cotton canopy was shaped chiefly by species that were consistently more abundant (such as coccinellids, followed by red imported fire ants, big-eyed bugs, and lacewings), and that varied from season to season (Fig. 3.1 and 3.2). The significant changes in species abundance in 2003 and 2004 in Bt relative to non-Bt cotton appear to be due to insecticide use. Differences were detected by PRC analyses (Figs. 3.1 and 3.2) and univariate analyses (Tables 3.3 to 3.5) in both years on sample weeks following lambda-cyhalothrin applications (Table 3.2).

Early-season management of heliothine populations was generally done with a selective insecticide (spinosad). Spinosad is selective to lepidopteran larvae, and has limited impact on common natural enemies in cotton (Tillman and Mulrooney 2000, Cisneros et al. 2002). However, in 2003, two applications of lambda-cyhalothrin were made at Chula farm (C) following spinosad. These insecticide applications reduced predator numbers in the non-Bt cotton. As the average is produced from counts on all three fields, this single-field treatment led to average decreased species abundance in non-Bt cotton field immediately following insecticide application (Fig. 3.2). The opposite pattern occurred in 2004, although the same pattern for higher species abundance in Bt-cotton was expected, since the broad-spectrum pyrethroid lambda-cyhalothrin was sprayed in all three non-Bt cotton fields. However, the greatest statistical contributors to the change in predatory abundances depicted by the PRC in 2004 were *H. convergens* and *H. axyridis*. These two lady beetles shifted populations between Bt and non-Bt [having the highest and lowest species weights (Fig. 3.1)], apparently due to differential susceptibility to pyrethroid insecticides (Torres and Ruberson 2005). Pyrethroid applications eliminated *H. axyridis* populations, which are highly susceptible to lambda-cyhalothrin (Tillman and Mulrooney 2000, Torres and Ruberson 2005), and allowed populations of *H. convergens*, which were found to be resistant to pyrethroids (Torres and Ruberson 2005), to flourish. These observations point out that care must be taken when interpreting field results to assess risk of transgenic plants to natural enemy communities in commercial fields that usually rely on insecticides to manage pest populations not targeted by the transgenic plants.

Prey shortages resulting from reduced numbers of lepidopteran larvae in Bt-crops are considered to interfere with the third trophic level (Luttrell et al. 1995, Schuler et al. 1999, Groot and Dicke 2002). However, this does not seem to be a problem for the predatory community in

cotton for at least two reasons. First, the predator community in cotton is dominated by generalist species (Table 3.5). Second, despite Bt-cotton reducing caterpillar populations, the abundance of lepidopteran eggs remains about the same (CHAPTER 5), and eggs are a significant food resource for many predators. Further, many other leaf-feeding lepidopteran species only partially susceptible to Cry1Ac occur in Bt-cotton fields (e.g., *Pseudoplusia* and *Spodoptera* spp.) to various extents throughout the season, and these may be easier prey for predators than are heliothines, which are partially protected inside bolls, squares, or terminal tissues.

The predator community in cotton can change in many ways under grower agronomic practices, but heavy insecticide use in cotton is the most recognized factor interfering with tritrophic interactions (Bartlett 1968, Kerns and Gaylor 1993, Wu and Guo, 2003, Naranjo et al. 2003 and 2004). Our results suggest that within each season a minor number of predator species in the cotton canopy can dramatically shift abundance between Bt or non-Bt cotton (Tables 3.3, 3.4, and 3.6) and thereby dictate the differences between Bt and non-Bt fields. Significant contrasts occurred throughout seasons (Tables 3.3, 3.4 and 3.6) for a few species (Figs. 3.1 to 3.3), but these differences did not persist when data for all three seasons were pooled (Table 3.5). This pattern of ephemeral scattered and inconsistent differences in species abundance between Bt and non-Bt crop fields has been reported in other systems, such as potatoes (Riddick et al. 2000, Reed et al. 2001) and corn (Pilcher et al. 1997, Al-Deeb and Wilde 2003), as well as in cotton (Men et al. 2003). This fact partially explains the inconsistent results on the impact of Bt relative to non-Bt crops observed in short-term field experiments. The 40 significant contrasts observed on specific sample dates or weeks within the seasons were reduced by 72.5% when data from all three seasons were pooled (Table 3.5). And of the 11 remaining significant contrasts, 72.7%

involved just one species (*H. convergens*), and this difference is explainable as a result of insecticidal release from interspecific competition (Torres and Ruberson 2005).

The results of pitfall trap collections revealed no change in the ground-dwelling predator community by univariate or by PRC analyses (Table 3.5 and Fig. 3.3). The predominant predators representing the ground-dwelling community (cicindelines, araneids, staphylinids, carabids, and some dermapterans species) are not expected to suffer quite as strongly from insecticides applied to the cotton canopy, and the target foliar herbivores are not likely primary prey for epigeal predators. Our results agree with those reported for transgenic Bt-corn (Cry1Ab) (Lozzia 1999, Al-Deeb and Wilde 2003, Candolfi et al. 2004, French et al. 2004) and Bt-potato (Cry3Aa) (Riddick et al. 2000, Reed et al. 2001, Duan et al. 2004), and together with these other results suggest no adverse effects of Bt transgenic crops on ground-dwelling predator communities. This trend, however, is not as clear for predator species that share canopy and ground habitats. For example, *G. punctipes* is a typical predator in the cotton canopy, and significant numbers captured in pitfall traps favored non-Bt cotton in 2004 (Table 3.5). Higher numbers of *G. punctipes* were collected in pitfall traps in the sampling weeks 4 and 11 July 2004, following treatment with lambda-cyhalothrin (Tables 3.2 and 3.6). The increased appearance of *G. punctipes* in pitfall traps may have been a result of lambda-cyhalothrin application forcing predators to forage on the ground or fall from the plants into the traps after foliar sprays since few individuals were enough to generate significant differences.

Any disruption in the herbivore/plant relationship can cascade up to the third trophic level. However, typical predator communities in the cotton ecosystem were affected more by insecticide use than by Bt-cotton. Trophic effects can occur under many other conventional pest control methods, including pest resistance traits in cotton plants introduced by conventional breeding

(Schuster and Calderon 1986, Cortesero et al. 2000). In such complex trophic webs as the cotton ecosystem, changes in the predatory arthropod community can fluctuate due to introduction of one or more practices that affect herbivore population. Those predator/prey relationships adapted to disruptive agronomic practices will persist, while those prone to change will be more erratic. The particular or small changes in arthropod community found in small-plot studies tend to be magnified by the relatively brief evaluation period and the limited size of experimental plots, perhaps more so than by use of insecticide, etc., which does not represent a realistic grower situation. In our study, these small changes occurred during limited periods within the season, but did not persist in our long-term evaluations of large fields under grower practices. Some of the changes are easily explained and others less so. The fourth trophic level also may play a role that has not been explored yet. Generalist parasitoids of predators surviving on different species may switch to a more common host as host species abundance fluctuates, perhaps providing some stability to population abundance or enhancing differences.

Negative impacts on populations of predators considered important for cotton pest management, such as big-eyed bug (*G. punctipes*), insidiosus flower bug (*O. insidiosus*), coccinellids (*Scymnus* sp. and *H. axyridis*), lacewings (*C. rufilabris* and *Micromus* sp.), a tiger beetle (*M. carolina*), and red imported fire ant (*S. invicta*), were not observed in our study, and these species were often abundant in all locations and years. Other species were more variable in abundance, and changes in their populations could be expected to be due to other factors than the single factor of Bt-cotton adoption.

Our results suggest that Bt-cotton use, coupled with judicious insecticide selection when economic thresholds are exceeded, has no adverse effect on the predator community. Not surprisingly, significant reductions in predator species abundance occurred mid to late season

after broad-spectrum insecticide applications (lambda-cyhalothrin, zeta-cypermethrin, and dicrotophos). The results reported here for three successive seasons conducted in grower fields showed that variation in relative predator abundance among dates could be common (Table 3.3, 3.4, and 3.6) for some species, but become null over long-term analyses (Table 3.6). These findings reiterate suggestions of Candolfi et al. (2004) and O'Callaghan et al. (2005) that population-level and large-scale effects, evaluated over sufficiently long periods to consider environmental variability, should be the ultimate endpoint of concern in risk assessment trials, despite the challenges and limitations of field work.

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Table 3.1. Study site descriptions of cotton fields, near Tifton, GA, 2002-2004.

Year	Field	Previous crop	Planting date	Geographic localization	Area (ha)	
					Bt	Non-Bt
2002	Chula	Peanut	14 May	31° 51'N, 83° 55'W	6.0	6.5
	Marchant	Cotton	12 May	31° 48'N, 83° 55'W	5.5	5.0
	Ty Ty	Peanut	25 April	31° 45'N, 83° 63'W	5.8	6.9
2003	Chula	Cotton	9 May	31° 51'N, 83° 55'W	6.0	5.5
	Marchant	Peanut	7 May	31° 48'N, 83° 56'W	5.0	7.5
	Old House	Cotton	13 May	31° 23'N, 83° 32'W	5.5	6.5
2004	Chula	Sorghum	12 May	31° 51'N, 83° 55'W	15.0	11.0
	Marchant	Tobacco	10 May	31° 48'N, 83° 55'W	6.0	5.0
	Frazier	Cotton	6 May	31° 23'N, 83° 39'W	11.0	6.5

Table 3.2. Timing, materials, and rates of insecticide applications for management of pest infestations in the experimental fields, 2002-2004.

Dates (Fields)[a]	Non-Bt cotton	Bt-cotton	Targeted pest[d]
2002			
Application date			
2-14 May (C,M,T)[c]	aldicarb 15G (560 g/ha)[b]	aldicarb 15G (560 g/ha)	Thrips
8-9 July (C,M,T)[c]	spinosad (100 g/ha)	-[e]	Heliothines
10-12 August (C,M)	lambda-cyhalothrin (34 g/ha) + thiodicarb (680 g/ha)	-	Heliothines
14 August (T)	-	dicrotophos (390 g/ha)	Stinkbugs
5-7 September (C,M)	pyriproxifen (60 g/ha)	pyriproxifen (60 g/ha)	Whiteflies
2003			
Application date			
7-14 May (C,M,O)	aldicarb 15G (560 g/ha)	aldicarb 15G (560 g/ha)	Thrips
8 July (O)	spinosad (100 g/ha)	-	Heliothines
13 July (M)	spinosad (100 g/ha)	-	Heliothines
14 July (C)	lambda-cyhalothrin (30 g/ha)	-	Heliothines
21 July (C)	lambda-cyhalothrin (45 g/ha)	-	Heliothines
3-5 August (M,O)	lambda-cyhalothrin (45 g/ha)	-	Heliothines + stinkbugs
30 August (O)[3]	bifenthrin (70 g/ha)	bifenthrin (70 g/ha)	Stinkbugs
2004			
Application date			
6-12 May (C,M,F)	aldicarb 15G (560 g/ha)	aldicarb 15G (560 g/ha)	Thrips
2-7 July (C,M,F)	lambda-cyhalothrin (45 g/ha)	-	Heliothines
15 July (C,M)	spinosad (100 g/ha)	-	
29 July (C)	-	dicrotophos (420 g/ha)	Stinkbugs
5 August (C)	zeta-cypermethrin (160 g/ha)	-	Heliothines + stinkbugs
17 August (M,F)	zeta-cypermethrin (210 g/ha)	zeta-cypermethrin (210 g/ha)	Heliothines + stinkbugs
31 August (M)[3]	-	acephate (810 g/ha)	Stinkbugs

[a] C = Chula; M = Marchant; T = Ty Ty; O = Old House; and F = Frazier fields.
[b] Rate in grams of active ingredient per hectare.
[c] Insecticide applied after sampling termination.
[d] Thrips (*Frankliniella occidentalis*, *Frankliniella fusca* and *Thrips tabaci*), Heliothine (*Helicoverpa zea* and *Heliothis virescens*), Stinkbugs (*Nezara viridula* and *Euschistus servus*). Thresholds: Thrips (preventive treatment); Heliothine = 8-10% of plants with eggs or small larva on terminals; Stinkbugs = 18-20% bolls of ~2.5 cm diameter with internal damage; whiteflies = plants infested and honeydew on plants (GA Pest Management Handbook 2004).
[e] No treatment was applied.

Table 3.3. Seasonal mean of arthropod predators (nymphs or larvae + adults) per 40-drop cloth samples throughout the cotton season, from 1st week of June to 4th week of August in *Bt* and non-Bt cotton fields near Tifton, GA, 2002-2004.

	2002			2003			2004		
Predators	*Bt*	Non-Bt	F^a	Bt	Non-Bt	F^a	*Bt*	Non-Bt	F^a
Chrysoperla rufilabris	2.4 ± 0.63	0.9 ± 0.21	1.70	4.5 ± 0.97	6.3 ± 1.27	0.76	4.8 ± 0.83	5.6 ± 0.82	3.44
Coccinella 7-punctata	14.9 ± 4.80	27.2 ± 8.03	1.37	10.4 ± 2.64	12.1 ± 2.82	0.14	4.9 ± 0.98	4.7 ± 1.27	0.88
Coleomgilla maculata	1.4 ± 0.57	2.0 ± 0.64	0.41	5.7 ± 1.38	2.1 ± 0.53	10.66**[3,7-10]	5.0 ± 1.00	2.6 ± 0.68	15.11**[6-10]
Diomus spp.	0.4 ± 0.31	0.6 ± 0.30	0.55	11.3 ± 2.47	10.6 ± 2.08	0.00	7.8 ± 1.46	12.4 ± 2.45	2.54
Doru taeniatum	1.6 ± 0.52	1.3 ± 0.48	0.04	1.3 ± 0.46	0.97 ± 0.31	0.21	1.5 ± 0.52	1.1 ± 0.40	0.15
Geocoris floridanus	-	-	-	0.25 ± 0.22	0.08 ± 0.05	0.29	0.05 ± 0.04	0.05 ± 0.05	0.01
Geocoris punctipes	26.7 ± 3.84	41.4 ± 6.87	1.09	25.6 ± 4.18	35.9 ± 6.95	1.71	57.0 ± 9.28	55.0 ± 8.78	0.04
Geocoris uliginosus	2.1 ± 0.47	2.4 ± 0.51	0.11	0.5 ± 0.23	0.6 ± 0.21	0.16	4.9 ± 0.88	6.5 ± 1.18	0.66
Harmonia axyridis	15.4 ± 4.79	20.1 ± 4.09	0.93	22.2 ± 4.28	21.2 ± 5.02	1.20	40.8 ± 8.27	26.0 ± 7.89	3.16**[6-7]
Hippodamia convergens	7.5 ± 1.06	14.7 ± 1.99	4.12*[6-7]	2.9 ± 0.89	3.5 ± 1.09	1.66	3.2 ± 0.73	26.4 ± 6.07	8.25**[6-10]
Micromus sp. larvae	5.7 ± 1.31	4.2 ± 1.96	0.11	13.5 ± 2.15	20.4 ± 5.89	1.48	5.7 ± 1.26	6.8 ± 1.67	0.98
Nabis spp.	7.7 ± 1.58	6.7 ± 1.40	0.34	16.9 ± 2.86	14.4 ± 2.38	1.69	12.5 ± 2.65	8.2 ± 1.76	1.47
Notoxus monodon	14.2 ± 2.91	12.9 ± 2.76	0.16	5.4 ± 1.25	4.4 ± 0.75	0.04	4.7 ± 1.08	3.1 ± 0.75	0.16
Orius insidiosus	27.8 ± 7.31	20.9 ± 4.98	0.06	38.7 ± 5.81	36.7 ± 4.83	0.00	37.5 ± 8.82	58.6 ± 12.74	2.29
Podisus maculiventris	0.4 ± 0.11	0.8 ± 0.24	0.37	4.2 ± 0.74	5.7 ± 0.89	1.11	1.3 ± 0.34	1.7 ± 0.35	3.35
Scymnus spp.	70.0 ± 11.16	51.2 ± 6.14	4.32*[9-10]	47.9 ± 9.02	50.2 ± 10.68	0.03	55.2 ± 10.34	49.6 ± 7.15	0.00
Solenopsis invicta	431.0 ± 53.92	230.0 ± 26.76	2.92	258.4 ± 27.88	201.1 ± 31.08	1.47	262.0 ± 21.6	421.2 ± 39.90	2.12
Sinea spp. + *Zelus* spp.	1.3 ± 0.29	1.4 ± 0.25	0.03	2.0 ± 0.60	1.98 ± 0.48	0.36	3.2 ± 0.54	2.6 ± 0.49	0.73
Spiders	65.7 ± 8.04	59.2 ± 6.49	0.11	149.5 ± 19.24	117.8 ± 13.71	2.81	96.0 ± 10.22	84.1 ± 10.78	3.39
Stiretrus anchorago	-	-	-	0.06 ± 0.02	-	-	0.05 ± 0.04	0.04 ± 0.03	0.50
Syrphid fly larvae	2.9 ± 1.11	2.7 ± 1.07	0.03	2.19 ± 0.83	2.25 ± 0.86	0.00	2.1 ± 0.72	2.4 ± 1.03	0.01

[a]ANOVA results (*F*-test) from repeated-measures procedure of SAS. Superscript values after significance asterisks indicate the sampling dates out of 12 dates that were significantly different between *Bt* and non-Bt cotton fields (*P<0.05, ** P<0.01).

Table 3.4. Seasonal mean of predatory insects (nymphs or larvae + adults) per whole cotton plant throughout the cotton season, from 3rd week of June to 1st week of September in *Bt* and non-Bt cotton fields near Tifton, GA, 2002-2004.

	2002			2003			2004		
Predators	*Bt*	Non-Bt	F^a	*Bt*	Non-Bt	F^a	*Bt*	Non-Bt	F^a
Chrysoperla rufilabris eggs	0.14 ± 0.05	0.11 ± 0.03	0.21	0.21 ± 0.04	0.20 ± 0.04	0.10	0.19 ± 0.03	0.26 ± 0.05	0.20
Chrysoperla rufilabris larvae	0.06 ± 0.02	0.06 ± 0.02	0.19	0.06 ± 0.01	0.09 ± 0.02	2.97	0.05 ± 0.013	0.05 ± 0.01	0.11
Coccinella septempunctata	0.10 ± 0.04	0.16 ± 0.05	1.83	0.08 ± 0.03	0.15 ± 0.08	0.41	0.03 ± 0.01	0.03 ± 0.02	0.01
Coccinellid egg masses	0.18 ± 0.07	0.16 ± 0.06	0.28	0.032 ± 0.007	0.030 ± 0.01	0.02	0.05 ± 0.02	0.096 ± 0.02	7.8*[3-4]
Coleomegilla maculata	0.009 ± 0.004	0.02 ± 0.008	0.41	0.05 ± 0.01	0.02 ± 0.008	1.60	0.02 ± 0.008	0.01 ± 0.005	3.10
Diomus spp.	0.05 ± 0.01	0.04 ± 0.01	0.44	0.08 ± 0.02	0.20 ± 0.05	2.66	0.05 ± 0.01	0.12 ± 0.02	4.42**[3,4-5]
Geocoris punctipes	0.12 ± 0.03	0.18 ± 0.05	0.55	0.17 ± 0.04	0.15 ± 0.03	0.11	0.18 ± 0.02	0.20 ± 0.04	0.04
Geocoris punctipes eggs	0.16 ± 0.03	0.22 ± 0.05	0.59	0.16 ± 0.03	0.14 ± 0.04	0.32	0.23 ± 0.03	0.27 ± 0.05	0.81
Geocoris uliginosus	0.01 ± 0.005	0.08 ± 0.02	7.24**[2-3]	0.00 ± 0.00	0.006 ± 0.003	2.59	0.03 ± 0.01	0.05 ± 0.01	1.28
Harmonia axyridis	0.19 ± 0.05	0.15 ± 0.05	0.81	0.08 ± 0.02	0.12 ± 0.04	0.62	0.26 ± 0.08	0.12 ± 0.03	4.01*[3,5]
Hippodamia convergens	0.09 ± 0.02	0.19 ± 0.03	3.66*[3,6,7]	0.02 ± 0.008	0.02 ± 0.006	0.02	0.05 ± 0.01	0.25 ± 0.06	5.31**[2-6]
Micromus sp. eggs	0.16 ± 0.03	0.13 ± 0.03	1.57	0.37 ± 0.06	0.24 ± 0.04	3.78*[5,7]	0.15 ± 0.07	0.27 ± 0.09	1.91
Micromus sp. larvae	0.04 ± 0.01	0.04 ± 0.01	0.00	0.09 ± 0.02	0.11 ± 0.04	1.49	0.05 ± 0.016	0.07 ± 0.02	0.25
Nabis spp.	0.08 ± 0.02	0.06 ± 0.009	0.68	0.08 ± 0.02	0.04 ± 0.009	6.47**[7-8]	0.03 ± 0.007	0.04 ± 0.01	0.24
Orius insidiosus	1.30 ± 0.32	0.87 ± 0.19	1.36	0.89 ± 0.16	0.83 ± 0.15	0.07	0.74 ± 0.19	0.76 ± 0.18	0.02
Podisus maculiventris	0.009 ± 0.005	0.003 ± 0.002	1.78	0.03 ± 0.01	0.06 ± 0.04	0.33	0.005 ± 0.003	0.007 ± 0.003	0.06
Scymnus spp.	0.60 ± 0.17	0.40 ± 0.12	0.37	0.51 ± 0.15	0.48 ± 0.17	0.12	0.34 ± 0.10	0.53 ± 0.15	2.76
Syrphid fly larvae	0.05 ± 0.03	0.03 ± 0.01	0.52	0.04 ± 0.02	0.02 ± 0.009	0.43	0.05 ± 0.02	0.05 ± 0.01	0.02

[a]ANOVA results from repeated-measures procedure of SAS. Superscript values after significance asterisks indicate the sampling dates out of 7 dates in 2002 and 8 dates in 2003 and 2004 that were significantly different between *Bt* and non-Bt cotton fields (*P<0.05, **P<0.01).

Table 3.5. Seasonal mean of predators collected throughout cotton seasons in 40-drop cloth, per plant and per pitfall trap from 2002 to 2004 near Tifton, GA.

	Drop cloth			Whole plant			Pitfall trap		
Predators	*Bt*	Non-Bt	F^{a} $(1\text{-}\beta)^{b}$	Bt	Non-Bt	F^{a} $(1\text{-}\beta)^{b}$	*Bt*	Non-Bt	F^{a} $(1\text{-}\beta)^{b}$
Carabidae[c]	-	-		-	-		0.07 ± 0.01	0.08 ± 0.02	0.07 (95.3)
Chrysoperla rufilabris eggs	-	-		0.18 ± 0.02	0.19 ± 0.03	0.01 (86.0)	-	-	
C. rufilabris larvae	3.9 ± 0.48	4.3 ± 0.55	0.33 (91.5)	0.06 ± 0.008	0.06 ± 0.01	0.06 (86.3)	-	-	
Cicindella punctulata	-	-		-	-		0.16 ± 0.03	0.18 ± 0.03	0.13 (100)
Coccinellid egg masses	-	-		0.05 ± 0.008	0.07 ± 0.01	3.47 (85.3)	-	-	
Coccinella septempunctata	10.1 ± 1.88	14.6 ± 2.98	1.13 (96.9)	0.07 ± 0.01	0.11 ± 0.03	0.93 (85.6)	-	-	
Coleomegilla maculata	4.1 ± 0.62	2.3 ± 0.36	3.62 (86.8)	0.03 ± 0.006	0.01 ± 0.004	1.99 (88.4)	-	-	
Diomus spp.	6.5 ± 1.05	7.9 ± 1.18	2.27 (96.8)	0.06 ± 0.009	0.12 ± 0.02	3.99 (85.2)	-	-	
Doru taeniatum	1.5 ± 0.28	1.1 ± 0.24	0.23 (96.6)	-	-		0.01 ± 0.01	0.01 ± 0.003	0.17 (100)
Euborellia annulipes	-	-		-	-		0.05 ± 0.01	0.06 ± 0.02	0.00 (100)
Geocoris punctipes eggs	-	-		0.19 ± 0.02	0.21 ± 0.03	1.35 (86.4)	-	-	
Geocoris punctipes	36.4 ± 3.86	44.1 ± 4.41	0.79 (94.59)	0.16 ± 0.02	0.17 ± 0.02	0.28 (85.1)	0.04 ± 0.007	0.06 ± 0.008	2.65 (100)
Geocoris uliginosus	2.5 ± 0.38	3.1 ± 0.49	0.68 (90.2)	0.01 ± 0.004	0.04 ± 0.008	$12.65^{*2\text{-}3}$ (84.0)	0.09 ± 0.02	0.12 ± 0.03	0.96 (92.3)
Harmonia axyridis	26.1 ± 3.63	22.6 ± 3.38	1.25 (98.7)	0.12 ± 0.02	0.17 ± 0.03	5.25 (86.3)	-	-	
Hippodamia convergens	4.5 ± 0.56	14.7 ± 2.31	$6.3^{*6\text{-}9}$ (84.9)	0.05 ± 0.009	0.15 ± 0.02	$6.80^{**2\text{-}5}$ (84.4)	-	-	
Labidura riparia	-	-		-	-		2.12 ± 0.45	1.71 ± 0.38	0.31 (97.2)
Megacephala caroline	-	-		-	-		7.84 ± 0.73	6.81 ± 0.71	0.61 (100)
Megacephala virginica	-	-		-	-		0.05 ± 0.009	0.07 ± 0.01	0.12 (100)
Micromus sp. eggs	-	-		0.20 ± 0.04	0.18 ± 0.04	0.14 (85.2)	-	-	
Micromus sp. larvae	8.3 ± 0.99	10.5 ± 2.17	0.03 (97.8)	0.08 ± 0.01	0.10 ± 0.03	3.33 (89.1)	-	-	
Nabis spp.	12.4 ± 1.44	9.7 ± 1.13	2.08 (86.1)	0.06 ± 0.008	0.04 ± 0.005	7.51^{*7} (83.3)	0.012 ± 0.01	0.016 ± 0.01	0.60 (100)
Notoxon monodum	8.1 ± 1.18	6.8 ± 1.06	0.27 (86.4)	-	-		-	-	

Table 3.5 Continued

Orius insidiosus	34.7 ± 4.27	38.7 ± 5.02	0.18 (97.2)	0.95 ± 0.13	0.82 ± 0.10	1.11 (84.9)	-	-	
Podisus maculiventris	1.9 ± 0.31	2.7 ± 0.38	1.55 (99.7)	0.02 ± 0.006	0.02 ± 0.01	0.17 (86.4)	-	-	
Scymnus spp.	57.7 ± 5.91	50.3 ± 4.70	0.38 (87.0)	0.48 ± 0.08	0.47 ± 0.08	0.01 (84.6)	-	-	
Solenopsis invicta	317.1 ± 22.65	284.1 ± 21.12	0.33 (81.9)	-	-		-	-	
Sinea spp. + *Zelus* spp.	2.2 ± 0.29	2.0 ± 0.24	0.01 (98.9)	-	-		-	-	
Spiders[d]	103.7 ± 8.37	87.1 ± 6.57	4.86 (100)	-	-		0.81 ± 0.09	0.66 ± 0.09	0.34 (100)
Staphylinidae[d]	-	-		-	-		0.21 ± 0.01	0.25 ± 0.02	1.08 (93.1)
Syrphid fly larva	2.4 ± 0.52	2.5 ± 0.57	0.00 (95.6)	0.04 ± 0.01	0.03 ± 0.007	0.23 (85.9)	-	-	

[a]ANOVA results (*F*-test) from repeated-measures procedure of SAS. Superscript values after significance asterisks indicate the sampling dates that were significantly different between *Bt* and non-Bt cotton ($P<0.05$).
[b] Power (proportion in 100 that *F*-test will detect 50% difference on abundance of collected taxa between Bt and non-Bt cotton).
[c] Only predatory taxa included.
[d] All collected taxa.

Table 3.6. Seasonal mean of predatory insects (adults) per pitfall trap, sampled weekly throughout cotton season from the 2nd week of June to 4th week of August in *Bt* and non-Bt cotton fields near Tifton, GA, 2002-2004.

	2002			2003			2004		
Predators	*Bt*	Non-Bt	F^a	*Bt*	Non-Bt	F^a	*Bt*	Non-Bt	F^a
Carabidae[b]	0.11 ± 0.03	0.08 ± 0.02	2.11	0.05 ± 0.012	0.12 ± 0.03	7.71*[10]	0.07± 0.02	0.04 ± 0.007	2.94
Cicindella punctulata	0.20 ± 0.06	0.29 ± 0.08	0.28	0.11 ± 0.03	0.16 ± 0.03	1.26	0.15 ± 0.05	0.10 ± 0.03	0.54
Doru taeniatum	0.005 ± 0.004	0.017 ± 0.001	0.58	0.001 ± 0.001	0.007 ± 0.004	1.30	0.04 ± 0.02	0.004 ± 0.004	1.03
Euborellia annulipes	0.13 ± 0.03	0.11 ± 0.07	0.25	0.00 ± 0.00	0.003 ± 0.003	1.00	0.04 ± 0.009	0.06 ± 0.02	0.33
Geocoris punctipes	0.06 ± 0.01	0.08 ± 0.02	0.54	0.02 ± 0.007	0.03 ± 0.009	0.87	0.03 ± 0.007	0.06 ± 0.01	20.21**[4-5]
Geocoris uliginosus	0.09 ± 0.04	0.10 ± 0.02	0.11	0.013 ± 0.007	0.012 ± 0.004	0.01	0.16 ± 0.05	0.24 ± 0.06	0.75
Labidura riparia	3.66 ± 1.35	3.49 ± 1.12	0.01	0.40 ± 0.10	0.22 ± 0.07	0.42	2.44 ± 0.33	1.55 ± 0.25	0.64
Megacephala carolina	4.59 ± 0.91	4.56 ± 0.88	0.01	6.75 ± 0.91	5.21 ± 1.08	1.18	11.87 ± 1.49	10.46 ± 1.44	0.63
Megacephala virginica	0.03 ± 0.009	0.02 ± 0.007	0.82	0.07 ± 0.02	0.11 ± 0.04	0.09	0.05 ± 0.01	0.07 ± 0.01	0.79
Nabis spp.	0.020 ± 0.008	0.017 ± 0.007	0.16	0.014 ± 0.05	0.016 ± 0.005	1.62	0.01 ± 0.002	0.02 ± 0.005	2.62
Spiders[c]	1.38 ± 0.26	1.20 ± 0.25	0.04	0.454 ± 0.07	0.458 ± 0.09	0.00	0.64 ± 0.08	0.39 ± 0.06	1.96
Staphylinidae[c]	0.15 ± 0.03	0.13 ± 0.02	1.05	0.18 ± 0.03	0.21 ± 0.03	1.88	0.29 ± 0.03	0.42 ± 0.05	1.90

[a]ANOVA results (*F*-test) from repeated-measures procedure of SAS. Superscript values after significance asterisks indicate the sampling dates out of 10 dates in 2002 season and out of 11 dates in 2003 and 2004 seasons that were significantly different between *Bt* and non-Bt cotton fields (*P<0.05, **P<0.01).
[b] Only predatory species.
[c] All collected taxa (11 species).

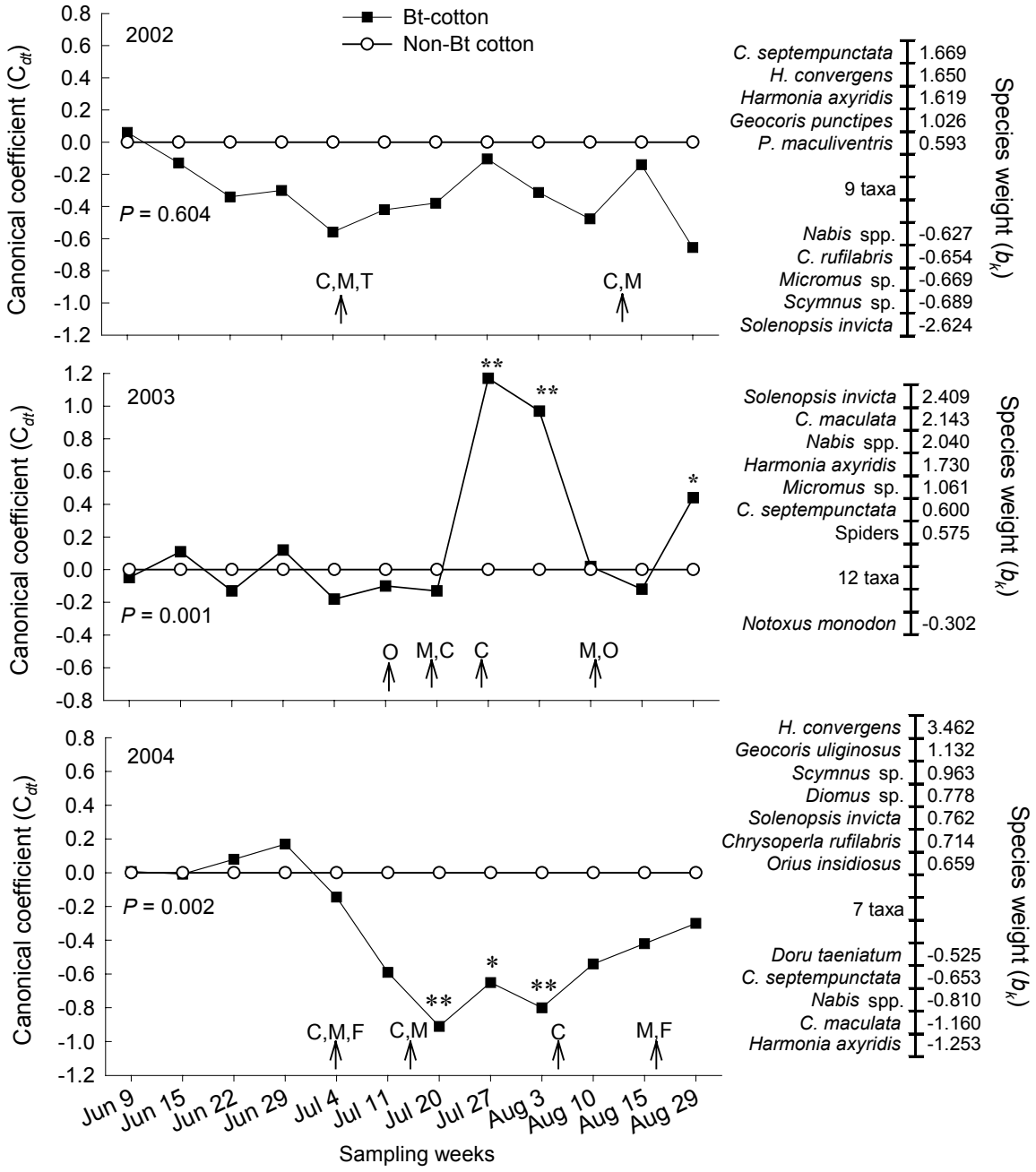

Fig. 3.1. Principal response curve (PRC) and species weight for predators collected in 40 drop cloth samples from 2002 to 2004, Tifton, GA. The PRC curves show the main effect of Bt-cotton on the predator community relative to non-Bt cotton (y = 0 line). The *P*-value indicates significance of the PRC diagram over all sample weeks based on *F*-type permutation test, and $^{**}P<0.01$ and $^{*}P<0.05$ indicate the significance for specified sampling date between cotton types. The arrows and letters denote insecticide applications on non-Bt fields only (C, Chula; M, Marchant; T, Ty Ty; O, Old House; F, Frazier, for more details see Table 3.2). The higher the weight, the more closely the taxon's response pattern follows the deviation pattern (from the non-Bt line) indicated on the PRC.

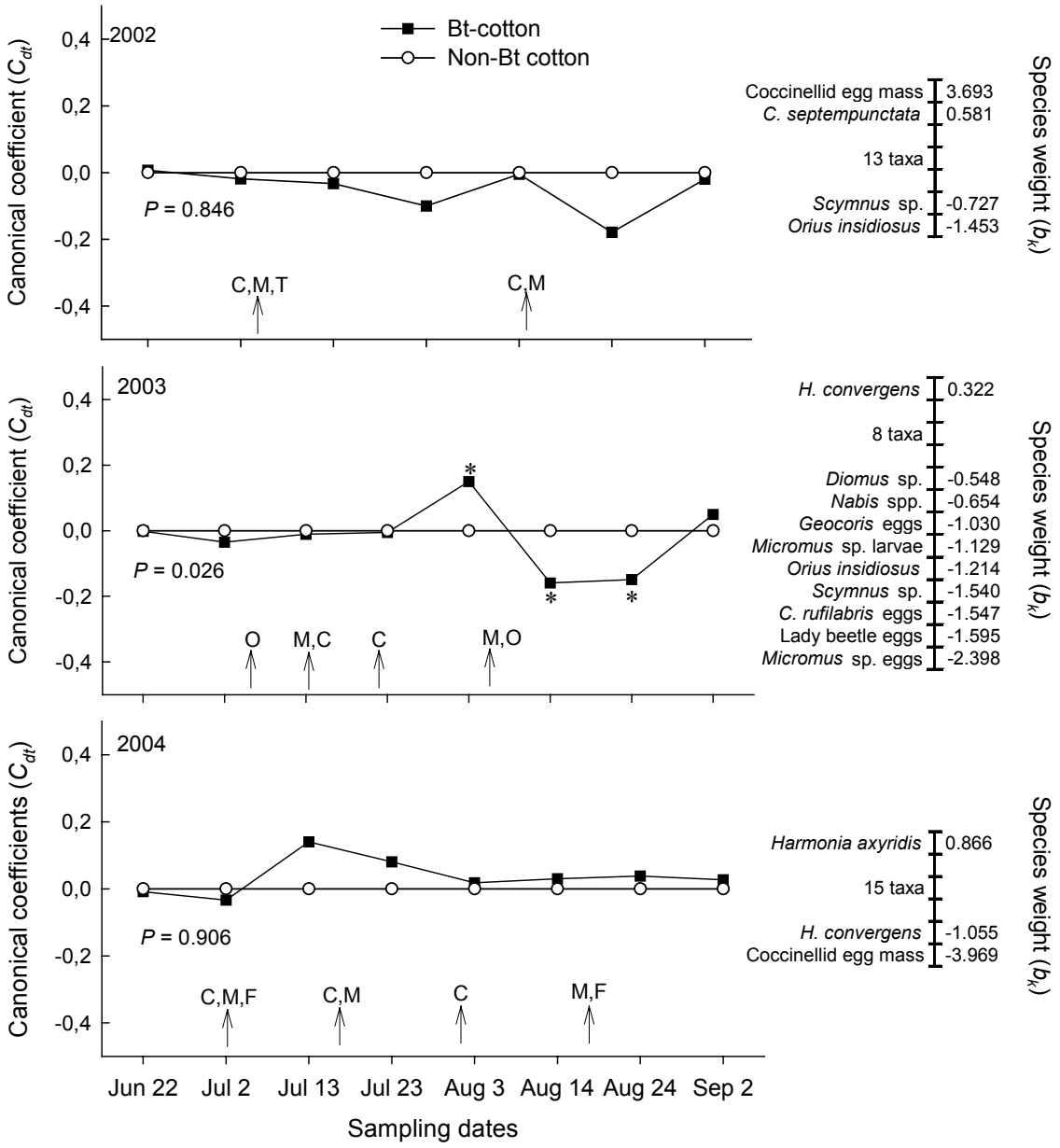

Fig. 3.2. Principal response curve (PRC) and species weight for predators collected in whole plant samples from 2002 to 2004, Tifton, GA. The PRC curves show the main effect of Bt-cotton on predator community relative to non-Bt cotton (y = 0 line). The *P*-value indicates significance of the PRC diagram over all sample dates based on *F*-type permutation test, and **P*<0.05 indicates the significance for specified sampling date between cotton types. The arrows and letters denote insecticide applications on non-Bt fields only (C, Chula; M, Marchant; T, Ty Ty; O, Old House; F, Frazier, for more details see Table 3.2). The higher the weight, the more closely the taxon's response pattern follows the deviation pattern (from the non-Bt line) indicated on the PRC.

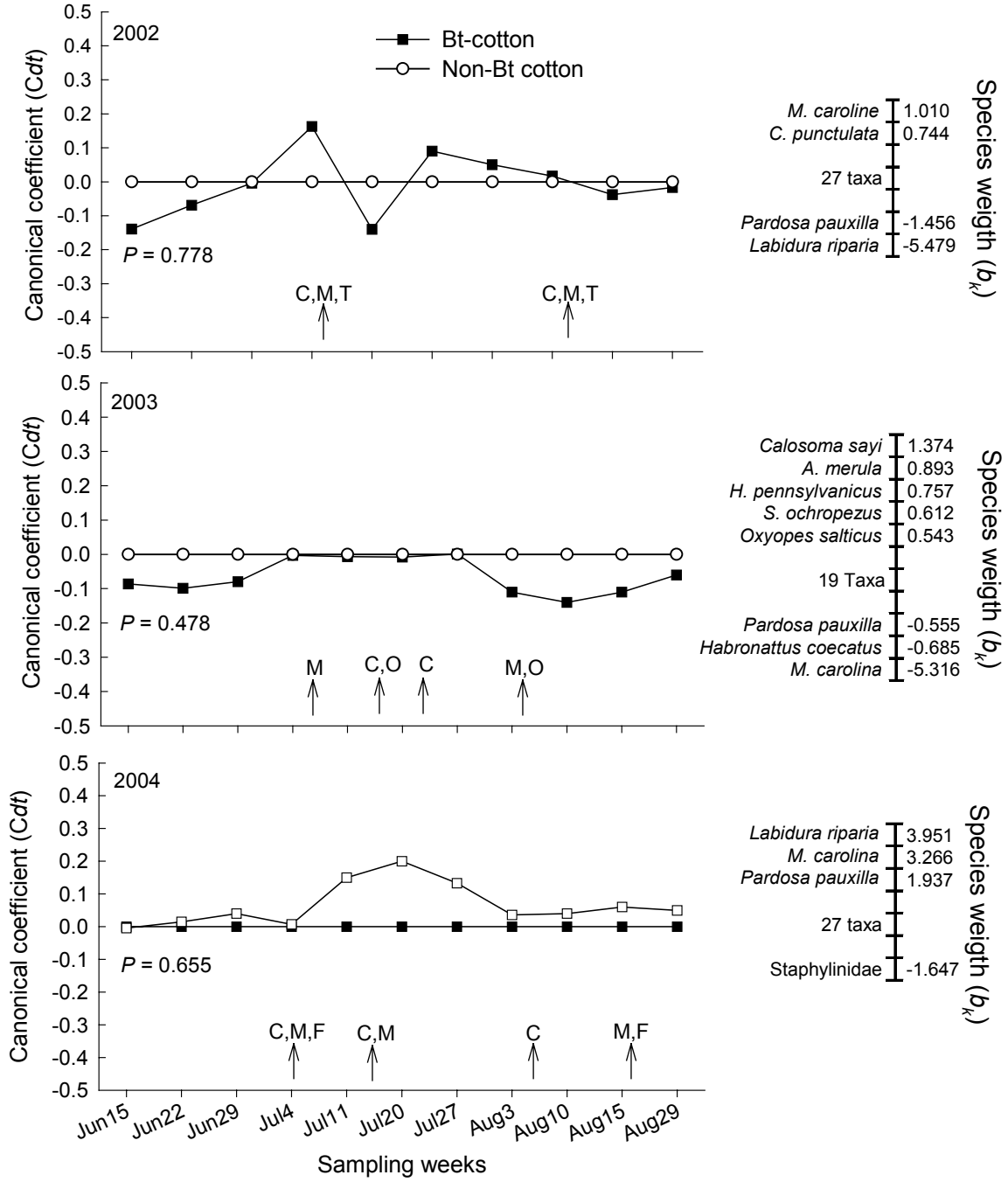

Fig. 3.3. Principal response curve (PRC) and species weight for predators collected in pitfall trap from 2002 to 2004, Tifton, GA. The PRC curves show the main effect of Bt-cotton on predator community relative to non-Bt cotton (y = 0 line). The *P*-value indicates significance of the PRC diagram over all sampling weeks based on *F*-type permutation test. The arrows and letters denote insecticide applications on non-Bt fields only (C, Chula; M, Marchant; T, Ty Ty; O, Old House; F, Frazier, for more details see Table 3.2). The higher the weight, the more closely the taxon's response pattern follows the deviation pattern (from the non-Bt line) indicated on the PRC.

CHAPTER 4

ABUNDANCE AND DIVERSITY OF GROUND DWELLING ARTHROPODS OF PEST MANAGEMENT IMPORTANCE IN BT AND NON-BT COTTON FIELDS[1]

[1]Torres, J.B. and J.R. Ruberson. To be submitted to *Agriculture, Ecosystems and Environment*.

Abstract

A survey of ground-dwelling arthropods was carried out weekly throughout each of three cotton seasons in commercial Bt and non-Bt cotton fields. Sixty-five taxa of ground-dwelling arthropods (carabids, cicindelines, staphylinids, dermapterans, heteropterans, and araneids) of importance for cotton pest management were collected. Species abundance and dynamics across cotton seasons were evaluated with univariate analysis of variance for higher taxa or multivariate principal response curve analysis for the whole community of 65 taxa. Diversity and richness indices, and accumulative species curves also were calculated. The analyses demonstrated no differences in the ground-dwelling arthropod communities between cotton types. One araneid species, *Pardosa pauxilla* Montgomery comprised ~80% of all araneids, *Labidura riparia* (Pallas) comprised ~96% of all dermapterans, *Megacephala carolina* L. comprised ~97% of cicindelines, and four carabid species, *Selenophorus palliatus* Fabr., *Apristus latens* LeConte., *Harpalus gravis* LeConte and *Anisodactylus merula* Germar, comprised ~80% of the total collected species of carabids. *M. carolina* outnumbered all collected species over three seasons. When only predatory species of carabids were considered, *A. merula*, *Calosoma sayi* Dejean, *Harpalus pennsylvanicus* DeGeer and *Stenolophus ochropezus* (Say) were predominant and similar between cottons. Numbers of dermapterans, staphylinids, araneids, and heteropterans varied among sample dates and across seasons, but did not differ between cottons. The high abundance and consistency of *M. carolina*, *S. palliatus,* and *P. pauxilla* in all fields and seasons on both cottons suggest that these species may be important for monitoring further changes in local communities due to agricultural practices.

Key words: Transgenic cotton, Cry1Ac, Carabidae, Cicindelinae, Staphylinidae, Labiduridae, Araneae, predatory heteropterans, *Falconia gracilis*.

INTRODUCTION

There is an increased interest in determining risks and benefits of agricultural practices for conservation of arthropod communities important for pest control and environmental health and sustainability. Impact of pest management practices and cropping systems on beneficial arthropods has been monitored using ground-dwelling arthropod communities (Eyre et al. 1989, Ellsbury et al. 1998, Carmona & Landis 1999, Rebek et al. 2002). Ground-dwelling arthropod communities are composed of a variety of species with different feeding behavior. Omnivorous and generalist species can be commonly found among ground-dwelling arthropods -- such as carabids, araneids, dermapterans, and cicindelines -- and their role in suppressing pests may be quite significant (Stinner & House 1990, Breene et al. 1993, Lövei & Sunderland 1996, Knisley & Schultz 1997). The conservation of certain groups of ground-dwelling arthropods inside crop fields has been tried through modified agricultural practices, with positive results in several cases (Stinner & House 1990, Nentwig et al. 1998).

The recent worldwide deployment of Bt-transgenic crops may impose potential risks on communities of ground-dwelling arthropods. Predator arthropods on the ground may have direct contact with activated Bt toxins released into the rhizosphere soil through plant root exudates and decaying plant material (Saxena et al. 1999, Zwahlen et al. 2003) or through species that are able to acquire and convey toxins to predators in the community (Saxena & Stotzky 2001, Wandeler et al. 2002). Despite disputed results on the amount of Bt toxins that accumulate and remain active in the soil during and after the crop season, and the adequacy of methods used in such studies (Sims & Holden 1996, Sims & Ream 1997, Head et al. 2002, Zwahlen et al. 2003, Hopkins & Gregorich 2003), the results indicate clear differences between laboratory and field experiments. Moreover, there are clear differences between purified toxins and toxins expressed

in the plants, plant and soil types, microorganisms present in the soil and their interactions with time after harvest, agricultural practices and environmental conditions. Bt toxin released into soil through root exudates is species specific, having been found for Bt transgenic corn, rice, and potato, but not for canola, tobacco, and cotton (Saxena et al. 2004). Head et al. (2002) found that Cry1Ac toxin in soil samples from different locations cultivated with Bt cotton over 3 to 6 seasons was at sufficiently low levels to express no biological activity after three months of cultivation. However, Gupta et al (2004) reported levels of Cry1Ac toxin in finer cotton roots comparable to that observed in leaves in the early season, and higher than leaves during late season with potential to be released into the soil through root fragments and contact with living organisms that feed on plant roots. For example, seven carabid species collected in field with Bt corn residues cultivated in previous years, contained Cry1Ab toxin in their bodies (Zwahlen & Andow, submitted). The degradation of the toxin synthesized by plants is expected to take place simultaneous to plant decomposition, which can take days to months depending on environmental conditions, and contact with ground-dwelling species during this period is likely.

Ground-dwelling arthropods are considered to be important not only for insect pest management, but also as predators of weed seeds, fungi, and other organisms competing with cultivated crops (Stinner & House 1990, Ball & Bousquet 2001). The use of Bt transgenic crops can benefit ground-dwelling arthropods by reducing use of broad-spectrum insecticides, or the Bt toxin can negatively affect them through contaminated prey and/or through plants and their products. For example, planting Bt potato increased abundance of ground-dwelling, generalist predator carabids and staphylinids by 65%, and of araneids by 56.8% in the plant canopy in Wisconsin and Oregon, respectively, compared to conventional potato fields treated with broad-spectrum insecticides (Hoy et al. 1998, Reed et al. 2001). The omnivorous feeding behavior of

ground-dwelling arthropods, however, can bring them into contact with prey conveying Bt toxin (Saxena & Stotzky 2001, Wandeler et al. 2002) or decaying plant material that contains the toxin. However, previous studies conducted in Bt corn (Cry1Ab or Cry3Bb1) fields found no effect of the Bt crop on abundance and diversity of carabids, mites, collembolans, and nematodes inhabiting soil on seasonal averages (Lozzia 1999, Saxena & Stotzky 2001, Al-Deeb et al. 2003, French et al. 2004). Despite the variability in methodologies used to collect samples, limited plot size, and short evaluation time in some of the studies, the results consistently indicate no significant changes suggesting of negative impacts of Bt corn on local epigeal communities.

Transgenic Bt cotton is completing one decade since field tests and the initiation of grower use. From 1996, the first season of Bt cotton in grower fields, it has been extensively planted across the US Cotton Belt with more than 58% of 5.85 million hectares of cotton cultivated with Bt-transgenic varieties in 2004 (NASS 2004). Planting transgenic cotton expressing Cry1Ac toxin from *Bacillus thuringiensis* (Berliner) to manage the bollworm complex (*Helicoverpa*, *Heliothis* and *Pectinophora*) has resulted in reduced insecticide use, with direct benefits to growers and the environment (Betz et al. 2000, Shelton et al. 2002). Transgenic plants, however, have sparked many questions concerning possible direct and indirect effects on nontarget organisms. No previous study has investigated the dynamics of ground-dwelling arthropods significant for pest management in the Bt-cotton ecosystem. The abundance and diversity of ground-dwelling arthropod communities possible in cotton fields offer a challenge to select an ecologically representative species or group. This limitation can be overcome by focusing on restricted taxa of interest depending on which question the study addresses. Therefore, in this study we surveyed carabids, cicindelines, staphylinids, dermapterans, heteropterans, and araneids - all of which are predators of interest for pest management in cotton. We used commercial cotton fields

representative of the size and agronomic practices for the region as the ultimate system to realistically evaluate impacts of transgenic crops on nontarget organisms (Marvier 2002, O'Callaghan et al. 2005). The primary objective of this survey was to determine if Bt-cotton and non-Bt cotton fields, cultivated and managed according to standard grower agricultural practices, support similar abundance, dynamics, and diversity of important ground-dwelling arthropod predators.

MATERIAL AND METHODS

Site description and pest management

This study was conducted in grower cotton fields cultivated with standard agricultural practices and located near Tifton, GA. The region is comprised of a mixed mosaic of agricultural habitats and forest remnants. Three pairs of Bt (DPL 458 or DPL 555) and non-Bt cotton (DPL 491) fields from 5.5 to 15.0 hectares each were monitored each season. Each pair of fields was separated from the others by 3.2 to 27 km, between the coordinates 31^{o} 39'N, 83^{o} 54^{o}W and 31^{o} 51'N, 83^{o} 55' N. At each location, adjacent fields of Bt and non-Bt cotton were separated from one another by a water ditch or road, and roads were between other adjacent crops such as peanut, tobacco, and watermelon. Other sides of the fields were surrounded by forest remnants. All fields were planted during the first or second week of May each year and received preventative in-furrow treatment to manage thrips [aldicarb 560 g (AI)/ha] and foliar insecticide applications as needed during the growing season to control bollworms, stinkbugs, and whiteflies. Based on scouting data, non-Bt fields received insecticide applications to control bollworms (*Helicoverpa zea* and *Heliothis virescens*) and both Bt and non-Bt fields were treated to control stinkbugs and whitefly infestations (Table 4.1). Whitefly control, however, was required only at the end of the season in the first week of September 2002, which had no influence on our data because sampling

was terminated on 31 August 2002. The non-Bt cotton field at Chula was treated twice for bollworms within 15 days in July 2003 due to rainfall right after the first application. The second application was at an elevated rate to manage small to medium bollworm larvae. The frequency of rain during July 2003 caused variation in dates and frequency of sprays applied to each field. In 2004, after a one-year rotation with tobacco in Marchant, the selected fields returned to the same locations as were used in 2002, only changing crop arrangement inside the cultivated area. The Chula fields remained in the same location all three seasons, but experimental fields were rotated inside a cultivated area of approximately 40 hectares surrounded by forest remnants. The third pair of fields, Ty Ty, Old House, and Frazier was set in different locations each season.

Ground-dwelling arthropod sampling procedure

A convenient pitfall trap was made using 500-ml plastic cups (9cm diameter X 12cm depth) (Solo® P-16, Solo cup company, Urbana, IL). On each side of the cup we made two holes of 2-cm diameter approximately 5 cm from the bottom and covered with mesh in order to drain excess water from irrigation or rainfall. As retention liquid, we used water mixed with Tween20 at 0.2% to break surface tension, and as preservative we used 4-5 pellets per cup of Diamond Crystal® water softener salt, with softener care™ additive (Cargil Co., Minneapolis, MN). Each pitfall cup was installed inside a larger and deeper plastic base-cup (10cm diameter X 15cm depth) (PackerWare®, Lawrence, KS), that had no bottom to permit drainage and installed previously across the fields. The pitfall cups and their bases were built so that the pitfall cups fit snugly inside the base-cup, with the rim of the lining cup held in place slightly below the top edge of the base cup. The upper edge of the base cup was level with the soil surface. Twenty traps were set up from border to border of all fields in 10 equally-spaced, prefixed stations. The sampling stations were set up right after seedling emergence. At each station, two traps were installed

within cotton rows and with five rows between traps at the same station. The traps were collected weekly (ca. one-week-long of running time) by replacing the cups and using the same base throughout the season. Collected traps were returned to the laboratory, where the contents were washed, removed, and stored in labeled 20-ml scintillation vials containing 70% ethanol. The data presented will focus on species representative of arthropod communities relevant to pest management of cotton for the region (e.g., carabids, cicindelines, dermapterans, staphylinids, heteropterans, and araneids).

Species identification and statistical analysis

In the laboratory, specimens were sorted into morphospecies of interest for this study, and all adult insects were identified to order, family, and species as possible. Identification of dermapterans to species was based on Hoffman (1987). Lindroth (1961-1969), Ciegler (2000), the University of Georgia Arthropoda Collection, Athens (GA), and the Florida State Collection of Arthropods, Gainesville (FL) were used for Carabidae species identification, and species nomenclature followed Ciegler (2000). Functional group designation of carabid species (carnivore or phytophage) was based on predominant feeding behavior reported in the literature (Table 4.2). Cicindelinae were identified based on Knisley & Schultz (1997), araneids were identified to species based on Kaston (1978) and Breene et al. (1993), and Staphylinidae were only sorted to family level. The vials containing the collected material are deposited at the Biological Control Laboratory (UGA-CPES), in Tifton, GA, and voucher specimens are deposited at the University of Georgia Collection of Arthropods (UGCA), Athens, GA.

Prior to analysis, data from individual pitfall traps were pooled within each week and for each field. These totals were standardized as the number of individuals per pitfall trap recovered out of 20 traps per week and per field, discarding traps lost to flooding or other random event, and

comprising three averages (ca., fields) for each sampling week. Seasonal averages were generated from each week averaged over the number of sample weeks (10 weeks in 2002 and 11 weeks in 2003 and 2004). Because the questions of interest were related to overall changes in the species community in Bt-cotton relative to non-Bt cotton fields, species were pooled to higher identified taxa and submitted to analysis of variance (ANOVA), which also avoided violation of ANOVA assumptions by considering species occurring only at very low densities. All data were log (x + 1) transformed prior to univariate analyses, but untransformed averages are presented. The results were submitted to one-way or two-factor repeated measures ANOVA, with repeated measures on sample weeks within seasons, and sample weeks and years (ca. seasons) for two factors, respectively; with fields as blocking factors since the arthropod sampling was conducted on the same fields over the season, and the same procedures were used each of the three years (2002-2004). These analyses were carried out using the Proc ANOVA of SAS (SAS Institute 1999-2001), adapting the PROFILE statement, as suggested by Cody & Smith (1997).

Because unequal numbers of pitfall traps were evaluated in each sample period, species accumulation curves were generated to establish the effect of sampling effort (10 weeks in 2002 and 11 weeks each in 2003 and 2004) and numbers of individuals collected (i.e., abundance) on species richness results, allowing comparisons of Bt and non-Bt cotton fields. The software program EstimateS (Colwell 2004) was used to calculate species accumulation curves for the whole community -- species richness through a Jackknife estimator, and diversity using the Shannon (H') and Simpson's indices for each field within each season (ca. 18 estimations, 9 Bt fields and 9 non-Bt fields) involving 100 randomizations of the samples (Colwell 2004).

Changes in species abundance of the ground-dwelling community were investigated using multivariate analysis through principal response curves (PRC), and considering each taxon

collected in Bt-cotton fields relative to non-Bt cotton, which was designated as the control. PRC analysis is a multivariate technique derived from redundancy analysis (RDA) that focuses on the proportion of the variance explained by variables of interest, in this case ground-dwelling species of economic interest for pest management collected in Bt-cotton on all sampling weeks throughout the cotton season. Parameters of the PRC were generated using CANOCO 4.5 for Windows (Lepš & Šmilauer 2003) through RDA least-squares estimates. By plotting values of c_{dt} for the treatment over sampling time, a PRC diagram is obtained that depicts species abundance changes in the community composition. We compared community abundance changes in Bt-cotton fields with the non-Bt cotton community as our standard ($c_{dt} = 0$). For each set of analyses, the null hypothesis that the PRC does not explain significant treatment variance was tested using an F-type test obtained by permutating whole time series in the partial RDA from which the PRC was obtained (Lepš & Šmilauer 2003). Random permutation through the Monte-Carlo method (999 permutations) was also performed for significant treatment PRC's using CANOCO 4.5 within each sampling date to test the null hypothesis that, on each sampling date, the principal response c_{dt} did not differ significantly between cotton types. Abundance values (predator species per pitfall trap) were log-transformed to reduce the effect of dominant species.

RESULTS

Ground-dwelling species abundance

A total of 38,980 ground-dwelling individuals comprising 65 taxa of interest in cotton pest management were collected across all seasons and fields during the study period. All of the specimens were identified to species except Staphylinidae, which were sorted only to family level. Species of geocorids, nabids, cicindelines, and dermapterans were collected in all fields, as were the most abundant species of carabids and araneids (Table 4.2). Among the most abundant

predatory ground-dwelling taxa none were unique to cotton genotype or year when greater than 5-10 individuals were collected in a single year, except one carabid, *Acupalpus testaceus* Dejean, that comprised 30 individuals collected only in 2004, but was found in both Bt and non-Bt cotton fields (Table 4.2). Seasonal averages for pitfall catches ranged from 9.1 to 16.3 ground-dwelling arthropods per trap across seasons and means always overlapped within 95% confidence intervals between Bt and non-Bt cotton fields (Table 4.2). Therefore, there is no evidence for difference in relative numbers captured per trap, and predominant species within groups were consistent between Bt and non-Bt cotton fields. With the exception of heteropterans and araneids, all other groups varied in abundance among seasons (year effect under two-way repeated measures ANOVA). A significant effect of years on abundance of dermapterans was observed ($F_{2, 8} = 22.63$, $p = 0.0002$), with relatively low densities of the predominant species, *L. riparia*, in 2003. Likewise, abundance of cicindelines varied across years ($F_{2, 8} = 14.13$, $p = 0.0024$), with abundance of *M. carolina* increasing late during 2004, similar to what was observed for staphylinids ($F_{2, 8} = 10.72$, $p = 0.0055$), which also were more abundant in 2004. All carabids, including predators and phytophagous species, tended to be more abundant in 2003 ($F_{2, 8} = 4.93$, $p = 0.0402$), especially some common species such as *S. palliatus* and *H. gravis*, while abundance of only predatory carabid species varied significantly among years ($F_{2, 8} = 22.26$, $p = 0.0005$). Because populations of each group varied differently in occurrence among years, the mean number of individuals captured per pitfall trap showed no significant effect of cotton type for any surveyed group within a season or over the 3-year period (one- and two-way repeated measures ANOVA, $p > 0.05$).

PRC analyses, in consonance with univariate analyses with higher taxa (Table 4.2), showed no statistically significant impact of Bt-cotton compared to non-Bt cotton (the standard reference)

on abundance of 65 taxa of ground-dwelling arthropods for data of all three years pooled (F = 2.86, p-value = 0.922; Fig. 4.1). Sample week (i.e., time) was the major contributor to variance in species abundance, with 73.8% of this variance explained by the first PRC axis. Although the second PRC axis explained an additional 11.2% of variance, the second PRC axis was not significant ($p > 0.05$). The interaction of sample week and cotton type explains 54.7% of the variance, whereas variance due to Bt cotton alone accounted for only 4.2%. This result reinforces the lack of effect of Bt cotton on abundance and dynamics of ground-dwelling arthropods in Bt relative to non-Bt cotton fields (Fig. 4.1). The contribution of each species to the community changes (response, c_{dt}) depicted by PRC diagrams can be also interpreted using the statistical weights (b_k) of each species, shown on the right side of the diagram (Fig. 4.1). Species with high weight values are most likely to exhibit population patterns that correspond to changes in abundance shown in the diagram, while low values contribute little to the overall community response indicating a weak association or a response pattern different than that displayed in the diagram (Van den Brink & Ter Braak 1999). Thus, of 65 taxa only those that make relatively important statistical contributions are shown on the right side of the diagram.

Seasonal patterns

Seasonal abundance of all carabids gradually declined throughout the growing season (repeated measures ANOVA, $F_{10, 40} = 19.08$, $p < 0.0001$), but equally between Bt and non-Bt cotton fields ($F_{1, 4} = 0.32$, $p = 0.6027$), and there were no cotton and year interactions ($F_{2, 8} = 0.22$, $p = 0.8049$) (Fig. 4.2). When only predatory carabids were considered, no changes in abundance were observed in any year over time (sampling week effect, $F_{10, 40} = 1.26$, $p = 0.2835$), nor were differences observed between cotton types ($F_{1, 4} = 0.42$, $p = 0.5529$). Likewise, cotton and year interaction was not significant ($F_{2, 8} = 0.00$, $p = 0.9983$). Cicindelines

became more abundant as June collections progressed and their abundance declined significantly later (sampling week effect, $F_{10, 40} = 40.98$, $p < 0.0001$), but equally between Bt and non-Bt cottons ($F_{1, 4} = 0.40$, $p = 0.5596$), and with no interactions between cotton and year ($F_{2, 8} = 0.273$, $p = 0.5107$). The other four taxa (dermapterans, araneids, staphylinids, and heteropterans) were similarly abundant in both cotton types ($p > 0.05$), with high variability among sample weeks throughout season. No pattern of abundance emerged, except in the case of heteropterans ($F_{10, 40} = 3.0$, $p = 0.0064$), which declined toward the end of the season (Fig. 4.2).

Experimental fields and species abundance and diversity

Field sizes ranged from 5.5 to 15 hectares between cottons and across years. Sampling area can be a concern regarding abundance and species diversity of ground-dwelling arthropods. The outcomes, however, show no significant effect of field area on number of species collected in either Bt ($r = 0.12$, $p = 0.7396$) or non-Bt cotton fields ($r = -0.55$, $p = 0.1178$). Similarly, the number of species collected was not influenced by numbers of individuals collected in Bt ($r = -0.05$, $p = 0.8972$) and in non-Bt ($r = 0.33$, $p = 0.3819$) cotton fields, or as a function of the number of pitfall traps recovered from each field (Bt, $r = -0.17$, $p = 0.6471$; and non-Bt, $r = 0.16$, $p = 0.6673$). These results suggest no interaction between final sampling efforts (i.e., number of pitfall traps recovered and abundance and diversity of ground-dwelling predator arthropods) and number of species sampled in each year and cotton type (Fig. 4.3). These results are supported by the species accumulation curves generated for each cotton type and year (Fig. 4.3). There is a clear difference in total number of individuals collected among years (Table 4.2) and between Bt and non-Bt cotton fields in 2004 (Fig. 4.3). However, the increase in species accumulation was not a linear relationship with sampling weeks or abundance (i.e., individuals collected). Thus, increases in number of individuals collected until sampling week 10 tends to reach a plateau and

did not result in significant differences in species richness, which is clearly seen in re-scaling sampling efforts for sampling weeks (Gotelli & Colwell 2001). The means of the estimated total species richness for the respective cotton types overlap within the 95% confidence intervals within seasons and across all years (Table 4.2). Further, when species accumulation curves were plotted for each cotton type and season there is a trend toward a plateau in the last sampling weeks, and this trend was similar for both cotton types (Fig. 4.3).

Although 65 taxa of ground-dwelling arthropods were identified from family to species level, relatively few taxa comprised the majority of the trapped specimens (Table 4.2). Among araneids, *Pardosa pauxilla* Montgomery was the most abundant species and accounted for 68.5 to 85% of the 11 species collected across years (Table 4.2). Cicindelinae and Dermaptera were chiefly represented by one species each, *Megacephala carolina* L. and *Labidura riparia* (Pallas), respectively, comprising >94% of all collected individuals of cicindelines and dermapterans. For example, *M. carolina* comprised 48.3% of specimens of all taxa collected in 2002, and outnumbered all other taxa together (>50%) in 2003 and 2004. Carabidae was the most speciose taxon with 44 species collected, but only four species -- *Selenophorus palliatus* Fabr., *Apristus latens* Lec., *Harpalus gravis* LeConte and *Anisodactylus merula* Germar -- accounted for more than 80% of all carabids collected. Of the most abundant carabids, one is omnivorous (*A. merula*), and the other three are predominantly seed feeders (Larochelle & Larivière 2003). Among those species with predatory habit, four species were more abundant and the most abundant species again was the omnivorous *A. merula*. The others were *Calosoma sayi* Dejean, *Harpalus pennsylvanicus* DeGeer and *Stenolophus ochropezus* (Say) (Table 4.2). Among predatory heteropterans, there was relative constancy in numbers caught among the species,

except for *Nabis* spp. and *Geocoris uliginosus* (Say), which were predominant in 2003 (85%) and 2004 (78.4%), respectively.

Diversity, dominance, and species richness, as measured by the Shannon (H') and Simpson's indices, and total species richness are shown in Table 4.2 as means of fields (ca. = 3 fields) within years for each cotton type. The 95% confidence intervals of the estimated means comparing cotton types within year always overlapped. Both Shannon and Simpson indices tended to be lower in 2004 because of large collection of individuals representing few species, as indicated in the species accumulation curves (Fig. 4.3). Total species richness ranged from 36.9 to 39 species (Table 4.2), although the number of species per field within season ranged from 25 species collected at Frazier field in 2004 to 35 at Marchant field in 2003, both in non-Bt cotton fields. Three new state records for Georgia were found: one spider, *Falconia gracilis* (Keyserling), an accidently introduced species in the US and already reported in Florida and Texas (Bonaldo 2000), was collected all 3 years with abundance increasing from 2002 to 2004; and two carabid species, *Apristus latens* Lec. and *Euryderus grossus* Say, based on the catalogue of Bousquet & Larochelle (1993) and Arnett (2000).

DISCUSSION

The cotton field size (areas from 5.5 to 15 ha) and agricultural practices (pest management – Table 4.1) of the 18 fields studied from 2002 to 2004 reflect local farmer cropping standards. Assessment on commercial scales provides the most realistic field experiments for studying nontarget impacts of transgenic crops on natural enemies (Marvier 2002, O'Callaghan et al. 2005). A concurrent study of the dynamics of foliage-dwelling predatory arthropods on Bt and non-Bt cotton, using the same fields, is reported in CHAPTER 3. These pooled data reasonably cover most of the possible changes in ground- and foliage-dwelling arthropode communities of

importance for pest management in the local cotton ecosystem. From these results, no effects of deploying Bt cotton on predatory arthropods were found for either epigeal or foliage-dwelling predators. Changes in densities of a few species of foliage-dwelling insects between Bt and non-Bt cotton fields within seasons are reported in CHAPTER 3. However, the detected changes were found to be related to other agricultural practices, such as insecticide use in non-Bt cotton, rather than Bt-cotton, and did not persist under long-term analysis.

Although relative abundance averaged per trap was similar between cotton types and did not correlate with species richness (Table 4.2 and Fig. 4.3), the total number of individuals collected per season increased from 2002 to 2004 (Table 4.2). That the total number of individuals trapped was smaller in 2002 is reasonable because only 860 traps were recovered over 10 sampling weeks compared to seasons 2003 and 2004 with 11 sampling weeks each. And number of individuals captured did not correlate with number of traps recovered in 2002 (Fig. 4.4), since few traps were lost in 2002 because of limited rainfall (ca., 144.5 mm and 25 days of raining) compared to the following years. In 2003 and 2004, 1058 and 1153 traps were evaluated, respectively, and the number of individuals collected was positively correlated with number of traps evaluated (Fig. 4.4). Differences in total number of individuals between 2003 and 2004 can be explained by an additional 95 pitfall traps recovered in 2004 than in 2003, but also from the strong relationship between total individuals captured as a function of number of traps recovered in 2004 (Fig. 4.4) during a similar number of sampling weeks (11 weeks) and possible effects of rainfall. Over the sampling period in 2003, 470.4 mm of rainfall was accumulated over 43 days, while in 2004 only 245.0 mm of rainfall accumulated throughout 28 days of raining. Flooding and mud inside pitfall traps were the major causes of trap losses,

resulting in relatively low total individuals captured and a trend toward reduced means of individuals caught per trap in 2003 compared to 2004 (Table 4.2).

All dermapteran species were less abundant in 2003 compared to the other seasons. One cause beside rainfall could be rotation of the third pair of fields (Table 4.1). The rainfall was greater and more temporally diffuse in 2003 compared to 2002 and 2004. The Ty Ty field and Frazier fields were major sources of *L. riparia* in 2002 and 2004, respectively, whereas the Old House field used in 2003 had relatively low dermapteran abundance. In contrast with dermapterans, the seed-feeding carabids *S. palliatus* and *H. gravis,* predominant species of our communities, were more abundant in 2003 than 2002 and 2004. The extended rain frequency in 2003 delayed weed control on two out of three locations in 2003 and, considering that these species feed on grass seeds (Larochelle & Larivière 2003), it is possible that weed seed was more abundant in 2003 due to limited herbicide use. Increase in weed biomass is correlated with population increases for many seed-feeding species of carabids in the genera *Harpalus* and *Amara* (see data and review in Brooks et al. 2003). Despite being the most abundant carabid species in our fields the life histories of *S. palliatus* and *H. gravis* are essentially unknown, and they could be favored by the high soil moisture due to extended rainfall in 2003, and the greater abundance of weeds that same year.

Only a few carabid species accounted for a large portion of all species collected in all 3 years. Rarity at a site may be from mass migration from nearby areas such as *A. testaceus* occurring only in 2004, but species such as *Lebia viridis* Say were collected only in 2002, although it was often seen foraging in the plant canopy during aphid infestation in 2003 and 2004, and was collected in drop cloth sampling. Five or fewer individuals of 28 taxa (26 carabids and 2 araneids) were collected, and they accounted for approximately 43% of all species. Rarity

in ground-dwelling arthropods can be related to many factors, including sampling methodology, spatial scale, sampling effort, and landscape. It is uncertain whether low arthropod densities rely on continual immigration with fields serving as islands, or if arthropods are simply able to persist at low densities (Halsall & Wratten 1988, Morrill et al. 1990). Usually multiple sampling methods are required to address abundance of rare species, and the efficiency of pitfall traps to detect species occurring at low densities is questionable (Greenslade 1964) because pitfall trap catchability is related to species activity (Morrill et al. 1990). In our survey half of the trapped carabid species with relatively low densities (ca., 13 out of 26) are small (body length <5mm) and capture efficiency may be related to their locomotion and patch occurrence. Small species associated with field edges may have difficulty reaching trapping stations inside the cotton fields, whereas large carabid species are able to forage extensive areas and, therefore, could more readily reach trapping stations inside the fields. Another explanation is that carabids usually exhibit predominance of four to five species comprising more than 80% of the total individuals as found here and reported elsewhere (Elsburry et al. 1998, Carmona & Landis 1999, Lozzia 1999, French et al. 2004). The degree of dominance suggests a community in an early successional stage, and carabids apparently follow this trend, especially in annual crop agroecosystems.

The 3-year data show that Bt-cotton fields sustain abundance and species richness of ground-dwelling arthropods of agronomic interest for cotton pest management at levels comparable to non-Bt cotton fields (Table 4.2 and Fig. 4.1-4.3). The average differences for means of each species abundance rated per pitfall trap (e.g., non-Bt mean *minus* Bt-cotton mean) produced values of 0.0129, 0.0204 and –0.006 individuals per pitfall trap in 2002, 2003, and 2004, respectively. These values corresponded to an average of 1.29, 2.03 and –0.63% of

difference and it agrees with the results from univariate and multivariate analyses for no significant differences between cotton types.

The potential of carabids as natural control agents for crop pests and weeds is recognized (Lövei & Sunderland 1996, Ball & Bousquet 2001, Larochelle & Larvierei 2003) and efforts to conserve them through modification of agricultural practices have yielded positive results (Lövei & Sunderland 1996, Nentwig et al. 1998). All species representing the six higher taxa considered in this study (Table 4.2) are insect predators, except carabids that have many seed-feeding species (Larochelle & Larivière 2003). A conservative approach was taken to functionally sort carabids as predators to avoid mischaracterizing abundant species as potential natural enemies of insect pests in cotton fields. Although a large number of carabid species collected in this study are carnivorous, the most abundant species were those that feed on seeds, plant tissues, or pollen (Table 4.2). Predation on the weed seed bank is important, and no effect of Bt-cotton was observed for these herbivorous species.

Abundance of predatory heteropterans per pitfall trap decreased over the season (Fig. 4.2), and less evident variation was observed for araneids and dermapterans. Predatory heteropterans sampled are predominantly plant foragers, except *G. uliginosus* that is predominant on the ground (Crocker & Whitcomb 1980). The collection decline of predatory heteropterans is consistent in Bt-cotton fields, but abundance was highly variable in non-Bt cotton fields (Fig. 4.2). This may be a response to the increased plant foliage area with seasonal progression, increasing the area for foliar foraging on both cottons and reducing activity on the ground, thereby lessening chance of capture by pitfall. The greater variability of population dynamics in non-Bt cotton fields was in conjunction with insecticide use on plant foliage (ca., first week of July and August) (Table 4.1). Therefore, the variability in population dynamics of ground-

dwelling taxa, such as heteropterans, araneids, and dermapterans, that also forage in the plant canopy are likely due to the impact of foliar insecticide. Strict epigeal species, such as most carabids, cicindelines, and staphylinids, would not be as readily affected by foliar insecticides. These three last taxa had more consistent population dynamics even in non-Bt cotton fields more often treated with insecticides (Table 4.1). Araneid species found in this study are reported to be important biological control agents in cotton fields, foraging on the ground and on plant foliage (Breene et al. 1993). Among dermapterans, the omnivorous predator *L. riparia* was very abundant and has potential as a biological control agent, along with *P. pauxilla* and *P. malvina*. Among cicindelines, *M. carolina* outnumbered all other taxa and showed a clear pattern of abundance (Table 4.2, Fig. 4.2). According to Knisley & Schultz (1997), *M. carolina* is a voracious and gregarious predator, that can be quite abundant, as was the case in our fields.

The specific mechanisms underlying predator abundance are often difficult to identify because abundance is not always strongly correlated with a specific factor, such as prey or plant density or diversity. The ability to feed on different prey and on different trophic levels creates a complex system that can simultaneously suffer high or low magnitude of effects because of a suite of factors. Numbers of lepidopteran larvae per drop cloth and cotton aphid per leaf evaluated on the same days as pitfall trap collections within seasons and across years did not significantly correlate with numbers of predatory carabids, dermapterans, araneids, and heteropterans per pitfall trap. However, an exception was found for cicindeline abundance, which had a significant negative correlation with number of lepidopteran larvae (Bt, $r = -0.69$, $p < 0.0001$; and non-Bt, $r = -0.49$, $p = 0.003$) and positive correlation with aphids per leaf in both Bt ($r = +0.73$, $p < 0.0001$) and non-Bt cotton fields ($r = +0.44$, $p = 0.0207$). Cicindeline and aphid populations peaked between the last week of June and the first week of July and declined

subsequently, while cicindeline and lepidopteran populations generated the opposite pattern. Lepidopteran larvae, consisting of species partially susceptible to Bt-toxin in Bt-cotton fields, and susceptible and unsusceptible species in non-Bt cotton fields, became more abundant late in the season when cicindeline abundance decreased (Fig. 4.2). The poor relationship between ground-dwelling arthropods and these two major prey items in the cotton ecosystem is expected due to generalist feeding behavior of epigeal predators, and the diversity of other prey items found in cotton fields.

The adoption of transgenic cotton has been widely considered detrimental for insect predators and parasitoids by directly eliminating prey/host availability or rendering prey/hosts unsuitable (reviews in O'Callaghan et al. 2005). In this study, abundance and diversity of ground-dwelling arthropods, focused on predators commonly found in cotton fields, were not affected during three successive years by planting transgenic Bt-cotton. The alternate hypothesis elaborated for this study, that diversity and abundance of ground-dwelling predator arthropods could be reduced in Bt-cotton, was not supported. Indeed, the use of transgenic cotton can generate changes in abundance and diversity of arthropods as a result of reduced applications of broad-spectrum insecticides in the Bt-cotton ecosystem, making the system more salubrious for communities of ground-dwelling predators.

Collembolans are widely used to measure anthropogenic impacts on soil communities (Rebek et al. 2002). However, there have been recent developments in the use of a variety of invertebrate groups for biomonitoring, including cicindeline (Pearson & Cassola 1992, Rodriguez et al. 1998) and carabid beetles (Eyre et al. 1989). Considering the relative ease of collection, the body size of the abundant species, consistent trapping in all locations, and well-defined taxonomy of the cicindeline *M. carolina* (Knisley & Schultz (1997), the carabid *S. palliatus* (Ciegler 2000),

the dermapteran *L. riparia* (Hoffman 1987), and the araneid *P. pauxilla* (Breene et al. 1993), we should consider these species as important for further local monitoring for ecosystem effects, and for population comparisons representing four important ground-dwelling taxa in modified crop systems of Georgia's Coastal Plain.

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Table 4.1. Time and insecticide applied to manage pest infestations in Bt and non-Bt cotton fields near Tift County, GA, 2002-2004.

Dates (Fields)[1]	Non-Bt cotton	Bt-cotton	Targeted pest[3]
2002			
Spraying date	aldicarb 15G (560 g/ha)[2]	aldicarb 15G (560 g/ha)	Thrips
8-9 July (C,M,T)[c]	spinosad (100 g/ha)	-	Heliothines
10-12 August (C,M)	lambda-cyhalothrin (34 g/ha) + thiodicarb (680 g/ha)	-	Heliothines
14 August (T)	-	dicrotophos (390 g/ha)	Stinkbugs
5-7 September (C,M)	pyriproxifen (60 g/ha)	pyriproxifen (60 g/ha)	Whiteflies
2003			
Spraying date	aldicarb 15G (560 g/ha)	aldicarb 15G (560 g/ha)	Thrips
8 July (O)	spinosad (100 g/ha)	-	Heliothines
13 July (M)	spinosad (100 g/ha)	-	Heliothines
14 July (C)	lambda-cyhalothrin (30 g/ha)	-	Heliothines
21 July (C)	lambda-cyhalothrin (45 g/ha)	-	Heliothines
3-5 August (M,O)	lambda-cyhalothrin (45 g/ha)	-	Heliothines + stinkbugs
30 August (O)[3]	bifenthrin (70 g/ha)	bifenthrin (70 g/ha)	Stinkbugs
2004			
Spraying date	aldicarb 15G (560 g/ha)	aldicarb 15G (560 g/ha)	Thrips
2-7 July (C,M,F)	lambda-cyhalothrin (45 g/ha)	-	Heliothines
15 July (C,M)	spinosad (100 g/ha)	-	
29 July (C)	-	dicrotophos (420 g/ha)	Stinkbugs
5 August (C)	zeta-cypermethrin (160 g/ha)	-	Heliothines + stinkbugs
17 August (M,F)	zeta-cypermethrin (210 g/ha)	zeta-cypermethrin (210 g/ha)	Heliothines + stinkbugs
31 August (M)[3]	-	acephate (810 g/ha)	Stinkbugs

[1] C = Chula; M = Marchant; T = Ty Ty; O = Old House; and F = Frazier fields.
[2]Rate in grams of active ingredient per hectare.
[3]Insecticide application after terminating experimental sampling; Thrips (*Frankliniella occidentalis*, *Frankliniella fusca* and *Thrips tabaci*), Heliothine (*Helicoverpa zea* and *Heliothis virescens*), Stinkbugs (*Nezara viridula* and *Euschistus servus*). Thrips threshold (preventative treatment); Heliothine threshold = 8-10% of plants with eggs or small larvae on terminals; and stinkbug threshold = 18-20% bolls of ~2.5 cm diameter with internal damage; whitefly threshold = plants infested and honeydew on plants (Guillebeau, 2004).

Table 4.2. Totals and means per pitfall trap (Bt = 1569 traps; and non-Bt = 1501 traps), functional group, abundance and diversity indices of ground-dwelling arthropods collected in three pairs of Bt and non-Bt commercial cotton fields during the season 2002 (*n* = 10 sampling weeks) and 2003 and 2004 (*n* = 11 sampling weeks), Tift County, GA.

		2002			2003			2004		
Taxa	Group[a]	n	Bt	Non-Bt	n	Bt	Non-Bt	n	Bt	Non-Bt
Araneae										
Clubionidae										
Castianeira nr. *floridana*	C[1]	70	0.11	0.12	5	0.003	0.005	9	0.006	0.009
Corinidae										
Falconia gracilis (Keyserling)	C[12]	6	0	0.032	14	0.013	0.010	20	0.017	0.018
Lycosidae										
Hogna sp.	C[1]	15	0.017	0.016	7	0.006	0.004	13	0.012	0.008
Pardosa milvina (Hentz)	C[1]	26	0.027	0.033	35	0.030	0.030	110	0.110	0.068
Pardosa pauxilla Montgomery	C[1]	684	1.16	0.93	476	0.398	0.405	424	0.455	0.250
Schizocosa sp.	C[1]	23	0.026	0.042	8	0.007	0.007	15	0.008	0.016
Oxyopidae										
Oxyopes salticus Hentz	C[1]	14	0.017	0.022	8	0.002	0.016	0	-	-
Salticidae										
Habronattus coecatus (Hentz)	C[1]	19	0.021	0.032	7	0.011	0.003	26	0.025	0.016
Tetragnathidae										
Tetragnatha laboriosa Hentz	C[1]	0	-	-	0	-	-	1	0.002	0
Theredíidae										
Lactrodectus sp.	C[1]	8	0.006	0.012	0	-	-	0	-	-
Thomisidae										
Xysticus sp.	C[1]	3	0.005	0.002	0	-	-	1	0	0.002
Dermaptera										
Forficulidae										
Doru taeniatum (Dohorn)	C[2]	6	0.005	0.017	5	0.001	0.007	23	0.033	0.004
Carcinophoridae										
Euborellia annulipes (Lucas)	C[2]	97	0.13	0.11	2	0	0.006	56	0.036	0.065
Labiduridae										
Labidura riparia (Pallas)	C[2]	2,329	3.66	3.49	367	0.04	0.22	2,406	2.447	1.547
Coleoptera										
Staphylinidae		134	0.19	0.13	200	0.181	0.206	356	0.196	0.419

Table 4.2. Continued.

Cicindelidae										
Megacephala carolina Linnaeus	C^3	4,654	4.59	4.56	6,903	6.755	5.207	13,524	11.877	10.462
Cicindela punctulata Oliver	C^3	259	0.21	0.29	156	0.112	0.157	157	0.157	0.104
Megacephala virginica Linnaeus	C^3	21	0.20	0.018	99	0.071	0.108	73	0.054	0.071
Carabidae										
Acupalpus testaceus Dejean	C^5	0	-	-	0	-	-	30	0.024	0.023
Agonum aeruginosum Dejean	U	0	-	-	0	-	-	1	0.002	0
Amara crupeolata Putzeys	$P^{4,5}$-C^5	10	0.008	0.009	3	0.002	0.004	10	0.005	0.011
Amara impuncticolis (Say)	P^5-$C^{5,12}$	0	-	-	10	0.007	0.103	6	0.008	0.004
Amara sp.	P^5	26	0.012	0.018	19	0.017	0.015	10	0.005	0.015
Anisodactylus merula (Germar)	C^5-P^5	12	0.019	0.005	50	0.023	0.064	132	0.077	0.014
Apenes sinuatus (Say)	U	0	-	-	1	0.002	0	0	-	-
Apristus latens (LeConte)	U	230	0.16	0.32	88	0.092	0.076	129	0.161	0.048
Ardistomis schaumii LeConte	U	0	-	-	0	-	-	2	0.003	-
Aspidoglossa subangulata (Claudoir)	P^5	0	-	-	1	0.002	0	0	-	-
Bembidion semistriatum (Haldeman)	U	2	0	0.002	0	-	-	0	-	-
Calleida decora (Fabricius)	C^5	1	0.008	0	0	-	-	3	0	0.005
Calosoma sayi Dejean	C^5	16	0.018	0.003	41	0.012	0.063	26	0.033	0.009
Calosoma scrutator (Fabricius)	C^5	1	0	0.002	0	-	-	0	-	-
Chlaenius aestivus Say	C^8	8	0.008	0.014	5	0.004	0.007	2	0.003	0
Chlaenius sericeus sericeus (Foster)	C^5	0	-	-	0	-	-	1	0.002	0
Chlaenius tricolor Dejean	$C^{5, 8}$	0	-	-	5	0.003	0.005	0	-	-
Clivina americana Dejean	U	0	-	-	0	-	-	3	0.003	0.001
Clivina bipustulata (Fabricius)	U	2	0.010	0	13	0.015	0.009	0	-	-
Clivina sp.	?	1	0.002	0	0	-	-	0	-	-
Dicaelus elongatus Bonelli	C^5	0	-	-	0	-	-	1	0	0.002
Dyschirius filiformis LeConte	U	0	-	-	2	0.005	0	5	0.005	0.003
Dyschirius haemorrhoidalis (Dejean)	U	1	0.002	-	0	-	-	0	-	-
Euryderys grossus Say	$P^{5,8}$	1	0	0.012	0	-	-	0	-	-
Galerita bicolor Drury	C^5	0	-	-	0	-	-	2	0.002	0
Harpalus caliginosus (Fabricius)	C^5-$P^{5, 8}$	10	0.018	0.013	6	0.008	0.005	6	0.005	0.006
Harpalus gravis LeConte	U	9	0.015	0.013	520	0.621	0.358	20	0.022	0.011
Harpalus pennsylvanicus (De Geer)	C^5-P^5	25	0.031	0.025	10	0.003	0.019	6	0.009	0
Lebia analis Dejean	C^5	0	-	-	1	0.002	-	1	0.002	0
Lebia ornata Say	C^5	0	-	-	1	0	0.002	0	-	-
Lebia viridis Say	C^2	6	0.010	0.006	0	-		0	-	-
Leptotrachelus dorsalis (Fabricius)	C^5	0	-	-	0	-	-	3	0.002	0.003
Loxandrus velocipes Casey	U	2	0.005	0	0	-	-	0	-	-
Morion monilicornis (Latreille)	U	1	0	0.002	0	-	-	0	-	-
Nemotarsus elegans LeConte	U	0	-	-	1	0.002	-	0	-	-
Platynus decentis (Say)	C^5	0	-	-	1	0	0.002	0	-	-
Scarites quadriceps Claudoir	C^6	0	-	-	0	-	-	3	0	0.005

Table 4.2. Continued.

Scarites subterraneus Fabricius	C[5]-P[8]	2	0.005	0	0	-	-	0	-	-
Selenophorus palliatus (Fabricius)	P[7]	679	0.66	0.94	1669	1.99	1.15	323	0.28	0.26
Semiardistomis viridis (Say)	U	0	-	-	0	-	-	1	0.002	0
Stenolophus conjunctus (Say)	C[5]	0	-	-	1	0	0.002	0	-	-
Stenolophus ochropezus (Say)	C[9,12]-P[5]	5	0.010	0.10	13	0.008	0.020	10	0.012	0.006
Tetragonoderus fasciatus (Haldeman)	U	6	0.007	0.004	0	-	-	0	-	-
Tetragonoderus intersectus (Germar)	U	31	0.032	0.044	43	0.026	0.044	20	0.008	0.029
Heteropteran										
Geocoridae										
Geocoris punctipes (Say)	C[10]	65	0.064	0.082	24	0.016	0.030	55	0.032	0.052
Geocoris uliginosus (Say)	C[10]	94	0.092	0.105	16	0.012	0.012	243	0.164	0.245
Nabidae										
Nabis spp.	C[11]	17	0.024	0.017	233	0.151	0.272	12	0.004	0.017
Total/seasonal mean per trap		9,631	11.4 (7.8-15.1) [b]	11.5 (8.4-14.6)	11,069	11.7 (8.9-14.5)	9.1 (6.5-13.1)	18,280	16.3 (13.0-19.6)	14.0 (10.5-17.3)
Total species richness (Jacknife estimator)			37.5 (34.3-40.7)	39.0 (35.5-42.5)		37.9 (35.4-40.4)	37.5 (34.9-40.1)		36.9 (34.6-39.2)	38.4 (34.6-42.2)
Diversity (H') index			1.42 (0.92-1.91)	1.56 (1.37-1.74)		1.28 (0.40-2.15)	1.55 (0.83-2.26)		1.03 (0.39-1.67)	1.01 (0.33-1.68)
Simpson's index			2.47 (1.99-2.96)	2.80 (2.14-3.46)		2.41 (2.20-2.62)	2.78 (2.19-3.37)		1.88 (1.62-2.14)	2.05 (1.63-2.47)

[a]Functional group based on predominant feeding behavior as Carnivorous (C), Phytophagous (P), Unknown (U) and respective reference source: [1]Breene et al. (1993), [2]Hoffman (1987), [3]Knisley and Schultz (1997), [4]Johnson & Cameron (1969), [5]Larochelle & Larivière (2003), [6]Best & Beegle (1977), [7]Lindroth (1961-1969), [8]Ball & Bousquet (2001), [9]Jo & Smitley (2003), [10]Crocker & Whitcomb (1980), [11]Lattin (1989), [12]Frank & Shrewsburry (2004), [12]Bonaldo (2000).

[b]Values between parenthesis stand for 95% confidential intervals of means from untransformed data, and p-values comparing mean abundance per trap within group between cotton types are offered in the text.

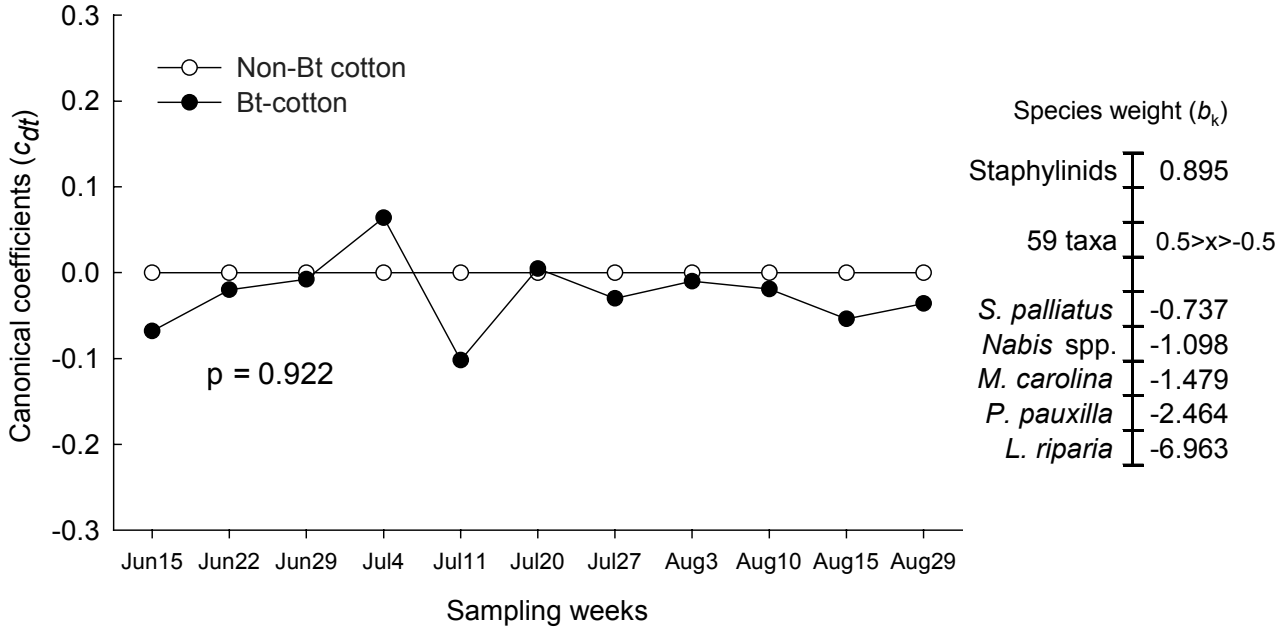

Fig. 4.1. Principal response curve (PRC) and species weight for species abundance collected in pitfall trap throughout cotton seasons 2002 to 2004, Tift County, GA. The PRC curve shows the main effect of Bt-cotton on predator community relative to non-Bt cotton (y = 0 line). The P-value indicates significance of the PRC diagram over all sampling dates based on *F*-type permutation test. The higher species weight, the more the actual response pattern of the species is likely to follow the pattern of the PRC. For a complete list of all species included in the analysis see Table 2.

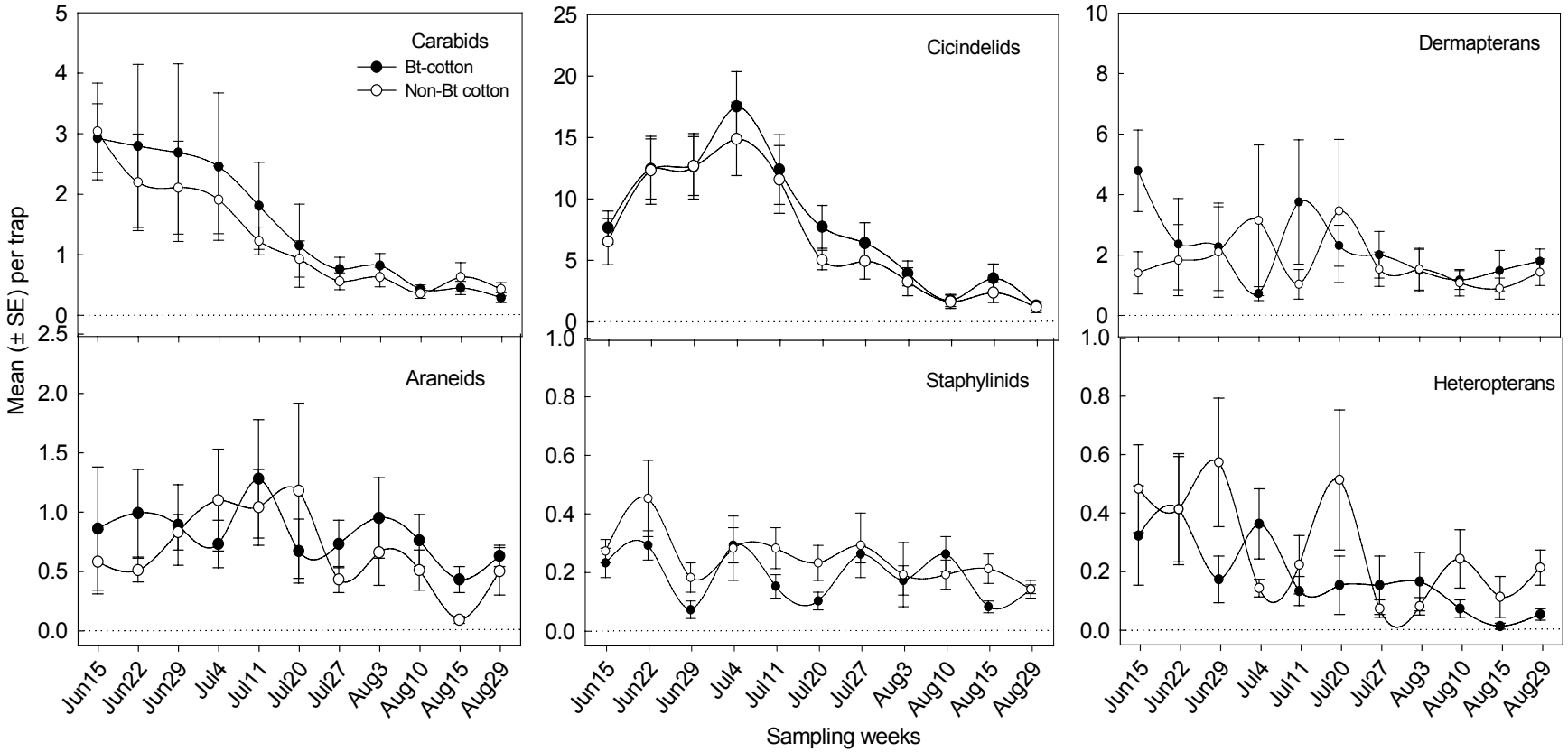

Fig. 4.2. Dynamics of ground-dwelling arthropods rated per pitfall trap collected on Bt and non-Bt cotton fields throughout cotton seasons 2002-2004, Tift County, GA. Note that y-axis scale differs among taxa as result of differences on abundance.

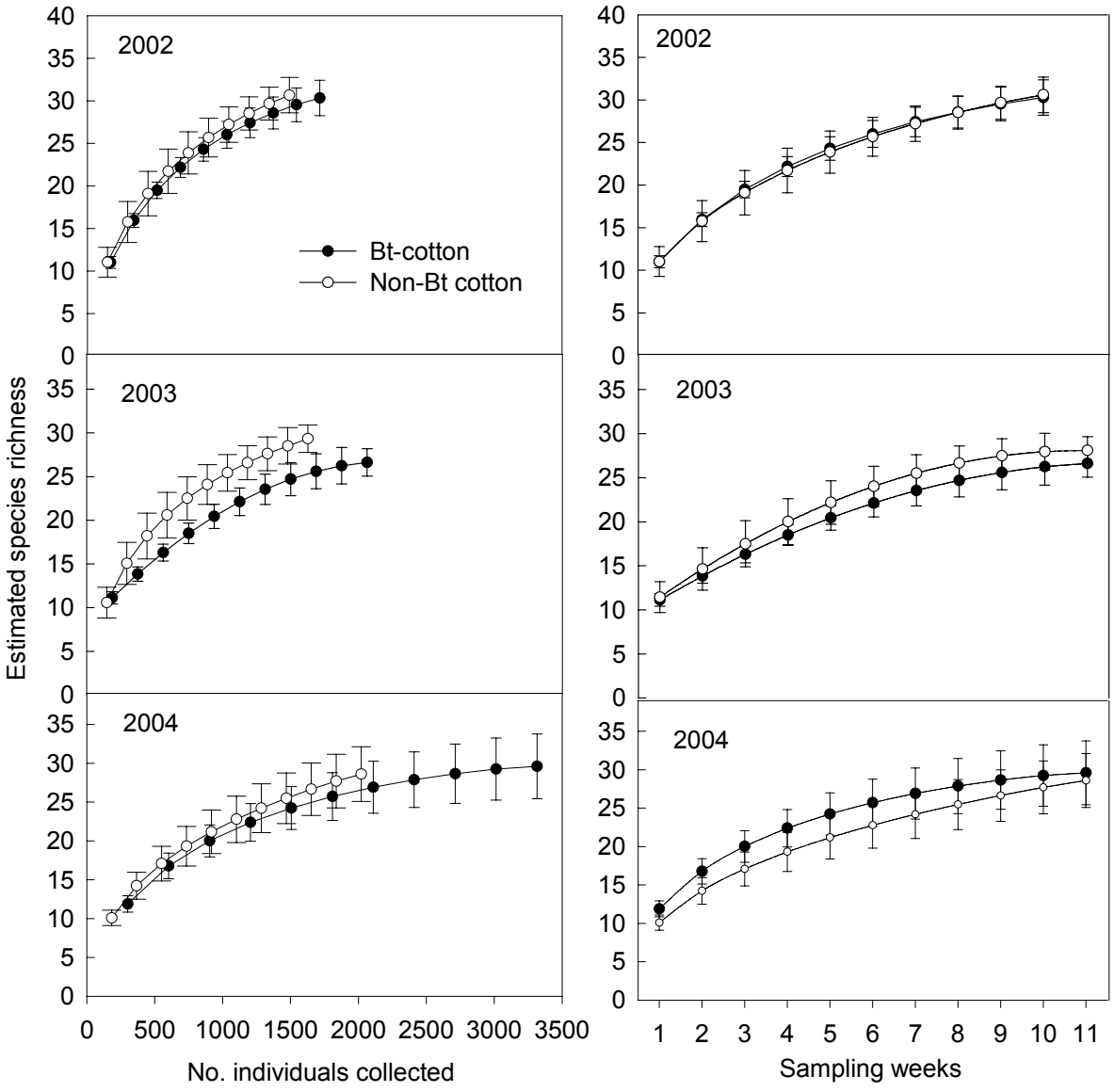

Fig. 4.3. Comparison of species accumulation curves of ground-dwelling arthropods in Bt and non-Bt cotton fields based on number of individuals collected and number of sampling weeks for each season from 2002 to 2004.

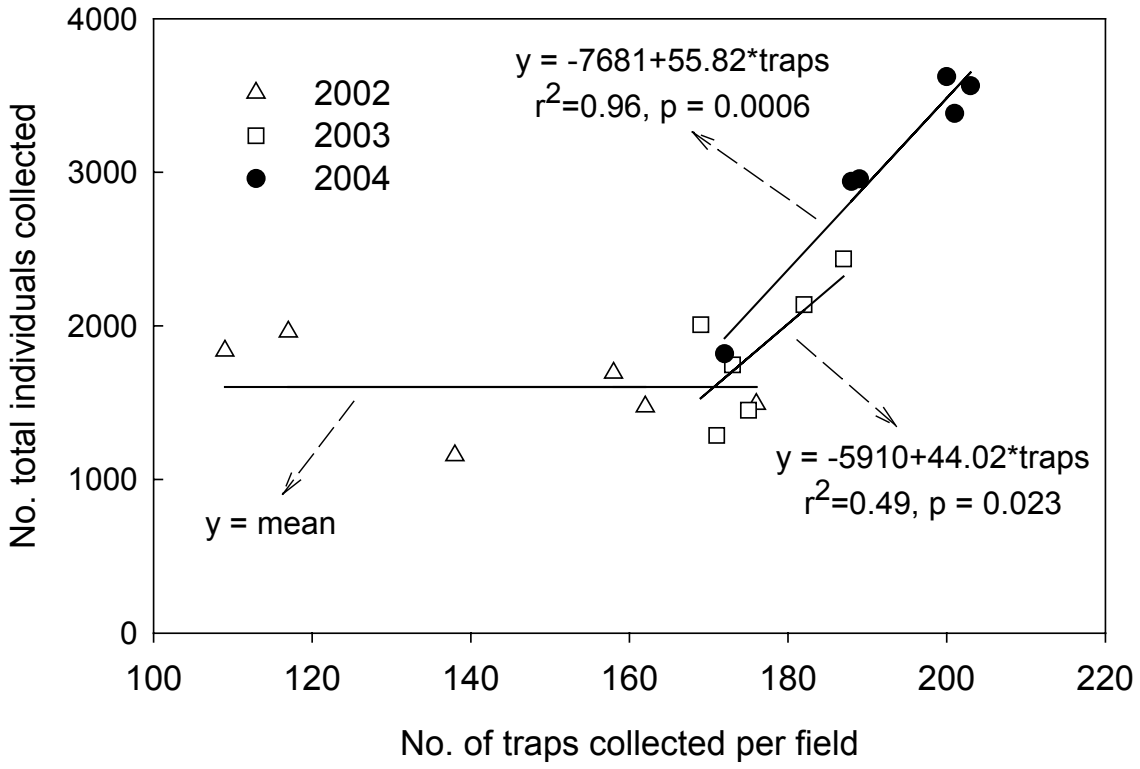

Fig. 4.4. Seasonal relationship between individuals collected and number of traps recovered pooled per field of Bt and non-Bt cotton during 2002-2004.

CHAPTER 5

SPATIAL AND TEMPORAL DYNAMICS OF BOLLWORM AND THREE PREDATORS' EGGS IN BT AND NON-BT COTTON FIELDS[1]

[1]Torres, J.B. and J.R. Ruberson. To be submitted to *Entomologia Experimentalis et Applicata*.

Abstract Host plants exhibiting insect resistance traits have long been known to influence within-plant distributions of pests and their natural enemies. Sites and timing of egg deposition are particularly important for synchrony of predators and their prey. Temporal and spatial distribution of eggs of cotton bollworms [*Heliothis virescens* (F.) and *Helicoverpa zea* (Boddie)], and of the predators *Geocoris punctipes* (Say) (Heteroptera: Geocoridae), *Chrysoperla rufilabris* (Burmeister) (Neuroptera: Chrysopidae), and *Micromus* spp. (Neuroptera: Hemerobiidae), were determined in three cotton seasons, from 2002 to 2004, by collecting and examining plants throughout each season. Comparisons also were made between Bt and non-Bt cotton to investigate possible changes in oviposition behavior on Bt cotton. Egg densities for predators and bollworms varied among years, but were similar on Bt and non-Bt cottons. Oviposition of bollworms and *G. punctipes* correlated spatially within plants, with most eggs laid on structures in the top five nodes of cotton plants and on the three outermost leaves on lateral branches regardless of cotton type. Bollworm oviposition dynamics exhibited two peaks within the season (early July and early August). Eggs of all species collected from the field and incubated in the laboratory had high hatching rates throughout each season. Oviposition dynamics of green lacewings correlated significantly with bollworm oviposition, but the correlation was delayed 10 days for big-eyed bug oviposition (despite similarities in egg distribution within plant structures). Brown lacewing oviposition did not correlate with that of bollworms. Further, Bt cotton plants exerted no significant effect on temporal or spatial patterns of oviposition of bollworms or selected predators, indicating no change in oviposition behavior of bollworm moths within plant structures after almost one decade of widespread planting of Bt cotton.

Key words: Host plant resistance, Cry1Ac protein, big-eyed bug, green lacewing, brown lacewing, *Trichogramma*, *Telenomus reynoldsi*, transgenic plants, nontarget effect.

INTRODUCTION

The budworm-bollworm complex, *Heliothis* and *Helicoverpa* (Lepidoptera: Noctuidae) (hereafter "bollworm"), is one of the most serious pest lepidopteran groups for several crops, including cotton (Fitt, 1989; Luttrell et al., 1994). Despite many control practices targeting this pest complex in cotton, the use of insecticides is often required to obtain a profitable yield, although efficacy of insecticides is hampered in some areas by insecticide resistance (Wolfenbarger et al., 1981, Plapp et al., 1990, McCaffrey 1998). Almost one decade after Bt-transgenic cotton (cotton plants genetically modified with a Cry toxin gene from the bacterium *Bacillus thuringiensis* Berliner; hereafter referred to as "Bt cotton") was first cultivated commercially, Bt cotton has become a major tool for managing bollworms in major cotton-producing countries (James, 2004). Although resistance to the Cry1Ac toxin of Bt has not been detected in field populations of *Heliothis* or *Helicoverpa*, populations with high levels of resistance to Bt toxins have been selected in the laboratory (Gould et al., 1995; Fengxia et al., 2003). Therefore, cropping practices to avoid or at least delay the spread of resistance in field populations have been recommended (Roush, 1997).

Because the expression of Cry1Ac toxin in Bt cotton varies among plant structures (Greenplate, 1999), changes in behavior of the larvae within the plant are a concern, and have been investigated as a potential mechanism contributing to control failures and selection for resistance. Neonate bollworm larvae are able to recognize Bt plants, and increasingly move from previous feeding sites; and older larvae are less susceptible to Cry1Ac toxin (Parker & Luttrell, 1999; Gore et al., 2001; 2002; Zhang et al., 2004). Studies on oviposition behavior of bollworm moths have demonstrated that ovipositing moths clearly prefer the upper third of cotton plants for egg placement (Fye, 1972; Wilson et al., 1980; Mabbett & Nachapong, 1984; Farrar & Bradley,

1985). This behavior results in contact of neonate larvae with terminal plant tissues that have higher Cry1Ac toxin expression. However, bollworm moths laying eggs on flower structures and other structures with low toxin expression within the plant canopy increase the likelihood of survival for their offspring (Gore et al., 2001; 2002). Oviposition in flower structures, for example, allows initial larval development to an older stage more tolerant of Cry1Ac, and can lead to control failures and increase resistance selection. Therefore, a behavioral change in bollworm egg placement may facilitate escape from exposure to the highest dosages of Cry1Ac toxin expression.

The tops of cotton plants, the preferred oviposition site of bollworm moths, is also the preferred foraging location of immatures and adults of many species of their predators in the plant canopy, including the big-eyed bug, *Geocoris punctipes* (Say) (van den Bosch & Hagen, 1966; Wilson & Gutierrez, 1980; Nuessly & Sterling, 1994). This pattern, however, may change for both pest and predator if the environment of the plant canopy is changed with the expression of a new plant trait for pest resistance. Morphological traits are the most often responsible for altering pest and predator behavior on plants exhibiting insect resistance (e.g., Butter & Singh, 1996), but changes in plant physiology also can affect prey and predator behavior (Cortesero et al., 2000).

Spatial patterns of predator oviposition are critical for ensuring interaction between immature predators and their prey (Sadeghi & Gilbert, 2000), and can be very important for escaping competition among predator species (Schellhorn & Andow, 1999). Predators in cotton fields tend to have weak temporal associations with pest populations (Ellington et al., 1997), suggesting that other important factors besides pestiferous prey underlie predator dynamics in cotton fields. Among these factors, alternative prey, host plant quality and plant phenology may be quite significant. In addition, for predators that supplement their diets with plant products,

plant species and site selection within the plant canopy for oviposition can be important by providing close contact of their offspring with plant tissues or structures that facilitate acquisition of the food supplement (sap, pollen, nectar, etc.). Evaluation of site-specific predator oviposition on plants in the field may provide a more accurate understanding of the temporal and spatial association of predators with the host plant and prey.

Cotton supports a large suite of predator species. *Geocoris punctipes* is one of the most abundant Geocoridae ("big-eyed bugs") of the US Cotton Belt, and the species also is common in Central and South America (Sweet, 2000). Besides big-eyed bugs and other predators, lacewings also are important predators in cotton fields (López et al. 1996). From natural populations or from inundative releases (Nuessly & Sterling, 1994; Lingren et al., 1968; López et al. 1996), predators can significantly reduce bollworm populations, and populations of other cotton pests not targeted by Bt toxin, reducing frequency of insecticide sprays (Wuo & Guo, 2003; Hagerty et al., 2005). Given the continuing need for effective biological control in Bt cotton, it is important to determine whether Bt cotton affects behaviors of the natural enemies relative to pest prey such that secondary pest problems could result. In this study, we investigated the site- and time-specific oviposition dynamics of bollworms, and of predators (big-eyed bugs, and green and brown lacewings) common in cotton fields in relation to Bt cotton plants.

MATERIALS AND METHODS

Study area and pest management

Three paired Bt and non-Bt cotton fields, located in different farms across years (farm names: Marchant, Old House, Frazier, Ty Ty, and Chula), were surveyed from 2002 to 2004. All farms were located in Tift County, GA, between coordinates 31^{o} 45'N, 83^{o} 63'W - 31^{o} 51'N, 83^{o} 55'W, and were 3 to 17 km from one other. All fields were planted in the first and second week of

May all three years. The fields ranged in size from 5.5 to 15 ha each. The fields at the Old House and Chula locations were completely surrounded by hardwood/conifers, and the Ty Ty, Frazier, and Marchant locations were partially surrounded by hardwood/conifers, and the remaining edges were surrounded by pasture and paved roads.

During the first cotton season, the fields were planted with Bt-cotton variety DPL 458 and non-Bt cotton DPL 491. In 2003 and 2004, all fields were cultivated with the Bt-cotton variety DPL 555RR and non-Bt cotton variety DPL 493. The Bt-cotton variety DPL 458 was the second most planted Bt-cotton variety in 2002 and, DPL 555 was the most widely-planted variety in Georgia in 2003 and 2004 (http://www.ams.usda.gov/cotton/mncs/index.htm).

Crop management followed standard agronomic practices, including insecticide applications based on scouting data. Insecticide applications were based on established economic thresholds for pest infestations in both Bt and non-Bt fields for Georgia (GA Pest Management Handbook, 2004). All fields received preventative in-furrow treatments at planting to control thrips all three years [Temik® 15G at 560 g (AI)/ha]. Based on scouting data, non-Bt fields received insecticide applications to control bollworm larvae, and both field types (Bt and non-Bt) were sprayed to control stink bugs and whitefly infestations. In 2002, non-Bt fields received one application of spinosad [Tracer® at 100 g (AI)/ha] to control an infestation of bollworm eggs and larvae on 8 July (Ty Ty) and on 9 of July (Chula and Marchant). A second insecticide application with lambda-cyhalothrin [Karate® at 34 g (AI)/ha] + thidiocarb [Larvin® at 680 g (AI)/ha] was made on 10 and 12 August (Chula and Marchant) to control a second peak of bollworm larvae and an initial infestation of stink bugs. The Bt-cotton field located in Ty Ty received one application of dicrotophos [Bidrin® at 390 g (AI)/ha] on 14 August to control stink bugs. Whitefly control [pyriproxifen at 60 g (AI)/ha] was required only at the end of 2002 (ca. after opening bolls - first

week of September), which had no influence on our data because sampling was terminated the last week of August in 2002. During 2003, foliar applications of spinosad [Tracer® 100 g (AI)/ha] were applied on 8 and 13 July in Old House and Marchant fields, respectively, to control bollworm larvae. The non-Bt cotton field at the Chula farm was treated with lambda-cyhalothrin [Karate® at 30 g (AI)/ha] on 14 July. Due to rainfall right after treatment of the non-Bt cotton field at the Chula farm on 14 July, a second application was necessary on 21 July with lambda-cyhalothrin [Karate® at 45 g (AI)/ha] to control the same infestation. The second insecticide applications to non-Bt cotton fields at Marchant and Old House were made with lambda-cyhalothrin [Karate® at 40 g (AI)/ha] on 3 and 5 August, respectively.

During 2004, the non-Bt cotton fields at Frazier, Chula, and Marchant were treated with lambda-cyhalothrin [Karate® at 45 g (AI)/ha] on 2, 5 and 7 July, respectively. A second insecticide treatment to control bollworms was required and made with spinosad [Tracer® at 100 g (AI)/ha] on 15 July in Marchant and Chula fields. Both the Bt-cotton and non-Bt cotton fields at Chula were treated on 29 July and on 5 August with dicrotophos [Bidrin at 420 g (AI)/ha] and zeta-cypermethrin [Mustang Max® at 160 g (AI)/ha], respectively, to control stink bugs, and stink bugs plus bollworm larvae. The Frazier and Marchant Bt and non-Bt fields were treated with zeta-cypermethrin [Mustang Max® at 210 g (AI)/ha] to control stink bugs and bollworms on 17 August.

Bollworm and predator (big-eyed bug, green lacewing and brown lacewing) egg survey

To determine egg distribution and oviposition dynamics on plants over the cotton season, plants were harvested in plastic bags from each of the cotton fields at approximately 10-d intervals (hereafter "sampling dates") in each growing season. Transparent plastic bags, 110-cm wide by 125-cm long (Stone Container Corporation, Mansfield, OH), were used to bag the

plants. The bottoms of the bags were cut off and bags were tied around the base of the cotton plant and pressed on the ground around the plant 5-10 d prior to collection. On the day of collection, the plastic bags were pulled quickly over the plant, tied at the top and the cotton plant was cut off at ground level and transferred to the laboratory for examination. Plants were inspected within 24 h of collection using a 10x magnifying light. The specific locations of eggs of bollworms and big-eyed bugs were recorded in reference to nodes on the principal stem (node 0 to terminal) and leaves on the branches, plant structure, and location in the structure. Eggs of green lacewing and brown lacewing were scattered within plants (personal observations) and were not mapped. Following mapping of the eggs, each egg was removed carefully by cutting a small piece of the substratum or through stalk (green lacewings) and incubated in 1.5-ml centrifuge tubes in the laboratory to evaluate egg fate. A total of 414 and 424, 581 and 607, and 516 and 517 Bt and non-Bt cotton plants, respectively, were collected and evaluated in 2002, 2003, and 2004.

Statistical analysis

Prior to analyses, number of eggs collected was transformed into a standardized unit. The total of eggs were averaged per plant and per field (n =3), due to variability in the number of Bt and non-Bt plants evaluated for each of the three field pairs (minimum of 20 plants per field and sampling date). Further, the number of eggs per plant was tested for normality (Kolmogorov-D:Normal test) and homogeneity of variance (Bartlett's test), and square root (x + 0.5) or log transformations were used when necessary; however, untransformed means are presented in tables and figures. The results were submitted to repeated measures analysis of variance (ANOVA) with one (sampling dates) or two (sampling dates and years) repeated factors. Data also were submitted to 3-way ANOVA with two repeated factors (sampling dates and years), and egg location on

plants and type of cotton as pre-determined factors. Fields were considered as a blocking factor because plant collections were carried out in the same field across the seasons. Analysis was conducted using a mixed model for repeated measures procedure of SAS (SAS Institute 1999-2001). Correlations between bollworm and predator egg dynamics were assessed using concurrent dynamics or predator egg numbers 10, 20 and 30 days following bollworm oviposition (big-eyed bug, green and brown lacewings) to explore a possible time-delay in predator oviposition relative to timing of bollworm oviposition. In addition, orthogonal contrasts were performed to test the null hypothesis that on each sampling date within the season the densities of bollworms and predator eggs per plant did not differ significantly between Bt and non-Bt cottons, when cotton type was a statistically significant factor, by repeated measures ANOVA. In addition, the average number of bollworm and big-eyed bug eggs was compared among plant thirds (ca., upper, middle, and lower) on each cotton type using Tukey's test.

Bollworm and big-eyed bug egg counts per node and mapped from the uppermost node downward to cotyledon node, and from the outermost leaf of the vegetative/fruiting branches toward the interior of the plants, were averaged per field (n=3 each year) and transformed to percentage of eggs per structure from the total of eggs collected. Further, the percentage of eggs per node was regressed against node position vertically in the plant (uppermost node = 1 and downward) or against leaf position in the branch using Proc Reg of SAS (SAS Institute 1999-2001) to compare egg distribution within plant structures between Bt and non-Bt cottons. Comparisons of the slopes of regression lines between Bt and non-Bt cottons were made using Proc Mixed to test the equality of slopes (SAS Institute 1999-2001).

RESULTS

A total of 1,511 and 1,548 Bt and non-Bt cotton plants were collected from commercial fields and searched completely for bollworm and predator (*G. punctipes*, *C. rufilabris*, and *Micromus* sp.) eggs.

Bollworm oviposition

We found 642 bollworm eggs on field-collected plants. Repeated measures ANOVA showed that the number of bollworm eggs differed among years ($F_{1, 8} = 5.29$, $P = 0.0344$), but not between cotton types ($F_{1, 4} = 1.30$, $P = 0.3184$) for three years pooled data or for each year ($P>0.05$). Considering year-to-year variation, more bollworm eggs were recovered from Bt-cotton than non-Bt cotton plants in 2003 and 2004 (Table 5.1), specifically during the second peak of oviposition (Figure 5.1). Bollworm eggs per plant varied significantly over sampling dates ($F_{7, 28} = 10.68$, $P < 0.0001$), as did the distribution of eggs among plant thirds ($F_{2, 8} = 314.15$, $P < 0.0001$). Hence, only sampling dates and plant thirds interactions varied significantly ($F_{14, 8} = 6.33$, $P = 0.0066$).

Bollworm oviposition produced two oviposition peaks each season – in early July and early August (Figure 5.1). Because the second oviposition peak contributed nearly twice as many eggs as the first peak (Figure 5.1), the second oviposition peaks in 2003 and 2004 contributed to a significantly higher seasonal average of bollworm eggs in Bt-cotton compared to non-Bt cotton (see row comparisons in Table 5.1).

For both cotton types, and during both oviposition peaks, there were proportionally more eggs laid in the upper third of the plants than in the other sections (Figure 5.2). Oviposition in the middle and lower thirds remained relatively low throughout the season, independent of oviposition peak and availability of other plant structures as plants developed. This explains the

interaction between sampling dates and plant thirds. As the structures of the upper thirds of plants were the preferred parts, the top 5-10 nodes averaged approximately 80 to 95% of bollworm eggs laid on both cottons. As oviposition was concentrated in the top nodes, a quadratic model was the best fit to represent the decrease in percentage of eggs laid per node from top to bottom of plants, and the pattern was similar for both cottons (Proc Mixed of SAS for equality of slopes, $t_{1, 236}$ = 0.08, P = 0.9332). Likewise, a similar pattern was observed between cotton types (Proc mixed of SAS for equality of slopes, $t_{1, 80}$ = -0.25, P = 0.8028) for eggs laid on vegetative/fruiting branches where terminals (bud and outermost leaf on the branch) were the location of 77 and 80% of the eggs collected on Bt and non-Bt cottons, respectively (Figure 5.3).

Bollworm moths showed similar oviposition preference for plant structures in both cotton types (Table 5.2). Plant terminals (ca. bud-pinhead and upper expanded leaves) in the upper third of the plant were the preferred sites for oviposition, followed by fruit structures (flower structures) (Table 5.2). Over three seasons only three eggs were found on bracts of developed bolls, which were included with fruit structures, while bracts of squares (flower buds) were the preferred location in the fruit structures. Although the dry flower petals are also a fruit structure, they were treated separately and hosted more eggs than other boll components, but with significantly fewer eggs relative to the other upper plant structures (Table 5.2).

Bollworm eggs collected throughout the season and incubated in the laboratory showed relatively high viability, with more than 82% of eggs hatching. Eggs not hatching were either parasitized by *Trichogramma* sp. or failed to hatch for unknown reasons. Parasitism by *Trichogramma* sp. accounted for 13.3 and 12.5% of egg mortality, and non-viability reduced egg hatch 3.7 and 2.3% in Bt and non-Bt cotton, respectively.

Big-eyed bug oviposition

Examination of whole Bt and non-Bt cotton plants throughout each cotton season allowed us to map within-plant the distribution of 589 big-eyed bug eggs and to determine big-eyed bug oviposition dynamics. From these eggs, only three yielded nymphs of the big-eyed bug, *Geocoris uliginosus* (Say); therefore, all recovered eggs are treated as belonging to *G. punctipes*. *G. uliginosus* is a big-eyed bug typically found on the ground, but it also can be found on cotton plants, especially when abundant in the field, as was the case in whole-plant and drop-cloth samples during 2004 compared to 2002 and 2003 (CHAPTER 3).

The average number of eggs per plant was quite variable within and among years (Figure 5.1 and Table 5.1), but repeated-measures ANOVA did not detect any difference in average eggs per plant between cotton types in any year ($P = 0.2712$) or for data from all years pooled ($P = 0.3605$) (see statistics in Table 5.1). The egg densities per plant increased progressively on both cottons across sampling dates in 2002 ($F_{6, 24} = 5.09$, $P = 0.0017$) and in 2004 ($F_{7, 28} = 3.59$, $P = 0.0070$), and for all years pooled data ($F_{7, 28} = 9.38$, $P < 0.0001$). The number of eggs per plant on each sampling date in 2003, although tending to increase with seasonal progression, was quite variable with no significant difference between sampling dates for either cotton type ($F_{7, 28} = 1.79$, $P = 0.1291$). For seasonal averages, nearly 28% more big-eyed bug eggs were found per plant in 2004 in both cotton types compared to 2002 and 2003, generating a significant effect of years ($F_{2, 8} = 5.25$, $P = 0.035$) (Table 5.1).

Eggs of big-eyed bugs were found in much higher densities in the upper third of cotton plants ($F_{2, 4} = 293.84$, $P = 0.0001$), and varied according to the sampling dates ($F_{7, 4} = 29.08$, $P = 0.0028$) (Figure 5.2). At the beginning of the season, big-eyed bug eggs occurred at low densities, similar across plant thirds; but as the season progressed, so too did egg densities, with more eggs

found in the upper third of the plants (Figure 5.2). Vertical egg distribution within plants showed a linear decrease from the plant apex toward the lowest node on the plant stem (Figure 5.4), and exhibited a similar vertical distribution pattern between Bt and non-Bt cottons (Proc Mixed, comparing slopes on node height, $t_{1, 236} = 0.77$, $P = 0.4439$). The 5th and 10th nodes from the top accounted for more than 46% and 73% of eggs found in both cottons, respectively. However, the distribution of eggs on fruiting/vegetative branches was concentrated on the plant periphery, with the three outermost nodes/leaves on fruiting/vegetative branches accounting for nearly 90% of the eggs, and showing a quadratic decrease toward the plant's interior (Figure 5.4). Similar to the vertical distribution on nodes of the main stem, big-eyed bug egg distribution on fruiting/vegetative branches was similar between Bt and non-Bt cottons (Proc Mixed, comparing linear slopes on leaf/node of branches, $t_{1, 373} = -1.36$, $P = 0.1748$).

Within plant structures, big-eyed bugs preferred leaves as oviposition sites on both cottons (Table 5.3), and more than 93% of the eggs were found on the lower surface of leaves. Further, big-eyed bugs preferred laying their eggs along veins of the leaves. Plant terminals ('pinhead structures'), fruit structures (i.e., squares, flowers, bracts, bolls, and open lint), and stems were also sites for egg laying, but in very low frequencies (Table 5.3).

Eggs of big-eyed bugs collected and incubated in the laboratory exhibited relatively high percentage of egg hatching (>74%) from both cottons. Among the remaining eggs, 13.3 and 12.8% were parasitized by *Telenomus reynoldsi* Gordh in Bt and non-Bt cotton, respectively. Eggs not hatching or parasitized were considered nonviable (12.1 and 12.8% for Bt and non-Bt). Nonviable eggs were usually white or pale, with no sign of development (e.g., red eyespots), and eventually collapsed.

Green lacewing oviposition

Plant inspection resulted in 595 green lacewing eggs counted (Bt = 289 and non-Bt = 306). Densities of green lacewing eggs were not different between cotton types ($F_{1, 4}$= 0.01, P = 0.938), although some difference was found among years ($F_{2, 8}$= 3.87, P = 0.066). The partial year effect is due to significantly fewer eggs being found in non-Bt cotton fields in 2002 compared to 2003 and 2004, while no difference was found among years in Bt-cotton fields. Sampling dates also exerted a limited effect ($F_{7, 28}$= 2.26, P = 0.059), with green lacewing oviposition fluctuating throughout the season – and peaking in the first week of July and of August (Figure 5.1). However, among all remaining factors, only the interaction between years and sampling dates was significant ($F_{13, 58}$= 5.7, P = 0.0001). Despite fewer eggs occurring in 2002 compared to subsequent years in non-Bt cotton, no difference was observed between Bt and non-Bt cotton (Table 5.2), and similar dynamics were exhibited in both cottons (Fig. 5.1), generating the significant interaction between years and sampling dates.

Green lacewing oviposition peaks occurred simultaneously with bollworm egg peaks (Fig. 5.1) resulting in a positive and significant correlation (Table 5.2). Consideration of later predator oviposition (10, 20, and 30 d following bollworm oviposition) did not yield any enhancement of the relationship between predator and bollworm oviposition dynamics (Table 5.4).

In the laboratory, more than 93% of the collected chrysopid eggs hatched successfully, while 1% and 2.3% were found non-viable, and 5.8% and 1.8% were parasitized by *Telenomus* sp. in Bt and non-Bt cotton, respectively.

Brown lacewing oviposition

Plant inspection resulted in collection of 606 brown lacewing eggs (Bt = 312, and non-Bt = 294). Repeated measures ANOVA failed to detect significant variation between cotton types in

hemerobiid egg dynamics ($F_{1,4}$= 0.14, P = 0.727), but a significant difference was found among years ($F_{2, 8}$= 31.57, P = 0.0002), and across sampling dates throughout the season ($F_{7, 28}$= 6.61, P = 0.0016). Relatively few eggs per plant were found on both cotton types in 2002 relative to 2003 and 2004 (Table 5.1). Brown lacewing eggs per plant increased slowly throughout the season, with significantly higher densities at the end of the season, but similar numbers in both cottons (Figure 5.1). Among the interaction factors, only year and sampling dates produced significant effect ($F_{13, 58}$= 6.09, P = 0.0247) because egg densities were low in 2002 and did not fluctuate across season, while in 2003 and 2004, oviposition increased significantly toward the end of the season.

More than 91% of brown lacewing eggs collected from plants and incubated in the laboratory hatched in both cottons. The remaining collected eggs were parasitized at rates of 7.9% and 3.5% by *Trichogramma* sp. or were considered non-viable (0.90 and 1.1%) from Bt and non-Bt cotton fields, respectively.

DISCUSSION

Bollworm moths and predators (big-eyed bugs and lacewings) apparently did not discriminate between Bt and non-Bt cotton in the field, laying similar numbers of eggs on both cotton types. The large size of the fields minimized intra-field movement; thus, our results strongly suggest that these predators are developing populations equally in both cotton types, which agrees with surveys of immature or adult predators conducted simultaneously in these same fields (CHAPTER 3). Bollworm oviposition patterns observed in our study did not support the possibility of a behavioral change toward egg placement on lower plant structures to escape high Bt-toxin expression in the peripheral, younger plant parts. These findings agree with results reported by Parker & Luttrell (1998) in a study of oviposition behavior of *Heliothis virescens* (F.).

They used field cages with Bt-cotton mixed from 0 to 100% with non-Bt cotton in 1994, prior to commercial release of Bt cotton. Evaluating the top five nodes, the authors reported no difference in tobacco budworm egg distribution between cotton types or among the plant structures selected for oviposition by artificial moth infestations in caged plants. Parker and Luttrell (1998) also reported no difference in egg densities on plant terminals (only the upper three nodes) between Bt and non-Bt cotton fields during 1995, as we found from 2002 to 2004. However, those authors did not evaluate entire plants, and eggs laid on structures lower in the plant, where Cry1Ac toxin expression is reduced, were not considered. Although Parker & Luttrell's studies were conducted prior to large-scale use of Bt-transgenic cotton with no previous exposure of bollworms to Bt-cotton plants in the field, their results provide an excellent standard for further monitoring of bollworm oviposition behavior since moths with no prior experience with Bt cotton did not discriminate between Bt-transgenic and regular cotton. Therefore, any further change in bollworm moth oviposition to Bt-cotton relative to the findings of Parker & Luttrell (1998) would indicate that populations are beginning to behave differently in relation to Bt-cotton. Our results match those of Parker and Luttrell (1998), indicating that no such changes have occurred in the past decade of Bt-cotton use.

The periodic monitoring of pest behavior is not only important for bollworms in cotton but also for other important pests targeted by Bt transgenic crops. Bt-transgenic cotton exhibits physiological changes other than direct toxicity that can affect life history traits of larval bollworm. Although an oligophagous species (Fitt, 1989), *H. virescens* moths responded positively to extracts of their suitable host plants, and did not fly upwind in response to odors of a resistant tobacco cultivar or to extracts of non-host plants (Tingle et al., 1990), suggesting that specific volatiles play a role in host location and probably for oviposition. Also, after landing on a

host plant, *H. virescens* is able to discriminate among host plants through chemicals on the leaf surface using chemoreceptors in the tarsi (Ramaswamy et al. 1987). The insertion of Bt genes into cotton plants is known to have induced changes in important secondary compounds related to herbivore-cotton plant interactions (Jallow et al., 1999; Zhang et al., 1999; Ding et al., 2001; Yan et al., 2004). For instance, alpha-pinene and beta-pinene are among the major volatile compounds triggering antennal response in Old World bollworm, *Helicoverpa armigera* Hübner (Jallow et al., 1999; Yan et al., 2004). These compounds are 5.5 and 2.85 times higher in Bt-cotton than in non-Bt cotton (Yan et al., 2004) and could induce more oviposition on Bt-cotton than in regular cotton in the field. In addition, condensed tannins that play a role in cotton resistance to arthropod pests were significantly lower in Bt-cotton (Zhang et al., 1999).

Adult moth behavioral adaptation to differential Cry1Ac expression among plant structures expressed as site selection for oviposition might be a phenomenon that never occurs because there are many additional factors underlying oviposition behavior in the field (Thompson & Pellmyr, 1991; Renwick & Chew, 1994). The phenology of bollworm moths during the cotton season indicates that they complete generations on other crops or alternative native hosts before and during the cotton season (Fitt, 1989), thereby escaping continuous selection pressure from Bt toxins in cotton. Moreover, bollworm moths are active in short-range movement among crops, but can also migrate long distances, such as into the US from Mexico and among US states, to cope with food and environmental changes (Westbrook et al., 1990; 1998). Nevertheless, widespread planting of Bt-transgenic cotton may contribute to oviposition modifications, and any behavior change that could interfere with the efficacy of Bt crops to control bollworms would be a great threat. Therefore, further studies of bollworm oviposition in the years ahead and in different

cotton regions, and comparisons with the results reported here and those of Parker & Luttrell (1998), will be important to detect possible behavioral changes in this key cotton pest.

Regardless of cotton genotype, two major differences were observed in bollworm egg dynamics. First, higher egg densities were observed in both cottons in 2002 compared to the following two seasons (Table 5.1). Second, higher egg densities were noted in Bt-cotton fields than in non-Bt fields during the second peak of oviposition in 2003 and 2004. Higher egg counts in 2002 are probably a result of high bollworm moth abundance that year. The seasonal moth average [*Helicoverpa zea* (Boddie)] from four counties around our fields during the same survey period was 32.5% greater in 2002 (mean ± SE, 173.0 ± 24.2 moths per pheromone trap) compared to 2003 (116.8 ± 19.8) (Ruberson et al., 2003; Diffie et al., 2004). Second, the difference in bollworm egg densities between Bt and non-Bt cotton observed during the second oviposition peak in 2003 and 2004 (Figure 5.1) does not necessarily indicate that moths preferred Bt cotton to lay their eggs. Differences in egg numbers between cotton types might be related to insecticide use to control bollworms and stink bugs. All non-Bt cotton fields in 2003 and 2004 required second insecticide applications, which used the broad-spectrum insecticides lambda-cyhalothrin in 2003, and lambda-cyhalothrin and zeta-cypermethrin in 2004. No pyrethroids were used in Bt-cotton fields in 2003 or 2004. Therefore, considerable insecticide pressure was imposed on all life stages of bollworms in non-Bt cotton fields 2003 and 2004, besides the overall repellency of lambda-cyhalothrin to insects and mites. In addition, one Bt-cotton field (Chula field) was treated with organophosphate (dicrotophos) on 29 July 2004 to control stink bugs immediately before the second bollworm oviposition peak. Broad-spectrum organophosphate insecticides such as dicrotophos have been known to induce lepidopteran outbreaks in cotton because of the elimination of natural enemies in treated fields (Eveleens et al., 1973). Predation in cotton fields

can account for considerable reduction of bollworm eggs in the tops of cotton plants, and is similar between Bt and non-Bt cotton fields (Obrycki et al., 2004). The abundance of key bollworm egg predators [*G. punctipes*, *Orius insidiosus* (Say) and *C. rufilabris*] on the two sampling dates following dicrotophos application on Bt cotton (e.g., Chula field) averaged 0.13 predators per plant, compared to 0.41 and 0.43 predators per plant in untreated Bt-cotton and non-Bt cotton fields, respectively. At the same time, bollworm egg densities averaged 1.11 eggs per plant in the dicrotophos-treated Bt-cotton field (Chula field) compared to 0.11 and 0.19 eggs per plant in untreated Bt-cotton and non-Bt cotton fields, respectively. A similar trend was reported by Mellett et al. (2004), who found two times more eggs of the Old World bollworm, *H. armigera*, in cotton fields following two foliar sprays with endosulfan compared to Bt and non-Bt untreated fields. Therefore, the absence of pyrethroid pressure on bollworm populations after the first oviposition peak in Bt-cotton fields and the use of an organophosphate insecticide to control stink bugs, with the strongly detrimental impact of the insecticide on predators, might have increased egg counts by relieving predation pressure on eggs in Bt cotton during the second peak of oviposition.

Among the predators evaluated, only oviposition by green lacewings was correlated with bollworm oviposition (Table 5.4), while oviposition by big-eyed bugs exhibited a 10-d delay relative to bollworm oviposition. Brown lacewing oviposition did not correlate at all with bollworm oviposition. Bollworm moths showed two well-defined peaks of oviposition, whereas the big-eyed bugs and brown lacewings tended to progressively increase their oviposition throughout the season. Lack of correlation between oviposition of *G. punctipes* and brown lacewings with bollworm eggs would be expected. Big-eyed bugs are generalist feeders, use a variety of arthropod as prey in cotton fields, and increase oviposition steadily throughout the

season (Figure 5.1). Moreover, strong association of big-eyed bug eggs and bollworm eggs is of risk for the predator. The incubation period of big-eyed bugs is around three times longer than for bollworms, thus big-eyed bug nymphs would always be behind relative to the availability of bollworm prey (eggs and young larvae). Nevertheless, in general, both big-eyed bug and bollworm eggs overlapped considerably in their spatial distribution within cotton plants, bringing the predator in close contact with potential prey (Figures 5.3 and 5.4 and Tables 5.2 and 5.3). This pattern may be an explanation for greater mortality of bollworm eggs toward the tops of the plants (Nuessly & Sterling, 1994), which correlates with the preferred foraging location of *G. punctipes* and other small predatory heteropterans (e.g., *Orius*) within cotton plants (Wilson & Gutierrez et al., 1980).

Green and brown lacewings are more specialized on aphids, and their population dynamics tend to be more closely related to aphid abundance (Agnew et al. 1981, Szentkirályi 2001). Although green lacewings prey on eggs and larvae of bollworms, the positive correlation of green lacewing and bollworm eggs seems more accidental than biologically meaningful. In our fields, infestations of cotton aphids (*Aphis gossypii* Glover), a preferred prey of green and brown lacewings, peaked in late June and early July, providing abundant food for population growth and coinciding with the first bollworm and green lacewing egg peaks. The generation time of the green lacewings would place their next ovipositional peak about the time of the second bollworm egg peak in late July and early August.

All studied predators had already deposited eggs in the fields by the time of the first sampling date all three seasons, but at very low densities that were similar in both cottons (Figure 5.1). In this context, reduced insecticide use in Bt-cotton fields opens opportunities for conservation of these predator populations. For this reason, cropping systems favoring early

cotton field colonization and further reproduction by these predators will be important to foster presence of predators when oviposition by major pests such as bollworms occurs and young bollworm larvae first appear in cotton fields. The data indicate that factors other than bollworm eggs determine dynamics of the three studied predators in cotton fields. Previous studies found that *G. punctipes* lay eggs on various surfaces, but tend to prefer certain plants when given a choice, and preferentially oviposit on leaves rather than other plant structures (Naranjo, 1987), and this result was not related to prey availability (Naranjo & Stimac, 1987). Among factors influencing oviposition of predatory heteropterans, plant type and plant structure seem to be very important (Naranjo & Stimac, 1987; Coll, 1996; Pfannenstiel & Yeargan, 1998; Evangelista et al., 2003), especially for those species with strong plant feeding behavior. Plants preferred for oviposition by some predatory heteropterans have been found to correlate with nymphal (development and survival) and adult (longevity and fecundity) performance (Coll, 1996; Evangelista et al., 2003). Therefore, *G. punctipes* laying eggs on soft and young cotton plant tissues may be advantageous for the predator for two reasons: first, soft and young cotton plant tissues may facilitate acquisition of moisture and nutrients from plant feeding by young nymphs; and second, the spatial match with a highly suitable prey – bollworm eggs -- in cotton plant terminals (Figures 5.3 and 5.4).

It is apparent that Bt cotton plants exerted no significant effect on temporal or spatial patterns of oviposition of bollworms or selected predators, indicating no change in oviposition behavior of bollworm moths within plant structures after almost one decade of widespread planting of Bt cotton. Further, the lack of differences in oviposition by predators throughout the season and over three years between Bt and non-Bt cotton suggests that population dynamics of important predators species are not impaired by Bt cotton.

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Table 5.1 Seasonal means (± SE) of eggs of bollworms (*Heliothis* and *Helicoverpa*), big-eyed bug (*Geocoris punctipes*), green lacewing (*Chrysoperla rufilabris*) and brown lacewings (*Micromus* sp.) per plant of Bt and non-Bt cotton.

Cotton seasons	Bt-cotton	Non-Bt cotton	
Bollworms			Statistics[1]
2002	0.337 ± 0.045	0.335 ± 0.092 a	F = 0.00, P = 0.983
2003	0.155 ± 0.051	0.078 ± 0.028 b	F = 2.41, P = 0.043
2004	0.226 ± 0.081	0.130 ± 0.038 b	F = 5.31, P = 0.023
Statistics[2]	F = 2.05, P = 0.136	F = 6.44, P = 0.002	
Big-eyed bug			
2002	0.163 ± 0.030 b	0.191 ± 0.050 ab	F = 0.41, P = 0.551
2003	0.165 ± 0.039 b	0.142 ± 0.042 b	F = 0.39, P = 0.566
2004	0.231 ± 0.032 a	0.267 ± 0.050 a	F = 0.81, P = 0.417
Statistics[2]	F = 4.77, P = 0.008	F = 4.91, P = 0.007	
Green lacewing			
2002	0.14 ± 0.05	0.11 ± 0.04 b	F = 0.21, P = 0.669
2003	0.21 ± 0.04	0.20 ± 0.04 ab	F = 0.11, P = 0.661
2004	0.19 ± 0.03	0.26 ± 0.06 a	F = 0.20, P = 0.678
Statistics[2]	F = 1.06, P = 0.354	F = 2.59, P = 0.041	
Brown lacewing			
2002	0.06 ± 0.03 b	0.03 ± 0.01 b	F = 1.57, P = 0.578
2003	0.37 ± 0.06 a	0.24 ± 0.04 a	F = 3.42, P = 0.138
2004	0.15 ± 0.07 b	0.26 ± 0.10 a	F = 1.91, P = 0.238
Statistics[2]	F = 9.24, P = 0.003	F = 5.27, P = 0.007	

[1]ANOVA results (F-test) from repeated-measures procedure of SAS.
[2]ANOVA results (F-test) from GLM procedure of SAS; different letters within column indicate that means are significantly different by Tukey's HSD test at 0.05 significance levels.

Table 5.2 Bollworms' eggs placement (means ± SE per season from 2002 to 2004) on different cotton plant structures, Tift County, GA.

	Cottons[1]	
Plant structures	Bt	Non-Bt
Bud (pinhead square)	15.1 ± 3.43 a	11.6 ± 3.35 a
Fruit structures	8.2 ± 1.99 ab	7.3 ± 2.04 a
Uppermost expanded leaf	4.7 ± 1.29 b	3.7 ± 0.94 ab
Mainstem leaf	5.8 ± 1.27 b	3.2 ± 0.83 b
Leaf petiole	1.8 ± 0.78 c	1.9 ± 0.75 bc
Dried petal (boll tag)	1.1 ± 0.42 c	1.2 ± 0.36 bc
Mainstem	0.9 ± 0.26 c	0.8 ± 0.22 c

[1]Means within column followed by different letters differ significantly by Duncan MRT at 0.05 significance levels. Fruit structures include squares, flowers, bracts, and bolls, but exclude dried petals; stem include main and branching stems.

Table 5.3 Seasonal means of *G. punctipes* eggs per field of Bt and non-Bt cotton collected throughout the 2002-2004 cotton seasons, according to plant structures, Tift County, GA.

Cotton	Main plant components	Eggs	Leaf components	Eggs
Bt	Leaf	24.8 ± 4.82	Bellow	23.8 ± 4.63
			Along vein	10.2 ± 1.76
			Edge	1.2 ± 0.74
			Petiole	1.3 ± 0.75
			Petiole junction	3.0 ± 0.78
			Middle leaf	8.1 ± 2.87
			Upper	1.1 ± 0.39
	Bud (pinhead)	2.2 ± 0.83		
	Upper expanded leaf	8.2 ± 0.28		
	Fruit structures[1]	1.4 ± 0.50		
	Stem	0.5 ± 0.28		
Non-Bt	Leaf	21.2 ± 3.10	Bellow	20.0 ± 3.14
			Along vein	11.1 ± 1.48
			Edge	0.6 ± 0.18
			Petiole	0.8 ± 0.28
			Petiole junction	2.6 ± 0.47
			Middle	5.0 ± 1.90
			Upper	1.5 ± 1.04
	Bud (pinhead)	1.8 ± 0.83		
	Upper expanded leaf	2.2 ± 0.66		
	Flower and bolls[1]	2.2 ± 1.01		
	Stem	0.3 ± 0.25		

[1]Fruit structures include squares, flowers, bracts, bolls, and open lint; stem include main and branch stems.

Table 5.4 Correlation coefficients between egg densities of the predators big-eyed bug, green lacewing and brown lacewing with bollworms eggs from 2002 to 2004 showing the time delay in the predator egg dynamics relative to those of bollworm eggs.

	Bollworm eggs (Jun 20 – Sep 4)	
Big-eyed bug eggs	Bt-cotton	Non-Bt cotton
Jun 20 – Sep 4	-0.021 (P = 0.861)	0.068 (P = 0.577)
Jul 2-Sep 4	0.399 (P = 0.001)	0.659 (P < 0.0001)
Jul 13 – Sep 4	0.355 (P = 0.010)	0.664 (P < 0.0001)
Jul 23 – Sep 4	0.362 (P = 0.014)	0.669 (P < 0.0001)
Green lacewing eggs		
Jun 20 – Sep 4	0.25 (P = 0.040)	0.39 (P = 0.013)
Jul 2-Sep 4	0.16 (P = 0.234)	-0.13 (P = 0.321)
Jul 13 – Sep 4	0.08 (P = 0.567)	-0.12 (P = 0.409)
Jul 23 – Sep 4	-0.24 (P = 0.123)	0.10 (P = 0.513)
Brown lacewing eggs		
Jun 20 – Sep 4	0.17 (P = 0.161)	-0.16 (P = 0.197)
Jul 2-Sep 4	-0.08 (P = 0.506)	-0.12 (P = 0.312)
Jul 13 – Sep 4	-0.09 (P = 0.514)	-0.13 (P = 0.357)
Jul 23 – Sep 4	-0.11 (P = 0.472)	-0.19 (P = 0.207)

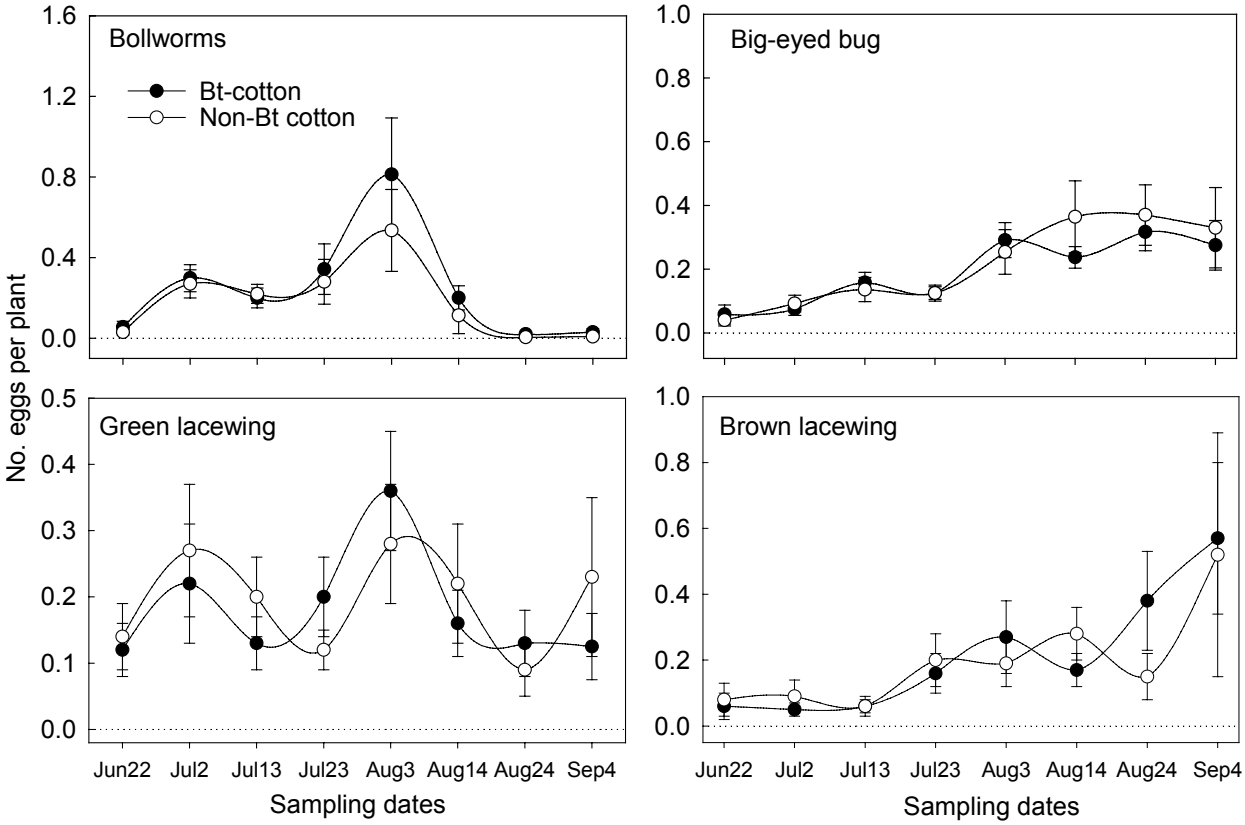

Figure 5.1 Oviposition dynamics within season (2002-2004; data pooled) of bollworms (*Heliothis* and *Helicoverpa* spp.) and the big-eyed bug, *Geocoris punctipes*, green lacewing, *Chrysoperla rufilabris*, and brown lacewing, *Micromus* sp., in Bt and non-Bt cotton fields, Tift County, GA. Note: scale of y-axis is in according to predator species.

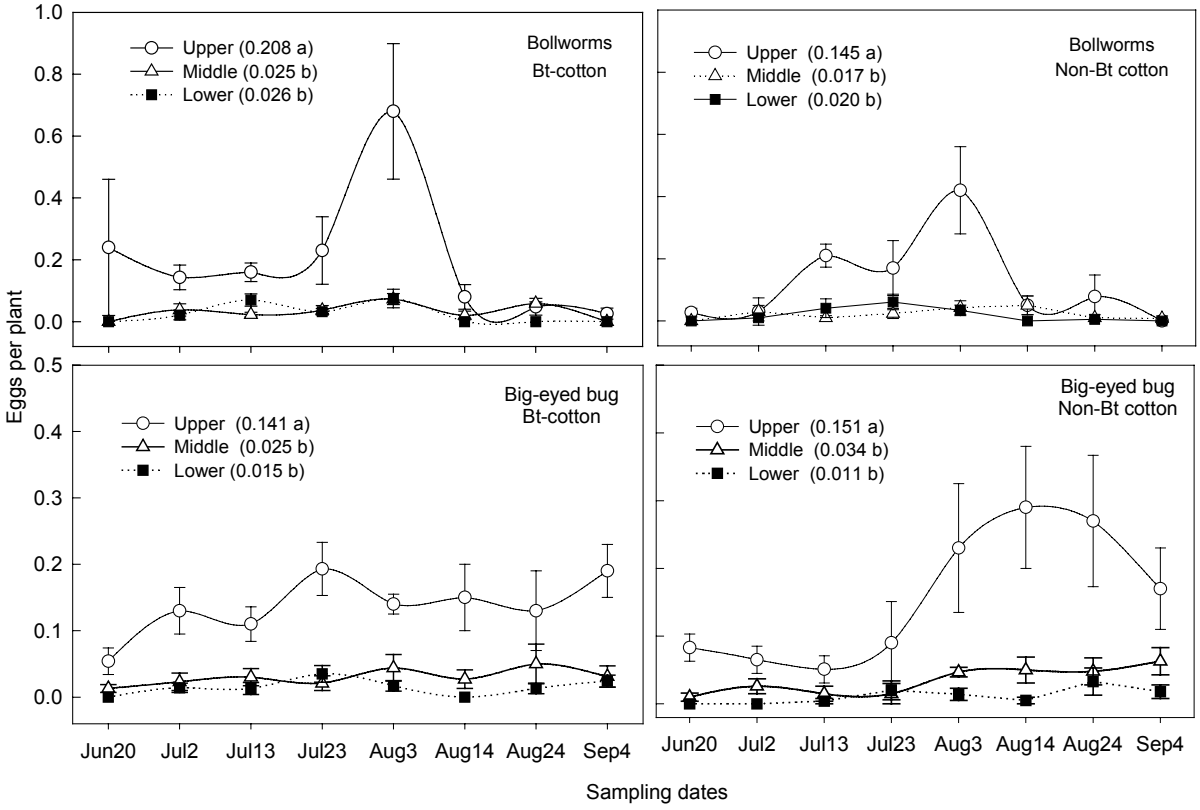

Figure 5.2 Average numbers of bollworm and big-eyed bug eggs within plant thirds and averaged across seasons (2002-2004).

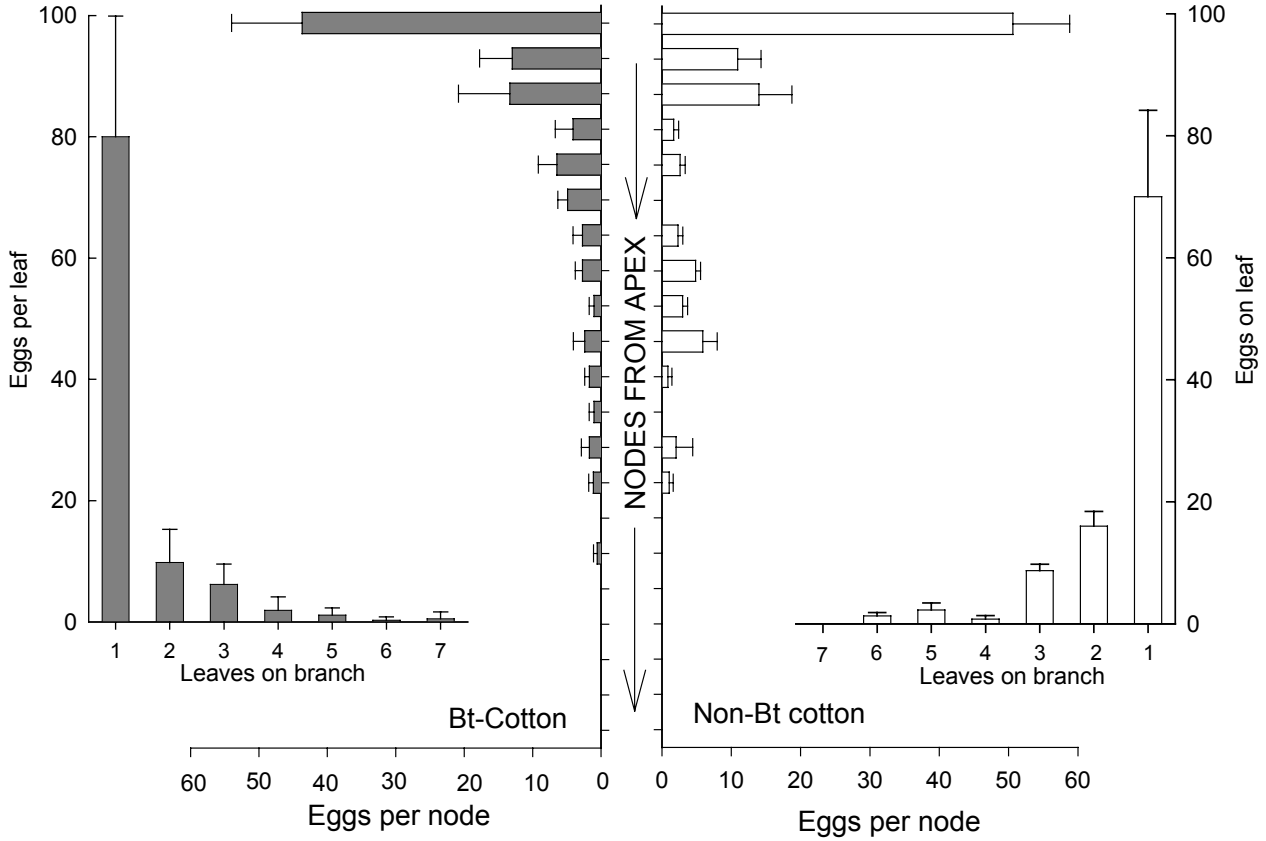

Figure 5.3 Vertical distribution of bollworm eggs (%) within Bt ($y = 29.12 – 4.58x + 0.167x^2$, $R^2 = 0.55$, $F = 71.65$, $P<0.0001$) and non-Bt ($y = 29.81 – 4.75x + 0.17x^2$, $R^2 = 0.44$, $F = 44.72$, $P<0.0001$) cotton plants based on plant node position from plant apex (node 1) to bottom (node 21), and on leaves/nodes of vegetative/fruiting branches from outside leaf (leaf 1 including bud) toward inside Bt ($y = 105.21 – 44.95x + 4.44x^2$, $R^2 = 0.78$, $F = 69.80$, $P<0.0001$) and non-Bt ($y = 95.17 – 39.12x + 3.78x^2$, $R^2 = 87.29$, $F = 87.29$, $P<0.0001$) cotton plants collected in the field during cotton seasons 2002 to 2004, Tift County, GA.

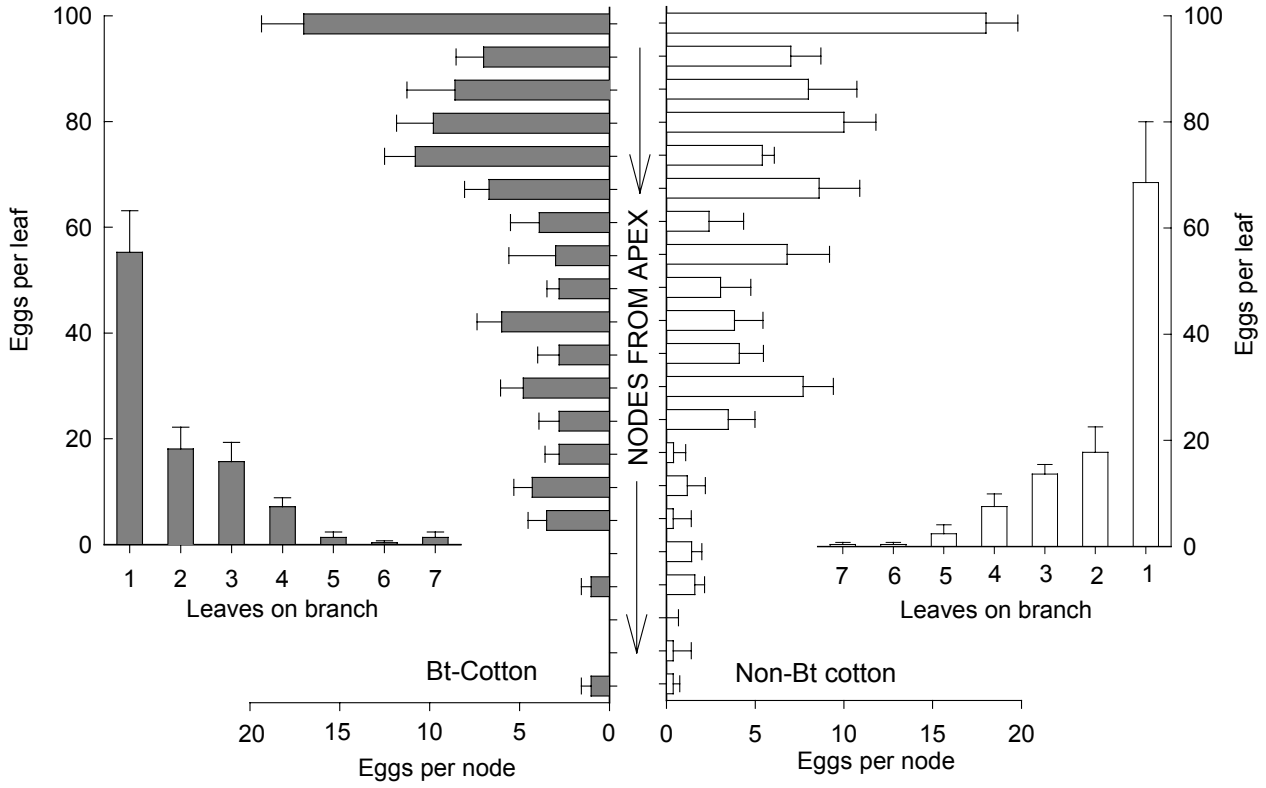

Figure 5.4 Vertical distribution (%) of *Geocoris punctipes* eggs on nodes within Bt ($y = 11.07 – 0.57x$, $r^2 = 0.29$, $F = 48.51$, $P<0.0001$) and non-Bt ($y = 12.88 – 0.67x$, $r^2 = 0.39$, $F = 51.25$, $P<0.0001$) cotton plants based on plant node position from plant apex (node 1) to bottom (node 21); and per leaves of vegetative/fruiting branches from outside (leaf 1 including branch bud) toward inside plant of Bt ($y = 70.26 – 24.89x + 2.17x^2$, $R^2 = 0.76$, $F = 96.82$, $P<0.001$) and of non-Bt ($y = 76.89 – 28.53x + 2.57x^2$, $R^2 = 0.78$, $F = 108.56$, $P<0.001$) cotton plants collected in the field during cotton seasons 2002 to 2004, near Tifton, GA.

CHAPTER 6

EXPRESSION OF *BACILLUS THURINGIENSIS* CRY1AC PROTEIN IN COTTON PLANTS, ACQUISITION BY PESTS AND PREDATORS: A TRITROPHIC ANALYSIS[1]

[1]Torres, J.B., J.R. Ruberson and M.J. Adang. To be submitted to *Ecological Entomology*.

Abstract. 1. Recent studies have provided evidence that Cry proteins from the bacterium *Bacillus thuringiensis* (Bt) expressed in transgenic plants can be acquired by non-target herbivores and predators. A series of studies was conducted to investigate how Cry1Ac protein from Bt transgenic cotton reaches the third trophic level and to measure the amount of protein that herbivores can acquire and expose to predators.

2. Cry1Ac protein in Bt-cotton plants (leaf), prey/herbivores (4 species), and immature or adult predators (7 species) was measured weekly during the cotton growing season using an immunological assay (ELISA). The amount of Cry1Ac in cotton plants decreased over the season, with a seasonal mean of 0.24 μg Cry1Ac g^{-1} fresh tissue. Among the prey/herbivores tested, Cry1Ac was detected only in lepidopteran larvae, with levels differing among species. Among predators assayed, Cry1Ac was detected in *Podisus maculiventris* adults and *Chrysoperla rufilabris* larvae from one and two late-season sample dates, respectively.

3. *Spodoptera exigua* larvae–fed Bt-cotton conveyed from 67 to 78% of the original Cry1Ac present in Bt-cotton plants to the third trophic level, but only 14% of the Cry1Ac detected in *S. exigua* was subsequently found in the predator *P. maculiventris*. *Podisus maculiventris* consumed ~100 mg of *S. exigua* larvae fed-Bt cotton during 24 h, while *Orius insidiosus*, *Geocoris punctipes* and *Nabis roseipennis* consumed 0.3, 1.5 and 3 mg, respectively, and failed to acquire detectable levels of Cry1Ac protein from prey fed Bt-cotton. None of these omnivorous predators acquired Cry1Ac protein when confined on Bt-cotton plants deprived of prey.

4. Cry1Ac protein ingested by the predator *G. punctipes*, drinking from different concentrations (0, 2, 4, 8, 16 and 32 ppm) was detectable at a lower threshold of 4 ppm, and was detectable up to 48 h in the body and 72 h in the feces after drinking from the highest protein concentration.

5. Predator and Bt-protein interactions in cotton fields showed some movement of Cry1Ac to the third trophic level. Predatory heteropterans can pick up Cry1Ac from prey fed Bt-cotton, but the acquisition is dependent on predator species and amount of prey consumed by the predator. The type and availability of prey capable of acquiring the protein, coupled with the generalist feeding behavior of the most common predators in the cotton ecosystem, probably constrain the flow of Cry1Ac through trophic levels.

Key words. Transgenic plants, risk assessment, food web, non-target effects, predatory Heteroptera, phytophagy.

INTRODUCTION

The environmental advantages of using formulations of the bacterium *Bacillus thuringiensis* (Bt) over other insecticides for pest control are well known (Glare & O'Callaghan 2000). However, field cultivation of Bt-transgenic plants that continuously produce Bt protein throughout the growing season is relatively new and has fuelled many concerns. Bt-proteins present in transgenic corn have been detected in root exudates in soil (Saxena *et al*., 1999); in pollen drift to areas adjacent to fields (Jesse & Obrycki, 2000); in spider mites, thrips and leafhoppers fed Bt-corn (Dutton *et al*., 2004); in honeydew produced by planthoppers fed on Bt-rice (Bernal *et al.*, 2002); and in nontarget chewing herbivores (Howald *et al.*, 2003; Dutton *et al.*, 2003). The acquisition of Bt-proteins by non-target herbivores and by lepidopterans with low susceptibility to Bt transgenic crops indicates that Bt-proteins can be transferred among trophic levels, and may interfere with established food webs. However, each system has its peculiarities, being affected by the Bt-protein and promoter used to drive gene expression, plant species and tissues, background cross, rainfall, soil type, and soil fertility (Sachs *et al.*, 1998; Greenplate, 1999; Adamczyk & Sumerford, 2001). For example, Cry1Ab expression levels in corn are two-fold higher than Cry1Ac in cotton, and the same trend occurs when both toxins are inserted in cotton plants (Perlak *et al*., 1990; Sachs *et al.*, 1998). In addition, Cry1Ac present in Bt-cotton terminal foliage can range from 19.1 μg/g dry weight in cotton cultivated in Georgia to 125.6 μg/g dry weight in cotton cultivated in Mississippi, and vary between years and locations (Greenplate, 1999). Cry1Ac protein expression is clearly influenced by species of plant and environmental factors, and these may differentially affect tritrophic associations (plant-herbivore-natural enemy).

The cotton ecosystem supports a substantial complex of arthropod pests and natural enemies. Three major groups of predatory insects (heteropterans, coleopterans, and neuropterans) are recognized as important natural enemies of key and secondary pests in cotton, and these predators are capable of consuming non-pest arthropods to sustain their populations (López *et al.*, 1996 and references therein). Herbivores in cotton may not be susceptible to Bt-proteins, but still may acquire Bt-protein from the plant and convey it to higher trophic levels. Conveyance of Bt-proteins in the prey/host body to predators and parasitoids has been investigated as a potential route for non-target impact of Bt-transgenic plants (Raps *et al.*, 2001; Head *et al.*, 2001; Bernal *et al.*, 2002; Dutton *et al.*, 2003; Schuler *et al.*, 1999 and 2001). The risk of Bt-protein exposure to predators and parasitoids has been studied in transgenic corn under controlled conditions (Hilbeck *et al.*, 1999; Head *et al*., 2001; Raps *et al.*, 2001; Dutton *et al.*, 2002). In the cotton ecosystem, it is possible that species moderately or not susceptible to Cry1Ac can acquire the protein from the plants and expose it to the third trophic level. Species of *Spodoptera* and *Pseudoplusia* occurring in cotton fields are only partially affected by Bt-cotton (Stewart *et al.*, 2001) and, hence could convey Cry1Ac to their predators. In addition, omnivorous predators that occasionally feed on plants may be directly exposed to Bt-proteins, as well. Although carnivory is the rule for coccinellids, chrysopids, and predatory heteropterans, omnivory can be broadly present within these groups, and direct feeding on plants or their products, such pollen and nectar, has been considered an important life history strategy (Coll & Guershon, 2002; Eubanks *et al.*, 2003). Plant feeding is common in predatory heteropterans.

In the current study, we investigated if the Cry1Ac protein expressed in transgenic Bt-cotton plants is moved from plants to herbivores, and subsequently to their predators in the cotton system. Therefore, a series of experiments were conducted with three objectives: (1) to

investigate the amount of Cry1Ac protein moving through trophic levels in Bt-cotton fields; (2) to determine the acquisition rate of Cry1Ac by predatory heteropterans from lepidopteran prey fed-Bt cotton, and from direct feeding on Bt-cotton plants; and (3) to verify that heteropteran predators are capable of ingesting and excreting Cry1Ac, using the big-eyed bug *G. punctipes*. The studies were conducted in the laboratory, greenhouse, and in the field. To our knowledge, this project represents the first study covering all segments of trophic interactions in the cotton ecosystem, from the plant to the third trophic level, quantifying levels of Bt-protein present in each trophic level under controlled and field conditions. Also, the field results cover whole crop seasons, as has been strongly recommended in risk assessment guidelines (Schuler *et al.*, 2001; Dutton *et al.*, 2003).

MATERIAL AND METHODS

Insects

The insects used in the laboratory and greenhouse experiments were cultured in the Biological Control Laboratory (University of Georgia, Tifton) or were acquired from field collections. Adults of *G. punctipes* and *O. insidiosus* were reared using corn earworm eggs [*Helicoverpa zea* (Boddie) (CEW)] as prey (obtained from the USDA-ARS-CPMRL, Tifton, GA). To obtain enough *O. insidiosus* adults to conduct the experiments (~150 adults per treatment), predators were collected from silks of non-Bt corn at the Lang Farm (University of Georgia, Tifton GA). Spined soldier bugs, *Podisus maculiventris* (Say), originated from a collection of females on peach trees near Plains, GA, and were cultured in the laboratory using beet armyworm larvae, *Spodoptera exigua* (Hübner) (BAW), as prey. Damsel bugs, *Nabis roseipennis* Reuter, were collected from a non-Bt cotton field at the Coastal Plain Experiment Station, Tifton, GA. Adults of *N. roseipennis* from field collections were maintained in the

laboratory in cages containing pieces of green been and 2-5-d-old beet armyworm larvae. Field-collected predators, when used in the experiments, were held in the laboratory for one week to verify predator health (pathogen- and parasitoid-free) and to starve the predators to uniform hunger levels prior to the experiments.

Beet armyworm larvae were reared on a standard artificial diet for selected lepidopteran species (Burton 1969). Moths were maintained in plastic cages with white paper towels for an oviposition substrate, and were fed 10% honey/water solution. Eggs laid on the paper were collected and incubated. For the experiments, neonate larvae were caged on Bt and non-Bt cotton plants at various intervals in the greenhouse to produce larvea/prey of appropriate size for each predator species (see below).

Cry1Ac purified toxin

Activated Cry1Ac protein (65 kDa) was prepared from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-73 obtained from the *Bacillus* Genetics Culture Collection (Columbus, Ohio) as previously described by Luo *et al.* (1999). The protein used was kindly provided by Dr. Juan Luis Jurat-Fuentes (Department of Entomology, University of Georgia, Athens) at a concentration of 1.6 mg/ml, and stored at –25^{o}C until needed. The original concentration was used to prepare specified dilutions in distilled water immediately before being offered to the predators.

Cry1Ac toxin in cotton plants, prey and predators in cotton fields

Cotton aphids, lepidopteran larvae and predators (immature or adult) were collected in three pairs of Bt and non-Bt cotton fields (5 to 11 ha) from different locations near Tifton, GA, in 2004 using drop cloth samples (dislodging insects from two rows onto a 1-m-long white canvas cloth laid between the cotton rows). The fields were representative of cotton production in the

region, and sampling focused on several of the most common predators found in cotton fields (Knutson & Ruberson, 1996).

The cotton fields [*Gossypium hirsutum* (L.)] were planted with Bt-cotton (DPL 555) and non-Bt cotton (DPL 493) during the second week of May and sampled throughout the season until the fourth week of August in 2004. Leaf material was collected from 6-7 randomly-selected plants in each Bt-cotton field. On each plant, a leaf disc was collected by snapping a centrifuge tube cap down between the main veins of the uppermost fully expanded leaf of the plant. Abundant potential prey species for predators were also assayed for toxin content. Larvae of several lepidopteran species (Noctuidae) variably susceptible to the Cry1Ac protein [soybean looper, *Pseudoplusia includens* (Walker) (n=67 larvae), southern armyworm, *Spodoptera eridania* (Cramer) (n=108 larvae), and beet armyworm, *Spodoptera exigua* (Hübner) (n=35 larvae)] were collected in drop cloth samples throughout cotton growing season and assayed to Cry1Ac protein. Individuals of the cotton aphid, *Aphis gossypii* (Glover) (4094 mg of sample) were brushed from infested upper leaves. Simultaneously, important predators in the cotton ecosystem were collected in drop cloth samples. Lady beetle collections focused on *Harmonia axyridis* (Pallas) (n= 122 larvae) because it is a common species through most of the season in the sampled cotton fields, whereas other species are more sporadic. Adults of common predatory heteropterans [*G. punctipes* (n=231), *Orius insidiosus* (Say) (n= ~5000), *N. roseipennis* (n=71) and *P. maculiventris* (n=32) and larval lacewings [*Chrysoperla rufilabris* (Burmeister) (n=116) and *Micromus* sp. (n=115)] also were collected. Immediately after collection, specimens were chilled in a cooler until return to the laboratory. In the laboratory, the material was stored in centrifuge tubes at –25^{o}C until protein extraction and the enzyme-linked immunosorbent assay (ELISA) assays were run. Plant material and *G. punctipes* were collected weekly throughout the

season, while other predators and prey were not as consistently abundant, and were sampled as they occurred.

Toxin acquisition by predatory heteropterans from prey and plant

Cotton plants expressing the gene for the Bt-protein Cry1Ac (variety DPL 555) and a non-transgenic variety (DPL 5415) were used in this experiment. The plants were cultivated in pots (15 cm diam. and 15 cm deep) filled with high porosity potting soil BM6™ (Berger Peat Moss Saint-Modeste, Quebec), mixed with 14-14-14 controlled-release fertilizer (Osmocote™, Scotts-Sierra Horticultural Products Company, Marysville, OH), and maintained under greenhouse conditions of 26 ± 4.0 ^{o}C (mean ± SD) and ~14 h of light. Plants were used when they were 26-32 days old, comparable in size, and had 7-8 fully-expanded leaves.

Prey (neonate BAW larvae) were caged on cotton leaves using organdy fabric sleeve cages. The appropriate prey size for each predator species was achieved by offering BAW larvae of different ages to the respective predators. BAW larvae offered to big-eyed bugs, *G. punctipes*, and insidiosus flower bugs, *O. insidiosus*, were fed for 1 d on plants (caged for 24 h on plants before offering to the predators). BAW offered to damsel bugs, *N. roseipennis* and to spined soldier bugs, *P. maculiventris*, were 3- and 9-d-old plant-fed larvae, respectively. The predators, however, were caged on plants with or without prey for 24 h. The number and weight of prey offered to the predators were obtained before confining them on the plants. The number of larvae consumed and predator weights before and after caging were used to estimate the amount of fresh material consumed by individual predators. The treatments consisted of predators caged on (i) plants without prey (BAW larvae), or (ii) plants with prey. As non-Bt controls, predators were simultaneously caged on non-Bt cotton plants either with or without prey. To encourage prey and plant feeding, and to standardize predator hunger, predators were deprived of prey for 24-36h

before placement in cages. Big-eyed bugs (16), damsel bugs (15), and spined soldier bugs (10) were singly caged with 20 BAW larvae of appropriate size, while insidiosus flower bugs were caged in groups of 20 predators per cage, containing 60 1-d-old BAW larvae. Cages consisted of 500-ml styrofoam cups, with bottoms removed, wrapped in knee-high nylon stretch hose, and tied to the cotton leaf petioles. Only predators which were alive 24 h after caging were assayed for Cry1Ac protein. The numbers assayed for each predator were: 12 (Bt) and 13 (non-Bt) *G. punctipes*, 12 (Bt) and 10 (non-Bt) *N. roseipennis*, 9 (Bt) and 9 (non-Bt) *P. maculiventris*, and 165 (Bt) and 148 (non-Bt) *O. insidiosus*.

We caged 16 *G. punctipes*, 10 *P. maculiventris*, 10 *N. roseipennis* and 120 *O. insidiosus* in the treatments with predators caged on Bt and non-Bt cotton plants deprived of prey. From this initial sample size, again, only predators which were alive 24 h after caging were assayed and comprised 13 *G. punctipes* from each cotton type, 10 (Bt) and 9 (Non-Bt) *P. maculiventris*, 10 (Bt) and 9 (non-Bt) *N. roseipennis*, and 102 (Bt) and 112 (non-Bt) *O. insidiosus*.

The material representing all three trophic levels of this association (cotton leaf, prey, and predator) was collected at the end of the exposure period (24 h after caging predators on plants) and assayed for Cry1Ac protein. Plant material consisted of a leaf disc collected by snapping the centrifuge tube cap down between the main veins of the leaf inside the cage. Living prey (BAW larvae caged on plants), plant material and predators were collected and stored in a freezer at -25^{o}C until the ELISA assays were run.

Toxin ingestion by the predatory big-eyed bug G. punctipes

In the laboratory, a study of ingestion of various dosages of Bt-protein was conducted using adult *G. punctipes* to verify the ability of this predatory heteropteran to ingest Cry1Ac protein. Male and female *G. punctipes* were starved for 48 h before beginning the experiment. Five

concentrations of purified Bt Cry1Ac protein (2, 4, 8, 16, 32 ppm) were offered to predators for one hour in a droplet of 1µl of protein-water per predator. The volume and exposure time were determined in a previous test using only distilled water to determine the volume of water ingested before substantial evaporation could alter the concentrations. Distilled water droplets were offered to the predators assigned as the control treatment. Fifteen bugs were individually placed in plastic petri dishes (2 cm diam., 1 cm high) and allowed to acclimate for about one hour. A 1-µl volume of the appropriate protein-water solution was then placed in each petri dish using a micropipettor. All predators were observed to ensure that drinking occurred for more than one minute. Individuals that failed to drink for at least one minute were discarded; hence, the final sample size ranged from 10-12 individuals for each concentration. After 1 h, all predators that drank were transferred to centrifuge tubes and stored at –25^{o}C until protein extraction and ELISA assay.

Fate of Cry1Ac protein in G. punctipes' *body and feces*

Female and male *G. punctipes* (5-10 d old) were starved for 36-48 h to enhance thirst levels. The predators were individually placed in 2-cm diameter petri dishes. After 0.5 h of resting in the petri dishes, a 1-µl droplet of purified Cry1Ac protein in distilled water was offered to each predator. We used 16 and 32 ppm Cry1Ac protein concentrations based on the ingestion test previously conducted. The unused portion of the Cry1Ac-water dilutions was also stored at -25^{o}C and assayed to verify the protein levels in the dilutions. As above, only predators observed to drink from the droplet for 1 min were used in the analyses. Exposure of the droplet to predators did not run beyond 1h to avoid significant change in droplet volume and concentration.

To investigate the fate of Cry1Ac protein ingested by *G. punctipes*, predators' bodies and feces were frozen at various intervals following drinking: immediately after drinking (<1h), 12,

24, 48, and 72 h after drinking. Feces were collected during the intervals 0-12h, 12-24h, 24-48h and 48-72h following drinking. Predators not immediately frozen or used to assay feces were maintained in centrifuge tubes, with 5-6 bugs per tube. The opening of the tubes was covered by screen mesh, secured by a ring inside the tube. The screen mesh permitted ventilation and held the prey (corn earworm eggs) in place. Moisture was provided to predators in the tubes using a micropipette tip containing cotton saturated with water. At each post-drinking interval, predators were transferred to a clean centrifuge tube and stored at –25^{o}C until the ELISA assays. In order to detect and quantify Cry1Ac protein, ELISA assays were run separately for predator bodies and feces. For protein extraction from feces, the centrifuge tubes containing the material were washed with 100 µl of extraction buffer and the contents pooled in a single sample for each time interval. Likewise, all predators' bodies for each collection interval were pooled into a single sample for the assays, and variability was derived from the OD results of multiple samples of the extracted solution (i.e., subsamples represented by each well in the plate were used to generate variability estimates).

Bt-toxin (Cry1Ac) analysis

Cry1Ac protein was quantified in the plant material, prey, and predators for all experiments described above. All frozen material, except for *G. punctipes* feces, was thawed, weighed, placed in a 1.5-ml centrifuge tube, and mixed with phosphate-buffered saline solution and Tween 20 (1xPBST) (Agdia® Inc., Elkhart, IN). Non-fat dried milk (0.4% w/v) and Tween 20 (0.5% v/v) were added to PBST to compose the final extraction buffer, which was mixed with sample material at a rate of 1:10 (w/v). Extraction of Cry1Ac from plant material was conducted by macerating the leaf material in buffer in a 10-ml tube. Prey and predator materials were macerated using 10-ml tubes or 1.5-ml centrifuge tubes, depending on the volume produced by

the sample. The extract supernatants were transferred to clean 1.5-ml centrifuge tubes and stored at -25^{o}C until the ELISA assay (1-2 weeks later). On the day of protein assay, samples were thawed at room temperature, and centrifuged at 5000 rpm for 1 min and loaded at rate of 100 µl per test well.

Cry1Ac levels in the samples were assayed using antibody-coated wells of PathoScreen® plates for Bt-Cry1Ac/Cry1Ab ELISA in a kit using peroxidase enzyme conjugate (Agdia® Inc., Elkhart, IN). Standards of Cry1Ac at concentrations 0.625, 1.25, 2.5, 5, 10, 20 and 40 ng/ml (ppb) were used to build a standardized optical density curve for estimating protein content of material from field collections and greenhouse experiments. For Cry1Ac detection in *G. punctipes* body and feces, the standards were calibrated at concentrations of 0.312, 0.625, 1.25, 2.5, 5.0 and 10 ng/ml (ppb). Absorbance measurements were taken with an EL_x808 microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT) reading at 450 nm. To read the results at 450nm, 50µl of a 3M sulfuric acid solution was added to each well immediately after reading. Using the optical density results generated from the standards, an assay curve was built and the concentrations of Cry1Ac protein were determined for each sample by comparing the sample's reading with the optical density reading of the standard curve of Cry1Ac pure protein, and correcting for the appropriate dilution and unit (µg Cry1Ac g^{-1} of fresh weight). Because there was no initial weight measurement for *G. punctipes* feces, the results for feces were only interpreted through optical density (OD_{450nm}) readings in the standards and sample material.

Statistical analysis

Predator body weight changes, number of BAW larvae consumed, and fresh weight of prey consumed were determined for each predator species caged with BAW larvae and cotton plants in the greenhouse. The data were square-root (x + 0.5) transformed and submitted to Student's *t*-

test (using the Proc TTEST of SAS; SAS Institute 1999-2001) to compare predator weight and prey consumption when caged on Bt and non-Bt plants for each predator species. Changes in Cry1Ac detected in Bt-cotton plants from the fields across sample dates, and Cry1Ac levels in the bodies of *G. punctipes* as a function of Cry1Ac-water dilution concentrations were analyzed using regression analysis with Proc GLM of SAS (SAS Institute 1999-2001). Optical density (OD) readings for Cry1Ac assayed in the feces of *G. punctipes* were submitted to two-way ANOVA (with the factors being concentration and time interval after drinking), using Proc GLM of SAS, and significantly different means were separated using Tukey's High Significant Difference test (HSD) (SAS Institute 1999-2001).

RESULTS

Field expression of Cry1Ac protein in cotton and toxin acquisition by prey and predators

From the 1st week of June to the last week of August, Cry1Ac protein in upper fully-expanded cotton leaves ranged from 0.20 to 0.29 μg g^{-1} of fresh tissue (Table 6.1), with a seasonal mean of 0.24 μg (Fig. 6.1 – commercial fields). A slight decrease in Cry1Ac level in the plants across progressive sample dates was observed ($\beta = -0.0045 \pm 0.001$) ($y = 0.274 - 0.0045x$, $r^2 = 0.16$, $F = 9.01$, df = 1, 47, $P = 0.0043$). The highest and lowest protein levels in cotton plants were detected in the 2nd week of June and July, respectively. Among the sampled canopy-dwelling herbivores, no Cry1Ac protein was detected in *A. gossypii*. However, all three assayed lepidopteran species exhibited detectable levels of Cry1Ac. *Spodoptera eridania* was the lepidopteran species most commonly collected on Bt-cotton, except on the 1st-sample date (Table 6.1). The other two species, *P. includens* and *S. exigua*, were common during the middle and later portions of the season. Cry1Ac levels detected in these species were quite variable during the season and among species. The seasonal mean for Cry1Ac was higher in *S. exigua*,

followed by *S. eridania* and *P. includens* (Fig. 6.1 – commercial fields). Nearly 50, 42 and 17% of the original Cry1Ac level detected in the cotton plants was detected in *S. exigua*, *S. eridania* and *P. includens*, respectively. Among the seven representative predator species collected during the season and assayed for Bt-protein, Cry1Ac was only detected in *C. rufilabris* larvae and *P. maculiventris* adults (Table 6.1). The presence of Cry1Ac protein was detected in two out of seven weeks for *C. rufilabris* (late in the season) and one week out of six for *P. maculiventris* (also late in the season). The level of toxin observed in predators that were positive for toxin was only 8.3% (*P. maculiventris* adults) and 29% (for *C. rufilabris* larvae) of the amount found in the plants. The timing of Cry1Ac presence in predators coincided with abundant populations of *P. includens*.

Toxin acquisition by prey and predatory heteropteran

Bt-protein was measured in all three trophic levels in the greenhouse cage experiments. In Bt-cotton plants (1st trophic level), Cry1Ac was 0.18 ± 0.03 (mean ± SD of μg g^{-1} of fresh weight) and decreased to 0.14 ± 0.01, 0.14 ± 0.02 and 0.12 ± 0.03 in 10-d-, 4-d- and 2-d-old BAW larvae fed Bt-cotton plants (2nd trophic level, Fig. 6.1 - greenhouse). Cry1Ac in the third trophic level was only detected in the predator *P. maculiventris*. The level of Cry1Ac in *P. maculiventris* was 0.02 ± 0.004 μg Cry1Ac g^{-1} of fresh body weight. The estimated fresh weight of prey (9-d-old BAW) consumed by *P. maculiventris* during the 24-h experimental period was similar whether larvae were fed Bt or non-Bt cotton plants (Table 6.2). However, to achieve similar amount of food ingestion, *P. maculiventris* consumed almost twice as many BAW larvae fed on Bt-cotton compared with non-Bt fed larvae to compensate for the smaller size of BAW larvae fed on Bt-cotton plants (Table 6.2). Cry1Ac protein was not detected in adult *N. roseipennis*, *G. punctipes*, or *O. insidiosus* that preyed on BAW larvae fed Bt-cotton. Nor was

Cry1Ac detected in any of the predators fed BAW larvae that consumed non-Bt cotton. The estimated fresh prey consumption and predation rate by these three predator species preying on BAW larvae did not differ for prey fed either Bt- or non-Bt cotton plants ($P > 0.05$). The estimated fresh consumption of BAW by *P. maculiventris* (which had detectable Cry1Ac levels) was approximately 32, 68, and 338 times greater than the amount of prey consumed by *N. roseipennis*, *G. punctipes*, or *O. insidiosus*, respectively (Table 6.2).

Toxin ingestion by Geocoris punctipes

Adults of the big-eyed bug *G. punctipes* were able to ingest detectable levels of Cry1Ac protein diluted in water (Fig. 6.2). The lower threshold of Cry1Ac ingestion for ELISA detection was 4 ppm in the tested range of 2 to 32 ppm. The levels of Cry1Ac detected in the predator body decreased linearly as a function of the concentrations from 4 to 32 ppm ($F = 125.5$, $P < 0.0001$, df = 1, 10) at a proportion of –0.0087 (± 0.007) μg Cry1Ac g^{-1} of fresh body weight (Fig. 6.2). The amount of Cry1Ac toxin detected in the predatory body was nearly 100 times less than the original amount of Cry1Ac in the concentration offered to the bug.

Fate of Cry1Ac protein in G. punctipes*' body and feces*

Cry1Ac protein exposed in 16 and 32 ppm concentrations to bugs was detected in the predators' bodies and feces in amounts proportional to the concentration offered and related to duration time passed since drinking (Fig. 6.3A-B). Adult *G. punctipes* had detectable protein in their bodies up to 24 and 48 h after drinking from 16- and 32-ppm concentrations of purified Cry1Ac protein, respectively. The ELISA did not detect any Cry1Ac in the predators' bodies 72 h after drinking from either 16- or 36-ppm concentrations. The amount of Cry1Ac measured in *G. punctipes* immediately after drinking 16- and 32-ppm concentrations (<1h later) was ~ 46% (mean ± SD; 0.35 ± 0.003 μg/g fresh body weight) and ~ 58% (0.57 ± 0.002 μg/g fresh body

weight) of the original amount available in the dilutions, respectively. The levels of Cry1Ac protein decreased linearly as the post-drinking interval increased from ~1 h to 72 h (16 ppm; $y = 0.035 – 0.0011x$, $r^2 = 0.95$, $F = 133.10$, $P < 0.0001$; 32 ppm; $y = 0.055 – 0.00037x$, $r^2 = 0.87$, $F = 67.88$, $P < 0.0001$; Fig. 6.3A). Although, no Cry1Ac was detected in predators' bodies 72 h after drinking from either concentration offered, the results demonstrate that Cry1Ac levels in bugs fed 16 ppm concentration declined approximately 3 times as rapidly as levels in bugs that drank from the 32 ppm concentration ($\beta_{16ppm} \div \beta_{32ppm}$).

Optical density (OD) readings from ELISA assays (Fig. 6.3B) indicated detectable levels of Cry1Ac in feces of *G. punctipes* during the four intervals after drinking from both concentrations tested (16 and 32 ppm). Two-way ANOVA indicated that concentration had a high effect on the levels of Cry1Ac detected in big-eyed bug feces (average OD across time intervals was 0.42 ± 0.10 and 1.58 ± 0.48 for 16 and 32 ppm, respectively; $F = 565.13$, df = 1, 16, $P < 0.0001$) as did time interval following drinking; with significant interactions between concentration and time intervals ($P < 0.0001$). Feces collected during the 1-12 h post-drinking interval produced the lowest level of Cry1Ac for bugs drinking the 32 ppm concentration, but did not differ from levels observed in the 24-48 and 48-72 h intervals for bugs drinking 16 ppm. The peak of Cry1Ac excretion in the big-eyed bug feces was observed during the 12-24 h interval for both concentrations.

DISCUSSION

Among important predators in the cotton ecosystem, *G. punctipes* was able to ingest Cry1Ac protein in its purified form, and *P. maculiventris* was able to acquire toxin through prey fed Bt-plants in confined conditions and in the field. The same patterns are found for *Chysoperla carnea* (Stephens) fed Cry1Ab-sucrose diet (Romeis *et al.*, 2004), and for *C. rufilabris* that

consumed prey that fed on Bt-cotton in the field (Table 6.2). The ability to ingest Cry1Ac at detectable levels in a purified form directly from water dilutions opens opportunities to directly test ingestion toxicity of Cry proteins to these predators. Direct toxicity has been used as a basic laboratory screening of selectivity (Sims, 1995; 1997; Romeis *et al*., 2004). For example, Romeis *et al.* (2004) offered Cry1Ab in sucrose diet to second-instar larvae of *C. carnea* and demonstrated no direct effect of purified Cry1Ab on development of lacewing larvae, although Cry1Ab was detected in larvae fed Cry1Ab-sucrose diet. The study by Romeis et al. (2004) demonstrated the lack of toxicity of Cry1Ab for *C. carnea* larvae, in contrast with previous results indicating negative effects (Hilbeck *et al*., 1998). Consumption of detectable levels of Cry1Ac by *G. punctipes* will allow doses of Cry-proteins much higher than usually expressed in transformed plants to be tested, permitting generation of direct and quick information on the safety of the protein. Protein detection in predator feces will also permit tracing the fate of the protein in the body and excreta of bugs fed Cry protein.

The high levels of Cry1Ac in *G. punctipes* feces, however, do not exclude the possibility that some of the protein was broken down during ingestion and discarded or used in an altered form in the bug. There is no published information regarding the fate of Cry proteins in heteropterans. Cry1Ac was not detected 48 and 72 h, respectively, after drinking from Cry1Ac-water at concentrations of 16 and 32 ppm. Considering the short time for digestion in heteropterans (liquid feeders), it is possible that Cry1Ac ingested by *G. punctipes* may be restricted to the digestive tract and eliminated in the feces. However, Cry1Ac traces could remain in the digestive system at levels not detectable by ELISA (0.5 ppb) but be concentrated sufficiently in the feces to produce detectable levels.

Insect feces seem to accumulate ingested Cry protein. *Geocoris punctipes* feces, as is the case for other predatory heteropterans, consist of semi-liquid excreta, which can lose water quickly, increasing the concentration of undigested material in the feces. Overall, insect excretion is slower than ingestion and undigested contents are more concentrated than was the original food (Chapman, 1998). This may explain why levels of Cry protein detected in the feces were higher than levels in the original food. For example, Cry1Ab in feces of *Spodoptera littoralis* (Boisduval) fed-Bt corn for 24 h was tenfold higher than levels in the larvae (Raps *et al*., 2001). Further study of predator excretion products may provide insights into toxin ingestion and processing.

The levels of Cry1Ac in the body of *G. punctipes* decreased around 100 times from the original concentrations provided to them in the droplets (e.g., 32/0.27 = 118.5) (Fig. 6.3). This dilution effect may explain the negative results obtained when this predator consumed prey fed Bt-cotton in the greenhouse, as well as the negative results from field samples. Based on our results, and assuming no loss of Cry1Ac during prey consumption, the predator would have to consume a minimum of 24 mg of BAW to acquire sufficient Cry1Ac to be detectable at the lower detection limit for the ELISA (0.5 ppb). The 12 *G. punctipes* assayed consumed a total of 17.88 mg of prey [individual predators consumed 1.49 mg fresh weight of BAW larvae (Table 6.2)]. Only *P. maculiventris*, which individually consumed 101.4 mg fresh weight of BAW, consumed sufficient prey material to acquire detectable levels of Cry1Ac (Table 6.2).

No aphid species have been reported to acquire Cry protein by feeding on plants. Our findings with *A. gossypii* collected in Bt-cotton fields agree with those reported for *Rhopalosiphum padi* L. and *Rhopalosiphum maidis* (Fitch) (Raps *et al*., 2001; Dutton *et al*., 2002; Head *et al*., 2001). Further, we found that predatory heteropterans -- a group of predators

well known for their plant feeding behavior -- in the greenhouse failed to acquire Cry protein from direct feeding on Bt-cotton plants (Fig. 6.1 – greenhouse). Unlike aphids, feeding by predatory heteropterans is not confined to phloem, where Cry-protein would not be expected to occur (Raps *et al.*, 2001). Plant feeding by predatory heteropterans is believed to occur by insertion of stylets randomly into plant tissue and removal of liquid contents and materials liquefied by the action of salivary enzymes, such as amylase and proteinases that are found in salivary glands of *O. insidiosus*, *P. maculiventris,* and *G. punctipes* (Stamopoulos *et al.*, 1993; Cohen, 1996; Zeng & Cohen, 2000). Therefore, Cry1Ac protein could be picked up as a component of digested cell debris and other material. In our trials, however, if it was acquired at all, the toxin level was not enough to be detected through the ELISA assays. Armer *et al*. (2000) also reported no ingestion of Cry3A from direct feeding on Bt-potato plants by *Orius tristicolor* (White), *Nabis* sp., and *Lygus hesperus* Knight, although the last species is a phytozoophagous species that is recognized as a pest in some crops. It would be reasonable to expect Cry3A to be detected at least in *L. hesperus* fed Bt-potato plants because the salivary glands of this bug produce pectinase (Strong & Kruitwagen, 1968), which is responsible for digesting plant cell walls.

The wide size range of heteropteran predators used in the greenhouse cage experiments was predetermined to more broadly assess the exposure risk of this important predator group to Cry1Ac in Bt-transgenic cotton. We would expect larger predators to consume more prey, and thereby acquire more toxin and increase exposure risk. Because the estimated amount of BAW larvae fresh weight consumed played a role in Cry1Ac acquisition and detection it would be expected that if Cry1Ac were to be detected in any of the heteropteran predators tested, it would be found in *P. maculiventris*, one of the largest predatory heteropterans found in cotton. The

sizes of predators caged on Bt-cotton deprived of prey in this study [*P. maculiventris* (84.4 ± 18.5 mg), *N. roseipennis* (7.8 ± 4.1 mg), *G. punctipes* (4.6 ± 0.8 mg) and *O. insidiosus* (0.27 ± 0.05 mg)] covered a large portion of predatory heteropterans not only common in cotton fields, but in other crop ecosystems as well. In addition, the species studied exhibit feeding behavior and enzymatic profiles representative of extra-oral and gut digestion found among predatory taxa in the Pentatomomorpha (*Podisus* and *Geocoris*) and Cimicomorpha (*Nabis* and *Orius*) (Cohen, 1996).

Major prey items available in the cotton canopy to important insect predators and assayed for Cry1Ac are shown in Table 6.1. Although aphids failed to acquire toxin, the lepidopteran larvae did. Larvae of *S. exigua*, *S. eridania*, and *P. includens* can expose their predators to ~50, 42, and 17% of the original Cry1Ac levels in Bt-cotton plants (Fig. 6.1 – commercial fields). The amount of Cry protein conveyed to the third trophic level seems to be dependent on herbivore species. Nearly 21% of original Cry1Ab expressed in Bt-corn (event N4640Bt) was detected in *S. littoralis* larvae, while 73% was detected in the spider mite, *Tetranychus urticae* (Koch) (Dutton *et al.*, 2002). Studying four herbivores fed Bt-corn (Bt11), Dutton *et al.* (2004) found no Cry1Ab in the aphid *R. padi*, and the highest amount of Cry1Ab was detected in the spider mite, *T. urticae* (5.56 μg g^{-1} fresh weight), followed by the thrips, *Frankliniella tenuicornis* (Uzel) (0.91 μg), and the leafhopper, *Zyginidia scutellaris* (Herrich-Schaefer) (0.20 μg). Larvae of the sawfly *Athalia rapae* (L.) exhibited 18% of Cry1Ac that was present in the plants when fed Bt-rape expressing Bt-protein Cry1Ac, and almost the same amount was detected in their feces, but feeding on Bt-rape had no effect on the sawfly's fecundity and fertility (Howald *et al.* 2003). In addition, a large decrease in Cry1Ab in lepidopteran larvae fed Bt-corn (MON 810) compared to the original levels of Cry1Ab in transgenic Bt-plants was reported by Head *et al.* (2001). Cry1Ab

in the European corn borer, *Ostrinia nubilalis* (Hübner), *H. zea*, and *Agrotis ipsilon* (Hufnagel) larvae fed Bt-corn was, respectively, 143, 67 and 59 times less in the larvae than the original level of Cry1Ab in the transgenic Bt-corn. Mean values of Cry1Ab in the larvae found by Head *et al.* (2001) were 0.07, 0.15 and 0.17 ppm (μg/g or μg/ml) of the fresh weight of *O. nubilalis*, *H. zea*, and *A. ipsilon*, respectively. We observed a similar range of variation in Cry1Ac levels among the three lepidopteran species fed Bt-cotton in our field collections, from 0.04 to 0.12 μg Cry1Ac g^{-1} fresh weight (Fig. 6.1 - commercial fields).

Despite detecting Cry1Ac in *C. rufilabris* larvae and in *P. maculiventris* in the field, it is not apparent that these predators are adversely affected by the protein. Seasonal means per 40-drop cloth samples over three successive cotton growing seasons covering multiple generations of both predators were similar in Bt and non-Bt cotton fields, including the 2004 season when *C. rufilabris* (mean ± SE for Bt vs. non-Bt cotton: 3.9 ± 0.48 vs. 4.3 ± 0.55; $F = 0.33$, df = 1, 215, $P = 0.5989$) and *P. maculiventris* (1.9 ± 0.31 vs. 2.7 ± 0.38; $F = 1.55$, df = 1, 215, $P = 0.2809$) were positive to Cry1Ac (CHAPTER 3). The ingested Cry1Ac by predators such as heteropterans may be handled as other undigested/unused material from the diet and excreted. This possibility was indicated in this study with the predator *G. punctipes* (Fig. 6.2B), as in the feces of *A. rapae* larvae fed-Bt rape (Howald *et al.*, 2003) and in honeydew produced by brown planthopper, *Nilaparvata lugens* (Stål) fed-Bt rice (Bernal *et al.*, 2002). The latter two herbivores, despite acquiring Cry1Ac and Cry1Ab, respectively, did not differ in their life history characteristics compared to bugs free of Cry proteins.

To date, studies of ingestion of Cry proteins by predators through diets, prey fed-Bt plants, or Bt plant products have yielded no evidence of adverse effects of Bt-proteins on predators. These studies have been done with green lacewings, *C. carnea*, that were fed: Bt-sucrose diet

(Sims, 1995; 1997; Romeis *et al.*, 2004), pollen of Bt-corn (Pilcher *et al.*, 1997), and aphids fed Bt-corn (Lozzia *et al*., 1998; Meier & Hilbeck, 2001); with lady beetles *Coleomegilla maculata* Timberlake and *Hippodamia convergens* Guerin-Meneville fed pollen of Bt-plants, prey fed Bt-plants, or prey fed diet containing Cry proteins (Sims, 1995; 1997; Pilcher *et al.*, 1997); and with predatory bugs, *O. insidiosus*, *Orius majusculus* (Reuter) and *Cyrtorhinus lividipennis* Reuter that consumed prey fed on Bt plants (Zwahlen *et al.*, 2000; Pilcher *et al.*, 1997; Bernal *et al.*, 2002). Therefore, the ability of herbivorous prey to acquire Bt-proteins from host plants and the resulting exposure to predators seems to have no adverse effects on predator populations. The presence of Cry protein in the lepidopteran larvae does not necessarily imply negative impacts on the third trophic level in cotton fields. Most of the common predatory arthropods in cotton are generalists and can feed on herbivores free of Bt-protein, which could moderate adverse effects, if such exist. This is supported by the demonstration that negative effects on green lacewing larvae attributed to Bt-proteins (Hilbeck *et al.*, 1999) were later shown to be due to suboptimal prey quality rather than Cry1Ab protein (Dutton *et al.*, 2002). Further, direct toxicity of Cry proteins to predators has not been reported (Sims, 1995; 1997; Romeis *et al.*, 2004).

Cry1Ac was detected in the larvae of *C. rufilabris* late in the season when aphids, a common prey for lacewings and one that does not acquire Bt-protein, were scarce, and Cry1Ac-containing lepidopteran larvae were abundant. Therefore, late in the season predation on lepidopteran larvae could be enhanced because of reduced numbers of alternative prey not conveying Cry1Ac protein. Nordlund & Morrison (1990) found that *C. rufilabris* preferred *Heliothis virescens* (F.) larvae to cotton aphids. The availability of lepidopteran larvae, a preferred prey of *P. maculiventris*, also probably is the source of Cry1Ac detected in this predator, since they do not acquire Cry1Ac directly by plant feeding (Fig. 6.1 – greenhouse). In

the early and middle portions of the cotton season, *P. maculiventris* may consume primarily herbivores feeding on weeds, or other prey that does not accumulate Cry1Ac (e.g., coccinellid larvae). However, lepidopteran larvae partially susceptible to Cry1Ac usually become more abundant in the middle or late in the season, replacing the alternative prey free of Cry1Ac as they become scarce. Greater numbers of prey containing Cry1Ac increase the probability of Bt-protein accumulating in predators. Therefore, there is an important seasonal element to dynamics of prey and predators in the cotton ecosystem (in this case, between Bt-cotton, lepidopteran larvae eating Bt-cotton plants, and *C. rufilabris* larvae and *P. maculiventris* adults) that is highly relevant to assessing the possible risks of exposure to Cry proteins. These findings reinforce the criticisms made by Schuler *et al.* (2001) and Dutton *et al.* (2003) that field experiments should consider more than one generation of the organisms during the crop season to ascertain risks.

The amount of Cry1Ac protein in field cotton plants decreased slightly across the cotton season (Table 6.2), but toxin levels were sufficient to move up through trophic levels in the cotton ecosystem. Many factors contribute to Cry1Ac expression in transgenic Bt-cotton. Detailed studies on environmental and plant factors affecting Cry1Ac expression in the field were conducted by Greenplate (1999) and Adamczyk and Sumerford (2001). Their results indicated that Bt-cotton grown in Georgia expressed the lowest rate of Cry1Ac in terminal foliage relative to cotton cultivated in six other Southeastern US States. Adamczyk and Sumerford (2001) found a decrease of Cry1Ac expression in 13 Bt-cotton varieties across the cotton season in Mississippi ranging from ~1.5 to 0.5 ppm (μg/g or ml) per fresh weight of tissue. Considering that the toxin levels in Mississippi Bt-cotton should be around 6.57 times higher than those detected in Georgia Bt-cotton (Greenplate, 1999), the Cry1Ac levels in our

sampled plants across the growing season fit the expected values. The lower Cry1Ac expression in the Georgia cotton does not compromise its value for pest management since the lethal concentration for neonate larvae of the major target lepidopterans is much lower (Perlak *et al.*, 2001).

Attempts to determine the safety of Bt-transgenic plants for non-target organisms, especially natural enemies, have often been conducted in laboratories or focused on just one trophic level. Although there is some agreement on identification of potential negative interactive effects, it is not yet possible to predict with certainty the impact of Bt-proteins in the field because most conclusions rely heavily on artificial conditions that are unrealistic. Although recommending a hierarchy of studies from lab to the field, and from small to large scale studies (Schuler *et al.*, 1999; 2001; Dutton *et al.*, 2003), the conditions in standard farm studies can make such studies difficult, as they are susceptible to many sources of uncontrolled variation. Nevertheless, by using stepwise tri-trophic experiments extending from the lab to field, our results suggest that predatory heteropterans can ingest purified Cry1Ac protein in concentrations above 4 ppm and in some cases can acquire toxin from prey fed on Bt-cotton. However, in greenhouse experiments we found that Cry1Ac acquisition from prey fed Bt plants, however, was dependent on the amount of prey consumed (Table 6.2 and Fig. 6.1 – greenhouse). Also, predator exposure to toxin can be dependent on prey species, as we found that lepidopteran species collected in the field contained different levels of Bt-protein (Table 6.1 and Fig. 6.1 – commercial fields). We also found that no predatory heteropterans assayed were able to pick up Cry1Ac by direct feeding on plants. The amount of Cry1Ac acquired from plants by herbivores and conveyed to the third trophic level is prey/species specific, and ELISA results proved that the predators *C. rufilabris* larvae and *P. maculiventris* were able pick up Cry1Ac conveyed by prey fed Bt-cotton in the field. In

conclusion, despite continuous expression of Cry1Ac by Bt-cotton plants, the degree to which toxin reaches the third trophic level in cotton fields seems to be related to the community structure and dynamics of lepidopteran larvae and their predators, coupled with availability of alternative prey free of Cry1Ac-protein.

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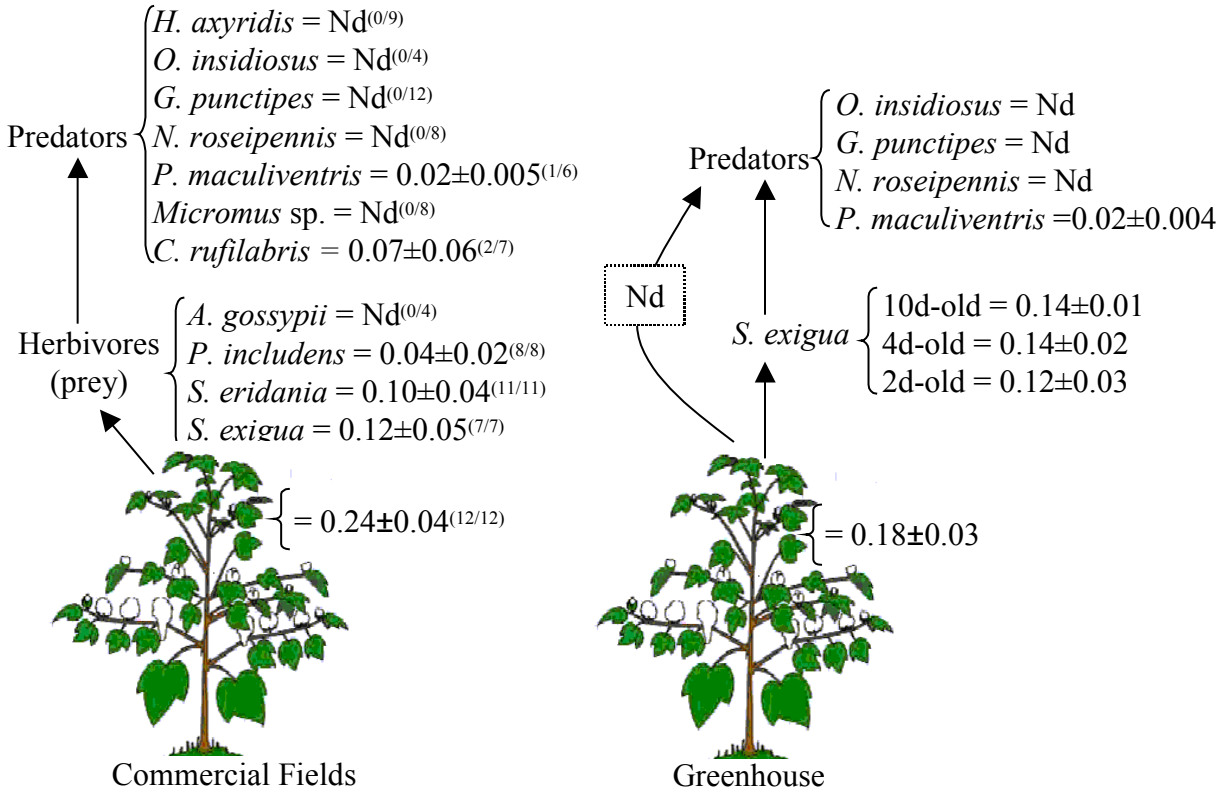

Fig. 6.1. Levels (µg Cry1Ac g^{-1} fresh weight) of *Bacillus thuringiensis* Cry1Ac protein in Bt-cotton plants (DPL 555), herbivores, and predators (representing the three trophic levels in the cotton ecosystem) in commercial fields and under greenhouse conditions. Seasonal mean is presented for material collected from cotton fields throughout the growing season; Nd = not detected; numerators on superscript values for the field results represent the number of sample dates tested positive to Cry1Ac on which the respective organisms were found and sampled in the field out of the 12 sample weeks.

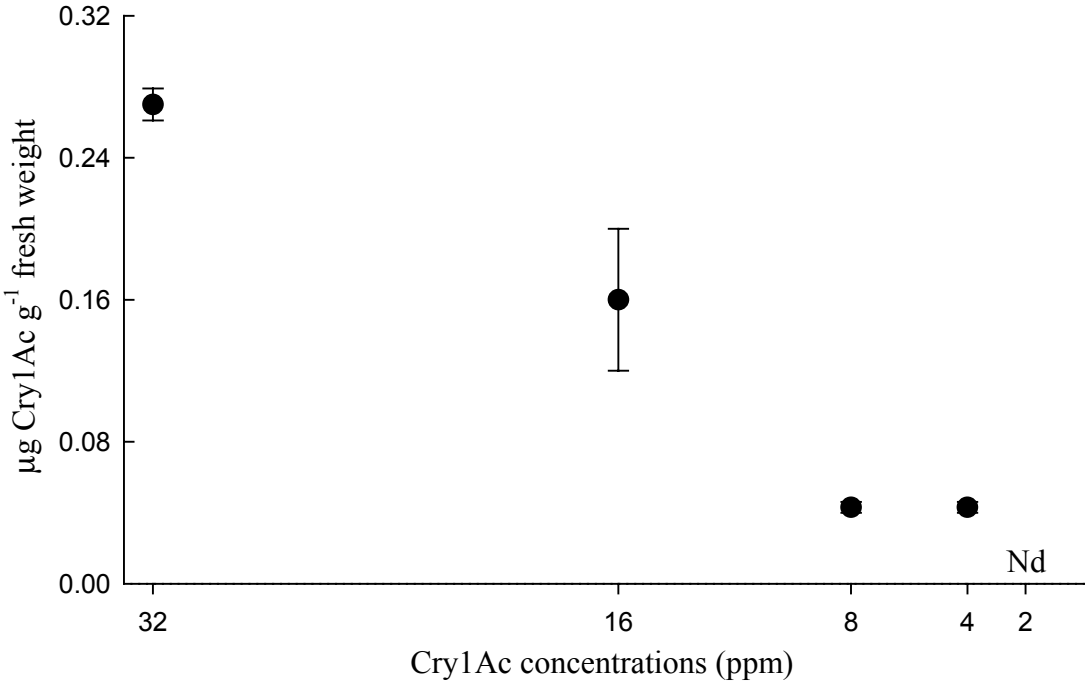

Fig. 6.2. Amount of Cry1Ac protein detected in bodies of *Geocoris punctipes* after drinking Cry1Ac protein-water (concentrations from 2 to 32 ppm). Nd = not detected at 0.5 ppb of standard detection limit.

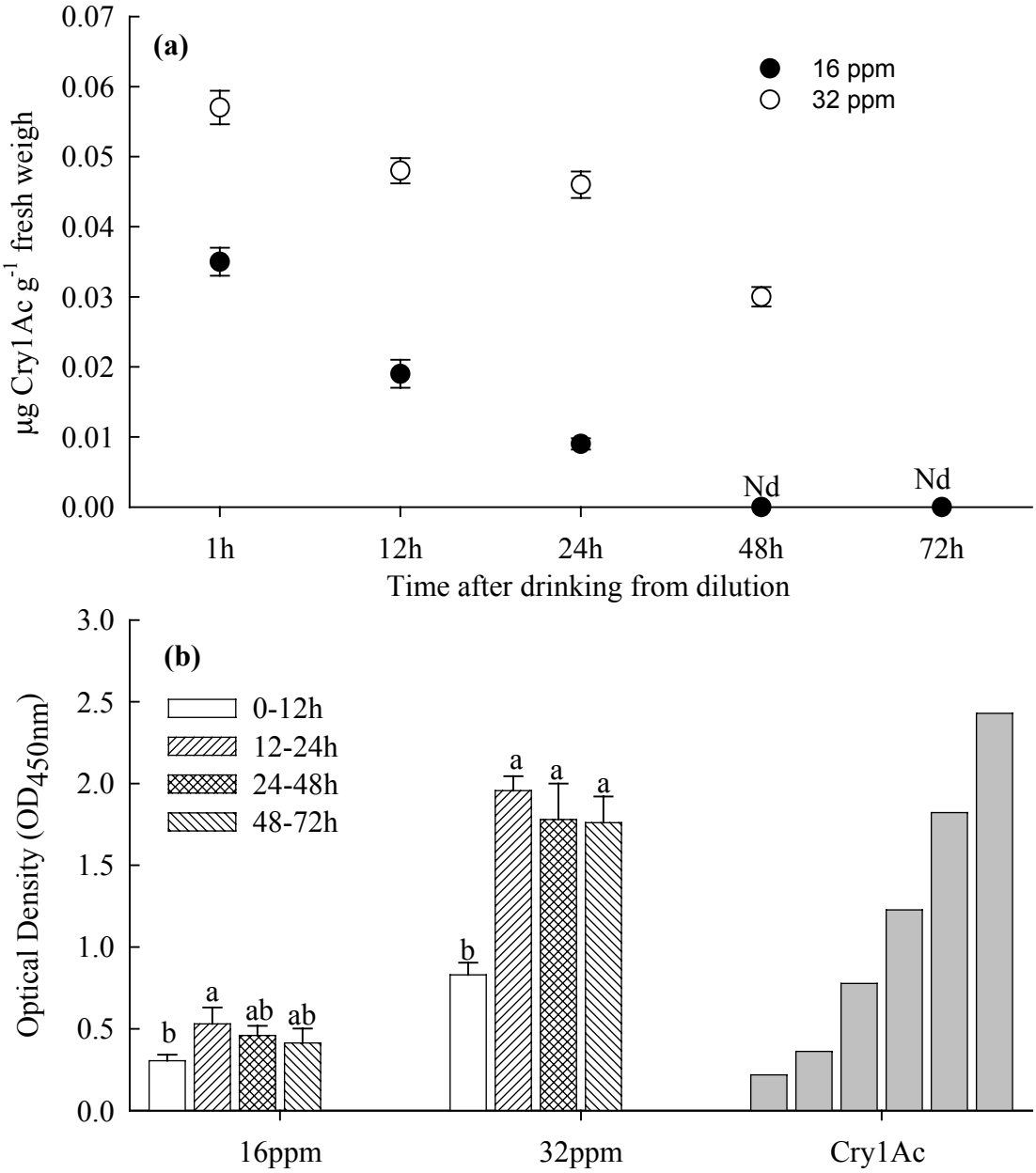

Fig. 6.3. (a) Mean (± SD) Cry1Ac protein concentrations in the bodies of *Geocoris punctipes* in five intervals after drinking from purified Cry1Ac-water dilutions of 16 and 32 ppm (Nd= not detected). (b) Optical density (OD) readings of ELISA assays for detection of Cry1Ac protein in *G. punctipes* feces at different intervals after drinking from 1µl of purified Cry1Ac-water dilution (16 and 32 ppm), corrected for distilled water drinking bugs. Gray bars from left to right represent OD readings for standards consisting of purified Cry1Ac (0.312, 0.625, 1.25, 2.5, 5.0 and 10 ng/ml). Bars under same letter do not differ among time intervals within the same concentration at 0.05 significance levels (Tukey's HSD test).

Table 6.1. Means of Cry1Ac protein (μg Cry1Ac g^{-1} fresh weight) in uppermost fully-expanded Bt-cotton leaves, selected herbivores and predators collected throughout the cotton growing season. Tift County, GA. 2004.

	Sample weeks											
	June				July				August			
Sources	7-9	14-16	21-23	28-30	5-7	12-14	19-21	26-28	2-3	9-11	23-25	28-29
Bt-cotton DPL 555	0.25	0.29	0.27	0.27	0.26	0.20	0.21	0.27	0.25	0.24	0.22	0.22
Aphis gossypii	-[a]	-	Nd[b]	Nd	Nd	Nd	-	-	-	-	-	-
Spodoptera eridania	-	0.17	0.15	0.06	0.11	0.12	0.09	0.06	0.12	0.08	0.07	0.10
Pseudoplusia includens	-	-	-	-	0.03	0.02	0.03	0.02	0.05	0.09	0.02	0.04
Spodoptera exigua	-	-	-	-	0.13	0.09	0.08	0.13	0.14	0.07	0.21	-
Chrysoperla rufilabris	-	-	-	Nd	Nd	Nd	Nd	Nd	0.12	-	0.012	-
Hemerobiid larvae	-	-	-	Nd	Nd	Nd	Nd	Nd	-	Nd	Nd	Nd
Geocoris punctipes	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Orius insidiosus	-	-	-	-	-	-	Nd	Nd	Nd	Nd	-	-
Nabis roseipennis	-	-	-	-	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Podisus maculiventris	-	-	-	Nd	-	-	Nd	Nd	Nd	0.02	Nd	-
Harmonia axyridis	-	-	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	-

[a]Signifies absence in the field or collected individuals did not compose a minimum of 10mg sample to permit testing. [b]Nd = Specimens tested but no Cry1Ac protein detected at a standard detection limit of 0.5 ppb.

Table 6.2. Body weight changes, number of *Spodoptera exigua* larvae (BAW) killed and estimated fresh weight of BAW consumed by individual predators caged on Bt- or non-Bt cotton plants under greenhouse conditions (mean ± SD: 27.1 ± 4°C and ~14h of light).

Predator	Cotton	Weight gain (mg)[a]	BAW killed (no.)[b]	Prey consumed (mg)[c]
P. maculiventris	Bt	24.9 ± 3.49	10.1 ± 1.28	101.3 ± 14.96
	Non-Bt	27.2 ± 5.51	5.7 ± 0.49	104.3 ± 12.03
		t = -0.17, p = 0.8660	t = 3.28, p = 0.0053	t = -0.61, p = 0.5522
N. roseipennis	Bt	1.39 ± 0.21	6.1 ± 1.41	3.18 ± 0.70
	Non-Bt	1.28 ± 0.36	5.9 ± 0.81	2.65 ± 0.65
		t = 0.96, p = 0.3530	t = -0.19, p = 0.8485	t = 0.56, p = 0.5853
G. punctipes	Bt	0.83 ± 0.14	8.0 ± 0.81	1.49 ± 0.16
	Non-Bt	0.64 ± 0.29	6.7 ± 0.71	1.84 ± 0.18
		t = 0.83, p = 0.4321	t = 1.02, p = 0.3213	t = -1.62, p = 0.1155
O. insidiosus	Bt	0.0417 ± 0.011	3.4 ± 0.37	0.30 ± 0.03
	Non-Bt	0.0200 ± 0.015	2.9 ± 0.31	0.38 ± 0.04
		t = 1.27, p = 0.2954	t = 0.94, p = 0.3601	t = -1.57, p = 0.1356

[a] Difference between individual predator weight before and after caging with prey on respective cotton types.

[b] Average number of *Spodoptera exigua* larvae killed by individual predators. BAW larvae were offered to predators at different ages (*P. maculiventris* = 9 d-old; *N. roseipennis* = 3 d-old; *G. punctipes* and *O. insidiosus* = 1 d-old).

[c] Fresh weight of prey material consumed per individual predator, considering the number of prey killed and predator weight change during the exposure period.

CHAPTER 7

NEITHER PREY FED BT-COTTON NOR BT-COTTON PLANTS AFFECT THE OMNIVOROUS PREDATOR BIG-EYED BUG *GEOCORIS PUNCTIPES* (SAY) IN THE FIELD[1]

[1]Torres, J.B. and J.R. Ruberson. To be submitted to *Oecologia.*

Abstract Continuous expression of *Bacillus thuringiensis* (Bt) toxin in Bt-transgenic cotton affords frequent contact of Bt toxin with omnivorous predators, such as the big-eyed bug *Geocoris punctipes* (Say), which is an important predator of pests in cotton fields, through prey fed Bt-cotton or possibly through plant feeding. To assess the significance of this risk, the relative impact of Bt-cotton plants and prey fed Bt-cotton on development and reproduction of this omnivorous predator was studied in the field. We used two prey types to assess direct plant- and indirect prey-mediated effects on the predator: 1) larvae of the beet armyworm, *Spodoptera exigua* (BAW), were used as low-quality, herbivorous prey capable of conveying Bt-toxin to the third trophic level, and 2) eggs of the corn earworm, *Helicoverpa zea* (CEW), a high quality prey incapable of transferring toxin across trophic levels. The experiment was conducted during 2003 and 2004 beginning with newly-hatched big-eyed bug nymphs and ending when the last female died. The combination of prey and Bt-cotton plants did not exert interactive effects on development and reproduction of the predator. The prey effect was independent of host plants. Delayed development and smaller adults with no difference between cotton genotypes were observed for nymphs fed BAW larvae. Reproductive output and longevity were similar between cottons for both prey types, and were consistently lower for predators fed BAW larvae. Cry1Ac was detected in Bt-cotton, BAW larvae fed Bt-cotton offered to the predators, but not in the predators. The results do not indicate any lethal or sublethal effect of transgenic Bt-cotton or of Cry1Ac conveyed through prey on development and reproduction of *G. punctipes* in the field.

Keywords Predatory heteropteran, transgenic Bt plants, nontarget impact, Cry1Ac, phytophagy.

INTRODUCTION

Insecticide-transgenic plants have become important components of maize and cotton production in various areas of the world (James 2004). These plants have been modified with genes from other species (in most cases from the bacterium *Bacillus thuringiensis* Berliner; hereafter referred to as Bt) that constitutively express toxins. In crop systems where such plants are used, omnivorous generalist predators are exposed to natural and transgenic plant defenses through direct feeding on plants and via herbivorous prey. Numerous tritrophic studies have elucidated host plant traits that may affect the life histories of natural enemies when implemented in pest management programs (Hagen 1986, Bottrell et al. 1998). The widespread adoption of plants expressing transgenic toxins adds another element of complexity to the multitrophic interactions in agroecosystems that may exert a variety of effects (Obrycki et al. 2004). There are indications of some insecticide-transgenic plants exerting at least indirect adverse effects on omnivorous predators (Bouchard et al. 2003, Bell et al. 2003). Given the high dose strategy being used in the Bt-transgenic plants, the season-long constitutive expression of the toxins, and the dual routes of exposure to omnivores, a significant concern is whether Bt-transgenic plants exert negative effects on the life histories of important omnivorous predators.

Hilbeck et al. (1999) observed that chrysopid larvae fed lepidopteran larvae reared on meridic diet treated with various Cry proteins suffered higher mortality and reduced body weights that were correlated with toxin dosage in the diet. However, in studies with the same chrysopid species, Romeis et al. (2004) fed toxin directly to the predators and found no effect on immature development or survival. They further demonstrated that prey quality significantly affected the predator's development and survival, whereas the Bt toxin (Cry1Ab) itself did not. Similarly, other laboratory studies have indicated that there are no short-term indirect effects of

prey fed Bt-plants on some predatory heteropterans (Pilcher et al. 1997, Zwahlen et al. 2000, Al-Deeb et al. 2001, Bernal et al. 2002). However, there are no long-term studies of the effects of Bt toxins on life histories of omnivorous predators that account for prey quality, direct and indirect routes of exposure, and the effects of field conditions, all of which are highly relevant to understanding environmental impacts of insecticide-transgenic plants. Our study was designed to address the question of direct and indirect effects of Bt-transgenic cotton on an important omnivorous predator, *Geocoris punctipes* (Say), under field conditions, accounting for effects of prey quality and multiple routes of exposure, as well as environment.

Omnivorous predators are able to use food resources from different trophic levels, which buffers them against the unpredictability of spatial and temporal occurrence of food resources in patchy environments (Pimm and Lawton 1978, Coll and Guershon 2002). The ability to switch among prey and to persist on plant material allows omnivorous predator populations to persist under conditions inimical to specialized entomophages, and to be present when alternative prey begin colonizing a habitat and occur at low densities. These attributes may be particularly valuable for predators in ephemeral agricultural systems, where prey resources can be quite variable (Coll and Guershon 2002). Generalists (including omnivores) tend to be most effective at maintaining incipient pest populations at low levels, but do not necessarily exhibit numerical responses to particular prey, as is the case with specialists (Whitcomb and Godfrey 1991, Symondson et al. 2002). This can be exemplified in cotton fields with the omnivorous generalist predator big-eyed bug *G. punctipes* that feeds on a diverse prey suite varying in quality (Crocker and Whitcomb 1980) and use host plants as a food supplement (Stoner 1970, Naranjo and Stimac 1985). Three years of field data show that *G. punctipes* dynamics do not correlate with populations of high-quality prey (heliothine eggs) in cotton fields (Torres 2005). *G. punctipes*

populations increase steadily as the cotton season progresses, regardless of presence of high-quality prey. Big-eyed bugs also clearly preferred plant terminals and young leaves -- soft plant tissues that could serve as ready food supplements for nymphs -- for oviposition, indicating the importance of cotton plants in the ecology of these predators.

Despite its potential advantages, omnivory also brings with it the risk of exposure to a wide range of suitability in dietary constituents that may include detrimental components (Bozer et al. 1996, Weiser and Stamp 1998). Thus, the fitness of omnivores can vary widely depending on habitat and available prey and plant resources (Coll 1998). Plants can exert effects on omnivorous predators directly and indirectly through prey (Coll and Guershon 2002). There is a large body of literature addressing the direct effects of plant feeding on life histories of omnivorous heteropteran predators (e.g., chapters in Alomar and Wiedenmann 1996). Prey-mediated effects of plant allelochemicals also have been reported (Orr and Boethel 1986, Bozer et al 1996, Weiser and Stamp 1998). Thus, omnivorous predators are exposed to plant defensive compounds via two routes, which may intensify pressure on these species.

To examine the effects of Bt-cotton plants and prey quality interactions on life-history parameters of the omnivorous predator, the big-eyed bug *G. punctipes*, we conducted experiments over two growing seasons using bugs caged on plants in the field. We used a combination of Bt and non-Bt cotton plants, and of prey, with one prey treatment capable of conveying Bt Cry1Ac toxin to upper trophic levels [*Spodoptera exigua* (Hübner) larvae] and the other prey item incapable of transferring toxin [*Helicoverpa zea* (Boddie) eggs]. This design allowed us to differentiate direct and indirect effects of the toxin in the host plant from effects attributable to prey quality under field conditions.

Study system

Cotton is a key crop in a number of countries, and the adoption of Bt-transgenic cotton varieties has been extensive (James 2004). In the United States, approximately 50% (www.ams.usda.gov/cotton/mncs/index.htm) of the total cotton production is planted with Bt-transgenic varieties. All of the Bt-transgenic varieties currently in use express the Cry1Ac toxin, which is specific for lepidopteran larvae, although its efficacy varies with species (Stewart et al. 2001). Caterpillars that are partially susceptible to Bt toxins exhibit delayed development and smaller size (Jenkins et al. 1993; Mascarenhas and Luttrell 1997), and are more readily predated (Roush 1996). An example of a lepidopteran that is only partially susceptible to Cry1Ac is the beet armyworm, *S. exigua*, a noctuid pest of cotton and other crops in North America. The beet armyworm also is typically controlled in cotton in the southeastern US by the activity of natural enemies (Ruberson et al. 1994). The partial susceptibility of this species, coupled with its importance in cotton and the important role of predators in suppressing it, make it an ideal and relevant organism for testing the transfer of Bt toxin across trophic levels in a crop system.

Geocoris punctipes is an important omnivorous predator in the eastern half of the US Cotton Belt. The efficacy of this predator against various pests has been documented (Lingren et al. 1968, Ali and Watson 1982, Ruberson et al. 1994). However, prey vary considerably in their suitability for predator development, survival, and reproduction – lepidopteran eggs are typically much more suitable than are lepidopteran larvae and aphids (Dunbar and Bacon 1972a, Lawrence and Watson 1979, Cohen & Debolt 1983, Eubanks and Denno 2000, Torres et al. 2004). *Geocoris punctipes* also feeds frequently directly on plants (Stoner 1970, Tillman and Mullinix 2003), and can acquire direct benefits from phytophagy (Naranjo and Stimac 1985), but also can be affected when feeding on plant exhibiting herbivore resistance (Roger and Sullivan

1986). The broad prey range of *G. punctipes* and its frequent phytophagous behavior can place this predator in indirect contact with Bt-toxins through contaminated prey, and its active plant-feeding behavior may provide direct contact to toxins in plants. Nymphal stages of *G. punctipes* are adversely affected when fed prey contaminated with a commercial Bt formulation (Herbert and Harper 1986), whereas adults were not so strongly affected. Thus, there is potential for the Bt cotton to adversely affect this important predator.

MATERIAL AND METHODS

Field description

Two parallel experimental plots of cotton, 20 rows wide and 85 m long, were planted at the Coastal Plain Experiment Station (CPES), Tifton, GA. Two varieties of cotton were used in the study: non-Bt cotton variety DPL 5690 and transgenic Bt-cotton variety DPL 458. Field plots were planted on 8 May 2003 and on 2 June 2004. All plots were treated with aldicarb to suppress thrips (Temik 560 g a.i./ha) at planting. Temperature was monitored using a WatchDog logger (Spectrum™ Technologies, Inc.) stored inside the cages holding nymphs and prey (see below) set to record at 30-min intervals, and rainfall data were obtained from a local weather station of the Coastal Plain Experiment Station (www.georgiaweather.net).

Prey types

Two different prey types were used in the study to permit differentiation of the effects of prey quality and of Bt toxin. The first prey type, first-instar larvae of the beet armyworm, *S. exigua* (hereafter BAW), is able to survive ingestion Cry1Ac toxin (Stewart et al. 2001) and can expose predators indirectly to the toxin. This prey also is a relatively low-quality prey for *Geocoris* spp. (Torres et al. 2004). The second prey type was eggs of the corn earworm, *Helicoverpa zea* (hereafter CEW), which do not expose the predator indirectly to Cry1Ac toxin,

and which are high-quality food for *Geocoris* spp. The beet armyworm neonate larvae (<24h after hatching) were produced in the Biological Control Laboratory of the CPES and corn earworm eggs were provided by USDA-ARS-CPMRL, Tifton, GA. The prey were offered *ad libitum* to the predators and were changed at 2-d intervals.

Newly-hatched nymphs (<24h) of *G. punctipes* were caged on Bt and non-Bt-cotton plants in the field plots on 17 June 2003 (32-d-old plants). The nymphs were caged in organdy bags (~30 cm long by 15 cm wide) with 5 nymphs per bag (15 replicates), for a total of 75 nymphs per treatment. The bags containing predator nymphs and prey were tied to the leaf petiole enclosing the uppermost fully-expanded cotton leaf. At the beginning of the experiment, bags containing prey and predators were attached to the plants and supported with bamboo rods because the plants were not strong enough to hold the bags unaided, especially under wind and rain conditions. The experiment consisted of two host plant types (Bt and non-Bt cotton) and two prey types (BAW and CEW) arranged in a 2x2 design. Nymphal developmental times, mortality, and adult weight at emergence were monitored. Adults were paired on the day of emergence and maintained under the same treatments experienced as nymphs. Pairs were held individually in 500-ml styrofoam cups with the bottoms removed and wrapped in knee-high stretch hose. The cages enclosed one leaf each and were tied to the cotton leaf petioles. To facilitate location and counting of eggs in the cages, a small square of cotton batting (~1cm^2) was inserted in each cage as an oviposition substrate; in practice, eggs were also laid on the cup wall, knee-high stretch hose and on the cotton leaves. Eggs were collected and prey replaced every other day. Eggs were counted using a 10x magnifying lamp, and the eggs were subsequently incubated in plastic cups with a piece of green bean pod to determine egg viability.

The same treatments and procedures used in 2003 were repeated in 2004, except that the timing of nymphal placement in the cages coincided with the appearance of nymphs in feral populations in the field (i.e., 22 June, parallel survey of bugs in the plots). To avoid possible effects of plant age and phenology, cotton planting in 2004 was determined based on predator dynamics from previous studies to roughly produce plants at same age used in the previous year (28-d old plants). Newly-hatched (<24h) big-eyed bug nymphs obtained from the laboratory colony were caged on Bt and non-Bt cotton plants in the field on 30 June 2004. As in 2003, nymphs were caged in organdy bags with five nymphs per cage (15 replicates), and a total of 75 nymphs per treatment. The prey used, cage types, and data collection followed the same procedures used in 2003.

During the week that caged nymphs became adults on both cotton plants and prey (i.e., 1st week of August), feral adults from the Bt and non-Bt cotton fields were collected. The collection was carried out with drop cloths (1-m long white canvas cloth laid on the ground between two cotton rows, and plants on the adjacent rows are shaken vigorously over the cloth). Adults that fell on the cloth were collected. Twenty females and 20 males were collected in each cotton type and taken to the laboratory where they were weighed, and subsequently released in their plots of origin. The weight of feral adults that developed on available prey in the respective cotton types was compared to the weight of adults that had developed in the cages of the various treatments.

Toxin (Cry1Ac) in trophic levels of cotton ecosystem in 2004

To verify exposure of Cry1Ac to predators, materials representing the three trophic levels (plant, prey and predators) were assayed for Cry1Ac using enzyme-linked immunosorbent assay (ELISA). Cotton leaves and unconsumed BAW (2-3 days old) inside cages were collected and frozen from 2 July to 22 September 2004 covering the period of the predators' nymphal

development and the peak of their adult reproduction. Predator adults assayed consisted of 14-15 males remaining from pairs in which the female died prior to 30 September (14 in Bt-CEW, and 15 in Bt-BAW). The materials were assayed to determine the levels of Cry1Ac toxin. The cotton leaf sample was collected by folding the cotton leaf along the main vein and pressing the lid of a microcentrifuge tube through the two leaf layers, and samples were taken twice a week from Bt-cotton leaves caged with predators and BAW or CEW.

All frozen materials were thawed and weighed in a 1.5-ml centrifuge tube and mixed with phosphate-buffer saline solution in Tween20 (1xPBST) (Agdia® Inc., Elkhart, IN). Non-fat dried milk (0.4% w/v) and Tween20 (0.5% v/v) were added to PBST to compose the final extraction buffer, which was mixed with sample material at a rate of 1:10 (w/v). The toxin levels in the samples were assayed using antibody-coated wells in PathoScreen® plates for Bt-Cry1Ac/Cry1Ab, part of an ELISA kit using peroxidase enzyme conjugate (Agdia® Inc., Elkhart, IN). Standards of Cry1Ac at concentrations 0.625, 1.25, 2.5, 5, 10, 20 and 40 ng/ml (ppb) were used to build a standard optical density curve for estimating protein content of sampled material.

Statistical analysis

Nymphal survival was rated per cage (n = 5 nymphs in each cage) from a total of 15 cages, except in cases where cages were lost to damage or other factors. Big-eyed bug nymphal development time, survival, weights of newly-emerged adults, and adult reproductive parameters were submitted to a normality test (Kolmogorov-D:Normal test, Proc Univariate of SAS; SAS 1999-2001) and square-root (x + 0.5) transformed when needed to meet assumptions of analysis of variance (ANOVA). Because the variables required transformations due to the skewed distributions, the means are accompanied by confidence intervals rather than standard errors or deviations (Sokal and Rohlf 1995). The effect of prey (BAW and CEW) and plants (Bt and non-

Bt) were analyzed with 3-way ANOVA with prey, cotton, and year as main fixed factors, and the model was further reduced to two- or one-way ANOVA when appropriate. All analyses were performed using the Proc GLM of SAS (SAS 1999-2001) and significant treatment means were compared using the Tukey HSD test. Further, a retrospective power analysis for a 3-way ANOVA of major predator life history parameters (nymphal survival and developmental time, female longevity, and number of eggs produced) was conducted to detect an effect corresponding to a 20% difference between treatments (Marvier 2002). This analysis was conducted to avoid accepting a false null hypothesis of no difference between treatments having Bt and non-Bt cotton as main and fixed effect (Sahai and Ageel 2000). For all analyses, the effect size is given by *d* and defines the absolute difference (untransformed value) between treatments in the parameter of interest, determined using the within-population standard deviation.

RESULTS

Cry1Ac toxin in cotton plants and prey in 2004

Average levels of Cry1Ac toxin in cotton leaves from cages holding nymphs from 2 July to the first week of August and from the first week of August to 30 September (adult reproductive peak) were (mean ± SD) 0.23±0.04 and 0.25±0.03 μg Cry1Ac g^{-1} of fresh tissue, respectively. From this original amount of toxin expressed in Bt-cotton leaves nearly 81 and 76% was exposed to the predator nymphs and adults through the BAW larvae (0.18±0.03 and 0.19±0.02 μg Cry1Ac g^{-1} of fresh weight). Despite the amount of Cry1Ac toxin detected in the plants and prey, and directly and indirectly exposed to the big-eyed bug nymphs and adults, no toxin was detected in the bodies of adult predators.

Predator nymphal survival and development

The survival of big-eyed bugs throughout their preimaginal stages was variable within each treatment, ranging from 0 to 100% per cage (Table 7.1). In addition, 12 cages were lost (from 0 to 3 cages out of 15 cages per treatment) due to ant attack (mainly *Solenopsis invicta* Buren) among all treatments during the two seasons, and these replications were dropped from analyses. A 3-way ANOVA (cotton type, year, and prey type as main factors) indicated no differences in nymphal survival (mean ± 95% CI) between Bt- (44.8±7.36) and non-Bt cotton (46.7±7.7), with cotton as main effect ($F_{1, 100}$=0.14; P=0.7063, d =5.19; Power =0.8712), or between prey ($F_{1, 100}$=1.66; P=0.2913). However, nymphal survival varied significantly between years ($F_{1, 100}$=5.16; P=0.0252). On average, nymphal survival was lower in 2004 (mean ± SE = 40.7±3.71%, n = 57 cages with initial number of 285 nymphs) compared to 2003 (mean ± SE = 51.2±3.27%, n = 51 cages with initial number of 255 nymphs) across all treatments, but nymphs survived equally among treatments (Table 7.1) regardless of prey or cotton types used in 2003 (2-way ANOVA, $F_{1, 47}$ = 0.44; d =12.75; Power =0.9112) and 2004 (2-way ANOVA, $F_{1, 53}$=0.04; d =2.86; Power =0.9612).

Newly-hatched nymphs of big-eyed bugs caged either on Bt or non-Bt cotton exhibited similar developmental times, survival, sex ratio, and adult weight. On the other hand, prey type significantly affected nymphal developmental time, with nymphs fed BAW larvae requiring 3-7 additional days to reach the adult stage and weighing significantly less than nymphs fed CEW eggs (Table 7.1). From a complete model including cotton, year, gender, and prey as main factors, gender was dropped out after the first ANOVA run because it was not significant (gender, $F_{1, 280}$=0.41; P=0.5230). Among the remaining main factors, cotton type was not significant ($F_{1, 280}$=0.08; P=0.7762) but this factor was maintained in further analysis because it

significantly interacted with year. Then, using a 3-way ANOVA, nymphal developmental time (mean days ± 95% CI) did not differ between Bt (27.7±0.54) and non-Bt cotton (27.8±0.57) ($F_{1, 288}$=0.52; P=0.4748; d = 7.34; Power =1), but it varied as a function of year ($F_{1, 288}$=25.74; P<0.0001) and prey type ($F_{1, 288}$=254.24; P<0.0001). Nymphal development was 2 d faster in 2004 compared to 2003, and about 5 d faster when bugs were fed CEW eggs than BAW larvae. However, these differences were unaffected by being caged on either Bt or non-Bt cotton. The first level of interaction between year and prey was significant ($F_{1, 288}$=10.78; P=0.0016) with nymphs fed CEW eggs developing faster in 2004 than in 2003, but similar developmental times were observed for nymphs fed BAW larvae in both years.

The weight of newly-emerged adult predators varied as expected for gender (Male vs. Female, $F_{1, 280}$=405.63; P<0.0001), with males smaller than females (Table 7.1). Prey type also significantly affected adult weight (BAW vs. CEW, $F_{1, 280}$=183.5; P< 0.0001), as did year (2003 vs. 2004, $F_{1, 280}$= 8.15; P< 0.0001), and only the interactions between prey type and gender ($F_{1, 288}$=56.36; P<0.0001), and between year and gender ($F_{1, 288}$=4.81; P=0.0291) were also significant. Independent of gender, nymphs fed CEW eggs produced larger adults than those fed BAW larvae, and females fed CEW eggs were larger in 2003 than in 2004 (Table 7.1), but no effect of being caged on Bt-cotton was observed for either prey type [means for Bt = 3.5 mg and for non-Bt cotton = 3.6 mg ($F_{1, 107}$=1.09; P=0.2264)] (Table 7.1).

Adult weight for females and males caged on plants and fed exclusively with BAW larvae or CEW eggs and weights of feral adults (n = 20 adults for each gender per cotton type), collected in the same plots in the field feeding on any available prey, showed no significant effect of cotton genotype ($F_{1, 181}$=1.87; P=0.2731). No significant interaction was found between cotton type and gender ($F_{1, 181}$=1.61; P=0.2931), or cotton type and prey ($F_{1, 181}$=1.72;

P=0.1812). However, adult weight was significantly affected by gender as a main effect ($F_{1, 181}$=332.00; P<0.0001), and by prey type ($F_{2, 181}$=36.37; P<0.0001), and there was a significant interaction between prey and gender ($F_{2, 181}$=15.95; P<0.0001). The result indicated that adults reared on BAW larvae were smaller than those fed either CEW eggs or feral adults that consumed available prey in the field (Fig. 7.2). Also, nymphs fed CEW or feral bugs that consumed available prey in the field yielded larger females than nymphs reared on BAW larvae on both cotton types. However, adult males and males reared on BAW larvae in cages were smaller than adults reared on CEW eggs with both cotton types (Fig. 7.2).

Adult reproduction and longevity

All females (sample size specified in Table 7.2) produced viable eggs both years. However, of the 15 paired females per treatment in 2003, some were lost due to ant attacks inside the cages (mainly *S. invicta*). In 2004, only 12 females were paired in the BAW larvae treatments (Bt and non-Bt) and 14 females from CEW eggs in non-Bt cotton due to more variable nymphal survival. However, further reduction in sample size occurred due to ant attack or female escape. Egg hatching ranged from 62.4 to 92.8%. Reductions in hatching were due to various factors, one of which was the egg parasitoid, *Telenomus reynoldsi* Gordh & Coker. For both years and across plant/prey combinations, egg parasitism by *T. reynoldsi* ranged from 1.2% (14/1161 eggs from non-Bt cotton and BAW prey treatment) to 2.1% (75/3600 eggs from Bt-cotton and CEW eggs treatment).

Geocoris punctipes reared as nymphs and subsequently maintained as adults on BAW larvae or CEW eggs showed no measurable difference in number of eggs produced per female (3-way ANOVA, $df_{1, 77}$ =0.76; P =0.3863; d =35.52; Power =1) between Bt-cotton (mean ± 95% CI = 172.8±38.9) and non-Bt-cotton (159.3±36.0). Nor was female longevity affected (3-way

ANOVA, $df_{1, 77}$ =0.06; P =0.8093; d =14.89; Power =1) when compared between Bt-cotton (61.2±8.3) and non-Bt cotton (59.1±6.6). Other possible effects of cotton type with prey and year interactions were not significant (3-way ANOVA, P>0.05). Year had no significant effect on pre-oviposition periods and number of eggs per female (2-way ANOVA, P>0.05), but it had a marginal effect on female longevity ($F_{1,77}$=3.42; P=0.0684) and a highly significant effect on the duration of post-reproductive longevity ($F_{1,77}$=22.64; P<0.0001). Females fed CEW eggs lived longer -- 78.4 days in 2004 compared to 53.1 days in 2003 -- but for females fed BAW larvae there was no difference between 2003 and 2004 (55.2 vs. 47.9 days). Prey quality (BAW larvae vs. CEW eggs) was the most important factor interfering with all evaluated adult reproductive parameters (one-way ANOVA, Table 7.2) with no effects attributable to cotton type. Two-way ANOVA indicated that females fed CEW eggs initiated oviposition earlier ($F_{1,94}$=74.19; P<0.0001), produced more eggs per female ($F_{1,77}$=184.15; P<0.0001), and lived longer ($F_{1,77}$=7.40; P=0.0081) than females fed BAW larvae, regardless of which cotton they were provided (Table 7.2). An unexpected interaction was observed for prey type and year for number of eggs per female ($F_{1,77}$=9.17; P =0.0033) and female longevity ($F_{1,77}$=14.71; P =0.0003), with females fed BAW larvae producing more eggs (92.3 vs. 40.1 eggs per female) and living longer (55.3 vs. 47.9 days) during 2003 than 2004, while the opposite was observed for females fed CEW eggs (Table 7.2).

Among adult reproductive parameters, period of post-reproductive survival was longer in 2004 than in 2003. In 2004, a linear relationship was observed between the duration of post-reproductive survival and female longevity for all females reared on Bt and non-Bt cotton and feeding either on CEW eggs [y = -0.50 + 0.04 (± 0.007)x, r^2 = 0.58; F=31.71; P<0.0001)] or BAW larvae [y = -0.57 + 0.06 (± 0.01)x, r^2 = 0.69; F=34.91; P<0.0001)]. This relationship was

not significant for females in 2003 feeding on either prey. Females in 2004 that lived longer than 70 days experienced low temperatures because they were still alive later in the season than most females in 2003, which were caged earlier in that year (Fig. 7.1). For females in 2004 living less than 70 days (i.e., dying before November), post-reproductive longevity was short and similar to females in 2003. In contrast, those females surviving until November 2004 died up to 30 days after stopping oviposition.

DISCUSSION

Although it was apparent that Bt toxin passed from the plant to the herbivore-prey, there was no significant effect on the life history of *G. punctipes* attributable to Bt toxin. The most evident changes in nymphal development and adult reproduction of *G. punctipes* occurred when predators were reared on different prey types, with lower performance for those bugs feeding on BAW larvae compared to CEW eggs, regardless of cotton genotype. Variations in big-eyed bug life history due to the use of lepidopteran larvae or eggs as prey have been previously reported (Dunbar & Bacon 1972a, Lawrence & Watson 1979, Cohen & Debolt 1983, Torres et al. 2004). Therefore, there is no evidence of negative effect from Bt-BAW reared larvae or from direct feeding on Bt-cotton for this omnivorous predator. BAW larvae are only partially susceptible to the Cry1Ac toxin (Stewart et al. 2001), and are thus able to acquire the toxin from the plant and expose it to predators. Therefore, nymphs and adults of *G. punctipes* in the BAW treatment with Bt-cotton were exposed to Cry1Ac toxin through moribund or relatively healthy larvae containing the toxin. Nevertheless, despite consuming relatively large numbers of larvae containing Cry1Ac toxin throughout their lifetimes, development and reproduction of *G. punctipes* was unaffected by the presence of toxin in the prey.

No Cry1Ac toxin was detected in *G. punctipes* adults provided CEW eggs on Bt-cotton plants or in predators fed Bt-reared BAW larvae on Bt-cotton plants. This result was corroborated by another greenhouse study examining the acquisition of Cry1Ac from plants and prey by heteropteran predators (Torres 2005). In those studies the toxin was detected only in the largest predatory heteropteran, the pentatomid *Podisus maculiventris* (Say), and none was detected in *G. punctipes* (Torres 2005). In another study we demonstrated that *G. punctipes* is able to ingest purified Cry1Ac toxin that is detectable only above concentrations of 4 ppm (Torres et al. in preparation). These results clearly suggest that although *G. punctipes* can ingest Cry1Ac toxin, it does not acquire sufficient toxin from prey or from direct plant feeding to be detectable by ELISA. This result agrees with results reported for *Nabis* sp., *Geocoris* sp., *O. tristicolor* and *L. hesperus* confined on Bt-potato foliage and deprived of prey (Armer et al. 2000). The lack of effects of Cry1Ac for *G. punctipes* when provided Bt-fed BAW larvae may be due to removal of toxin from the predator's body. Although Cry1Ac can be ingested by *G. punctipes* from purified Cry1Ac-water concentrations higher than 4 ppm, we found that the toxin was largely eliminated through feces (CHAPTER 6) and not detectable 72 h after feeding either in the predators' bodies or in their feces.

Survival variability was certainly influenced by the variable environmental conditions during the experimental periods (Fig. 7.1). The year of the study (2003 or 2004) significantly affected nymphal survival across all treatments, and female longevity and post-reproductive survival for females fed CEW eggs. However, no interactions were observed between years and cotton types. Reduced nymphal survival in 2004 may have resulted from many causes, but temperature and moisture play significant roles in development and survival of insects in the field. Nymphal survival was low in the first and second instars, when dehydration risk would be

the greatest (data not shown). Rainfall during the period from placement of neonate predators in cages until the last nymph molted to the adult stage was 211.1 mm in 2003, compared to only 68.6 mm in 2004. There also were more hours of high temperatures in 2004 than in 2003 (average of 38.1°C for maximum temperature during the period compared to 35°C in 2003; Fig. 7.1). Although capable of tolerating relatively high temperatures, *G. punctipes* requires a reliable source of free water because the predators have low resistance to water loss (Cohen 1982).

Caging nymphs 13 days later in 2004 accelerated nymphal development ($F_{1, 294}$=13.0; P=0.0004) (28.3 days in 2003 vs. 26.9 days in 2004) in all four treatments. More rapid preimaginal development in 2004 can be explained by accumulated thermal units during the nymphal period. Considering the period during which preimaginal development occurred in both seasons, and using an estimated lower developmental threshold for nymphs of 13.3°C (estimated from Dunbar & Bacon 1972b), nymphs were exposed to similar degree-days (DD; 336°C in 2003 vs. 343.3°C in 2004). The DD result indicates that nymphs in 2004 were exposed to sufficient thermal units in a shorter period to complete development compared to 2003. This accelerated nymphal development, however, had a slight cost in adult weight for bugs developing more rapidly, with larger adults in 2003 than in 2004 (1-way ANOVA, $F_{1, 294}$=8.61, P=0.0036) across all treatments independent of gender, prey, and cotton type (Table 7.1).

Although body weight sometimes correlates with female fecundity in predatory heteropterans (Honek 1993), the variation in body weight was not enough to produce significant correlations (P>0.05) between number of eggs per female or weight and female longevity in our study. Independent of weight and within prey types, females fed high-quality prey (CEW eggs) in 2004 tended to be more fecund than in 2003, which is due to greater oviposition immediately after adult emergence compared to females in 2003 (Fig. 7.3). Indeed, increased longevity of

females fed CEW eggs in 2004 may explain the greater fecundity, because number of eggs per female correlated significantly and positively with female longevity reared either on BAW (r=0.19, *P*=0.006) or on CEW (r=0.49, *P*<0.0001) independent of cotton genotype.

Since the predator diet (prey and plant) was similar between seasons, it appears that the temperature decline during November (Fig. 7.1) was the major factor affecting the duration of the post-reproductive period. The accumulated DD over the reproductive temperature threshold (18^{o}C, Davis 1981) during the adult period was quite similar (2003 = 651.1 and, 2004 = 645.0^{o}C), although the last surviving females in 2003 and 2004 died on 10 and 28 November, respectively. The average extended period of living females in 2004, however, exposed them to temperatures below that favorable for reproduction, but not low enough to cause mortality, resulting in longer post-reproductive survival of females in 2004 compared 2003.

The results reported here with close control of prey and plant types available to predators, in combination with natural environmental variability in the fields, support the data from several field surveys of predators in Bt and non-Bt cotton that found no effect of Bt-cotton on populations of predatory heteropterans, including *G. punctipes* (Flint et al. 1995, Lutrell et al. 1995, Armstrong et al. 2000, Hagerty et al. 2005, Torres & Ruberson 2005). The present study further demonstrates that there is no measurable life-history impact of Cry1Ac at the individual level. The ability of predators to compensate for variable prey was strongly indicated by the size of feral predators collected in our experimental Bt and non-Bt cotton fields (Fig. 7.2). The quality of food resources available to preimaginal predators can be indirectly assessed by comparing the size of field-collected females with experimental ones. In this instance, feral females were significantly larger than experimental females reared on BAW larvae, and similar in size to those fed CEW eggs (Fig. 7.2), a high-quality prey for big-eyed bugs. The absence of a

treatment without prey, as is often used in evaluations of host plant effects on zoophytophagous predators, was not adopted in our study because in the field *G. punctipes* can feed on a wide variety of prey, and the cotton ecosystem usually supports a diverse fauna of potential prey.

The Bt-cotton plants and Bt-fed prey did not interact to produce measurable effects on the life history of the omnivorous, predatory big-eyed bug *G. punctipes*, regardless of prey quality. Nor have any effects on populations of *G. punctipes* been observed in Bt-cotton. The diversity of prey typically available in cotton ecosystems probably compensates readily for reductions in the numbers of caterpillars that are targeted by Cry1Ac toxin. Also, the field densities of lepidopteran eggs, the bollworm stage preferred by big-eyed bugs, are not directly affected by Bt-cotton (CHAPTER 5). Indeed, field-collected big-eyed bug females were similar in weight to bugs caged under the same field conditions and fed abundant, high-quality prey (CEW eggs) (Fig. 7.2). Considering the detrimental impact on predatory heteropterans of broad-spectrum insecticides used to manage bollworms infestations in non-Bt cotton (Eveleens et al. 1973, Naranjo et al. 2003, Hagerty et al. 2005), the use of Bt-cotton seems to be a suitable strategy for conserving big-eyed bugs in cotton ecosystems to help manage pest populations not targeted by Bt-cotton, as has been demonstrated with commercial formulations of Bt (Ali & Watson 1982). This is important because, although Bt transgenic cotton has provided excellent control of the tobacco budworm [(*Heliothis virescens* (Fabr.)], bollworms (*H. zea*) and some other lepidopterans can exceed economic thresholds in Bt cotton fields when predatory heteropterans are disrupted with use of broad-spectrum insecticides (Hagerty et al. 2005).

Based on our results, we reject the hypothesis that modified Bt-cotton expressing Cry1Ac toxin, and other physiological changes to insect resistance traits induced by the Bt toxin (Zhang et al. 1999), affect the life history of the omnivorous predatory heteropteran *G. punctipes*.

Although the predators were exposed to plant and prey tissues that contained significant levels of Bt toxins throughout the course of their lives, no adverse effects attributable to the Bt toxins were detected. Thus, the Cry1Ac toxin does not present direct or indirect risks to this important omnivorous predator.

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Table 7.1 Survival (%), development from newly hatched nymphs to adult emergence (days), fresh body weight (mg), and sex ratio of *Geocoris punctipes* reared on *Helicoverpa zea* eggs (CEW) or *Spodoptera exigua* first instar larvae (BAW). Predators were caged on Bt or non-Bt cotton plants in the field during the 2003 and 2004 cotton seasons.

Cotton	Prey	Survival (95% CI)[a]	Days to adult emergence (95% CI)	Body Weight (95% CI)		Sex ratio
				♀	♂	
Season 2003 – nymphs caged on 17 June (plants 32- d old)						
Bt	CEW	45.9 (30.1 - 63.0)	27.2 b[b] (26.7 – 27.6)	5.29 a (4.9 – 5.6)	3.16 a (2.9 – 3.4)	0.53
	BAW	51.6 (39.1 - 64.1)	29.9 a (29.2 – 30.5)	3.83 b (3.6 – 4.0)	2.90 b (2.6 – 3.2)	0.62
Non-Bt	CEW	61.3 (43.5 - 70.2)	26.4 b (25.9 – 26.8)	4.92 a (4.7 – 5.2)	3.32 a (3.1 – 3.4)	0.55
	BAW	45.2 (28.2 – 59.3)	30.5 a (29.8 – 31.1)	3.83 b (3.6 – 4.1)	2.98 b (2.7 – 3.2)	0.58
Season 2004 – nymphs caged on 30 June (plants 28-d old)						
Bt	CEW	46.6 (37.1 - 58.9)	24.3 b (23.6 – 25.0)	4.86 a (4.5 – 5.2)	3.00 a (2.8 – 3.1)	0.56
	BAW	37.3 (14.7 – 55.9)	29.1 a (27.4 – 30.8)	3.44 b (3.1 – 3.7)	2.67 b (2.4 – 2.9)	0.54
Non-Bt	CEW	36.9 (19.2 – 54.6)	23.8 b (22.8 – 24.7)	4.34 a (3.9 – 4.8)	3.18 a (2.9 – 3.4)	0.50
	BAW	42.8 (30.2 - 55.3)	30.8 a (29.6 – 32.2)	3.45 b (3.0 – 3.8)	2.88 b (2.6 – 3.1)	0.45

[a]95% confidential intervals of mean.
[b]Means followed by the same letters within column and season do not differ significantly by Tukey HSD test (P>0.05).

Table 7.2 Means (95% Confidential intervals) of reproductive characteristics of *Geocoris punctipes* fed *Helicoverpa zea* eggs (CEW) or beet armyworm (BAW) first instar larvae caged on Bt or non-Bt cotton plants in the field [temp. ^{o}C (2003, mean = 22.8, min = 6.8 and, max = 39.3); 2004, mean = 22.4 (min = -1.5 and max = 43.4) and natural photoperiod].

Year	Characteristics[a]	Bt-cotton		Non-Bt cotton		One-way ANOVA
		CEW eggs *(n=10)*	BAW larvae *(n=10)*	CEW eggs *(n=9)*	BAW larvae *(n=13)*	Statistics $[F_{df}]^{p}$
2003	Age at 1st oviposition[b]	5.6 b (5.2 – 7.0)	9 a (6.3 – 11.7)	5.1 b (4.7 – 5.5)	9.3 a (7.0 – 11.7)	$F_{3, 44}=6.48^{0.0009}$
	Nº of eggs per female	217.5 a (154.8 – 280.7)	94.9 b (80.0 – 112.5)	255.8 a (202.3 – 356.3)	89.1 b (72.1 – 106.5)	$F_{3, 39}=24.10^{<0.0001}$
	Female longevity (days)	48.7 a (34.4 – 62.9)	55 a (45.7 – 64.3)	57.6 a (46.8 – 68.4)	54.5 a (49.3 – 61.2)	$F_{3, 39}=1.22^{0.3147}$
	Post-reproductive period	1.8 a (0.4 – 3.2)	3.2 a (0.9 – 5.4)	0.8 a (0.4 – 2.0)	1.8 a (0.9 – 2.7)	$F_{3, 39}=2.09^{0.1169}$
		(n = 12)	*(n = 9)*	*n (=13)*	*(n = 8)*	
2004	Age at 1st oviposition[b]	5 b (4.3 – 5.6)	9.5 a (7.8 – 11.2)	5.4 b (4.6 – 6.1)	8.2 a (4.8 – 11.6)	$F_{3, 44}=28.41^{<0.0001}$
	Nº of eggs per female	300 a (240.4 – 359.5)	38.5 b (25.5 – 51.6)	209.2 a (145.2 – 273.2)	41.8 b (27.6 – 56.1)	$F_{3, 38}=41.49^{<0.0001}$
	Female longevity (days)	85.4 a (69.2 – 102.7)	49.7 b (32.4 – 67.1)	72 a (53.4 – 90.6)	45.8 b (32.4 – 59.4)	$F_{3, 38}=5.91^{0.0021}$
	Post-reproductive period	12 a (3.8 – 20.1)	7.5 a (0.1 – 15.3)	7 a (2.0 – 12.1)	7 a (0.6 – 14.6)	$F_{3, 38}=0.69^{0.6184}$

[a] Means followed by the same letter within rows do not differ significantly (One-way ANOVA; Tukey HSD test; $P>0.05$).

[b] Time from adult emergence to initial oviposition.

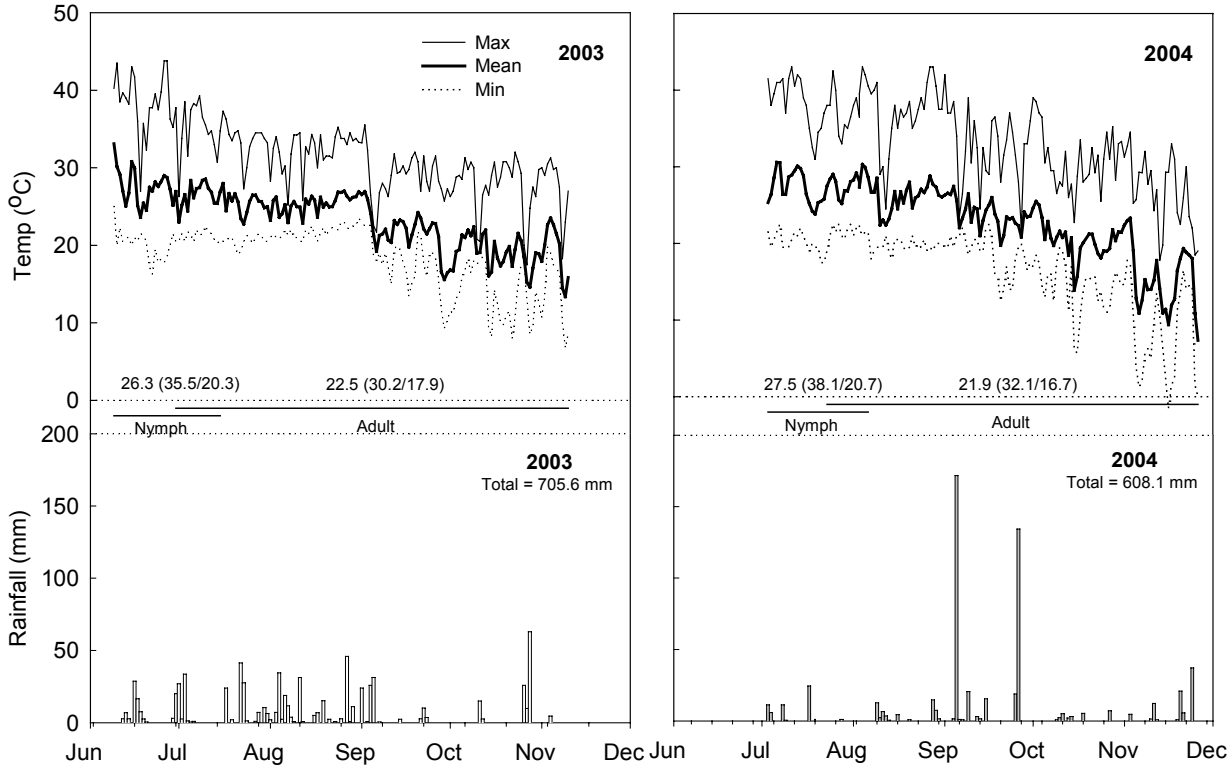

Fig. 7.1 Temperature and rainfall data measured during the experimental periods in 2003 and 2004. Horizontal lines represent the time period of nymphal and adult stages in the field, and the numbers stand for average temperature for that period and numbers inside parentheses indicate maximum and minimum average temperatures.

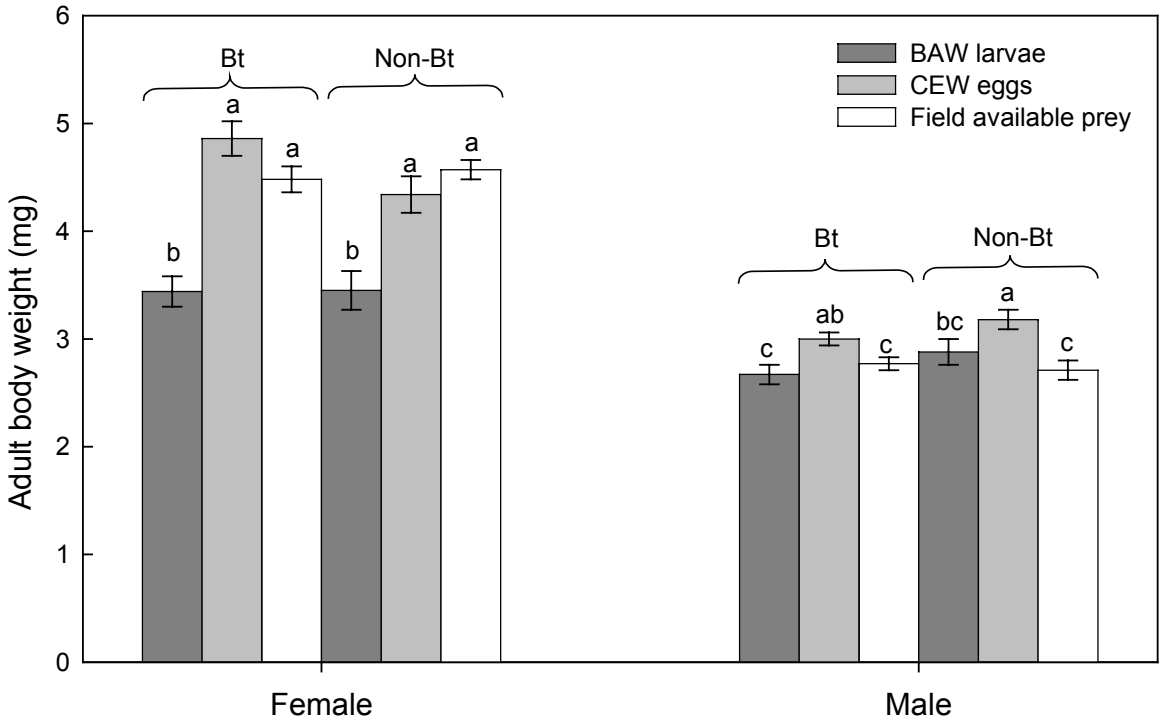

Fig. 7.2 *Geocoris punctipes* adult fresh body weight (±SE) reared in cages on Bt and non-Bt cotton plants, and fed beet armyworm neonate (BAW) larvae or corn earworm (CEW) eggs. Caged predators are compared with feral predators collected from both cotton fields, and fed on available field prey. Bars under different letters are different across all prey but within same gender by Tukey HSD test at 0.05 levels of significance.

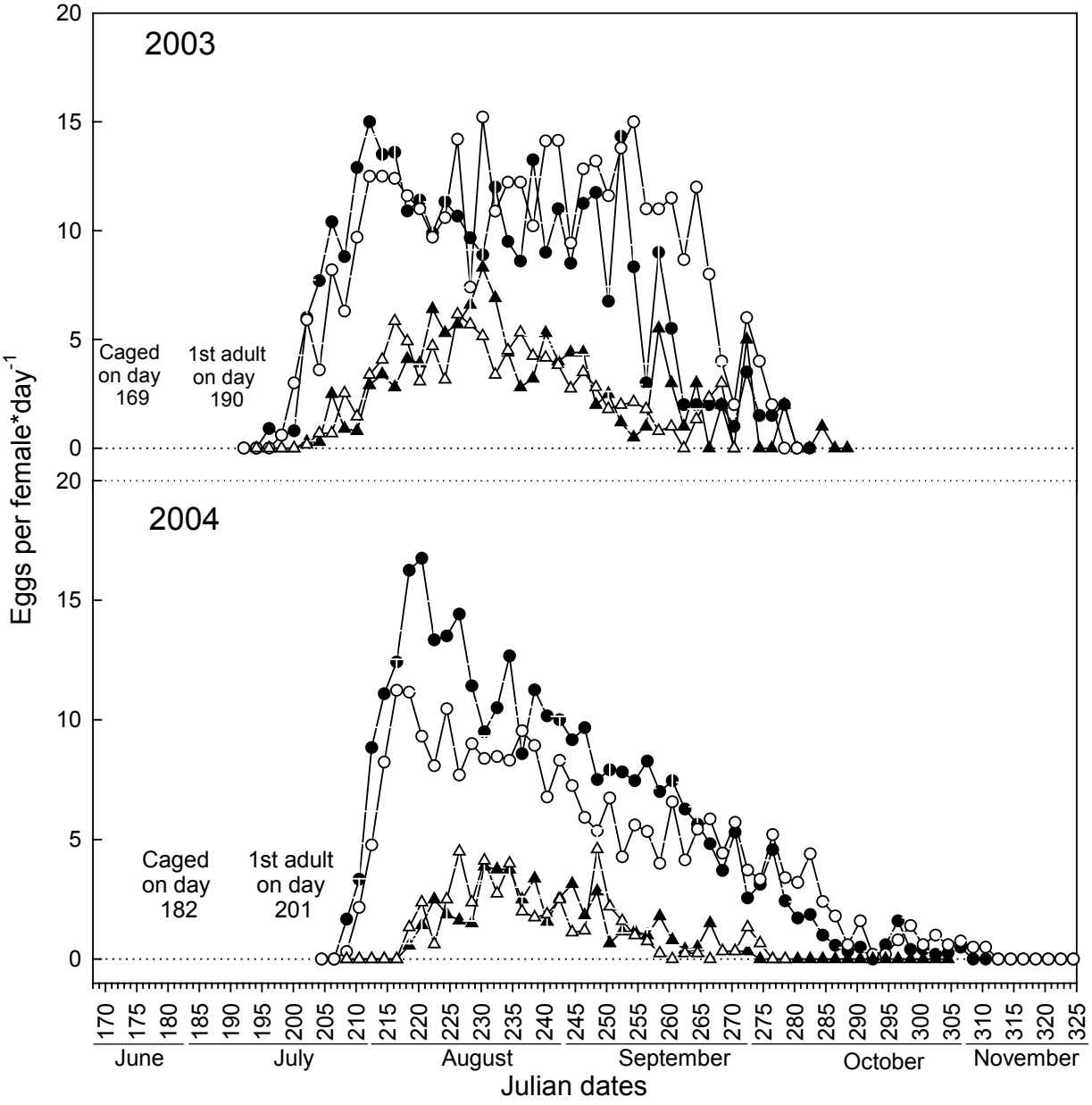

Fig. 7.3 Age-specific oviposition of *Geocoris punctipes* females fed *Spodoptera exigua* (BAW) larvae (triangle) or *Helicoverpa zea* (CEW) (circle) eggs, and caged on Bt (closed symbol) and non-Bt cotton (open symbol) during two seasons in the field.

CHAPTER 8

CONCLUSIONS

Bt transgenic plants successfully control some important lepidopteran pests of agricultural commodities in the US. For instance, the effectiveness of Bt cotton to control bollworms, a key group of cotton pests, has led to widespread adoption of this technology not only in the US but also in other regions of the world. Direct measurable economic benefits for cotton production and indirect benefits by reducing environmental contamination, for example, have contributed to the adoption of Bt cotton. The large-scale insertion of Bt-transgenic cotton in the ecosystem raised questions about potential nontarget impacts. From the perspective of cotton pest management, a major concern is the potential impact on natural enemies that help suppress other important pests not targeted by Bt toxins. Therefore, our research was focused on the interactions of Bt cotton with arthropod predators common in cotton fields.

Arthropod predator communities were surveyed from 2002 to 2004 in commercial fields of non-Bt and Bt-cotton using whole-plant inspections, drop-cloth sampling and pitfall traps focused on taxa that are sedentary on the plant, free living in the plant canopy, and ground dwelling, respectively. The results showed significant differences between cotton types infrequently on sampling dates, and as seasonal averages for a few taxa on either cotton, but the differences generally did not persist when data were pooled across seasons for immatures and adults of the 105 taxa sampled. Significant differences in abundance for those few species (primarily the coccinellid *Hippodamia convergens*) for which differences were observed were related to insecticide use rather than Bt-cotton. Abundance, dynamics, diversity, and species richness of

arthropod predators, measured and analyzed through different methods, demonstrated that there is no detectable negative impact on predatory arthropod communities of Bt cotton compared to non-Bt cotton.

To validate our field results and generate answers to more specific questions, experiments were conducted under more controlled environments in the greenhouse and in the laboratory. Greenhouse experiments, in agreement with field results indicating toxin movement through trophic levels (Bt-cotton plants – herbivores – predators), showed that lepidopteran larvae could convey toxin expressed in Bt cotton to predators in the third trophic levels. The acquisition of toxin by predators in the third trophic level, however, is dependent on the abundance of lepidopteran larvae in the field and the amount of prey consumed by the predator. The predatory heteropteran, *P. maculiventris*, and larvae of the green lacewing, *C. rufilabris*, tested positive for Cry1Ac toxin from field collections when lepidopteran larvae were abundant. Results of greenhouse experiments corroborated the field results. Small predatory heteropterans such as *Geocoris*, *Orius*, and *Nabis*, and those predators that do not rely on lepidopteran larvae as prey, such as brown lacewings, *Micromus* spp., and larvae of the ladybeetle *Harmonia axyridis* were not positive for Cry1Ac from field collections. Small predatory heteropterans, common in cotton fields and tested in this reseach, were not positive to Cry1Ac toxin either from field collection or from confining predators directly on Bt cotton with and without caterpillar prey fed Bt cotton in the greenhouse. Cry1Ac detection in these small predators was limited by the amount of prey consumed; small predators did not consume enough prey material to acquire detectable amounts of toxin. Although it did not test positive for Cry1Ac when it consumed prey fed Bt cotton, the small heteropteran predator *G. punctipes* was able to acquire toxin from purified concentrations. However, levels of toxin ingested to be detectable through immunological assay required feeding

on concentrations equal to or higher than 4 ppm, an amount much higher than is expressed in the plants or conveyed through lepidopteran larvae fed Bt-cotton. Analysis of predator bodies and their feces after being fed Cry1Ac toxin concentrations showed that most of the toxin is excreted through their feces and toxin could not be detected in the predator body or feces 72 h after feeding. The results strongly suggest that predatory heteropterans might acquire Cry1Ac toxin from prey fed Bt cotton, but the toxin is eliminated quickly without apparent adverse effect. The method used and the results obtained open a methodological opportunity to test direct toxicity of any Bt toxin for these predators using controlled concentrations much higher than that available through plant or their prey in the field to ascertain direct toxicity. In addition, further fine-tuning of this method may allow labeling of predators with Cry1Ac toxin to conduct mark-recapture studies of predator activity and movement in the field.

Because conventional resistant plants can act directly or indirectly by altering behavior of herbivores and predators, we investigated the oviposition pattern on Bt and non-Bt cotton of bollworms and three predators common in cotton fields. Although Bt-cotton has been widely used for nearly a decade, there is no evidence sign that bollworms, big-eyed bugs (*G. punctipes*), green lacewings, or brown lacewings have altered ovipositional site selection in response to Bt cotton. Bollworms and big-eyed bugs preferred plant terminals for laying their eggs spatially overlapping their oviposition sites within plant structures on both cottons. However, predator oviposition does not appear to be synchronized temporally with bollworm oviposition, but is coincidentally correlated.

Omnivory and the generalist feeding behavior of *G. punctipes* are considered important life history strategies to sustain predator populations in ephemeral crop systems with unpredictable food resources. To address plant- and prey-mediated effects on the omnivorous predator *G.*

punctipes, we investigated development and reproduction of the predator reared on Bt and non-Bt cotton plants and two prey types (conveying Cry1Ac toxin and free of toxin) in the field. Contrary to conventional resistance traits introduced into cultivated plants that have been reported to exert negative effects on *G. punctipes* through plant feeding or indirectly through prey fed resistant plants, neither Bt-cotton nor prey fed Bt-cotton caused negative effects on life history parameters of this omnivorous and important predator during two growing seasons.

These results provide clarification from broader to more specific interactions of predators and Bt-cotton, especially as the effects relate to predator abundance, dynamics, diversity, and tritrophic associations, but further study is warranted to address additional questions. Among them, what is the fate of the Cry1Ac toxin ingested by predators? Is the toxin excreted as the original structure or is it modified during passage through the predator's gut? Is the toxin partially metabolized or, possibly, used as nutrient? Could the Cry1Ac toxin display biological activity after passing through the predator's digestive tract? Further, the deployment of new varieties of novel gene constructs and modes of action will continue to raise questions concerning environmental impacts, and will continue to alter the pest complexes. The need for understanding food webs and tritrophic interactions in agricultural systems will become more acute.