Another View on Bt Proteins – How Specific are They and What Else Might They Do?

A. HILBECK* AND J.E.U. SCHMIDT

Institute of Integrative Biology, ETH Zurich, Switzerland

Biopestic. Int. 2 (1): 1-50 (2006)

ABSTRACT The entomopathogenic bacterium *Bacillus thuringiensis* (Bt) and its toxins are extensively used for pest control purposes in agriculture, forestry and public health programmes since the 1930. In addition to spray formulations, transgenic plants containing Bt genes for the expression of the toxins (Bt plants) are commercially available since the mid 1990s and are grown on an increasing percentage of the global agricultural area. A main reason for the importance of Bt as a pesticide is the assumed environmental safety concluded from the high specificity of its endotoxins (Cry proteins) towards a limited number of target organisms, mostly distinct groups of pest insects. While the mode of action of the Cry toxins in these susceptible target insects is well studied, Bt experts claim that several details are still not understood well enough. Although there is considerable experience with the application and the environmental safety of Bt sprays, a number of research papers were published in the past that did report adverse effects on non-target organisms. These and the widespread use of transgenic Bt plants stimulated us to review the published laboratory feeding studies on effects of Bt toxins and transgenic Bt plants on non-target invertebrates. We describe those reports that documented adverse effects in non-target organisms in more detail and focus on one prominent example, the green lacewing, *Chrysoperla carnea*. Discussing our findings in the context of current molecular studies, we argue firstly that the evidence for adverse effects in non-target organisms is compelling enough that it would merit more research. We further conclude from our in-depth analysis that the published reports studying the effects of Bt toxins from Bt pesticides and transgenic Bt plants on green lacewing larvae provide complementary and not contradictory data. And, finally, we find that the key experiments explaining the mode of action not only in this particular affected non-target species but also in most other affected non-target species are still missing. Considering the steadily increasing global production area of Bt crops, it seems prudent to thoroughly understand how Bt toxins might affect non-target organisms.

KEY WORDS: *Bacillus thuringiensis*, Cry proteins, mode of action, specificity, transgenic Bt plants, unexpected effects

INTRODUCTION

Bacillus thuringiensis – A Microbial Insecticide

Bacillus thuringiensis Berliner, commonly abbreviated as Bt, is a gram-positive, facultative aerobic, rod-like and motile bacterium, which has gained outstanding significance as a microbial pesticide throughout the 20th century (Entwistle *et al.*, 1993; de Maagd *et al.*, 2003). While about 100 bacteria were identified as exo- and endopathogens

* Corresponding author: E-mail: angelika.hilbeck@env.ethz.ch

0973-483X/06/1-50©2006 (KRF)

of arthropods (Thacker, 2002), only a few are used in pest management (e.g. *Bacillus popilliae, Bacillus sphaericus*, *Serratia entomophila*) and only one, namely Bt, has achieved significant attention and commercial success (Rodgers, 1993; Brar *et al.*, 2006). Bt has been reported to occur in samples from various and mostly insect-rich environments, such as grain stores and stored products, different composts and soils, the phylloplane of different plants, insect cadavers, and faeces of herbivorous vertebrates, but also from aquatic environments (Bernhard *et al.*, 1997; Martínez and Caballero, 2002). Because of its apparent opportunity to colonise such diverse habitats, Bt is often referred to as ubiquitous and globally distributed (de Maagd *et al.*, 2001). Within the Bacillaceae family, close genetic relations between Bt and the mammal pathogen *Bacillus anthracis*, and especially to *Bacillus cereus*, a bacterium that causes food poisoning, are considered (Priest, 2000; de Maagd *et al.*, 2003).

The growth cycle of Bt consists of a vegetative and a stationary phase (Lambert and Peferoen, 1992). Cells can grow in a vegetative mode as long as nutrients are sufficiently available, but form endospores within sporangia under unfavourable environmental conditions. The spores are able to survive until the conditions have improved and vegetative reproduction becomes feasible again. Coinciding with the sporulation process, Bt produces crystalline parasporal inclusion bodies that consist of a large amount of one or more proteins of the crystal (Cry) or the cytotoxic (Cty) type (Crickmore *et al.*, 1998; de Maagd *et al.*, 2003). Apart from these proteins, which have attracted most attention for their insect toxicity, the chemical arsenal of Bt strains is much broader and includes diverse substances with different characteristics, specificities and modes of action (Lereclus *et al.*, 1993; Schnepf *et al.*, 1998; de Maagd *et al.*, 2003). To date, 335 different Cry d-endotoxins have been described (Crickmore *et al.*, 2005), most of which share a structure consisting of three globular domains and have a size of either ca. 130 kDa or 70 kDa (Dean *et al.*, 1996; Schnepf *et al.*, 1998; de Maagd *et al.*, 2003). The three domains have distinct roles as part of the commonly accepted mode of action in target pest species (see below), but details of their functions are still being investigated.

Despite considerable research and literature about Bt, the evolutionary role of the toxins still remains subject of discussion (de Maagd *et al.*, 2001). While dead insects, which were killed by the toxins, provide a suitable nutrient source for the spores to germinate and continue vegetative growth, a possible role of the toxins in interactions between microorganisms in soil or in dead organic matter was also proposed (Addison, 1993).

Brief History of the Use of Bt Spray Formulations

Bt was first isolateded in 1901 by bacteriologist S. Ishiwata as "*Bacillus sotto*" after being recognized as the cause of the sudden-collapse disease ("sotto") in larvae of the silkworm moth *Bombyx mori* (L.) (Lepidoptera: Bombycidae) (Beegle and Yamamoto, 1992). At that time, it was considered a threat to Japan's silk industry and its potential as a microbial pest control agent was not yet realized (Glare and O'Callaghan, 2000). Only in 1915, Bt was scientifically described and given its valid name by German scientist Ernst Berliner, who had discovered it in 1911 in dead flour moth caterpillars killed by the "Schlaffsucht" disease. As it had now been found in a pest insect, its insect pathogenic characteristics made the bacterium attractive for use as a pesticide. Efforts to develop methods for its culture and application against flour moths started soon after Berliner's publication. Later, trials were conducted to explore the suitability of Bt as a microbial insecticide against the European corn borer, *Ostrinia nubilalis* (Glare and O'Callaghan, 2000). In 1938, the first commercial formulations of Bt consisting mainly of sporulated cells, were available under the product name "Sporeine" in France (Lambert and Peferoen, 1992). However, the mode of action in target species (see below) was not described before 1956 (Crook and Jarrett, 1991), when Bt attracted more and more interest because of the increasing environmental problems with synthetic insecticides. For many years, Bt, namely the potent subspecies *B. thuringiensis kurstaki* (Btk), was only used to control Lepidoptera. Strains of Btk still form the basis for many spray formulations and had an important role in the creation of transgenic Bt plants. Screening programs, however, have identified many other subspecies and

strains of Bt, with *aizawai, israelensis, tenebrionis* being the most important ones (Crickmore *et al.*, 1998; de Maagd *et al.*, 2003), and additional possibilities for its use were discussed (Feitelson *et al.*, 1992). In particular, the isolation of coleopteran and dipteran-active strains was important in the subsequent development of control strategies against beetle pest species in agriculture and against dipteran disease vectors in public health programmes (Keller and Langenbruch, 1993; Becker and Margalit, 1993).

In all Bt strains, the genes that produce the proteinaceous crystal are located on plasmids. Bt proteins were originally classified into one of four classes known as CryI, CryII, CryIII and CryIV according to their insecticidal activities (Höfte and Whiteley 1989): CryI and CryII proteins are active against lepidopteran and/or dipteran species, CryIII are active against Coleoptera and CryIV are active against Diptera. Crickmore *et al.* (1998) introduced a new nomenclature based on relationships in the amino acid sequences of the toxins. However, the spectrum of Cry proteins and their assumed specific toxicity is much more diverse, including other arthropods, nematodes, flatworms and protozoa (Feitelson *et al.*, 1992). Most Bt strains are able to produce several different crystal proteins and the same protein can be found in different strains or subspecies (Koziel *et al.*, 1993; Schnepf *et al.*, 1998). The diversity of Bt toxins is used for controlling important pests in agriculture and forestry (mainly herbivorous larvae of moth and beetle species) and disease vectors (mosquitoes). However, shortcomings of the use of Bt are low persistence of the toxins under UV light and difficulties to control certain important pests like stem borers such as the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae), in maize in Europe and North America, but also other species in tropic regions (Rice and Pilcher, 1998; Hilbeck, 2002).

TRANSGENIC Bt PLANTS

The interactions between insects and plants have long been recognized as complex and very important for ecosystems (Schoonhoven *et al.*, 1998). To protect themselves against the damage of herbivorous arthropods, plants have developed a plethora of defence strategies ranging from mechanical to chemical. Various substances that are produced by secondary metabolic pathways are toxic to insects, but their biosynthesis is often poorly understood or too complex to be used for genetic engineering of insect-resistant plants. Several strategies have been followed to generate resistance in crop plants involving conventional plant breeding, and, more recently, in vitro techniques (e.g. electrofusion of protoplasts) and genetic engineering to create plants that express insecticidal or entomopathogenic proteins (Jouanin *et al.*, 1998). In genetic engineering genes from other species are isolated and transferred into the plant genome. This involves the use of the gall-forming bacterium *Agrobacterium tumefaciens* as vector for genetic information or the application of ballistic methods like so-called “gene-guns”.

Two main approaches to create plants expressing insecticidal proteins were followed (Jouanin *et al.*, 1998), both targeting the digestive system of insect pests (Schuler *et al.*, 1998). One approach is based on plant-derived genes, e.g. the genetic information that code for enzymes like proteinase inhibitors, amylase inhibitors, cholesterol oxidase or lectines (e.g. the snowdrop lectin (GNA) of *Galanthus nivalis* L.; Amaryllidaceae). The other approach uses genes of *B. thuringiensis* that code for their insect-toxic proteins – mostly the *cry*-genes (Schuler *et al.*, 1998). Only this approach has led to the commercially available transgenic insecticidal crop plants that are currently cultivated. The most common Bt toxins expressed by these plants include the lepidopteran-active Cry1Ab in maize (*Zea mays* L., Poaceae), the lepidopteran-active Cry1Ac in maize and cotton (*Gossypium* spp., Malvaceae) and the coleopteran-active Cry3Bb in maize (Andow and Hilbeck, 2004). More and more transgenic crop plants carry now two or more different Bt transgenes combined in their genomes. In addition to the commercially available Bt crop plants, various Bt toxins are expressed in transgenic varieties of an array of other crop plant species from several families that are not yet approved (Hilbeck, 2001; e.g. Cry3Bb in eggplants, *Solanum melongena* L., Solanaceae, Arpaia *et al.*, 1997). Since the first commercial releases 10 years ago, the area of agricultural land planted with transgenic insect resistant plants for

pest control has increased considerably (James, 2005).

One reason for the interest in transgenic Bt plants is that they are often assumed to be harmless to beneficial insects, including predators and parasitoids, and to other non-target species based on the commonly accepted mode of action known from bacterial Bt toxins (Shelton *et al.*, 2002). Other benefits claimed for transgenic plants are: less effort required for monitoring of target pests (Obrycki *et al.*, 2001), reduced applications of broad-spectrum insecticides and increased or more secure yields due to season-long control of important pest species. Transgenic Bt plants are different from conventional Bt spray formulations. First, while Bt toxins can only persist for a short time on the surface of plants after spray applications, transgenic Bt plants express the Bt toxins throughout their entire lifetime. Bt spray formulations contain bacterial cells, spores and inactive protoxins which must be activated in a complex biochemical process as described below, during which the molecular weight of the proteins is reduced, e.g. from 130-140 kDa to 60-65 kDa for the Cry1 toxins. The structure of some activated proteins have been described (de Maagd *et al.*, 2003). In contrast, transgenic plants express Bt toxins in a more activated form of differing molecular weights (69 kDa in the toxin Cry1Ab) (Hilbeck, 2001).

Effects of Bt plants on non-target species became a major concern after some publications reporting adverse effects on organisms outside of the known range of target insects (Table 3). Several reviews were published recently where the authors evaluated often the same studies but arrived at different conclusions (Lövei and Arpaia, 2005; O'Callaghan *et al.*, 2005, Romeis *et al.*, 2006).

In this paper, we also review recent peer-reviewed literature, in which effects of Bt toxins and transgenic Bt plants on non-target organisms were investigated. However, we focus on those studies, that reported statistically significant differences between a Bt treatment and the corresponding control. While we do acknowledge that there are several studies, concerned with possible implications of Bt toxins on vertebrates, in this review paper, we address invertebrates only as they fall in our area expertise.

MODE OF ACTION OF Bt CRY TOXINS IN INVERTEBRATES

Current Understanding in Target Pest Insects

According to the commonly accepted understanding of its mode of action, the insecticidal activity of Bt is triggered when spores and toxin crystals are ingested (Höfte and Whitley, 1989; Gill *et al.*, 1992; Knowles and Dow, 1993; Knowles, 1994; Schnepf *et al.*, 1998; Whalon and Wingerd, 2003). To solubilise the toxin crystal, pH conditions in the midgut must be suitable, whereas differences in solubility are known for the different toxin families. Cry1 and Cry2 proteins need a higher pH, which is realised in the more alkaline gut milieus of their target insect groups, Lepidoptera and Diptera, while coleopteran active Cry3 proteins are solubilized at a pH closer to neutrality, reflecting the typical gut conditions in their target species. Protease enzymes of the insect gut digest a portion of the solubilized protoxin by removing amino acid sequences from its C- and N-terminal ends and release a protease resistant polypeptide, the so called d-endotoxin, which represents the biologically active fragment. Through this process, the mass of the original protoxin (ca. 130-140 kDa) is reduced to ca. 60-65 kDa in the active toxin. The C-terminal domain of the biologically active toxin binds to specific receptors on the membranes of brush border epithelium cells of the target insect's midgut followed by the insertion of the hydrophobic region of the toxin molecule into the cell membrane. This induces a change in the membrane permeability and the osmotic balance, the formation of transmembrane pores and, subsequently, cell lysis in the gut wall, which allows gut contents to leak into the haemocoel (Dean *et al.*, 1996).

The infested target insect then dies from starvation and lethal septicaemia and, if bacterial spores are present, the abundance of nutrients stimulates germination and the beginning of vegetative bacterial growth. The most frequently expressed advantage of using Bt for pest control is its presumed specificity to certain insect species, the so-called target species, while all other organisms (referred to as "non-target species") are not affected. This specificity in activity is assumed to be caused by the existence of the toxin specific receptors in the brush border epithelium and the pH content in

the gut (Höfte and Whiteley, 1989). Different target species exhibit different numbers of binding sites and the toxin affinity does not appear to be constant for all insects (van Rie *et al.*, 1989)

'Specificity' and 'Susceptibility'

The known mode of action of Bt toxins stems largely from investigations with economically important target pest species known to be susceptible at least to a significant degree in the context of crop production or forestry, such as lepidopteran and coleopteran pest species (e.g. *Heliothis virescens, Ostrinia nubilalis, Lymantria dispar, Leptinotarsa decemlineata*). Out of this tradition, terms like 'susceptibility' and 'selectivity' were defined for an economic, not an ecological context. The 'economic' definition only characterizes 'susceptible' species that can be killed quickly with few or one application measure only or – with regard to transgenic Bt plants – when taking a few bites of the Bt tissue. This requires an acute, lethal effect of a sufficiently high dose of Bt toxin. An 'ecological' definition of ‚susceptibility' or ‚selectivity' would also include species that exhibit long-term, sublethal and lethal effects. With the persistent and constitutive expression of activated Bt-toxins in transgenic Bt plants, ecological long-term effects became much more relevant for environmental risk assessment than with short-lived, inactive Bt-protoxins in microbial sprays. Chronic, sublethal effects can also cause severe adverse effects, even in a pest management context of crops. For instance, if the sublethal effect of prolonged development in a parasitoid or predator species causes temporal disruption in an important natural enemy – prey/host relationship, this can lead to serious consequences, possibly even more serious than a certain low-level lethal short-term effect. Research on modes of action in non-target insects, however, is an important gap of knowledge when considering the large areas planted to transgenic Bt crops and their persistent presence in the above- and below-ground agroecosystems.

Indications of Additional or Alternative Inter-actions

While the mode of action of natural and transgene produced Bt toxins is well documented in target pest species, authors of recent studies have pointed out that many of its details are still not thoroughly understood and that the interactions between Bt toxins and invertebrates may be more complex than thought before (see below). Summarizing recent molecular studies on Bt toxins and invertebrates conducted with nematode-active Bt toxins and the nematode *Caenorhabditis elegans* and with model Lepidoptera species, Crickmore (2005) indicates that modern approaches have revealed novel receptors and possible signal transduction pathways induced within the host following intoxication. Most notably, the author emphasises that (i) Bt toxin activity can be modulated by altered activation, referring to studies with midgut proteases; (ii) Bt toxin specificity is determined not only by the ability to bind to appropriate receptor molecules, but also by the ability to subsequently oligomerize and insert into the membrane; (iii) subtle differences in toxin structure could affect binding and that these differences could account for host specificity and (iv) sequestration of the peritrophic membrane could also apply for Bt toxins.

Very few studies have addressed the fate of ingested Bt toxin in non-target invertebrates. Brandt *et al.* (2004) conducted biochemical and immuno-cytochemical experiments with the bug *Lygus hesperus*, which is not susceptible to the Bt toxins Cry1Ac and Cry2Ab. When both toxins were fed to specimens in their activated form, proteolytic processing of the toxin within the digestive system of *L. hesperus* was observed, but excreted toxins retained their lepidopteran-active characteristics. On the other hand, Cry1Ac did not associate with *L. hesperus* tissues, while Cry2Ab did. The authors conclude, that binding alone is not sufficient for toxicity. Similar studies with invertebrate species, which exhibited non-target effects, would be of especially high value for understanding these observed effects. One crucial aspect in this context are possible structural changes of the ingested toxins in non-target species, which might, even if they are minor, change their binding affinity to membrane molecules (Crickmore, 2005) or other characteristics.

On the other hand, studies on possible additional or alternative effects of Bt toxins on target

species are also rare. Cerstiaens *et al.* (2001) injected different activated Bt toxins into the haemocoel of *Lymantria dispar* and *Neobelleria bullata* and found lethal or sublethal effects depending on the toxin applied. The authors indicate that the mode of action which is responsible for these results, must be different from the one occurring after ingestion. In-vitro toxicity of the Bt toxin Cry1C for *L. dispar* neuronal cells was demonstrated in this study and a connection with the observed effects is discussed. While such additional effects of Bt toxins might be masked by the immediate death of target species, they are important for a thorough understanding of the interactions between Bt toxins and invertebrates.

ADVERSE EFFECTS OF Bt TOXINS AND TRANSGENIC BT PLANTS ON NON-TARGET INVERTEBRATES

Published Studies on Non-target Effects of Bt Toxins in Biopesticides

Early feeding studies of invertebrates with Bt toxin conducted throughout the 1950 were mainly concerned with finding new susceptible pest species for a limited number of Bt strains (mostly lepidopteran-active). The first laboratory trials that aimed to assess the effects of non-target invertebrates started in the 1960s and involved beneficial species of economic significance like honey bees and earthworms (e.g. Smirnoff and Heimpel, 1961; Wilson, 1962). A tritrophic feeding experiment with Bt toxins under laboratory conditions was first performed by Yousten (1973) using the preying mantids *Tenodera sinensis* fed with *B. thuringiensis* subsp. *kurstaki* (Btk) fed cabbage looper, *Trichoplusia ni*, larvae. Moreover, first field studies were conducted to assess impacts on non-target arthropod (mainly lepidopteran) communities of areas treated with Bt sprays (Jaques, 1965).

Considerable laboratory testing of non-target effects was conducted with commercial spray formulations based on Btk which produces toxins of the Cry1 family (Krieg and Langenbruch, 1981; MacIntosh *et al.*, 1990), including commercial products and solutions that contained varying combinations of spores, crystals and toxins of different Bt subspecies. The resulting reports and publications were subsequently reviewed by several authors (e.g. Flexner *et al.*, 1986; Melin and Cozzi, 1990; Navon, 1993; Glare and O'Callaghan, 2000). Glare and O'Callaghan (2000) list more than 300 species of Lepidoptera and other invertebrates to which Btk was toxic. In general, the reported effects are contradictory: some studies documented a lack of effects while others found lethal or sublethal effects on various non-target invertebrates, including predators and, most notably, parasitoids. Sublethal effects were observed in context with different fitness parameters, e.g. fecundity, parasitation rate, host consumption, adult longevity, development time and sex ratios (Flexner *et al.*, 1986). While many studies were published in the form of reports or in journals that are difficult to access (see Flexner *et al.*, 1986 and Glare and O'Callaghan, 2000 for a review), we list some of the species, for which adverse effects of microbial Bt proteins were documented (Table 1). In some of these studies, an observed toxicity was attributed to the presence of β-exotoxins, but only Krieg and co-workers conducted more systematic studies in this context for adult honey bees (Krieg and Herfs, 1963; Krieg and Kulikov 1965; Krieg, 1963). Most of the older studies investigating impacts of Bt endotoxins on non-target species concentrated on lethal effects, which were reported only in a few cases. Also sublethal effects were reported but, to a much lesser extent (Glare and O'Callaghan, 2000).

Published Studies on Non-target Effects of Bt Toxins Expressed in Transgenic Plants

It was not until the mid and late 90s that effects of Bt toxins from transgenic plants on non-target organisms outside of the taxonomic order of the target pests were investigated in a meaningful way. At that time, based on the experience with microbial Bt sprays, the common assumption was that the Bt toxins expressed by transgenic Bt plants would not affect any organisms outside of the order of the target pest species, i.e., lepidoptera and herbivorous coleopteran (Sims, 1995). The publication of adverse effects of Bt toxins in predaceous green lacewing larvae (*Chrysoperla carnea*; Neuroptera: Chrysopidae) (Hilbeck *et al.*, 1998a,b and 1999) and larvae

Table 1. Summary of feeding studies with non-target invertebrate species that reported significant differences between a microbial Bt treatment and the corresponding control.

Species	Bt subspecies/ product	Observed effect	Experimental set up	Explanation for reported effects (if provided by the authors)	Reference
Herbivores:					
Hymenoptera					
Honey bee, *Apis mellifera* L. (Apidae)	Bt subspecies *kurstaki*	Increased mortality	Non-sporulated cultures fed to adults	Presence of β-toxins[1]	Krieg (1973)
	Bt subspecies *thuringiensis*	Increased mortality	Toxin in sugar solutions fed to adults	Presence of β-toxins	Krieg and Herfs (1963)[2]
	Bt subspecies *thuringiensis*	Total mortality	Diets containing the spore-crystal-exotoxin complex or the spore-crystal complex fed to adult workers	Presence of exotoxins	Martouret and Euverte (1964)
	Bt subspecies *thuringiensis*	Total (supernatant) or increased (spores) mortality	Sugar solutions containing spores or culture supernatant with β-exotoxins fed to adults	Presence of exotoxins[3]	Cantwell *et al.* (1966)
	Bt subspecies *thuringiensis*	Increased mortality	Non-sporulated broth cultures fed to adults	Presence of α-toxins[4]	Krieg (1973)
Diptera					
Fruit fly, *Drosophila melanogaster* (Meigen) (Drosophilidae)	Bt subspecies *thuringiensis*	Increased mortality	Toxin in substrate fed to neonate larvae		Krieg (1965)[5]
Chironomus srassicaudatus, *Chironomus decorus*, *Glyptotendipes paripes*, *Tanytaurus* sp. (Chironomidae)	Bt subspecies *israelensis*	Increased mortality			Ali (1981), Ali *et al.* (1981)
Predators:					
Coleoptera					
11-spot ladybird, *Coccinella undecimpunctata* L. (Coccinellidae)	Bt subspecies *entomocidus*	Prolonged larval development, reduced prey consumption	Toxin sprayed aphids fed to newly hatched larvae		Salama *et al.* (1982)

Species	Bt subspecies/ product	Observed effect	Experimental set up	Explanation for reported effects (if provided by the authors)	Reference
Neuroptera					
Green lacewing, *Chrysoperla (Chrysopa) carnea* Stephens (Chrysopidae)	Bt subspecies *entomocidus*	Prolonged larval development, reduced prey consumption.	Toxin sprayed aphids or toxin treated caterpillars (*Spodoptera littoralis*) fed to newly hatched larvae		Salama *et al.* (1982)
Parasitoids:					
Hymenoptera					
Cardiochiles nigriceps Viereck (Braconidae)	Bt subspecies *kurstaki* (commercial)	Shorter life spans	Toxin suspension fed to field collected adults	Starvation?[6]	Dunbar and Johnson (1975)
Cotesia (Apanteles) glomarata L. (Braconidae)	Bt subspecies *kurstaki* (Dipel)	Increased mortality after two weeks	Toxin fed to adults		Mück *et al.* (1981)
Cotesia melanoscelus (Ratzeburg) (Braconidae)	Bt subspecies *kurstaki* (commercial)	Increased parasitisation (synergism); prolonged development.	Susceptible (target species) host (*Lymantria dispar*) fed with toxin containing diet	Slower growth and longer persistence at sizes suitable for parasitisation	Weseloh and Andreadis (1982)
Cotesia rubecula (Marshall) (Braconidae)	Bt subspecies *kurstaki* (strain HD-1)	Increased mortality	Susceptible (target species) host (*Pieris rapae*) fed with toxin containing diet	Host suffering from acute intoxication	McDonald *et al.* (1990)
Hyposoter exiguae (Viereck) (Ichneumonidae)	Bt subspecies *kurstaki* (Dipel)	Increased mortality	Toxin suspension fed to adults	Spore-crystal complex responsible	Thomas and Watson (1986)
Microplitis demolitor Wilkinison (Braconidae)	Bt subspecies *thuringiensis*	Reduced reproduction potential	Host (*Spodoptera littoralis*) fed with toxin containing diet		Salama *et al.* (1982)
Pimpla turionella L. (Ichneumonidae)	Bt subspecies *kurstaki* (Dipel)	Midgut epithelium damage (but no observed effect on the adults)	Toxin fed to adults	Result of the ICP	Mück *et al.* (1981)
Rogas lymantriae Watanabe (Braconidae)	Bt subspecies *kurstaki*	Sex ratio skewed towards females	Susceptible (target species) host (*Lymantria dispar*) fed with toxin containing diet	Females lay more fertilized eggs in larger, untreated larvae.	Wallner *et al.* (1983)

Species	Bt subspecies/ product	Observed effect	Experimental set up	Explanation for reported effects (if provided by the authors)	Reference
Trichogramma cacoeciae Marchal (Tricho-grammatidae)	Bt subspecies *thuringiensis*	Reduced parasitisation capacity	Toxin suspension fed to adults	Presence of β-exotoxins	Hassan & Krieg (1975)
Zele chlorophthalma (Nees) (Braconidae)	Bt subspecies *entomocidus*	Reduced parasitisation rate, reduced emergence, reduced reproductive potential, retardation in development.	Host (*Spodoptera littoralis*) fed with toxin containing diet		Salama and Zaki (1983)
	Bt subspecies *thuringiensis*	Reduced reproduction potential	Host (*Spodoptera littoralis*) fed with toxin containing diet		Salama and Zaki (1983)

[1] Sporulated cultures did not show a similar effect (Krieg and Herfs, 1963).
[2] High doses of the spore-endotoxin complex are toxic.
[3] Crystals were not harmful.
[4] Sporulated cultures did not show a similar effect (Krieg *et al.*, 1980).
[5] No toxicity of the spore-endotoxin complex also at high doses.
[6] Not sure if ingestion took place.

of the Monarch butterfly (Losey *et al.*, 1999) surprised many scientists for different reasons and gave this field more momentum. Non-target effects of transgenic Bt plants finally made it on the agenda of mainstream research. Since then, research on such effects increased significantly until today. In this chapter, we will provide an overview of these studies conducted with Bt toxins and transgenic Bt plants since 1995 and discuss those reporting adverse effects on non-target organisms in more detail.

Table 2 lists a total of 60 non-target invertebrate species and two invertebrate groups of higher taxonomic order that were tested with regard to transgenic Bt plants and microbially produced Bt toxins in international peer-reviewed scientific journals. Twenty-four herbivore species from six insect orders and two herbivorous mites as well as 25 natural enemy species from four insect orders, three predatory mites and one spider were tested. Of these 25 natural enemy species, nine were hymenopteran primary parasitoids, one was a hymenopteran hyperparasitoid and the remaining were predators. Additionally, four detrivorous soil organisms (*Lumbricus terrestris*, *Folsomia candida, Porcellio scaber* and *Oppia nitens)*, unspecified nematodes and protozoa, the detritivorous cockroach *Blattella germanica* and, as a single aquatic organism, plankton-feeding larvae of *Aedes aegypti* were investigated. For the vast majority of these organisms only one publication was found, many only involving one experiment. Only five herbivore species, *Spodoptera littoralis* (5), *Apis mellifera* (4), monarch butterfly (4), spider mites (3), *Rhopalosiphum padi* (3) and 2 predators, *Chrysoperla carnea* (9) and *Coleomegilla maculata* (4), were subjected to experimentation in more than one publication found (Table 2). The reasons for this are arbitrary and based on professional preference rather than ecological necessity and justification. Further, only very few of the studied species are relevant for subtropical or tropical agroecosystems (e.g. *Cyrtorhinus lividipennis* and *Parallorhogas*

Table 2. List of invertebrates from different trophic levels tested for non-target effects of transgenic Bt plants (ticks represents the individual number of studies with the respective species published in international, peer-reviewed journals).

Taxon/Species	Trophic level (feeding type)		
	Herbivores	Predators, parasitoids	Other feeding type
PROTOZOA:			√ (*unclear*)
NEMATODA:			√ (*unclear*)
ANNELIDAE:			
Oligochaeta			
Lumbricus terrestris			√,√ (detritivore)
ARTHROPODA – CRUSTACEA:			
Isopoda			
Porcellio scaber			√,√ (detritivore)
ARTHROPODA – CHELICERATA:			
Araneae			
Araneus diadematus		√	
Acari			
Neoseiulus cucumeris		√	
Oppia nitens			√ (detritivore)
Phytoseiulus persimilis		√	
Rhizoglyphus robini	√		
Tetranychus urticae	√,√,√		
ARTHROPODA – INSECTA :			
Collembola			
Folsomia candida			√ (detritivore)
Blattodea			
Blattella germanica			√ (detritivore)
Thysanoptera			
Frankliniella tenuicornis	√		
Heteroptera			
Cyrtorhinus lividipennis		√	
Geocoris pallens		√	
Geocoris punctipes		√,√	
Lygus hesperus	√		
Nabis sp.		√,√	
Orius insidiosus		√,√	
Orius majusculus		√	
Orius tristicolor		√,√	
Zelus renardii		√	
Homoptera			
Aphis fabae	√		
Macrosiphum avenae	√		
Macrosiphum euphorbiae	√		
Myzus persicae	√		
Nilaparvata lugens	√		
Rhopalosiphum padi	√,√,√		
Neuroptera			
Chrysoperla carnea		√,√,√,√,√,√,√,√,√	
Micromus tasmaniae		√	

Taxon/Species	Trophic level (feeding type)		
	Herbivores	Predators, parasitoids	Other feeding type
Coleoptera			
Adalia bipunctata		√	
Anthonomus grandis	√		
Coleomegilla maculata		√,√,√,√	
Diabrotica undecimpunctata	√		
Hippodamia convergens		√,√	
Leptinotarsa decemlineata	√,√		
Poecilus cupreus		√	
Propylea japonica		√	
Hymenoptera			
Apanteles subandinus		√	
Aphidius nigripes		√	
Apis mellifera	√,√,√,√		
Athalia rosae	√		
Copidosoma floridanum		√	
Cotesia flavipes		√	
Cotesia marginiventris		√,√	
Cotesia plutellae		√	
Microplitis mediator		√	
Nasonia vitripennis		√	
Parallorhogas pyralophagus		√	
Tetrastichus howardi		√	
Lepidoptera			
Acherontia atropos	√		
Autographa gamma	√		
Danaus plexippus	√,√,√,√		
Galleria mellonella	√		
Manduca sexta	√		
Papilio polyxenes	√		
Pieris brassicae	√		
Pieris rapae	√		
Plutella xylostella	√		
Pseudoplusia includens	√		
Spodoptera littoralis	√,√,√,√,√		
Diptera			
Aedes aegypti			√ (plankton-feeder)
TOTAL:			
52 insect species from 10 orders;	**24 insect species from 6 orders;**	**25 insect species from 4 orders;**	**3 insect species from 3 orders;**
8 species from other invertebrate taxa;	**2 species from other invertebrate orders.**	**3 species from other invertebrate orders.**	**3 species from other invertebrate orders;**
2 invertebrate groups of higher taxonomic level.			**2 invertebrate groups of higher taxonomic level.**

pyralophagus), while the vast majority occurs in northern, temperate agro-ecosystems. In most publications, transgenic plant parts were used for testing, many used pollen from transgenic Bt maize and a few used microbially produced activated Bt proteins. Most of the studies focussed on lethal effects (parameters measured: mortality or survival), while some also reported parameters like development time, weight gain and fertility.

In 27 (50%) of the reviewed 54 studies, the authors reported negative effects on one or more of the tested parameters (Table 3); this also includes studies that reported no effects on other parameters. Positive effects were rare (Escher *et al.,* 2000; Deml *et al.,* 1999). Zemková Rovenská *et al.* (2005) reported a preference of spider mites, *Tetranychus urticae*, for transgenic Bt eggplants. The observed effects were often unpredictable in terms of degree and type of impact. Only the fact that Bt maize pollen containing the lepidopteran-specific Cry1Ab toxin adversely affected the caterpillars of the Monarch butterfly and other Lepidoptera species was hardly surprising. However, the actual surprise was the exposure route that had been overlooked until the publication by Losey *et al.* (1999). Since then, tests for Bt effects on Monarch butterfly caterpillars became a standard for regulatory approval in the USA (Oberhauser and Rivers, 2003).

Although there are many studies that reported no effects of Bt toxins and transgenic Bt plants on non-target arthropods in the current peer-reviewed literature, several significant examples exist where adverse effects on very different arthropod taxa were documented (Table 4a,b). Most studies were conducted with non-target herbivores, most prominently non-target Lepidoptera and transgenic Bt plants expressing the lepidopteran toxin Cry1Ab (Table 4a). This is the most proximate approach, because of the close taxonomic relation between the target and the non-target lepidopteran species. The most prominent study was that of Losey *et al.* (1999) reporting an increased mortality of Monarch butterfly (*Danaus plexippus*) larvae after feeding on their host plant, the common milkweed *Ascelapias syriaca*, dusted with pollen from Cry1Ab expressing maize plants. Toxicity of Bt pollen to Monarch caterpillars was also reported by Jesse and Obrycki (2000) in an independent study. As these results, in particular, became subject to debate between proponents and opponents of the technology and because of the cultural importance of the Monarch butterfly in North America, this system was extensively studied from different perspectives (Oberhauser and Rivers, 2003). Some of the subsequent experiments confirmed the toxicity of different tissues from Bt transgenic maize flowers (anthers, pollen) for caterpillars (Hellmich *et al.,* 2001; Anderson *et al.,* 2004), but also highlighted a lower risk for caterpillars when exposed to pollen from Bt maize varieties with low expression of the Bt toxin in pollen. However, effects of different Bt toxins were also found in other non-target Lepidoptera (Deml *et al.,* 1999; Felke *et al.,* 2002; Baur and Boethel, 2003). The mode of action of Cry1 toxins in non-target Lepidotera is presumed to be similar to that in target Lepidoptera. However, additional studies seem to be necessary to confirm this, in particular for non-target Lepidoptera that exhibited only sublethal effects. Most notably, Deml *et al.* (1999), who conducted an extensive study with native Bt toxins, found that also the coleopteran-active Cry3A toxins can have adverse effects on non-target Lepidoptera. Similarly, Hussein *et al.* (2005) and Hussein *et al.* (2006) reported deleterious effects on the polyphagous moth *Spodoptera littoralis* when caterpillars were fed Cry3A-expressing potato foliage. In studies with *S. littoralis* larvae feeding on lepidopteran-active Cry1Ab expressing maize, increased mortality, prolonged development time and reduced weight were reported in several studies (Dutton *et al.,* 2002; Dutton *et al.,* 2005; Vojtech *et al.,* 2005). In other studies, no or little effects of Cry1Ab toxin on this species were found (Höfte and Whiteley, 1989; Müller-Cohn *et al.,* 1996). The youngest larval stages were reported to be most sensitive (Hilbeck *et al.,* 1999; Dutton *et al.,* 2005). Further, the effects of transgenic Cry1Ab maize on *S. littoralis* mortality and development time were more pronounced in transgenic plants compared to a commercial spray formulation (Dutton *et al.,* 2005).

Some non-target effects found in non-lepidopteran herbivorous species (Table 4b), sucking insects in particular, were mostly attributed to

Table 3. List of invertebrate species from different trophic groups tested in laboratory feeding studies (ranked in chronological order) with regard to differences between a non-Bt control and microbial Bt-proteins (m) and/or transgenic Bt-plant material (tg).

Year	Non-target herbivores	Bt test material	Obs. Diff.	Predators	Bt test material	Obs. Diff.	Parasitoids-Hyperparsitoids	Bt test material	Obs. Diff.	Other feeding type	Bt test material	Obs. Diff.
1995	*Anthonomus grandis* (Sims, 1995)	Cry1Ac^{m}	0	*Hippodamia convergens* (Sims, 1995)	Cry1Ac^{m} (bi-troph.)	0	*Nasonia vitripennis* (Sims, 1995)	Cry1Ac^{m} (bi-troph.)	0	*Aedes aegypti* (Sims, 1995)	Cry1Ac^{m} (bi-troph.)	0
	Diabrotica undecimpunctata (Sims, 1995)	Cry1Ac^{m}	0	*Chrysoperla carnea* (Sims, 1995)	Cry1Ac^{m} (bi-troph.)	0						
	Leptinotarsa decemlineata (Sims, 1995)	Cry1Ac^{m}	0									
	Myzus persicae (Sims, 1995)	Cry1Ac^{m}	0									
	Apis mellifera (Sims, 1995)	Cry1Ac^{m}	0									
	Blattella germanica (Sims, 1995)	Cry1Ac^{m}	0									
1996	*Apis mellifera* (Arpaia, 1996)	Cry3B^{m}	0	*Hippodamia convergens* (Dogan *et al.*, 1996)	Cry3^{tg} (tri-troph)	0						

Year	Non-target herbivores	Bt test material	Obs. Diff.	Predators	Bt test material	Obs. Diff.	Parasitoids-Hyperparsitoids	Bt test material	Obs. Diff.	Other feeding type	Bt test material	Obs. Diff.
1997				*Coleomegilla maculata* (Pilcher *et al.*, 1997)	Cry1 Ab^{tg} (Pollen; bi-troph.)	0				*Folsomia candida* (Yu *et al.*, 1997)	Cry1Ac^{tg} Cry1Ab^{tg}	0
				Chrysoperla carnea (Pilcher *et al.*, 1997)	Cry1 Ab^{tg} (Pollen; bi-troph.)	0				*Oppia nitens* (Yu *et al.*, 1997)	Cry1Ac^{tg} Cry1Ab^{tg}	0
				Orius insidiosus (Pilcher *et al.*, 1997)	Cry1 Ab^{tg} (Pollen; bi-troph.)	0						
1998	*Rhopalosi-phum padi* (Lozzia *et al.*, 1998)	Cry1Ab^{tg}	0	*Chrysoperla carnea* (Hilbeck *et al.*, 1998b)	Cry1Ab^{tg} (tri-troph.)	-						
				Chrysoperla carnea (Hilbeck *et al.*, 1998a)	Cry1Ab^{m} (bi-troph.)	-						
				Chrysoperla carnea (Lozzia *et al.*, 1998)	Cry1Ab^{tg} (tri-troph.)	0						
				Coleomegilla maculata (Riddick & Barbosa, 1998)	Cry1Ab^{tg} (tri-troph.)	0						

Year	Non-target herbivores	Bt test material	Obs. Diff.	Predators	Bt test material	Obs. Diff.	Parasitoids-Hyperparsitoids	Bt test material	Obs. Diff.	Other feeding type	Bt test material	Obs. Diff.
1999	*Danaus plexippus* (Losey *et al.*, 1999)	$Cry1Ab^{tg}$ (pollen)	—	*Chrysoperla carnea* (Hilbeck *et al.*, 1999)	$Cry1Ab^{m}$, $Cry2A^{m}$ (tri-troph.)	-	*Cotesia plutellae* (Schuler *et al.*, 1999)	$Cry1Ac^{tg}$ (tri-troph.)	0			
	Ostrinia nubilalis (Deml *et al.*, 1999)	$Cry1Ac^{m}$ $Cry3A^{m}$	—									
	Acherontia atropos (Deml *et al.*, 1999)	$Cry1Ac^{m}$ $Cry3A^{m}$	0,0									
	Manduca sexta (Deml *et al.*, 1999)	$Cry1Ac^{m}$ $Cry3A^{m}$	–,–									
		$Cry1Ac^{m}$ $Cry3A^{m}$	–,–									
	Autographa gamma (Deml *et al.*, 1999)	$Cry1Ac^{m}$ $Cry3A^{m}$	0,0									
	Leptinotarsa decemlineata (Deml *et al.*, 1999)	$Cry1Ac^{m}$ $Cry3A^{m}$	–,0									
	Aphis fabae (Deml *et al.*, 1999)	$Cry1Ac^{m}$ $Cry3A^{m}$	0,0									
	Macrosiphum avenae (Deml *et al.*, 1999)											

Year	Non-target herbivores	Bt test material	Obs. Diff.	Predators	Bt test material	Obs. Diff.	Parasitoids-Hyperparsitoids	Bt test material	Obs. Diff.	Other feeding type	Bt test material	Obs. Diff.
2000	*Danaus plexippus* (Jesse & Obrycki, 2000)	Cry1Ab^{tg} (pollen)	—	*Orius tristicolor* (Armer *et al.*, 2000)	Cry3^{tg} (bi-troph)	0				*Porcellio scaber* (Escher *et al.*, 2000)	Cry1Ab^{tg}	0,+
	Papilio polyxenes (Wraight *et al.*, 2000)	Cry1Ab^{tg} (pollen)	0, —	*Geocoris punctipes* (Armer *et al.*, 2000)	Cry3^{tg} (bi-troph)	0						
	Tetranychus urticale (Lozzia *et al.*, 2000)	Cry1Ab^{tg}	0	*Geocoris pallens* (Armer *et al.*, 2000)	Cry3^{tg} (bi-troph)	0						
				Lygus hesperus (Armer *et al.*, 2000)	Cry3^{tg} (bi-troph)	0						
				Nabis sp. (Armer *et al.*, 2000)	Cry3^{tg} (bi-troph)	0						
				Orius majusculus (Zwahlen *et al.*, 2000)	Cry1Ab^{tg} (tri-troph)	0						
2001	*Danaus plexippus* (Hellmich *et al.*, 2001)	Cry1Ab^{m,tg} Cry1Ac^{m,tg} Cry9C^{m,tg} Cry1F^{m,tg} (pollen)	0,–	*Orius insidiosus* (Al-Deeb *et al.*, 2001)	Cry1Ab^{tg} (tri-troph) Mixed Cry toxins^{m} (Dipel) (tri-troph)	0	*Aphidius nigripes* (Ashouri *et al.*, 2001a)	Cry3A^{tg} (tri-troph)	-	*Lumbricus terrestris* (Saxena & Stotzky, 2001)	Cry1Ab^{tg} (root exudates, plant residues)	0

Year	Non-target herbivores	Bt test material	Obs. Diff.	Predators	Bt test material	Obs. Diff.	Parasitoids-Hyperparsitoids	Bt test material	Obs. Diff.	Other feeding type	Bt test material	Obs. Diff.
	Rhopalosiphum padi (Meier & Hilbeck, 2001)	Cry1Ab^{tg}	0	*Chrysoperla carnea* (Meier & Hilbeck, 2001)	Cry1Ab^{tg} (tri-troph.)	0,–				Nematodes (Saxena & Stotzky, 2001)	Cry1Ab^{tg} (root exudates, plant residues)	0
	Apis mellifera (Malone *et al.*, 2001)	Cry1Ba^{m}	0							Protozoa (Saxena & Stotzky, 2001)	Cry1Ab^{tg} (root exudates, plant residues)	0
	Macrosiphum euphorbiae (Ashouri *et al.*, 2001b)	Cry3A^{tg}	–									
2002	*Pieris brassicae* (Felke *et al.*, 2002)	Cry1Ab^{tg} (pollen)	—	*Coleomegilla maculata* (Lundgren and Wiedemann, 2002)	Cry3Bb^{tg} (pollen; bi-troph.)	0	*Parallorhogas pyralophagus* (Bernal *et al.*, 2002b)	Cry1Ab^{tg} (tri-troph.)	-,0	*Porcellio scaber* (Wandeler *et al.*, 2002)	Cry1Ab^{tg}	0; —
	Pieris rapae (Felke *et al.*, 2002)	Cry1Ab^{tg} (pollen)	—	*Coleomegilla maculata* (Duan *et al.*, 2002)	Cry3Bb1^{tg} (pollen; bi-troph.)	0						
	Plutella xylostella (Felke *et al.*, 2002)	Cry1Ab^{tg} (pollen)	—	*Orius tristicolor* (Ponsard *et al.*, 2002)	Cry1Ac^{tg} (tri-troph.)	—						

Year	Non-target herbivores	Bt test material	Obs. Diff.	Predators	Bt test material	Obs. Diff.	Parasitoids-Hyperparsitoids	Bt test material	Obs. Diff.	Other feeding type	Bt test material	Obs. Diff.
	Nilaparvata lugens (Bernal *et al.*, 2002a)	Cry1Ab^{tg} Cry1Ac^{tg}	0	*Zelus renardii* (Ponsard *et al.*, 2002)	Cry1Ac^{tg} (tri-troph.)	0						
	Rhopalosiphum padi (Dutton *et al.*, 2002)	Cry1Ab^{tg}	0	*Nabis* sp. (Ponsard *et al.*, 2002)	Cry1Ac^{tg} (tri-troph.)	0						
	Tetranychus urticale (Dutton *et al.*, 2002)		0	*Cyrtorhinus lividipennis* (Bernal *et al.*, 2002a)	Cry1Ab^{tg} Cry1Ac^{tg} (tri-troph.)	0						
	Spodoptera littoralis (Dutton *et al.*, 2002)		-	*Chrysoperla carnea* (Dutton *et al.*, 2002)	Cry1Ab^{tg} (tri-troph.)	0; –						
2003	*Apis mellifera* (Hanley *et al.*, 2003)	Cry1Ab^{tg}	0				*Cotesia marginiventri* (Baur & Boethel , 2003)	Cry1Ac^{tg} (tri-troph)	–	*Lumbricus terrestris* (Zwahlen *et al.*, 2003	Cry1Ab^{tg}	-
	Athalia rosae (Howald, 2003)	Cry1Ac^{tg}	0	*Geocoris punctipes* (Ponsard *et al.*, 2002)	Cry1Ac^{tg} (tri-troph.)	—						
	Galleria mellonella (Hanley *et al.*, 2003)	Cry1F^{tg}	—				*Copidosoma floridanum* (Baur & Boethel, 2003)	Cry1Ac^{tg} (tri-troph)	–; 0			
	Pseudoplusia includens (Baur & Boethel , 2003)	Cry1Ac^{tg}	-; 0									

Year	Non-target herbivores	Bt test material	Obs. Diff.	Predators	Bt test material	Obs. Diff.	Parasitoids-Hyperparsitoids	Bt test material	Obs. Diff.	Other feeding type	Bt test material	Obs. Diff.
2004	*Danaus plexippus* (Anderson *et al.*, 2004)	$Cry1Ab^{tg}$ (anthers)	–; 0	*Chrysoperla carnea* (Romeis *et al.*, 2004)	$Cry1Ab^{m}$ (bi-troph.)	0	*Cotesia flavipes* (Prütz & Dettner , 2004)	$Cry1Ab^{tg}$ (tri-troph.)				
	Rhizoglyphus robini (Carter *et al.*, 2004)						*Tetrastichus howardi* (Prütz *et al.*, 2004)					
2005	*Tetranychus urticae* (Zemková Rovenská *et al.*, 2005)	$Cry3Bb^{tg}$	+[1]	*Phytoseiulus persimilis* (Zemkova Rovenska *et al.*, 2005)	$Cry3Bb^{tg}$ (tri-troph.)	–	*Microplitis mediator* (Liu *et al.*, 2005a)					
	Spodoptera littoralis (Hussein *et al.*, 2005)	$Cry3Aa^{m,tg}$	0;—	*Propylea japonica* (Bai *et al.*, 2005)	$Cry1Ab^{tg}$ (pollen bi-troph.)	0; –	*Cotesia margini-ventris* (Vojtech *et al.*, 2005)					
	Spodoptera littoralis (Dutton *et al.*, 2005)	$Cry1Ab^{tg}$ $Cry1Ab^{m}$ (Dipel)	—;—	*Poecilus cupreus* (Meissle *et al.*, 2005)	$Cry1Ab^{tg}$ (tri-troph.)	–; 0						
	Spodoptera littoralis (Vojtech *et al.*, 2005)	$Cry1Ab^{tg}$	–;0									
	Frankliniella tenuicornis (Obrist *et al.*, 2005)	$Cry1Ab^{tg}$	0									

Year	Non-target herbivores	Bt test material	Obs. Diff.	Predators	Bt test material	Obs. Diff.	Parasitoids-Hyperparsitoids	Bt test material	Obs. Diff.	Other feeding type	Bt test material	Obs. Diff.
2006	*Spodoptera littoralis* (Hussein *et al.*, 2006)	Cry3Aa^{tg}	–	*Neoseiulus cucumeris* (Obrist *et al.*, 2006b)	Cry1Ab^{tg} (tri-troph.)	0	*Apanteles subandinus* (Davidson *et al.*, 2006)	Cry1Ac9^{tg}, Cry9Aa2^{tg} (tri-troph.)	0			
				Neoseiulus cucumeris (Obrist *et al.*, 2006b)	Cry1Ab^{tg} (pollen; bi-troph.)	—						
				Araneus diadematus (Ludy and Lang, 2006)	Cry1Ab^{tg} (pollen; bi-troph.)	0						
				Adalia bipunctata (Schmidt, 2006)	Cry1Ab^{m}, Cry3Bb^{m} (bi-troph.)	–; 0						
				Micromus tasmaniae (Davidson *et al.*, 2006)	Cry1Ac9^{tg}, Cry9Aa2^{tg} (tri-troph.)	0						
				Chrysoperla carnea (Rodrigo-Simon *et al.*, 2006)	Cry1Ac^{m}, Cry1Ab^{m} Cry2Ab^{m} (bi- and tri-trophic.)	0						

Obs. diff: observed difference: + = observed difference, positive for the test species; 0 = no difference observed; — = observed difference, negative for the test species.
[1] Preference of tested transgenic Bt cultivar.

pleiotropic effects of transgenic Bt plants. It was argued that the transgenic Bt cultivars used in the experiments provided the tested species with different conditions in terms of primary and secondary compounds than the non-transformed plants used as controls (Ashouri *et al.,* 2001b). Pleiotropic effects are also discussed with regard to differences in host plant preference in herbivorous mites (Zemková Rovenská *et al.,* 2005). However, further experimental investigations to establish the causes of the documented effects are still to be conducted. Similarly, reported effects of Cry1Ab on the detritivorous woodlouse, *Porcellio scaber* were mostly discussed in the context of nutritional quality of transgenic Bt plants (Escher *et al.,* 2000; Wandeler *et al.,* 2002), although Wandeler *et al.* (2002) also considered the possibility of Bt toxicity as an explanation for the reduced consumption rates observed.

Most studies with predators tested effects of Cry1Ab toxins and Cry1Ab-fed prey on larvae of the green lacewing *Chrysoperla carnea* (Hilbeck *et al.,* 1998a, b; Hilbeck *et al.,* 1999; Dutton *et al.,* 2002; Romeis *et al.,* 2004). Their results will be reviewed in more detail below, as this is the most prominent example of studies revealing different outcomes depending on different experimental methodologies and approaches. Moreover, Ponsard *et al.* (2002) found a reduction in longevity in two species of predatory bugs, *Orius tristicolor* and *Geocoris tristicolor*, when fed with caterpillars reared on Cry1Ac-expressing cotton plants (Table 4b). Higher mortality rates compared to the control were observed in larvae of the two-spot ladybird, *Adalia bipunctata*, when their food, flour moth eggs, had been treated with microbially produced trypsin-activated toxins (Schmidt, 2006; Schmidt *et al.,* submitted). In these experiments, mortality increased stronger in treatments with the lepidopteran-active Cry1Ab toxin than with the coleopteran active Cry3Bb. The reasons for this are unclear. However, earlier studies with earthworms reported reduced mortality at high toxins concentrations (Smirnoff and Heimpel, 1961). In another ladybird, *Propylaea japonica*, Bai *et al.* (2005) reported an increased longevity of females when fed with aphids and rice pollen of one Cry1Ab expressing variety (KMD-1) compared to females fed with aphids and rice pollen of non-Bt rice. A second Cry1Ab expressing rice variety (KMD-2), however, did not produce similar results. The authors could not offer an explanation for this result.

Effects of transgenic Bt plants on parasitoids are assumed to be more likely because of the closer trophic relationship between parasitoid larvae and their hosts compared to pedators and their prey (Bernal *et al.,* 2002b). Several studies with parasitoids reported lethal and sublethal effects of various parameters, when parasitoids developed within lepidopteran caterpillar hosts reared on Bt toxin containing diets (Bernal *et al.,* 2002b; Baur and Boethel, 2003; Prütz and Dettner, 2004; Liu *et al.,* 2005a). Sublethal effects included prolonged development time, reduced cocoon weights, reduced longevity, reduced fecundity and a shift in sex ratios. Liu *et al.* (2005b) studied the effect of a transgenic cotton variety (SGK321) expressing both a Cry1A toxin and the insect-active protease inhibitor CpTI on the parasitoid *Campoketis chlorideae* Uchida (Hymenoptera: Ichneumonidae). They reported reduced body weights of parasitoids, when their host caterpillars of the target species *H. armigera* fed on leaves of the transgenic cultivar 10-48 h after parasitisation. If hosts fed on the transgenic cultivar for more than 48 h, prolonged egg and larval development and decreased pupal and adult weight were observed. However, the reported effects cannot be linked to one of the transgene-expressed substances.

Only one publication was concerned with effects of transgenic Bt plants on a hyperparasitoid (secondary parasitoid). Prütz *et al.* (2004) reported reduced parasitisation, emergence and female weight in *Tetrastichus howardi* when developing in cocoons of the primary parasitoid *Cotesia flavipes,* which parasitized Bt maize fed *Chilo partellus* larvae.

The results of studies with predators and parasitoids are mostly discussed in context of reduced nutritional quality of toxin-affected prey and the known mode of action (e.g. Dutton *et al.,* 2003; Meissle *et al.,* 2005; Vojtech *et al.,* 2005). It is often stated, that direct toxin effects are “unlikely”, because Bt toxin specific receptors are only found in target organisms. However, some authors acknowledge that

Table 4a. Summary of feeding studies conducted with non-target Lepidoptera species that reported differences between a non-Bt control and microbial Bt proteins and/or transgenic Bt plant material.

Lepidoptera species	Source of Bt exposure	Statistically significant differences compared to Bt-free control[1] during/after exposure	Experimental set up with regard to reported effects	Duration of Bt exposure	Cause of reported differences suggested/ discussed by the authors	Reference
Deathhead hawkmoth, *Acherontia atropos* (L.) (Sphingidae)	Cry1Ac toxin produced from Btk strain HD-73; purified Cry3A toxin based on commercial formulation (Novodor).	Reduced food consumption, reduced growth[4].	10 day old larvae fed with toxin incorporated in Petri dishes artificial diet.	10 days during 2nd and 3rd instar.	Species-specific susceptibility of the insects to the toxins Reduced feeding and decreased utilization of food conta-ining an endo-toxin.	Deml *et al.* (1999)
Silver-Y moth, *Autographa gamma*, (L.) (Noctuidae)	Cry1Ac toxin produced from Btk strain HD-73; purified Cry 3A toxin based on commercial formulation (Novodor).	Increased mortality, increased food consumption (Cry3A), reduced growth (except Cry1Ab on dandelion)[4].	6, 8, 10 day old larvae fed with toxin incorporated in artificial diet or applied to dandelion (*Taraxacum officinale*) leaf discs in Petri dishes.	6 days during 2nd and 3rd instar.	Species-specific susceptibility of the insects to the toxins Reduced feeding and decreased utilization of food containing an endo-toxin.	Deml *et al.* (1999)
Monarch butterfly, *Danaus plexippus* (L.) (Nymphalidae)	Corn (event Bt11) expressing Cry1Ab toxin.	Increased mortality, reduced consumption.	Common milkweed (*Ascelapias syriaca*) leaf discs artificially dusted with pollen fed to caterpillars (3 days old) in Petri dishes.	4 days	Effect of Bt toxin in pollen.	Losey *et al.* (1999)
	Corn (events Bt176 and Bt11) expressing Cry1Ab toxin.	Higher mortality rates in caterpillars (after 48 hours, after 120 hours).	Common milkweed (*Ascelapias syriaca*) leaf discs naturally and artificially dusted with pollen fed to caterpillars (1st instar) in Petri dishes.	48 hours	Toxicity of Bt pollen.	Jesse and Obrycki (2000)
	Purified (trypsin-resistant core) toxins (Cry1Ab, Cry1Ac).	Increased mortality, reduced growth of 1st instar larvae (or larvae less affected).	Toxins incorporated into artificial diet fed to caterpillars.	During entire larval development.	Toxin effect.	Hellmich *et al.* (2001)

Lepidoptera species	Source of Bt exposure	Statistically significant differences compared to Bt-free control[1] during/after exposure	Experimental set up with regard to reported effects	Duration of Bt exposure	Cause of reported differences suggested/ discussed by the authors	Reference
	Purified corn pollen collected from Bt corn (event 176) containing Cry1Ab toxin.	Reduced growth.	Pollen applied to common milkweed (*Asclepias syriaca*) leaf discs fed to caterpillars.	During entire larval development.	Effect of pollen from Bt176 cultivar.	Hellmich *et al.* (2001)
	Purified corn pollen contaminated with corn tassel material both collected from Bt corn (events 176 and Bt11) containing Cry1Ab toxin and Bt corn (event Cbh351) containing Cry9C toxin.	Increased mortality.	Pollen and tassel material applied to common milkweed (*Asclapias syriaca*) leaf discs fed to caterpillars.	During entire larval development.	Effect of pollen in combination with tassel material from respective cultivars.	Hellmich *et al.* (2001)
	Corn (event Bt11) anthers containing Cry1Ab toxin.	Reduced feeding, prolonged development time, reduced weight in caterpillars Degree depends on number of anthers provided.	Anthers placed on leaf discs of common milkweed (*Ascelapias syriaca*) fed to caterpillars in Petri dishes.	Entire larval development (1^{st} to 5^{th} instar)[2].	Increased searching to avoid Bt ingestion (no direct toxicity proposed).	Anderson *et al.* (2004)
Cotton bollworm, *Helicoverpa armigera* (Hübner) (Noctuidae)	Cry1Ac toxin produced from Btk strain HD-73.	Reduced pupation rate, prolonged development time, reduced larval and pupal weight.	Caterpillars fed with toxin incorporated into artificial diet.	Entire larval development.	Effect of Cry1Ac toxin.	Liu *et al.* (2005a)
Tobacco hornworm, *Manduca sexta*, (L.) (Sphingidae)	Cry1Ac toxin produced from Btk strain HD-73; purified Cry3A toxin based on commercial formulation (Novodor).	Increased mortality, reduced food consumption, reduced growth[4].	10 day old larvae fed with toxin incorporated in artificial diet in Petri dishes.	10 days during 2^{nd} and 3^{rd} instar.	Species-specific susceptibility of the insects to the toxins Reduced feeding and decreased utilization of food containing an endotoxin.	Deml *et al.* (1999)

Lepidoptera species	Source of Bt exposure	Statistically significant differences compared to Bt-free control[1] during/after exposure	Experimental set up with regard to reported effects	Duration of Bt exposure	Cause of reported differences suggested/ discussed by the authors	Reference
European corn borer, *Ostrinia nubilalis* (Hübner) (Pyralidae)	Cry1Ac toxin produced from Btk strain HD-73; purified Cry3A toxin based on commercial formulation (Novodor).	Increased mortality, reduced food consumption (Cry3A), reduced growth[4].	5day old larvae fed with toxin incorporated in artificial diet in Petri dishes.	10 days during 2nd and 3rd instar.	Species-specific susceptibility of the insects to the toxins Reduced feeding and decreased utilization of food containing an endo-toxin.	Deml *et al.* (1999)
Large white, *Pieris brassicae* L. (Pieridae)	Corn (Bt176) pollen containing Cry1Ab toxin.	Reduced feeding, reduced growth, and higher mortality in caterpillars Degree depends on amount of pollen consumed.	Wild cabbage (*Brassica oleracea*) leaf disc artificially dusted with pollen fed to caterpillars in plastic boxes.	24 hours during 2nd instar[3].	Toxicity of Bt pollen.	Felke *et al.* (2002)
Small white, *Pieris rapae* L. (Pieridae)	Corn (Bt176) pollen containing Cry1Ab toxin.	Reduced feeding, reduced growth, higher mortality (and behavioural changes) in caterpillars Degree depends on amount of pollen consumed.	Wild cabbage (*Brassica oleracea*) leaf discs artificially dusted with pollen fed to caterpillars in plastic boxes.	24 hours during 2nd instar.	Toxicity of Bt pollen.	Felke *et al.* (2002)
Diamondback moth, *Plutella xyllostella* (L.) (Plutellidae)	Corn (Bt176) pollen containing Cry1Ab toxin.	Reduced feeding, reduced growth, and higher mortality in caterpillars Degree depends on amount of pollen consumed.	Wild cabbage (*Brassica oleracea*) leaf disc artificially dusted with pollen fed to caterpillars in plastic boxes.	24 hours during 4th instar.	Toxicity of Bt pollen.	Felke *et al.* (2002)
Soybean looper, *Pseudoplusia includens* (Walker) (Noctuidae)	Cotton (event 531, Nucotton-33B) leaves containing Cry1Ac toxin.	Increased mortality, prolonged development time, reduced prepupal weight[5].	Larvae fed with cut leaves in Petri dishes.	Entire larval development until prepupal stage.	Effect of transgenic cotton cultivar.	Baur and Boethel (2003)

Lepidoptera species	Source of Bt exposure	Statistically significant differences compared to Bt-free control[1] during/after exposure	Experimental set up with regard to reported effects	Duration of Bt exposure	Cause of reported differences suggested/ discussed by the authors	Reference
Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) (Noctuidae)	Corn (Bt11) expressing Cry1Ab toxin.	Increased mortality, prolonged development time to 2nd instar.	Caterpillars in cages clipped on leaves.	During 1st instar.	Toxicity of plant expressed activated Cry1Ab.	Dutton *et al.* (2002)
	Corn (Mon810) leaves/stems containing Cry1Ab toxin.	Increased mortality, prolonged development times, reduced larval weight.	Caterpillars reared on mixture of leaves and stems (2-4 week old plants).	Entire larval development.	Effect of Bt corn cultivar.	Vojtech *et al.* (2005)
	Corn (Bt11) expressing Cry1Ab toxin.	Increased mortality, increased development time.	Caterpillars reared in cages on leaves (1st and 2nd instar) and caged plants (from 3rd instar on).	Entire larval development.	Effect of the Cry1Ab toxin.	Dutton *et al.* (2005)
	Bt spray formulation (DIPEL).	Increased mortality, increased development time.	Caterpillars reared in cages on sprayed leaves (1st and 2nd instar) and caged sprayed plants (from 3rd instar on).	Entire larval development.	Effect of the Cry1Ab toxin.	Dutton *et al.* (2005)
	Potato (Newleaf Superior) leaves containing Cry3Aa toxin.	Reduced feeding in caterpillars, reduced size body of pupae, reduced fecundity in females.	Larvae (reared on artificial diet until 4th instar) fed with cut leaves.	During 5th and 6th (last) instar.	Reduced feeding: used Cry3Aa potato is less suitable a host plant; reduced body size: toxin could bind to midgut cells and reduce digestive function; reduced fecundity: unknown effect of Cry3A sequestration in females.	Hussein *et al.* (2005) Hussein *et al.* (2006)

[1] If several control treatments were included in the experiments, the statements refer to the treatment which, was most similar to the Bt treatment. [2] No effects documented when 3rd through 5th instar larvae were exposed. [3] No effects documented when 3rd instar larvae were exposed. [4] No effects of the purified Cry3A toxin on target *Leptinotarsa decemlineata* were reported in this study. [5] In larvae parasitized with *Copidosoma floridanum* (Encyrtidae) also an increased consumption was observed.

Table 4b. Summary of feeding studies conducted with nontarget non-Lepidoptera invertebrate species that reported differences between a non-Bt control and microbial Bt proteins and/or transgenic Bt plant material.

Taxon/species	Source of Bt exposure	Statistically significant differences compared to Bt-free control[1] during/after exposure	Experimental set up with regard to reported effects	Duration of Bt exposure	Cause of reported differences suggested/ discussed by the authors	Reference
Non-target herbivores:						
Acari						
Two-spotted spider mite, *Tetranychus urticae* Koch (Tetranychidae)	Eggplant expressing Cry3Bb toxin.	Higher host preference for Bt plant material.	Two-choice tests with adults on half leave discs in Petri dishes.	5 days (+ pre-experimental exposure).	Changes in primary or secondary metabolism.	Zemková Rovenská *et al.* (2005)
Homoptera:						
Black bean aphid, *Aphis fabae* (Scop.) (Aphididae)	Cry1Ac toxin produced from Btk strain HD-73; purified Cry3A toxin based on commercial formulation (Novodor).	Increased (Cry1Ac) or reduced (Cry3A) mortality.	Specimens fed with toxin incorporated in artificial diet in Petri dishes.	3 days	Species-specific susceptibility of the insects to the toxins Reduced feeding and decreased utilization of food containing an endotoxin.	Deml *et al*. 1999
Potato aphid, *Macrosiphum euphorbiae* (Thomas) (Aphididae)	Potato (Newleaf) plantlets containing Cry3A toxin.	Reduced growth, reduced fecundity, higher flight incidence of young alatae Differences in nutritional indices.	0-12 h old apterous adults caged on leaves.	7 days	Malnutrition through altered plant metabolism.	Ashouri *et al*. (2001a)
Grain aphid *Macrosiphum avenae* (Fabr.) (Aphididae)	Cry1Ac toxin produced from Btk strain HD-73; purified Cry3A toxin based on commercial formulation (Novodor).	Increased survival time.	Specimens fed with toxin incorporated in artificial diet in Petri dishes.	3 days	Species-specific susceptibility of the insects to the toxins Reduced feeding and decreased utilization of food containing an endotoxin.	Deml *et al*. 1999

Taxon/species	Source of Bt exposure	Statistically significant differences compared to Bt-free control[1] during/after exposure	Experimental set up with regard to reported effects	Duration of Bt exposure	Cause of reported differences suggested/ discussed by the authors	Reference
Predators:						
Acari						
Predatory mite, *Neoseiulus cucumeris* Oudemans (Phytoseiidae)	Corn (Bt11) pollen containing Cry1Ab toxin.	Increased development time in females, reduced fecundity.	Pollen fed to protonymphs in special cages.	Protonymphal and deutonymphal stage.	No toxin effect[3]. Unintended effect of transgenic plant due to breeding procedure after transformation. Pollen unsuitable food.	Obrist *et al.* (2006b)
Predatory mite, *Phytoseiulus persimilis* Athias-Henriot (Phytoseiidae)	Eggplant expressing Cry3Bb toxin.	Lower preference for Bt fed prey.	Choice-tests with eggplant-fed (entire life span) non-target acarine prey (*Tetranychus urticae*) in special set up.	3 days	Rejection of Bt in prey[2].	Zemková Rovenská *et al.* (2005)
Heteroptera:						
Bigeyed bug, *Geocoris punctipes* Say (Lygeidae)	Cotton (Nucotton-33B) leaves containing Cry1Ac toxin.	Decreased longevity.	Cotton-fed (24 hours) non-target lepidopteran prey (*Spodoptera littoralis*) fed to adult bugs (addition of cotton plant material in some trials).	Remaining life span after collection from field.	Toxicity of Bt proteins and possibly metabolites.	Ponsard *et al.* (2002)
Pirate bug, *Orius tristicolor* White (Anthocoridae)	Cotton (Nucotton-33B) leaves containing Cry1Ac toxin.	Decreased longevity.	Cotton-fed (24 hours) non-target lepidopteran prey (*Spodoptera littoralis*) fed to adult bugs (addition of cotton plant material in some trials).	Remaining life span after collection from field.	Toxicity of Bt proteins and possibly metabolites.	Ponsard *et al.* (2002)

Taxon/species	Source of Bt exposure	Statistically significant differences compared to Bt-free control[1] during/after exposure	Experimental set up with regard to reported effects	Duration of Bt exposure	Cause of reported differences suggested/ discussed by the authors	Reference
Coleoptera:						
Two-spot ladybird *Adalia bipunctata* L. (Coccinellidae)	Microbially produced trypsin-activated toxin solutions (Cry1Ab, Cry3Bb).	Increased larval/pupal mortality (Cry1Ab: 5, 25, 50µg/ml; Cry3Bb: 25µg/ml).	Toxin solutions in different concentrations sprayed on flour moth eggs fed to larvae in Petri dishes.	Entire larval development.	Unexpected toxicity.	Schmidt (2006); Schmidt *et al.* (submitted)
Carabid beetle, *Poecilus cupreus* L. (Carabidae)	Corn (Mon810) tissue containing Cry1Ab toxin.	Increased mortality[4].	Corn fed non-target lepidopteran prey (*Spodoptera littoralis*) fed to larvae (starting with neonate or 10 day old individuals) in glass tubes.	40 days (starting with neonate larvae); 20 days (starting with 10 day old larvae).	"Most likely" indirect effects due to poor prey quality. "Direct effects cannot be excluded".	Meissle *et al.* (2005)
Ladybird, *Propylaea japonica* (Thunberg)	Rice (KMD1, KMD2) pollen containing Cry1Ab toxin.	Reduced female longevity (only in KMD1 compared to non-Bt pollen)[5].	Anther powder (containing pollen) and aphids (additional food without treatment) fed to larvae in glass tubes.	Entire larval development.	Difference not discussed.	Bai *et al.* (2005)
Parasitoids: **Hymenoptera:**						
Cotesia marginiventris (Cresson) (Braconidae)	Cotton (event 531, Nucotton-33B) leaves containing Cry1Ac toxin.	Prolonged development time, reduced longevity in adults, fewer ova in females.	Cotton reared non-target lepidopteran hosts (*Pseudoplusia includens*) parasitized at end of 2nd instar.	Entire immature development.	Effect of host feeding on used transgenic cultivar.	Baur and Boethel (2003)
	Corn (event Mon810) leaves/ stems containing Cry1Ab toxin.	Increased mortality until cocoon formation, prolonged development time, reduced cocoon weight; sex ratio shifted towards females.	Non-target lepidopteran hosts (*Spodoptera littoralis*) reared on corn parasitized during 2nd instar.	Entire immature development? (diet of parasitized larvae not described).	Indirect effects due to low quality hosts;"direct effects cannot be excluded although very unlikely".	Vojtech *et al.* (2005)

Taxon/species	Source of Bt exposure	Statistically significant differences compared to Bt-free control[1] during/after exposure	Experimental set up with regard to reported effects	Duration of Bt exposure	Cause of reported differences suggested/ discussed by the authors	Reference
Copidosoma floridanum (Dalman) (Encyrtidae)	Cotton (event 531, Nucotton-33B) leaves containing Cry1Ac toxin.	Reduced emergence (depending on plant age).	Cotton reared non-target lepidopteran hosts (*Pseudoplusia includens*) parasitized as eggs.	Entire immature development.	Effect of host feeding on used transgenic cultivar (depending on plant age).	Baur and Boethel (2003)
Cotesia flavipes (Cameron) (Braconidae)	Corn (Bt176) stem piths infused with leaf suspension containing Cry1Ab toxin.	Reduced number of cocoons/pupae per host, reduced weight of coccons.	Cotton reared non-target lepidopteran hosts (*Pseudoplusia includens*) parasitized as eggs.	Entire immature development.	Indirect effects due to low quality hosts indicated.	Prütz and Dettner (2004)
Microplitis mediator (Haliday) (Braconidae)	Cry1Ac toxin produced from Btk strain HD-73.	Prolonged development time, reduced pupal weight, reduced adult weight, reduced longevity[6].	Lepidopteran host (*Helicoverpa armigera*) reared on artificial diet parasitized in 2nd (or 1st) instar.	Different (4-8 days) depending on applied concentration; diet after parasitation not specified.	Indirect effects due to low quality hosts.	Liu *et al.* (2005a)
Parallorhogas pyralophagus (Marsh) (Braconidae)	Corn (event CBH 351) tissue containing Cry9C toxin.	Increased immature mortality, increased immature development time, reduced female longevity.	Non-target lepidopteran hosts (*Eoreuma loftini*) (28-32 days old) fed with corn stalk tissue for 48 h parasitized.	Entire immature development (Bt toxin containing tissue in gut of paralysed host).	Lethal and sublethal host mediated effects of Bt toxin expressing maize tissue.	Bernal *et al.* (2002b)
Hyperparasitoids: Hymenoptera:						
Tetrastichus howardi (Olliff) (Eulophidae)	Corn (Bt176) stem piths infused with leaf suspension containing Cry1Ab toxin.	Reduced parasitisation, reduced emergence of adults (=reduced number of successfully parasitized cocoons), reduced weight of females.	Cocoon clusters from host (primary parasitoid *Cotesia flavipes* after emergence from its herbivorous host *Chilo partellus* reared within stem piths) parasitized.	Exposure to host during entire development (see *C. flavipes* above).	Indirect effect of transgenic Bt cultivar (mediated through primary parasitoid and its herbivorous host).	Prütz *et al.* (2004)

Taxon/species	Source of Bt exposure	Statistically significant differences compared to Bt-free control[1] during/after exposure	Experimental set up with regard to reported effects	Duration of Bt exposure	Cause of reported differences suggested/ discussed by the authors	Reference
Detritivores:						
Oligochaeta:						
Lumbricus terrestris L. (Lumbricidae)	Corn (event Bt11) expressing Cry1Ab toxin.	Reduced weight (200 days after the start)[7].	Leaf material fed to adults in soil filled glass tubes.	200 days	Potentially adverse effect of the Bt toxin or unanticipated changes in the plant quality or the bacterial community on plants due to the genetic transformation.	Zwahlen *et al.* (2003)
Isopoda:						
Common rough woodlouse, *Porcellio scaber* (Latreille) (Porcellionidae)	Corn (event Bt11) expressing Cry1Ab toxin.	Reduced mortality in juveniles, reduced weight gain in juveniles, faster weight gain in adults.	Leaves fed to adults and neonate juveniles in experimental boxes (Control non-Bt maize leaves).	120 days (juvenile mortality); 131 days (juvenile weight); 22 weeks (adult weight).	Better nutritional quality of Bt foliage supported by lower C:N ratio, lower lignin content and higher content soluble carbohydrates measured.	Escher *et al.* (2000)
	Corn (events Bt176, Bt11) expressing Cry1Ab toxin.	Reduced consumption (Degree depends on corn variety).	Senescent leaves fed to adults in experimental boxes.	20 days	Bt effects (differences between Bt varieties accounted for different toxin contents), higher lignin content or different microbial colonisation.	Wandeler *et al.* (2002)

[1] If several control treatments were included in the experiments, the statements refer to the treatment which, was most similar to the Bt treatment. [2] Referring to negative effects of coleopteran-active Bt toxins on the predatory mite *Metaseiulus occidentalis* (Chapman and Hoy, 1991). [3] Similar experiments with spider mite prey containing higher toxin concentrations revealed no statistically significant effects. [4] Neonate larvae were stronger affected than 10 day old larvae. [5] No significant difference of the KMD1-pollen+aphid diet compared to the KMD2-pollen and the aphid-only diet. No significant difference of the KMD2-pollen diet compared to the non-Bt-pollen diet. [6] Effects are more pronounced at higher toxin concentrations. [7] No similar differences were observed in the field.

transgenic plants express Bt toxins in a modified form (Hilbeck, 2001) and that plant-expresed Bt toxins can act in concert with other secondary defense compounds in the plants (Andow and Hilbeck, 2004). On the other hand, none of these studies confirmed or disproved that the effects observed in these non-target species are similar to those of target species and the mode of action was hardly ever studied in adversely affected non-target species. A first exploratory study in this regard was conducted by Rodrigo-Simon *et al.* (2006) using Bt fed green lacewing larvae (see below) but the studied predatory lacewings were not affected and only the protocols for target herbivores were used.

The Case Example of Bt Toxins and Green Lacewing Larvae

Because the green lacewing is the most intensively investigated non-lepidopteran, non-target organisms to date (Table 2), this case deserves a more detailed analysis. Six studies published on the effects of Bt toxins are often portrayed as supposedly contradictory while in reality the differences in the results can be explained through the differences in the methodologies used and the underlying research questions. In three studies, direct (bi-trophic) effects of microbially produced Bt toxins were tested (Hilbeck *et al.*, 1998b; Romeis *et al.*, 2004), and in four other studies the effects of prey-mediated (tri-trophic) exposure to Bt toxins from Bt maize (Hilbeck *et al.*, 1998a; Dutton *et al.*, 2002) or microbially produced Bt toxins and -protoxins (Hilbeck *et al.*, 1999) were examined (Table 5).

Bi-trophic Effects

Despite the different types of artificial diets used and parameters measured, a few components of the two studies by Hilbeck *et al.* (1998b) and Romeis *et al.* (2004) are comparable and yielded indeed similar results. Hilbeck *et al.* (1998b) detected a significant direct lethal effect of Bt toxins that began to manifest itself during the second larval stage but not during the first larval stage. Also, Romeis *et al.* (2004) could not observe adverse effects due to exposure to Bt toxin during this larval stage (Table 5, 5: 2.1, 5.2 and 5.3). The second instar was not studied.

Hilbeck *et al.* (1998b) used an artificial diet that was specifically developed for the commercial mass production of lacewing larvae for biocontrol purposes and allowed for continuous and complete development of the larvae from egg hatch to adult eclosion. This artificial diet was amended with Bt toxin (100µg/ml) and fed to the lacewing larvae throughout their entire juvenile feeding stage until pupation. The authors measured stage-specific mortality and development time. From the second instar on, lacewings exhibited a significantly higher mortality in the Bt treatment than in the control (Table 5, 5: 2.1) and, additionally, a significantly longer stage-specific development time. Notably, when only second and third instars were fed with Bt diet, but not the first instars, mortality in the Bt-treatment was still significantly higher than in the respective control but also significantly lower than in the Bt-treatment that included the first instar. Hence, early instars might be more susceptible than older ones, which is consistent with common knowledge about efficacy of Bt toxins on early larval stages.

Romeis *et al.* (2004) used sucrose solution as artificial diet in their trials. This diet does not allow a continuous and complete development of these predaceous larvae. Development of the larvae is arrested, but it allows them to survive periods of lack of prey longer than when sustaining themselves on water only (Limburg and Rosenheim, 2001). Lacewing larvae remained in the same larval stage and lived for up to 6 days longer than when being provided with water only. The only parameter measured was the time it took until the insects died. Romeis *et al.* (2004) added Bt toxins to the sucrose solution to see whether this caused a faster death or not. All test insects starved to death at the same speed regardless whether Bt toxin was added to the sucrose solution or not (Table 5, 5: 5.2). Also, the exposure to Bt sucrose solution during only a part of the first instar – 6 out of 11 days – did not result in a difference, when untreated flour moth eggs were provided afterwards. This high quality food allowed for recovery of the larvae without sustained consequences.

Rodrigo-Simon *et al.* (2006) provided lacewing larvae with a total of ca. 3-4 droplets of water containing Bt toxin at a low concentration,

Table 5. Comparative overview of six laboratory studies investigating the effects of Bt toxins on Green lacewing (*Chrysoperla carnea*) larvae.

Study	Study material	Design of individual experiments	First instar (L1)	Second instar (L2)	Third instar (L3)	Pupa (non-feeding)	Entire juvenile stage (L1-adult)
1 (Hilbeck *et al.*, 1998a)	Tri-trophic; Bt maize (Bt 11), isogenic control maize	***Experiment 1.1:*** Replications: 4 Treatments: 2 Larvae/trt.: 50 N = 400	Food: caterpillars (non-target species)	Food: caterpillars (non-target species)	Food: caterpillars (non-target species) Supplemented with flour moth eggs		
		Duration of Bt exposure	Entire stage	Entire stage	Entire stage		
		Parameters:					
		Mortality:	Bt: 24% Co: 10%	Bt: 40% Co: 21%	Bt: 11% Co: 7%	Bt: 0% Co: 2%	Bt: 60% Co: 37%
		Development time:	Bt: 5.0 days Co: 4.5 days	Bt: 6.5 days Co: 6.5 days	Bt: 7.3 days Co: 7.8 days	Bt: 12.5 days Co: 12.5 days	Bt: 31 days Co: 31 days
		Experiment 1.2: Replications: 4 Treatments: 2 Larvae/trt.: 50 N = 400	Food: caterpillars (non-target species)	Food: caterpillars (non-target species)	Food: caterpillars (non-target species)		
		Duration of Bt exposure	Entire stage	Entire stage	Entire stage		
		Parameters:					
		Mortality:	Bt: 29% Co: 10%	Bt: 45% Co: 20%	Bt: 11% Co: 7%	Bt: 8.0% Co: 2.5%	Bt: 66% Co: 38%
		Development time:	Bt: 5.8 days Co: 5.1 days	Bt: 7.5 days Co: 5.1days	Bt: 7.5 days Co: 6.5 days	Bt: 12.5 days Co: 12.5 days	Bt: 32 days Co: 29 days
2 (Hilbeck *et al.*, 1998b)	Bi-trophic; microbially produced Bt toxin (Cry1Ab) [100µg/ml], Bt-free control	***Experiment 2.1:*** Replications: 5 Treatments: 2 Larvae/trt.: 30 N = 300	Food: lacewing diet	Food: lacewing diet	Food: lacewing diet		

Study	Study material	Design of individual experiments	First instar (L1)	Second instar (L2)	Third instar (L3)	Pupa (non-feeding)	Entire juvenile stage (L1-adult)
		Duration of Bt exposure	Entire stage	Entire stage	Entire stage		
		Parameters:					
		Mortality:	Bt: 6% Co: 6%	Bt: 26% Co: 8%	Bt: 22% Co: 12%	Bt: 34% Co: 14%	Bt: 57% Co: 30%
		Development time:	Bt: 7 days Co: 7 days	Bt: 11 days Co: 10 days	Bt: 12 days Co: 10 days	Bt: 12 days Co: 12 days	Bt: 37.5 days Co: 37.5 days
		Experiment 2.2:					
		Replications: 5 Treatments: 2 Larvae/trt.: 30 N = 300	Food: flour moth eggs	Food: lacewing diet	Food: lacewing diet		
		Duration of Bt exposure	None	Entire stage	Entire stage		
		Parameters:					
		Mortality:	Bt: 2% Co: 2%	Bt: 15% Co: 6%	Bt: 7.5% Co: 5.0%	Bt: 7.5% Co: 4.0%	Bt: 29% Co: 17%
		Development time:	Bt: 4.5 days Co: 4.5 days	Bt: 4.3 days Co: 4.0 days	Bt: 7.5 days Co: 7.5 days	Bt: 12 days Co: 12 days	Bt: 28.0 days Co: 27.5 days
		Experiment 2.3:					
		Replications: 5 Treatments: 1 Larvae/trt.: 30 N = 300	Food: flour moth eggs	Food: lacewing diet	Food: lacewing diet		
		Duration of Bt exposure	None	Entire stage	Entire stage		
		Parameters:					
		Mortality :	ca. 1%	0 %	0.5 %	5%	8%
		Development time:	4.5 days	3.2 days	4.3 days	12 days	23 days

Study	Study material	Design of individual experiments	First instar (L1)	Second instar (L2)	Third instar (L3)	Pupa (non-feeding)	Entire juvenile stage (L1-adult)
3 (Hilbeck *et al.*, 1999)	Tri-trophic; microbially produced Bt toxins, Bt-free control	***Experiment 3:*** Replications: 4 Treatments: 8 Larvae/trt. : 30 N = 960	Food: caterpillars (non-target species)	Food: caterpillars (non-target species)	Food: caterpillars (non-target species)	Bt toxins, Bt-free	Larvae/trt.: 30
		Duration of Bt exposure	Entire stage	Entire stage	Entire stage		
		Parameters: Mortality:					
		Cry1Ab toxin [µg/ml]: 100, 50, 25	35, 18, 10%	25, 12.5, 17.5%	35, 46, 31%	40, 35, 24%	78, 69, 55%
		Cry1Ab protoxin [µg/ml]: 200, 100, 50	12.5, 12, 17.5%	16, 15, 20%	33, 25, 41%	14, 9, 20%	56, 46, 62%
		Cry2A protoxin [µg/ml]: 100	10%	14%	24%	15%	47.5%
		Control	6%	4%	13%	10%	26%
4 (Dutton *et al.* 2002)	Tri-trophic; Bt maize (Bt 11), isogenic control maize	***Experiment 4.1:*** Replications: 2 Treatments: 2 Larvae/trt.: 30 N = 120	Food: caterpillars (non-target species)	Food: caterpillars (non-target species)	Food: caterpillars (non-target species); replaced with flour moth eggs		
		Duration of Bt exposure	Entire stage	Entire stage	2 days		
		Parameters: Survival Rate : (Mortality):	Bt: 50% (50%) Co: 90% (10%)	Bt: 40% (60%) Co: 65% (35%)	Bt: 90% (10%) Co: 95% (5%)	=/< 2%	Bt: 20% (80%) Co: 60% (40%)
		Development time :	Bt: 5 days Co: 3 days	Bt: 8 days Co: 6 days	Bt: 5 days Co: 5 days		Bt: 24 days Co: 21 days
		Weight:	–	Bt: < 1mg Co: ca. 1mg	Bt: 2 mg Co: 2 mg		Bt: 9 mg Co: 10 mg

Study	Study material	Design of individual experiments	First instar (L1)	Second instar (L2)	Third instar (L3)	Pupa (non-feeding)	Entire juvenile stage (L1-adult)
		Experiment 4.2: Replications: 2 Treatments: 2 Larvae/trt.: 30 N = 120	Food: spider mites	Food: spider mites	Food: spider mites + flour moth eggs		
		Duration of Bt exposure	Entire stage	Entire stage	2 days		
		Parameters					
		Survival rate (Mortality):	Bt: 95% (5%) Co: 95% (5%)	Bt: 100% (0%) Co: 95% (5%)	Bt: 95% (5%) Co: 95% (5%)	=/< 2%	Bt: 95% (5%) Co: 90% (10%)
		Development time :	Co: 3 days Bt: 3 days	Bt: 3 days Co: 3 days	Bt: 4 days Co: 4 days		Bt: 19 days Co: 20 days
		Weight:	— Co: 95% (5%)	Bt: ca. 1 mg Co: ca. 1 mg	Bt: ca. 3 mg Co: ca. 3 mg		Bt: ca. 8 mg Co: ca. 8 mg
		Experiment 4.3: Replications: 2 Treatments: 2 Larvae/trt.: 30 N=120	Food: aphids	Food: aphids	Food: aphids		
		Duration of Bt exposure	None[1]	None[1]	None[1]		
		Parameters					
		Survival rate (Mortality):	Bt: 96% (4%) Co: 96% (4%)	Bt: 98% (2%) Co: 98% (2%)	Bt: 98% (2%) Co: 100% (0%)	=/< 2%	Bt: 95% (5%) Co: 92% (8%)
		Development time :	Co: 3 days Bt: 3 days	Bt: 3 days Co: 3 days	Bt: 4 days Co: 4 days		Bt: 20 days Co: 20 days
		Weight:	—	Bt: ca. 1 mg Co: ca. 1 mg	Bt: ca. 2 mg Co: ca. 2 mg		Bt: ca. 9 mg Co: ca. 9 mg
5 (Romeis *et al.* 2004)	Bi-trophic, microbially produced Bt toxin (Cry1Ab), Bt-free control	***Experiment 5.1:*** Replications: 1 Treatments: 2 Larvae/trt.: 40 N=80	Food: sugar solution	—	—		

Study	Study material	Design of individual experiments	First instar (L1)	Second instar (L2)	Third instar (L3)	Pupa (non-feeding)	Entire juvenile stage (L1-adult)
		Duration of Bt exposure	30 minutes	—	—		
		Parameter: Uptake rate (% weight difference)	Bt: 15.7% Co: 14.7%				
		Experiment 5.2: Replications: 6 Treatments: 5 (4 Bt concentrations+ Co) Larvae/trt.: 10 N=300	Food: sugar solution	—	—		
		Duration of Bt exposure	Provided until death	—	—		
		Parameter: Time to death (larval development stopped)	Bt (4 conc.): 9-10 days Co: 9.5 days				
		Experiment 5.3: Replications: 3 Treatments: 2 Larvae/trt.: 20 N=120	Food: Food: sugar solution + flour moth eggs	Food:flour moth eggs	Food:flour moth eggs		
		Duration of Bt exposure	6 days	None	None		
		Parameters: Survival rate	Bt: 87.9% (12.1%) Co: 84.7% (15.3%)	Bt: 96.1% (3.9%) Co: 96.0% (4.0%)	— —	— —	— —
		Development time:	Bt: 5.1 days (+6 Bt) Co: 5.1 days (+6 Bt)	Bt: 3.4 days Co: 3.4 days			
		Dry weight L3	—	— —	Bt: 1252mg Co: 1139mg		

Study	Study material	Design of individual experiments	First instar (L1)	Second instar (L2)	Third instar (L3)	Pupa (non-feeding)	Entire juvenile stage (L1-adult)
		Experiment 5.4:					
		Replications: 3 Treatments: 6 Larvae/trt.: 10 (flour moth eggs), 20 (caterpillars) N= 90 (flour moth eggs), N= 180 (caterpillars)	Food: caterpillars (non-target species) or flour moth eggs	Food: Bt- sugar solution sugar solution water	Food: Bt- sugar solution sugar solution water		
		Duration of Bt exposure	None	Yes, all larvae – duration unclear and different	Unclear (brief) - few larvae	—	—
		Parameter:					
		Survival rate (mortality) :					
		Flour moth eggs (L1)	Co: 98.9% (1.1%)*	—	—		
		Caterpillars (L1)	Co: 72.2% (27.8%)	—	—		
		Development time:					
		Flour moth eggs (L1)	Co: 3.7 days*	—	—		
		Caterpillars (L1)	Co: 5.7 days	—	—		
		Time to death:					
		Flour moth eggs (L1)	—	46 – 47 days	Not differentiated from L2		
			—	46 – 47 days			
			—	5.6 days (water)			
		Caterpillars (L1)	—	20 – 21 days	Not differentiated from L2		
			—	20 – 21 days			
			—	2.1 days (water)			
		Percentage to L3:					
		Flour moth eggs (L1)	—	—	37.9 – 46.7%		
		Caterpillars (L1)	—	—	0-2.4% (water)		

Study	Study material	Design of individual experiments	First instar (L1)	Second instar (L2)	Third instar (L3)	Pupa (non-feeding)	Entire juvenile stage (L1-adult)
6 (Rodrigo-Simon *et al*. 2006)	Tri-trophic; microbially produced Bt toxins: Cry1Ab, Cry1Ac, Cry2Ab [1-10µg/ml] Bt-free control	***Experiment 6.1:*** Replications: 3 Treatments: 9 Larvae/trt.: 10 N=270	Food: no information?	Food: Mixed 5 caterpillars (target species, stage unspecified) per day, flour moth eggs every other day (choice).	Food: Mixed 5 caterpillars (target species, stage unspecified) per day, flour moth eggs every other day (choice).		
		Duration of Bt exposure	Presumably none	Entire stage	Entire stage		
		Parameters:					
		Survival rate (Mortality):				L2-P	L2-A
		Cry1Ac (both conc.)	—	Not provided	Not provided	Bt: ca. 85-90% (ca. 10-15%) Co: ca. 85% (ca. 15%)	Bt: ca.65-70% (ca. 30-35%) Co: ca. 60% (ca. 40%)
		Cry1Ab (both conc.)	—	Not provided	Not provided	Bt: ca. 90-95% (ca. 5-10%) Co: ca. 90% (ca. 10%)	Bt: ca. 75-85% (ca. 15-25%) Co: ca. 70% (ca. 30%)
		Cry2Ab (both conc.)	—	Not provided	Not provided	Bt: 100% (0%) Co: 100% (0%)	Bt: ca. 75-85% (ca. 15-25%) Co: ca. 83% (ca. 17%)
						Pupa	L2-A
		Development time:					
		Cry1Ac (both conc.)	—	Bt: 2.1-2.3 d Co: 2 d	Bt: 5.2-5.8 d Co: 5.5 d	Bt: 12.8-13.7 d Co: 13.5 d	Bt: 20.2-21.8 d Co: 21 d
		Cry1Ab (both conc.)	—	Bt: 1.8-1.9 d Co: 2.3 d	Bt: 4.9-5.0 d Co: 5.4 d	Bt: 12.3-12.6 d Co: 12.8 d	Bt: 19.1-19.5 d Co: 20.5 d
		Cry2Ab (both conc.)	—	Bt: 1.8-2.0 d Co: 1.9 d	Bt: 5.5-5.7 d Co: 5.6 d	Bt: 13.8-14.3 d Co: 14 d	Bt: 21.1-22 d Co: 21.6 d

Study	Study material	Design of individual experiments	First instar (L1)	Second instar (L2)	Third instar (L3)	Pupa (non-feeding)	Entire juvenile stage (L1-adult)
	Bi-trophic; microbially produced Bt toxin (Cry1Ab) [4mg/ml], Bt-free control	***Experiment 6.2:*** Replications: 3 Treatments: 2 Larvae/trt.: 10 N=60	Food: ? no information	Food: Mixed 1 drop water w/wo Bt 24 h flour moth eggs 24 h starvation Procedure repeated 2-3 times until pupation.	Food: Mixed 1 drop water w/wo Bt 24 h flour moth eggs 24 h starvation Procedure repeated 2-3 times until pupation.		
		Duration of Bt exposure	Presumably none	Every 3rd day	Every 3rd day		
		Parameters:					
		Survival rate (Mortality):				L2-P	L2-A
		Cry1Ac	—	Not provided	Not provided	Bt: ca. 95% (ca.5%) Co: 100% (0%)	Bt: ca. 80% (ca.20%) Co: ca. 87% (ca.13%)
		Development time :				Pupa	
		Cry1Ac	—	Bt: 2.2 d Co: 2.1 d	Bt: 5.5 d Co: 5.8 d	Bt: 12.0 d Co: 12.3 d	Bt: 20.0 d Co: 19.9 d

*Data that were not included in tables or in the text were derived from figures of the publications. In these cases, a small inaccuracy in the data presented here is possible. Co = Control; Bt = Bt-Treatment. [1] According to Raps *et al.* (2001), the Cry1Ab toxin is not present in the phloem of transgenic corn, which means that it cannot be take up by aphids.

approximately 1 drop every 2-3 days until pupation. The larvae were raised on flour moth eggs interrupted by 24 h starvation periods prior to the administration of the water drop. Exposure began with the second larval stage. First instars were not exposed. No effects were observed.

Tri-trophic Effects

Both Hilbeck *et al.* (1998a) and Dutton *et al.* (2002) fed lacewing larvae with caterpillars that either had fed on Bt maize or isogenic maize. Hilbeck *et al.* (1998a) continued to feed lacewings with Bt prey until pupation, supplemented with meal moth eggs during the last instar (3 larval stages), while Dutton *et al.* (2002) stopped feeding Bt prey two days after larvae had reached the third instar and reared them exclusively on meal moth eggs until pupation (average 3 days, Table 6). Despite these differences, in both studies significantly more lacewing larvae died when they were raised with prey caterpillars that contained Bt toxin. Dutton *et al.* (2002) further conducted similar feeding studies with other types of prey, aphids and spider mites. For both prey types, lacewing larvae developed and died at similar rates regardless whether their prey had fed on Bt or isogenic maize (Table 6). For aphids, this can be explained, because as strict phloem-feeders they did not contain Bt toxin. Raps *et al.* (2001) and Head *et al.* (2001) did not detect any Bt toxin in the phloem of Bt maize or in aphids feeding on it. By contrast, spider mites did ingest the Bt toxin from the Bt maize but this did not induce higher mortality in the lacewing larvae. However, no studies on the biochemical processing of the Bt toxin in the spider mites and its sustained bioactivity were conducted.

Hilbeck *et al.* (1999) conducted further experiments where they fed lacewing larvae with prey caterpillars that had fed on artificial diet containing different concentrations of microbially produced Bt toxins. Again, significantly higher mortality rates in lacewings were observed that increased as the concentration of the Bt toxin in the diet for their prey increased (Table 6). While the prey caterpillars only showed significantly higher mortality of 42% at the highest Bt toxin concentration, lacewing larvae exhibited a lethal effect at all concentrations exceeding 70% when their prey had fed on the highest concentration diet. At the lower concentrations, caterpillars only exhibited sublethal effects, i.e., reduced weight, when feeding on the diet for several days. However, when designated as food for lacewings in the experiments, caterpillars were only allowed to feed for 12-24 h on the Bt diet. They did not exhibit noticable adverse effects at that time.

Rodrigo-Simon *et al.* (2006) raised lacewing larvae (again, beginning second instar) on five *H. armigera* larvae per day, supplemented with flour moth eggs every other day. First instars were not exposed. Also here, untreated flour moth eggs constituted a significant part of their diet, if not the main part. However, five *H. armigera* larvae per day seems rather few. In a study, where lacewing larvae were offered caterpillars and aphids in a choice and no-choice setup, Meier and Hilbeck (2001) reported consumption rates of 4-5 prey caterpillars in only 4 hours for second instar lacewing larvae when no other food was available. When a preferred food was offered, in that case aphids, second instar lacewing larvae still ate 2-3 prey larvae in 4 hours. Within the 4 hour period, third instar lacewing larvae on average ate only 6-9 prey larvae in a no-choice situation and 5-9 prey larvae in a choice situation. Hence, lacewing larvae in the Rodrigo-Simon *et al.* study (2006) were raised, to a substantional degree, on untreated flour moth egg diet offered every other day. Well-fed larvae can easily survive one day with a less preferred food type in limited supply, possibly allowing for a temporary recovery from the Bt-treatment.

DISCUSSION

Laboratory Studies with Bt Spray Formulation

Most laboratory testing on non-target effects was conducted with spray formulations that are based on *B. thuringiensis* subsp. *kurstaki* (Btk) producing toxins of the Cry1 family and with purified Cry1 toxins (MacIntosh *et al.*, 1990; Melin and Cozzi, 1990), fewer with Bt toxins active against other insect groups. Studies on non-target effects of Bt spray formulations showed conflicting results with no effects in some cases and lethal or sublethal effects in others. Many of the effects of Bt spray formulations observed in earlier studies were attributed to the occurrence of β-exotoxins, which

Table 6. Comparison of the individual similar components of five studies investigating non-target effects of Bt toxins and Bt-fed prey on Green lacewing (*Chrysoperla carnea*) larvae (Complementary to Table 5).

Experiment		1.1	4.1	1.1	4.1	5.4	2.3	5.4	2.1	5.2	2.1	5.2
Instar	Parameter	Bt-prey		Bt-free prey as control		Food type unclear	Flour moth eggs		Bt artificial diet		Control artificial diet	
L1	Mortality	24%	50%	10%	10%	27.8%	1-2%	1%	6%	—	6%	—
	Development time	5 days	5 days	4.5 days	3 days	5.7 days	4.5 days	3.7 days	7 days	—	7 days	—
	Time to death									9.5		9.5
L2	Mortality	40%	60%	21%	35%	—	—	—				
	Development time	6.5 days	8 days	6.5 days	6 days	—	—	—				
L1 – A*	Mortality	60%	80%	37%	40%	—	—	—				
	Development time	31 days	24 days	31 days	21 days	—	—	—				

1.1: Hilbeck *et al.* (1998a) ; 2.1 + 2.3: Hilbeck *et al.* (1998b); 4.1: Dutton *et al.* (2002); 5.2 + 5.4: Romeis *et al.* (2004). *Data that were not included in tables or in the text were derived from figures of the publications. In these cases, a small inaccuracy in the data presented here is possible..

are known to have a more general toxicity (Sebasta *et al.*, 1981; Melin and Cozzi, 1990), but this was seldom followed up. The awareness of the acute toxicity of β-exotoxins on non-target organisms has led to the proscription of formulations containing these substances (Lacey and Siegel, 2000). If adverse effects were observed, both direct (effect of the toxin) and indirect (effects of toxin affected prey/host, which provides a less suitable food source) were discussed (Flexner *et al.*, 1986). Despite a considerable number of studies reporting adverse effects on non-target invertebrates, Bt formulations based on δ-endotoxins (Cry proteins) are assumed to be highly specific and have negligible effects on non-target organisms because of their limited bioactivity under field conditions (Ignoffo and Garcia, 1978).

Laboratory Studies with Transgenic Bt Plants

Non-target invertebrates and Bt interactions. Overall, the results of the published laboratory studies on non-target effects in the context with transgenic Bt plants are inconsistent, and no coherent and predictable pattern of the observed Bt effects is emerging yet. All studies still represent pieces of a puzzle, the picture of which is not recognizable at this time. The majority of studies are isolated experiments following quite different methodologies. Further, the results of those studies, which tested a few organisms repeatedly (e.g. lacewing larvae), did not lead to a scientific consensus regarding the kind of impact transgenic Bt plants might exert and the responsible mode of action. In fact, similar to the cases described by Crickmore (2005) (see above), rather differing lines of interpretation complicated the situation further. As subtle differences in toxin structure could affect binding and host specificity (Crickmore, 2005), the uncertainty regarding structural differences of Bt toxins expressed by Bt transgenic plants when, in addition, passing through the digestive tract of herbivore insects could well explain effects found with non-target species. While Crickmore (2005) contemplates that too much research has been put on binding at the expense of other factors that might have an equally important role in determining the efficacy of a toxin, we argue that sublethal effects or lethal effects in non-target organisms at high toxin concentrations could also be triggered by other mechanisms that are masked in target species by the lethal effects induced through the commonly known mode of action.

Green lacewing larvae and Bt interactions – different interpretations of the same data and remaining gaps of knowledge. From their results from direct and prey-mediated Bt feeding trials, Romeis *et al.* (2004), Dutton *et al.* (2002) and Rodrigo-Simon *et al.* (2006) concluded that the

observed mortality in Bt-fed lacewing larvae is solely due to lower nutritional quality of the sublethally affected prey without the Bt-toxin having a role in it. We find this unlikely and a too limited interpretation. Firstly, the direct effects of the Bt toxin feeding study clearly document the sensitivity of *C. carnea* larvae, certainly at higher concentrations (Hilbeck, *et al.,* 1998b), and cannot be explained by reduced prey quality as Bt toxin was fed directly to the predator using a specific lacewing diet. The direct feeding trials by Romeis *et al.* (2004) and Rodrigo-Simon *et al.* (2006) complement the findings by Hilbeck *et al.* (1998b) in as much as they document that short term or intermittent exposure to Bt toxin at mostly low concentrations do not lead to measurable adverse effects, in particular, when lacewing larvae were subsequently raised on an optimal Bt-free diet. The totality of the data on lacewings, but also on other non-target species and Bt, indeed confirm earlier conclusions (Hilbeck, 2002; Andow and Hilbeck, 2004) that complex interactions are involved. These could involve other modes of action of the Bt toxins or its metabolites, and altered chemistry of Bt toxins when, firstly, expressed in a plant and, secondly, passing through the gut of a herbivore prey organism, including possibly one or all of the following: a) altered nutritional prey quality, b) toxicity of the Bt toxin or its metabolites, c) toxicity of natural plant secondary metabolites interacting with the Bt toxin/metabolites. To keep these processes apart experimentally is impossible, as too many possible interactions can be involved (Andow and Hilbeck, 2004).

However, the sustained scientific dissent highlights another understudied issue concerning the spread, processing, degradation and re-cycling of Bt toxins in above- and below-ground ecosystems and how its bioactivity can be affected by these processes. Only 10 years after large scale commercial production of Bt crops in some countries, the first studies were published that investigated the fate and spread of the expressed novel protein in the food chain and insect community of the Bt cropping system. Harwood *et al.* (2005) demonstrated that the Bt toxins have spread in the food chain and found surprisingly high concentrations in some higher trophic level organisms while not in others. Similar results were reported by Obrist *et al.* (2006a) for different non-target herbivores and predators. In spider mites, *Tetranychus urticae*, the authors even documented a concentration that was three times higher than in maize leaves the mites had fed upon. Zwahlen and Andow (2005) found the Bt toxin in field collected carabid beetles even if no Bt maize had been planted in the year before. Key experiments regarding the molecular characterization of Bt toxins – whether or not they are degraded – and their bioactivity are needed to better understand the spread of Bt toxins through food webs.

Only one study (Rodrigo-Simon, *et al.,* 2006) has so far considered the mode of action of Bt toxins in the gut of *C. carnea*. Based on their results from binding studies with lacewing midguts by following the procotols developed for caterpillars, Rodrigo-Simon *et al.* (2006) conclude that *C. carnea* larvae lack specific receptors for Cry1Ab and Cry1Ac. From this, we conclude that Bt toxins do not operate in predatory lacewing larvae like in herbivorous caterpillars, but the key experiments on what caused the significantly higher mortality in Bt-exposed lacewings larvae are still missing to date.

CONCLUSIONS

The reports of adverse effects of Bt toxins and transgenic Bt plants on arthropod species other than the target pest insects went surprisingly unnoticed by the scientific expert community studying Bt modes of action. Some experts simply attributed tri-trophic effects to poorer prey quality, while the bi-trophic direct adverse Bt effects were considered as “unlikely”, because it did not fit the commonly accepted model of Bt toxin mode of action in target species. While reports of unexplained effects on non-target species and the lack of explanations of their causes should call for more research, key experiments on alternative modes of action in non-target invertebrates are still missing. This is even more astonishing as the cultivation area of transgenic Bt crop plants increases continuously worldwide (James, 2005). Most of the tested species exhibiting unexplained effects are common members of the

insect community occurring in Bt fields and are exposed to Bt toxins (Harwood *et al.*, 2005; Zwahlen and Andow, 2005; Obrist *et al.*, 2006a). Crickmore (2005) emphasises that novel approaches should be applied in order to provide insights into the complex nature of the toxin-host interactions. A more detailed knowledge of Bt interactions could lead to the development of improved Bt biopesticides (Crickmore, 2005). We argue that this should not only be restricted to target insects but would also contribute to a better understanding of unexplained effects in non-target organisms. In fact, a recent paper explores the possibility of alternate modes of action in target insects (Zhang *et al.*, 2006). Additionally, there are emerging models for the mode of action in target insects that involve new elements like dual binding to aminopeptidases and cadherins, lipid targeting and more (e.g. Bravo *et al.*, 2004). Most recently, Broderick, *et al* (2006) reported that the presence of certain midgut bacteria is required for Bt toxins to unfold its activity in the investigated target insect. Again, we argue that this might also help explain some of the peculiar effects observed with non-target organisms. The currently existing model of Bt mode of action might have to be revised soon. We believe that including non-target organims into Bt research offers great opportunities to help improve our understanding on what else Bt might do. With this review, we hope to stimulate more research and thinking 'outside of the box'.

REFERENCES

Addison, J.A. (1993) Persistence and nontarget effects of *Bacillus thuringiensis* in soil: a review. *Can. J. Forest Res.*, **23**, 29–42.

Al–Deeb, M.A., Wilde, G.E. and Higgins, R.A. (2001) No effect of *Bacillus thuringiensis* corn and *Bacillus thuringiensis* on the predator *Orius insidiosus* (Hemiptera: Anthocoridae). *Environ. Entomol.*, **30**, 625–629.

Ali, A. (1981) *Bacillus thuringiensis* serosubspecies *israelansis* (ABG-6108) against chironomids and some nontarget aquatic invertebrates. *J. Invertebr. Pathol.*, **38**, 264-272.

Ali, A., Baggs, R.D. and Stewart, J.P. (1981) Susceptibility of some Florida chironomids and mosquitoes to various formulations of *Bacillus thuringiensis* serosubspecies *israelensis* de Barjay. *J. Econ. Entomol.*, **74**, 672-677.

Anderson, P.L., Hellmich, R.L., Sears, M.K., Sumerford, D.V. and Lewis, L.C. (2004) Effects of Cry1Ab expressing corn anthers on monarch butterfly larvae. *Environ. Entomol.*, **33**, 1109–1115.

Andow, D.A. (2003) Negative and positive data, statistical power, and confidence intervals. *Environ. Biosafety Res.*, **2**, 75–80.

Andow, D.A. and Hilbeck, A. (2004) Science–based risk assessment for non–target effects of transgenic crops. *BioScience*, **54**, 637–649.

Armer, Ch.A., Berry, R.E. and Kogan, M. (2000) Longevity of phytophagous heteropteran predators feeding on transgenic Btt-potato plants. *Entomol. Exp. Appl.*, **95**, 329-333.

Arpaia, S. (1996) Ecological impact of Bt–transgenic plants: Part 1. Assessing possible effects of CryIIIB toxin on honey bee (*Apis mellifera* L.) colonies. *J. Genet. Breed.*, **50**, 315–320.

Arpaia, S., Mennella, G., Onofaro, V., Perri, E., Sunseri, F. and Rotino, G.L. (1997) Production of transgenic eggplant (*Solanum melongena* L.) resistant to Colorado potato beetle (*Leptinotarsa decemlineata* Say). *Theor. Appl. Genet.*, **95**, 329–334.

Ashouri, A., Michaud, D. and Cloutier, C. (2001a) Recombinant and classically selected factors of potato plant resistance to the Colorado potato beetle, *Leptinotarsa decemlineata*, variously affect the potato aphid parasitoid *Aphidius nigripes*. *Biocontrol*, **46**, 401–418.

Ashouri, A., Michaud, D. and Cloutier, C. (2001b) Unexpected effects of different potato resistance factors to the Colorado potato beetle (Coleoptera: Chrysomelidae) on the potato aphid (Homoptera: Aphididae). *Environ. Entomol.*, **30**: 524–532.

Bai, Y.Y., Jiang, M.X. and Cheng, J.A. (2005) Effects of transgenic cry1Ab rice pollen on fitness of *Propylaea japonica* (Thunberg). *J. Pest Sci.*, **78**, 123–128.

Baur, M.E. and Boethel, D.J. (2003) Effect of Bt–cotton expressing Cry1A(c) on the survival and fecundity of two hymenopteran parasitoids (Braconidae, Encyrtidae) in the laboratory. *Biol. Contr.*, **26**, 325–332.

Becker, N. and Margalit, J. (1993) Use of *Bacillus thuringiensis israelensis* against mosquitoes and blackflies. *In* P.F. Entwistle, J.S. Cory, M.J. Bailey, and S. Higgs (eds.), *Bacillus thuringiensis, an*

Environmental Biopesticide: Theory and Practice. John Wiley & Sons, UK, pp. 147–170.

Beegle, C.C. and Yamamoto, T. (1992) History of *Bacillus thuringiensis* Berliner research and development. *Can. Entomol.*, **124**, 587–616.

Bernal, C.C., Aguda, R.M. and Cohen, M.B. (2002a) Effect of rice lines transformed with *Bacillus thuringiensis* toxin genes on the brown planthopper and its predator *Cyrtorhinus lividipennis*. *Entomol. Exp. Appl.*, **102**, 21–28.

Bernal, J.S., Griset, J.G. and Gillogly, P.O. (2002b) Impacts of developing on Bt maize–intoxicated hosts on fitness parameters of a stem borer parasitoid. *J. Entomol. Sci.*, **37**, 27–40.

Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D.J., Roberts, G.M., Pauli, S., Rodgers, P. and Burges, H.D. (1997) Natural isolates of *Bacillus thuringiensis*: worldwide distribution, characterization, and activity against insect pests. *J. Invertebr. Pathol.*, **70**, 59–68.

Brandt, S.L., Coudron. T.A., Habibi, J., Brown, G.R., Ilagan, O.M., Wagner, R.M., Wright, M.K., Backus, E.A. and Huesing, J.E. (2004) Interaction of two *Bacillus thuringiensis* δ–endotoxins with the digestive system of *Lygus hesperus*. *Curr. Microbiol.*, **48**, 1–9.

Brar, S.K., Verma, M., Tyagi, R.D. and Valéro, J.R. (2006) Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides. *Process Biochem.*, **41**, 323–342.

Bravo, A., Gomez, I., Conde, J., Munoz–Garay, C., Sanches, J., Miranda, R., Zhuang, M., Gill, S.S., Soberon, M. (2004) Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore–forming toxin to aminopeptidase N receptor leading to insertion into membrane micro–domains. *Biochimica et Biophysica Acta*, **1667**, 38–46.

Broderick, N. A., Kenneth F. Raffa, and Handelsman, J. (2006) Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity *Proc. Natl. Acad. Sci. USA*, 10.1073 (online)

Cantwell, G.E., Knox, D.A., Lehnert, T. and Michael A.S. (1966) Mortality of the honey bee, *Apis mellifera*, in colonies treated with certain biological insecticides. *J. Invertebr. Pathol.*, **8**, 228-233.

Carter, M.E., Villani, M.G., Allee, L.L and Losey, J.E. (2004) Absence of non–target effects of two *Bacillus thuringiensis* coleopteran active δ–endotoxins on the bulb mite, *Rhizoglyphus robini* (Claparède) (Acari, Acaridae). *J. Appl. Entomol.*, **128**, 56–63.

Cerstiaens, A., Verleyen, P., van Rie, J., van Kerkhove, E., Schwartz, J.–L., Laprade, R., de Loof, A. and Schoofs, L. (2001) Effect of *Bacillus thuringiensis* Cry1 toxins in insect hemolymph and their neurotoxicity in brain cells of *Lymantria dispar*. *Appl. Environ. Microbiol.*, **67**, 3923–3927.

Chapman, M.H. and Hoy, M.A. (1991) Relative toxicity of *Bacillus thuringiensis* var. *tenebrionis* to the two–spotted spider mite (*Tetranychus urticae* Koch) and its predator *Metaseiulus occidentalis* (Nesbitt) (Acari, Tetranychidae and Phytoseiidae). *J. Appl. Entomol.*, **111**, 147–152.

Crickmore, N. (2005) Using worms to better understand how *Bacillus thuringiensis* kills insects. *Trends in Microbiol.*, **13**, 347–350.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. and Dean, D.H. (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.*, **62**, 807–813.

Crickmore, N., Zeigler, D.R., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Bravo, A. and Dean, D.H. (2005) *Bacillus thuringiensis* toxin nomenclature http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore.Bt/

Crook, N.E. and Jarrett, P. (1991) Viral and bacterial pathogens of insects. *J. Appl. Bacteriol.*, **70**, 91–96.

Davidson, M.M., Butler R.C., Wratten, S.D. and Conner A.J. (2006) Impacts of insect–resistant transgenic potatoes on the survival and fecundity of a parasitoid and an insect predator. *Biol. Contr.*, **37**, 224–230.

de Maagd, R.A., Bravo, A. and Crickmore, N. (2001) How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends Genet.*, **17**, 193–199.

de Maagd, R.A., Bravo, A., Berry, C., Crickmore, N. and Schnepf, H.E. (2003) Structure, diversity, and evolution of protein toxins from spore–forming entopathogenic bacteria. *Annu. Rev. Genet.*, **37**, 409–433.

Dean, D.H., Rajamohan, F., Lee, M.K., Wu, S.–J., Chen, X.J., Alcantara, E. and Hussain, S.R. (1996) Probing the mechanism of action of *Bacillus*

thuringiensis insecticidal proteins by site–directed mutagenesis – a minireview. *Gene*, **179**, 111–117.

Deml, R., Meise, T., Dettner, K. (1999) Effects of *Bacillus thuringiensis* δ–endotoxins on food utilization, growth, and survival of selected phytophagous insects. *J. Appl. Entomol.*, **123**, 55–64.

Dogan, E.B., Berry, R.E., Reed, G.L. and Rossignol, P.A (1996) Biological parameters of convergent lady beetle (Coleoptera: Coccinellidae) feeding on aphids (Homoptera: Aphididae) on transgenic potato. *J. Econ. Entomol.*, **89**, 1105–1108.

Duan, J.J., Head, G., Mckee, M.J., Nickson, T.E., Martin, J.W. and Sayegh, F.S. (2002) Evaluation of dietary effects of transgenic corn pollen expressing Cry3Bb1 protein on a non–target ladybird beetle, *Coleomegilla maculata*. *Entomol. Exp. Appl.*, **104**, 271–280.

Dunbar, J.P. and Johnson, A.W. (1975) *Bacillus thuringiensis*: Effects on the survival of a tobacco budworm parasitoid and predator in the laboratory. *Environ. Entomol.*, **4**, 352-354.

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

Dutton, A., Klein, H., Romeis, J. and Bigler, F. (2003) Prey–mediated effects of *Bacillus thuringiensis* spray on the predator *Chrysoperla carnea* in maize. *Biol. Contr.*, **26**, 209–215.

Dutton, A., Romeis, J. and Bigler, F. (2005) Effects of Bt maize expressing Cry1Ab and Bt spray on *Spodoptera littoralis*. *Entomol. Exp. Appl.*, **114**, 161–169.

Entwistle, P.F., Cory, J.S., Bailey, M.J. and Higgs, S. (eds.) (1993) *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice.* John Wiley & Sons, UK.

Escher, N., Käch, B. and Nentwig, W. (2000) Decomposition of transgenic *Bacillus thuringiensis* maize by microorganisms and woodlice *Porcellio scaber* (Crustacea: Isopoda). *Basic Appl. Ecol.*, **1**, 161–169.

Feitelson, J.S., Payne, J. and Kim, L. (1992) *Bacillus thuringiensis*: insects and beyond. *Bio/Technology*, **10**, 271–275.

Felke, M., Lorenz, N. and Langenbruch, G.–A. (2002) Laboratory studies on the effects of pollen from Bt–maize on larvae of some butterfly species. *J. Appl. Entomol.*, **126**, 320–325.

Flexner, J.L., Lighthart, B. and Croft, B.A. (1986) The effects of microbial pesticides on non–target, beneficial arthropods. *Agric. Ecosyst. Environ.*, **16**, 203–254.

Gill, S.S., Cowles, E.A. and Pietrantonio, P.V. (1992) The mode of action of *Bacillus thuringiensis* endotoxins. *Annu. Rev. Entomol.*, **37**, 615–636.

Glare, T.R. and O'Callaghan, M. (2000) *Bacillus thuringiensis*: *Biology, Ecology and Safety*. John Wiley & Sons, UK.

Hanley, A.V., Huang, Z.Y. and Pett, W.L. (2003) Effects of dietary transgenic Bt corn pollen on larvae of *Apis mellifera* and *Galleria mellonella*. *J. Apicult. Res.*, **42**, 77–81.

Harwood, J.D., Wallin, W.G. and Obrycki, J.J. (2005) Uptake of Bt endotoxins by nontarget herbivores and higher order arthropod predators: molecular evidence from a transgenic corn ecosystem. *Mol. Ecol.*, **14**, 2815–2823.

Hassan, S. and Krieg, A. (1975) *Bacillus thruingiensis* preparations harmless to the parasite *Trichogramma cacoeciae* (Hym.: Trichogrammatidae). *Z. Pflanzenkrank. Pflanzen.*, **82**, 515-521.

Head, G., Brown, C.R., Groth, M.E. and Duan, J.J. (2001) Cry1Ab protein levels in phytophagous insects feeding on transgenic corn: implications for secondary exposure risk assessment. *Entomol. Exp. Appl.*, **99**, 37–45.

Hellmich, R.L., Sigfried, B.D., Sears, M.K., Stanley–Horn, D.E., Mattila, H.R., Spencer, T., Bidine, K.D., Daniels, M.J. and Lewis, L.C. (2001) Monarch larvae sensitivity to *Bacillus thuringiensis*–purified proteins and pollen. *Proc. Nat. Acad. Sci.USA*, **98**, 11925–11930.

Hilbeck, A. (2001) Implications of transgenic, insecticidal plants for insect and plant biodiversity. *Perspect. Plant Ecol. Evol. System.*, **4**, 43–61.

Hilbeck, A. (2002) Transgenic hostplant resistance and nontarget effects. *In* D. Letourneau and B. Burrows (eds.), *Genetically Engineered Organisms: Assessing Environmental and Human Health Effects.* CRC Press, USA, pp. 167–185.

Hilbeck, A., Baumgartner, M., Fried, P.M. and Bigler, F. (1998a) Effects of transgenic *Bacillus thuringiensis* corn–fed prey on mortality and development time of immature *Chrysoperla carnea*

(Neuroptera: Chrysopidae). *Environ. Entomol.*, **27**, 480–487.

Hilbeck, A., Moar, W.J., Pusztai–Carey, M., Filippini, A. and Bigler, F. (1998b): Toxicity of *Bacillus thuringiensis* Cry1Ab toxin to the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environ. Entomol.*, **27**, 1255–1263.

Hilbeck, A., Moar, W.J., Pusztai–Carey, M., Filippini, A. and Bigler, F. (1999) Prey–mediated effects of Cry1Ab toxin and protoxin and Cry2A protoxin on the predator *Chrysoperla carnea*. *Entomol. Exp. Appl.*, **91**, 305–316.

Höfte, H. and Whiteley, H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.*, **53**, 242–255.

Howald, R., Zwahlen, C. and Nentwig, W. (2003) Evaluation of Bt oilseed rape on the non-target herbivore *Athalia rosae*. *Entomol. Exp. Appl.*, **106,** 87-93.

Hussein, H.M., Habuštová O. and Sehnal, F. (2005) Beetle–specific *Bacillus thuringiensis* Cry3Aa toxin reduces larval growth and curbs reproduction in *Spodoptera littoralis* (Boisd.). *Pest Manag. Sci.*, **61**, 1186–1192.

Hussein, H.M., Habuštová O. Turanli, F. and Sehnal, F. (2006) Potato expressing beetle–specific *Bacillus thuringienis* Cry3Aa toxin reduces performance of a moth. *J. Chem. Ecol.*, **32**, 1–13.

Ignoffo, C.M. and Garcia, C. (1978) UV–photoinactivation of cells and spores of *Bacillus thuringiensis* and effects of peroxidase on inactivation. *Environ. Entomol.*, **7**, 270–272.

James, C. (2005) *Global Status of Commercialized Biotech/GM crops: 2005*. ISAAA Brief 34. International Service for the Acquisition of Agri–Biotech Applications, Ithaca, new York, USA.

Jaques, R.P. (1965) The effect of *Bacillus thuringiensis* Berliner on the fauna of an apple orchard. *Can. Entomol.*, **97**, 795–802.

Jesse, L.C.H. and Obrycki, J.J. (2000) Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly. *Oecologia*, **125**, 241–248.

Jouanin, L., Bonade–Bottino, M., Girard, C., Morrot, G. and Giband, M. (1998) Transgenic plants for insect resistance. *Plant Sci.*, **131**, 1–11.

Keller, B. and Langenbruch, G.–A. (1993) Control of coleopteran pests by *Bacillus thuringiensis. In* P.F. Entwistle, J.S. Cory, M.J. Bailey and S. Higgs (eds.), *Bacillus thuringiensis, An Environ–mental Biopesticide: Theory and Practice*. John Wiley & Sons, UK, pp. 171–191.

Knowles, B.H. (1994) Mechnisms of action of *Bacillus thuringiensis* δ–endotoxins. *Adv. Insect Physiol.*, **24**, 275–308.

Knowles, B.H. and Dow, J.A.T. (1993) The crystal δ–endotoxins of *Bacillus thuringiensis*: models for their mechanism of action on the insect gut. *BioEssays*, **15**, 469–476.

Koziel, M.G., Carozzi, N.B., Currier, T.C., Warren, G.W. and Evola, S.V. (1993) The insecticidal crystal proteins of *Bacillus thuringiensis*: past, present and future uses. *Biotechnol. Genetic Eng. Rev.*, **11**, 171–228.

Krieg, A. (1965) Über den Biotest von *Bacillus thuringiensis*-Exotoxin mit *Drosophila melanogaster*. *Entomol. Exp. Appl.*, **9**, 185-190.

Krieg, A. (1973) About toxic effects of cultures of *Bacillus cereus* and *Bacillus thuringiensis* on honey bees (*Apis mellifera*). *Z. Pflanzenkrank. Pflanzen.*, **80**, 483-486.

Krieg, A. and Herfs, W. (1963) The effects of *Bacillus thuringiensis* on honey bees. *Entomol. Exp. Appl.*, **6**, 1-9.

Krieg, A. and Kulikov, N. (1963) Wirkungen von Entobakterin-3 und Thuricide auf Bienen. *Pcelovodstvo*, **40**, 32-44 (russ.)

Krieg, A. and Langenbruch, G.A. (1981) Susceptibility of arthropod species to *Bacillus thuringiensis*. In: Burges, D (ed.) Microbial control of insects and plant diseases, 1970-1980. Academic, London.

Krieg, A., Hassan, S. and Pinsdorf, W. (1980) Comparison of the effects of the variety *israelensis* with other varieties of *B. thuringiensis* on non-target organisms of the order Hymenoptera: *Trichogramma cacoeciae* and *Apis mellifera*. *J. Pest Sci.*, **53**, 81-83.

Lacey, L.A. and Siegel, J.P. (2000) Safety and ecotoxicity of entomopathogenic bacteria. *In* J.–F. Charles, A. Delécluse and C. Nielsen–LeRoux (eds.), *Entomopathogenic Bacteria: From Laboratory to Field Application.* Kluwer Academic Publishers, The Netherlands, pp. 253–273.

Lambert, B. and Peferoen, M. (1992) Insecticidal promise of *Bacillus thuringiensis*. Facts and mysteries about a successful biopesticide. *BioScience*, **42**, 112–122.

Lereclus, D., Delecluse A and Lecadet M.M. (1993) Diversity of *Bacillus thuringiensis* toxins and genes. *In* P.F. Entwistle, J.S. Cory, M.J. Bailey, and S. Higgs (eds.), *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice.* John Wiley & Sons, UK, pp. 37–69.

Limburg, D.D. and Rosenheim, J.A. (2001) Extrafloral nectar consumption and its influence on survival and development of an omnivorous predator, larval *Chrysoperla plorabunda* (Neuroptera: Chrysopidae). *Environ. Entomol.*, **30**, 595–604.

Liu, X., Zhang, Q., Zhao, J.Z., Cai, Q., Xu, H. and Li, J. (2005a) Effects of the Cry1Ac toxin of *Bacillus thuringiensis* on *Microplitis mediator*, a parasitoid of the cotton bollworm, *Helicoverpa armigera*. *Entomol. Exp. Appl.*, **114**, 205–213.

Liu, X.–X., Sun, C.–G. and Zhang, Q.–W. (2005b) Effects of transgenic Cry1A+CpTI cotton and Cry1Ac toxin on the parasitoid, *Campoketis chlorideae* (Hymenoptera: Ichneumonidae). *Insect Sci.*, **12**, 101–107.

Losey, J.E., Rayor, L.S. and Carter, M.E. (1999) Transgenic pollen harms monarch larvae. *Nature*, **399**, 214.

Lövei, G.L. and Arpaia, S. (2005) The impact of transgenic plants on natural enemies: a critical review of laboratory studies. *Entomol. Exp. Appl.*, **114**, 1–14.

Lozzia, G.C., Furlanis, C., Manachini, B. and Rigamonti, I.E. (1998) Effects of Bt corn on *Rhopalosiphum padi* L. (Rhynchota Aphididae) and on its predator *Chrysoparla carnea* Stephen (Neuroptera Chrysopidae). *Bolletoni di Zoologia Agraria e di Bachicoltura Seria II*, **30**, 153–164.

Lozzia, G.C., Rigamonti, I.E., Manachini, B. and Rocchetti, R. (2000) Laboratory studies on the effects of transgenic corn on the spider mite *Tetranychus urticae* Koch. *Bolletoni di Zoologia Agraria e di Bachicoltura Seria II*, **32**, 35–47.

Ludy, C. and Lang, A. (2006) Bt maize pollen exposure and impact on the garden spider, *Araneus diadematus*. *Entomol. Exp. Appl.*, **118**, 145–156.

Lundgren, J.G. and Wiedenmann, R.N. (2002) Coleopteran–specific Cry3Bb toxin from transgenic corn pollen does not affect the fitness of a nontarget species, *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae). *Environ. Entomol.*, **31**, 1213–1218.

MacIntosh, S.C., Stone, T.B., Sims, S.R., Hunst, P.L., Greenplate, J.T., Marrone, P.G., Perlak, F.J., Fischhoff, D.A. and Fuchs, R.L. (1990) Specificity and efficacy of purified *Bacillus thuringiensis* proteins against agronomically important insects. *J. Invertebr. Pathol.*, **56**, 258–266.

Malone, L.A., and Pham-Delègue, M-H. (2001). Effects of transgene products on honey bees (*Apis mellifera*) and bumblebees (*Bombus* spp.). *Apidologie*, **32**, 287-304.

Marvier, M. (2002) Improving risk assessment for non target safety of transgenic crops. *Ecol. Applications*, **12**, 1119–1124.

Martínez, C. and Caballero, P. (2002) Contents of cry genes and insecticidal toxicity of *Bacillus thuringiensis* strains from terrestrial and aquatic habitats. *J. Appl. Microbiol.*, **92**, 745–752.

Martouret, D. and Euverte, G (1964) The effect of *Bacillus thuringiensis* Berliner preparations on the honey bee under conditions of forced feeding. *J. Invertebr. Pathol.*, **6**, 198-203.

McDonald, R.C., Kok, L.T. and Yousten, A.A. (1990) Response of fourth instar *Pieris rapae* parasitized by the braconid *Cotesia rubecula* to *Bacillus thuringiensis* subsp. *kurstaki* δ-endotoxin. *J. Invertebr. Pathol.*, **56**, 422-423.

Meier, M.S. and Hilbeck, A. (2001) Influence of transgenic *Bacillus thuringiensis* corn–fed prey on prey preference of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Basic Appl. Ecol.*, **2**, 35–44.

Meissle, M., Vojtech, E. and Poppy, G.M. (2005) Effect of Bt maize–fed prey on the generalist predator *Poecilus cupreus* L. (Coleoptera: Carabidae). *Transgenic Res.*, **14**, 123–132.

Melin, B.E. and Cozzi, E.M. (1990) Safety to nontarget invertebrates of lepidopteran strains of *Bacillus thuringiensis* and their α–exotoxins. *In* M. Laird, L.A. Lacey and E.W. Davidson (eds.), *Safety of Microbial Insecticides.* CRC Press, USA, pp. 149–167.

Mück, O., Hassan, S., Huger, A.M. and Krieg, A. (1981) Effects of *Bacillus thuringiensis* Berliner on the parasitic hymenoptera *Apanteles glomeratus* L. (Braconidae) and *Pimpla turionellae* (L.) (Ichneumonidae). *Z. Angew. Entomol.*, **92**, 303-314.

Müller–Cohn, J., Chaufaux, J., Buisson, Ch., Gilois, N., Sanchis, V. and Lereclus, D (1996) *Spodoptera littoralis* (Lepidoptera: Noctuidae) resistance to Cry IC and cross–resistance to other *Bacillus thuringiensis* crystal toxins. *J. Econ. Entomol.*, **89**, 791–797.

Navon, A. (1993) Control of lepidopteran pests with *Bacillus thuringiensis*. *In* P.F. Entwistle, J.S. Cory, M.J. Bailey, and S. Higgs (eds.), *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice.* John Wiley & Sons, UK, pp. 125–146.

O'Callaghan, M., Glare, T.R., Burgess, E.P.J. and Malone, L.A. (2005) Effects of plants genetically modified for insect resistance on nontarget organisms. *Annu. Rev. Entomol.*, **50**, 271–292.

Oberhauser, K.S. and Rivers, E.R.L. (2003) Monarch butterfly (*Danaus plexippus*) larvae and Bt maize pollen: a review of ecological risk assessment for a non–target species. *AgBiotechNet*, **5**, 1–7.

Obrist, L.B., Klein, H., Dutton, A. and Bigler, F. (2005) Effects of Bt maize on *Frankliniella tenuicornis* and exposure of thrips predators to prey–mediated Bt toxin. *Entomol. Exp. Appl.*, **115**, 409–416.

Obrist, L.B., Dutton, A., Albajes, R. and Bigler, F. (2006a) Exposure of arthropod predators to Cry1Ab toxin in Bt maize fields. *Ecol. Entomol.*, **31**, 143–154.

Obrist, L.B., Klein, H., Dutton, A. and Bigler, F. (2006b) Assessing the effects of Bt maize on the predatory mite *Neoseiulus cucumeris*. *Exp. Appl. Acarol.*, **38**, 125–139.

Obrycki, J.J., Losey, J,E,, Taylor, O.R. and Jesse, L.C.H. (2001) Transgenic insecticidal corn: beyond insecticidal toxicity to ecological complexity. *BioScience*, **51**, 353–361.

Pilcher, C.D., Obrycki, J.J., Rice, M.E. and Lewis, L.C. (1997) Preimaginal development, survival, and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. *Environ. Entomol.*, **26**, 446–454.

Ponsard, S., Gutierrez A.P. and Mills, N.J. (2002) Effect of Bt–toxin (Cry1Ac) in transgenic cotton on the adult longevity of four heteropteran predators. *Environ. Entomol.*, **31**, 1197–1205.

Priest, F.G. (2000) Biodiversity of entomopathogenic, endospore–forming bacteria. *In* J.–F. Charles, A. Delécluse and C. Nielsen–LeRoux (eds.), *Entomopathogenic Bacteria: From Laboratory to Field Application.* Kluwer Academic Publishers, The Netherlands, pp. 1–22.

Prütz, G. and Dettner, K. (2004) Effect of Bt–corn leaf suspension on food consumption by *Chilo partellus* and life history parameters of its parasitoid *Cotesia flavipes* under laboratory conditions. *Entomol. Exp. Appl.*, **111**, 179–187.

Prütz, G., Brink A. and Dettner, K. (2004) Transgenic insect–resistant corn affects the fourth trophic level: effects of *Bacillus thuringiensis*–corn on the facultative hyperparasitoid *Tetrastichus howardi*. *Naturwissenschaften*, **91**, 451–454.

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

Rice, M.E. and Pilcher, C.D. (1998) Potential benefits and limitations of transgenic Bt corn for management of the European corn borer (Lepidoptera: Cambidae). *Am. Entomol.*, **44**, 75–78.

Riddick, E.W. and Barbosa, P. (1998) Impact of Cry3A–intoxicated *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) and pollen on consumption, development, and fecundity of *Coleomegilla maculata* (Coleoptera: Coccinellidae). *An. Entomol. Soc. Am.*, **91**, 303–307.

Rodgers, P.B. (1993) Potential of biopesticides in agriculture. *Pestic. Sci.*, **39**, 117–129.

Rodrigo–Simon, A., de Maagd, R.A., Avilla, C., Bakker, P.L., Molthoff, J., González–Zamora, J.E. and Ferre, J. (2006) Lack of detrimental effects of *Bacillus thiuringiensis* Cry toxins on the insect predator *Chrysoperla carnea*: a toxicological, histopathological, and biochemical analysis. *Appl. Environ. Microbiol.*, **72**, 1595–1603.

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

Romeis, J., Meissle, M. and Bigler, F (2006) Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. *Nature Biotechnol.*, **24**, 1, 63–71.

Salama, H.S. and Zaki, F.N. (1983) Interaction between *Bacillus thuringiensis* Berliner an the parasites and predators of *Spodoptera littoralis* in Egypt. *Z. Angew. Entomol.*, **95**, 425-429.

Salama, H.S., Zaki, F.N. and Sharaby A.F. (1982) Effect of *Bacillus thuringiensis* Berl. on parasites and predators of the cotton leafworm *Spodoptera littoralis* (Boisd.). *Z. Angew. Entomol.*, **94**, 498-504.

Saxena, D. and Stotzky, G. (2001) *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. *Soil Biol. Biochem.*, **33**, 1225–1230.

Schmidt, J.E.U. (2006): The influence of transgenic plants expressing *Bacillus thuringiensis* δ–endotoxins on arthropod diversity and trophic interactions in crop ecosystems. Ph.D. Dissertation, ETH, Zürich.

Schmidt, J.E.U., Braun, C.U., Whitehouse, L.P. and Hilbeck, A. Effects of activated Bt transgene products (Cry1Ab, Cry3Bb) on the ladybird *Adalia bipunctata*. (Submitted)

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.*, **62**, 775–806.

Schoonhoven, L.M., Jermy, T. and van Loon J.J.A (1998): *Insect Plant Biology. From Physiology to Evolution*. Chapman and Hall, UK.

Schuler, T.H., Poppy, G.M., Kerry, B.R. and Denholm, I. (1998) Insect resistant transgenic plants. *Trends Biotechnol.*, **16**, 168–175.

Schuler, T.H., Poppy, G.M., Kerry, B.R. and Denholm, I. (1999) Potential side effects of insect–resistant transgenic plants on arthropod natural enemies. *Trends Biotechnol.*, **17**, 210–216.

Sebasta, K., Fargas, J., Horska, K and Vankova, J. (1981) Thuringiensin, the Beta–endotoxin of *Bacillus thuringiensis*. *In* H.D. Burges (ed.), *Microbial Control of Pests and Plant Diseases 1970–1980*. Academic Press, USA, pp. 249–281.

Shelton, A.M., Zhao, J.Z. and Roush, R.T. (2002) Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. *Annu. Rev. Entomol.*, **47**, 845–881.

Sims, S.R. (1995) *Bacillus thuringiensis* var. *kurstaki* [CryIA(c)] protein expressed in transgenic cotton: Effects on beneficial and other non–target insects. *Southwestern Entomol.*, **20**, 493–500.

Smirnoff, W.A. and Heimpel, A.M. (1961) Notes on the pathogenicity of *Bacillus thuringiensis* var. *thuringiensis* Berliner for the earthworm, *Lumbricus terrestris* Linnaeus. *J. Insect Pathol.*, **3**, 403–408.

Thacker, J.R.M. (2002) *An Introduction to Arthropod Pest Control*. Cambridge University Press, UK.

Thomas, E.M. and Watson, T.F (1986) Effect of Dipel (*Bacillus thuringiensis*) on the survival of immature and adult *Hyposoter exiguae* (Hymenoptera: Ichneuminidae). *J. Invertebr. Pathol.*, **47**, 178-183.

van Rie, J., Jansens, S., Höfte, H., Degheele, D. and van Mellaert, H. (1989) Specificity of *Bacillus thuringiensis* δ–endotoxins. *Eur. J. Biochem.*, **186**, 239–247.

Vojtech, E., Meissele, M. and Poppy, G.M. (2005) Effects of Bt maize on the herbivore *Spodoptera littoralis* (Lepidoptera: Noctuidae) and the parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae). *Transgenic Res.*, **14**, 133–144.

Wallner, W.E., Dubois, N.R. and Grinberg, P.S. (1983) Alteration of parasitism by *Rogas lymantriae* (Hymenoptera: Braconidae) in *Bacillus thuringiensis*-stressed gypsy moth (Lepidoptera: Lymantriidae) hosts. *J. Econ. Entomol.*, **76**, 275-277.

Wandeler, H., Bahylova, J. and Nentwig, W. (2002) Consumption of two Bt and six non–Bt corn varieties by the woodlouse *Porecllio scaber*. *Basic Appl. Ecology*, **3**, 357–365.

Weseloh, R.M. and Andreadis, T.G. (1982) Possible mechanism for synergism between *Bacillus thuringiensis* and the gypsy moth (Lepidoptera: Lymantriidae) parasitoid *Apanteles melanoscelus* (Hymenoptera: Braconidae). *An. Entomol. Soc. Am.*, **75**, 435-438.

Whalon, M.E. and Wingerd, B.A. (2003) Bt: mode of action and use. *Arch. Insect Biochem. Physiol.*, **54**, 200–211.

Wilson, W.T. (1962) Observations on effects of feeding large quantities of *Bacillus thuringiensis* Berliner to honey bees. *J. Insect Pathol.*, **4**, 269–270.

Wraight, C.L., Zangerl, A.R., Carroll, M.J. and Berenbaum. M.R. (2000): Absence of toxicity of

Bacillus thuringiensis pollen to black swallowtails under field conditions. *Proc. Nat. Acad. Sci. USA*, **97**, 7700–7703.

Yousten, A.A. (1973) Effect of the *Bacillus thuringiensis* delta–endotoxin on an insect predator which has consumed intoxicated cabbage looper larvae. *J. Invertebr. Pathol.*, **21**, 312–314.

Yu, L., Berry, R.E. and Croft, B.A. (1997) Effects of *Bacillus thuringiensis* toxins in transgenic cotton and potato on *Folsomia candida* (Collembola: Isotomidae) and *Oppia nitens* (Acari: Oribatidae). *J. Econ. Entomol.*, **90**, 113–118.

Zemková Rovenská, G., Zemek, R., Schmidt, J.E.U. and Hilbeck, A. (2005) Altered host plant preference of *Tetranychus urticae* and prey preference of its predator *Phytoseiulus persimilis* (Acari: Tetranychidae, Phytoseiidae) on transgenic Cry3Bb eggplants. *Biol. Contr.*, **33**, 293–300.

Zhang, X., Candas, M., Griko, N.B., Taussig, R., and Bulla, L.A.jr (2006) A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. *Proc. Nat. Acad. Sci. USA*, **103**, 9897–9902.

Zwahlen, C., Hilbeck, A., Howald, R. and Nentwig, W. (2003) Effects of transgenic Bt corn litter on the earthworm *Lumbricus terrestris*. *Mol. Ecol.*, **12**, 1077–1086.

Zwahlen, C. and Andow, D.A. (2005) Field evidence for the exposure of ground beetles to Cry1Ab from transgenic corn. *Environmental Biosafety Res.*, **4**, 1–5.

Zwahlen, C., Nentwig, W., Bigler, F. and Hilbeck, A. (2000) Tritrophic interactions of transgenic *Bacillus thuringiensis* corn, *Anaphothrips obscurus* (Thysanoptera: Thripidae), and the predator *Orius majusculus* (Heteroptera: Anthocoridae). *Environ. Entomol.*, **29**, 846–850.

Accepted 15 June 2006