

MINI REVIEW

A synthesis of laboratory and field studies on the effects of transgenic *Bacillus thuringiensis* (Bt) maize on non-target Lepidoptera

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Abstract

One of the major applications of transgenic crops in agriculture are the so-called *Bacillus thuringiensis* Berliner (Bt) plants, in particular Bt maizes, which produce insecticidal Cry proteins that target specific orders, such as the Lepidoptera or Coleoptera. We reviewed publications that reported on the direct toxic effects of Bt-maize and/or Cry proteins of current Bt-maize events on larvae of non-target butterflies and moths (Lepidoptera). In total, 20 peer-reviewed publications were identified, of which 16 papers contributed laboratory-based data and seven field-based data. An adverse effect on caterpillars was recorded in 52% of all laboratory-based and in 21% of all field-based observations. The variables most often studied and having the highest occurrence of effects were larval survival, body mass, and developmental time. Parameters of the adult stage were under-represented in the studies. Overall, 11 lepidopteran species were tested. The majority of the studies originated from the USA, with the Monarch butterfly being the most studied, whereas other species and other parts of the world were widely neglected. Laboratory experiments were often run under unrealistic conditions from an ecological point of view. Although the papers we reviewed indicated a potential hazard for Lepidoptera that are exposed to and feed on lepidopteran-specific Bt-maize pollen, a general conclusion on the level of risk for butterflies and moths cannot as yet be drawn. A comprehensive risk characterization would require thorough hazard identification, exposure assessment, and impact assessment. However, our review showed that even the basic level of hazard characterization is as yet incomplete. Reasons for this are the still-limited numbers of publications and concurrent lack of knowledge, the restriction of data to only a few species, the over-representation of North American species, and the identified limitations of both laboratory and field experiments. The findings of this review suggest that more realistic, ecologically meaningful, and detailed experiments and analyses are crucial to improve the present assessment of Bt-maize cultivation effects on Lepidoptera.

Introduction

One of the major applications of transgenic crops in agriculture are the so-called Bt plants (James, 2008). These plants contain genes modified from the soil bacterium *Bacillus thuringiensis* Berliner, and they express insecticidal proteins (Koziel et al., 1993; Van Rie, 2000). These bacte-

ria-derived toxins include various Cry proteins, and they are targeted against insect pests from the orders Lepidoptera, Coleoptera, or Diptera (Höfte & Whiteley, 1989). Possible harmful effects of Bt crops on the environment remain a major issue, and transgenic plants are required to undergo a risk-assessment procedure before release [EPA (US Environmental Protection Agency), 1998; CPB, 2000; European Parliament and Council, 2001; EFSA (European Food Safety Authority), 2006].

Several Bt crops have been developed or are under development, but at present cotton and maize are the

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only Bt crops being grown on a large scale (James, 2008). Possible exposure pathways of toxins of these Bt crops to non-target organisms, i.e., organisms that are not the intended target pest, include feeding on transgenic plant material and plant products, as well as the transfer of Bt toxins to higher trophic levels along the food web (Andow et al., 2006; Romeis et al., 2006). Current Bt-maize events express the Cry toxins in most plant tissues, including pollen. In contrast to cotton, maize is wind-pollinated (anemophilous), thus Bt toxins can be transported by wind-drifted pollen (Pleasant et al., 2001; Sears et al., 2001). The Bt-maize pollen can be deposited on other plants in and outside maize fields, and larvae of butterflies and moths (Lepidoptera) may consume the attached pollen when feeding on these (host) plants (Losey et al., 1999). Hence, concerns have been raised that populations of butterflies and moths may be adversely affected by cultivation of Bt-maize events producing Lepidoptera-specific Cry toxins (Losey et al., 1999; Obrycki et al., 2001; Dolezel et al., 2005). Potentially, cultivation of such Bt maize may put at risk Lepidoptera living in the agro-ecosystem itself as well as (rare or protected) species occurring in natural habitats nearby (Losey et al., 2003; Traxler et al., 2005; Hofmann & Schleichriemen, 2009).

Over the last decade, publications studying the possible side effects of transgenic Bt crops on non-target organisms have been repeatedly reviewed (Lövei & Arpaia, 2005; O'Callaghan et al., 2005; Romeis et al., 2006; Marvier et al., 2007; Icoz & Stotzky, 2008; Wolfenbarger et al., 2008; Lövei et al., 2009; Naranjo, 2009). Many of these reviews focused on natural enemies and/or soil organisms, but a review of side effects on butterflies and moths, specifically, is missing. Assessment of Bt effects on butterflies and moths appears especially relevant as the most widespread Bt-maize events and varieties grown worldwide express the Lepidoptera-specific Cry1Ab toxin. For example, in Europe large-scale growing of Bt maize has so far been mainly limited to Spain, but a substantial increase of Bt-maize cultivation in other European countries could expose other lepidopteran species and communities. Traxler et al. (2005) estimated that approximately 70% of the butterfly species of Austria (Papilionoidea and Hesperioidea) occur in arable land, and could be potentially exposed to the pollen of Bt maize at varying intensities, depending on their spatial and temporal overlap with pollen-shedding Bt-maize fields. Darvas et al. (2004) pointed out that a certain proportion of the protected Lepidoptera of Hungary would be exposed to Bt-maize pollen.

Conceptually, an environmental risk assessment includes several steps: hazard assessment, exposure assessment, impact assessment, and finally, risk characterization (Calow, 1998; Sears et al., 2001; Andow & Zwahlen, 2006).

Generally, the risk is ultimately viewed as the probability of an adverse outcome, which is in turn determined by the severity of the adverse effect and the probability of the effect to occur (following exposure). In this paper, we focus on the identification of hazard as a basic and important part of the risk assessment. To do so, we summarize the publications studying possible direct toxic effects of Bt maize and associated Cry toxins on exposed non-target caterpillars. We provide a detailed analysis of potential effects studied in laboratory and field studies, and discuss the results and some of the limitations of these studies.

Materials and methods

We analysed publications that tested the direct toxic effect of Bt-maize (pollen and anthers) and/or Cry proteins expressed in known Bt-maize lines on larvae of non-target butterflies and moths when exposed to the toxin during the larval stage. We ruled out studies with non-Bt-maize crops, such as cotton, potato, or oilseed rape (Yao et al., 2006, 2008; Chen et al., 2008) as these are not wind-pollinated and pollen drift to adjacent habitats is unlikely (although it should be kept in mind that larvae of some non-target lepidopteran species may feed on crop leaves, roots or dead organic matter, and may possibly be exposed through these pathways). We also excluded papers treating Lepidoptera that were the intended target of insect resistance in maize (Felke & Langenbruch, 2008), and excluded studies in which lepidopteran larvae fed on green maize plant tissue (Pilcher et al., 1997; Binning & Rice, 2002). We included observations studying the effects of artificial diets containing purified Cry proteins that are present in existing Bt-maize events (Hellmich et al., 2001). As we focused on papers investigating the direct toxic effects of Bt-maize pollen, anthers, and Cry proteins on Lepidoptera, publications were excluded that concentrated on exposure analysis (Schmitz et al., 2003; Gathmann et al., 2006a), that investigated oviposition behaviour (Tschenn et al., 2001; DiTommaso & Losey, 2003), that comprised comprehensive risk analyses for lepidopterans including an exposure analysis and risk characterization (Sears et al., 2001; Wolt et al., 2003, 2005; Peterson et al., 2006).

Only studies that had been published in peer-reviewed scientific literature and that contained original data from laboratory or field trials were considered. The selection and limitation to peer-reviewed publications was applied as a quality criterion. Furthermore, papers published in international journals are easily accessible to the scientific community. Only papers written in English were included (with one exception: Felke & Langenbruch, 2003; written in German with an English summary). Another German publication (Felke & Langenbruch, 2001) was not

considered, as the data of this paper were already included in Felke et al. (2002). To recover the relevant publications, searches in Web of Science (ISI) were carried out using multiple appropriate keywords, such as Lepidopt*, butterfly*, moth*, non-target*, Bt, *Bacillus thuringiensis*, Cry, maize, and corn. In addition, we searched our own databases and the NCEAS database (<http://delphi.nceas.ucsb.edu/btcrops/>), as well as the reference lists of published review articles (Dolezel et al., 2005; Lövei & Arpaia, 2005; O'Callaghan et al., 2005; Romeis et al., 2006; Marvier et al., 2007; Wolfenbarger et al., 2008; Lövei et al., 2009; Naranjo, 2009).

We identified 20 publications according to these criteria. Sixteen papers reported laboratory-based tests and seven papers field-based tests; we evaluated them separately (some papers contributed both laboratory- and field-based results). One paper (Dively et al., 2004) analysed pooled data from laboratory and field experiments and was assigned to the field-based studies, as the majority of the data resulted from field experiments. As publications contained different numbers of experiments with differing numbers of recorded variables (observations), we analysed and summarized the results in relation to the number of publications, experiments, and observations.

In assessing the experiments, we mostly followed the experimental description and analysis of the authors. In general, if the authors analysed trials and/or datasets separately, we considered the experiments to be independent and counted all results of these experiments. For instance, if the authors conducted trials on different dates, and presented and analysed these results by date, we counted all responses for the separate dates (e.g., Gathmann et al., 2006b). If different Bt events were tested in one experiment at a time, sometimes with one joint control treatment, the results (observations) were counted separately for each Bt event to be able to attribute the observed effects to different Bt-maize events (Jesse & Obrycki, 2000, 2002; Wraight et al., 2000; Stanley-Horn et al., 2001).

An observation was defined as a specific variable measured during one distinct test trial. An effect was judged to be present for a significance level $P < 0.05$ (based on the authors' own statistical analysis), and as not-present if $P > 0.05$. Given that the studies sometimes had low replication, resulting in a reduced statistical power to detect effects, we considered this a conservative approach, which possibly underestimated the frequency of adverse effects. Observations were only included if the actual data were presented and/or relevant statistics were provided. Observations were classified into the following categories: survival/mortality, records of larval food consumption, larval body mass, larval developmental time (to pupation), larval behaviour, pupal mass, duration of the pupal period (until

eclosion), adult sex ratio, adult body size, adult body mass, and abundance of eggs and larvae (the latter in field studies only).

Repeated measurements of a single variable were often taken during the course of experiments. For instance, survival/mortality was recorded regularly during the development of larvae; in this case we only counted the final value of the highest developmental stage at the end of the test. Similarly, if several values for consumption and/or larval body mass were given, we included only the last value of the most developed larval stage. Separate values presented for male and female adults (Lang & Vojtech, 2006) were pooled to adult stage (i.e., effect = present/absent). In contrast, if separate values were presented for the distinct lepidopteran developmental stages, i.e., larvae, pupae, and adults, we included the values for all of these developmental steps. Often, the pupal period was not measured directly, but two values were given for development duration: time until pupation and time until eclosion. In such cases, we counted both values, acknowledging that the two variables could be highly correlated if measured in this way. The measurement of lipid content in adults (Jesse & Obrycki, 2000) was not included in the analysis, because body mass was also recorded in this study and the mass of adult butterflies is mainly determined by body fat. In general, values of different variables may be inter-correlated and not independent of each other. We tested this possibility by calculating contingency coefficients (C), i.e., we tested whether the presence/absence of effects for a given variable were associated with the presence/absence for effects on other variables.

For some parameters, no exact data were given in the publications, and we estimated the respective values either from indirect information in the papers concerned, or by consulting other literature sources (e.g., on duration of developmental stages of species). This applied to the variables 'time to pupation' and 'time to eclosion' of larvae, as well as the duration of pollen shedding of the maize fields and concomitant exposure time of the larvae (for the latter, we assumed an average 8-day period, according to the mean anthesis duration of maize fields as reported by Treu & Emberlin (2000)).

Results

Laboratory studies

Overall, 16 peer-reviewed publications were found that studied the direct toxic effects of Bt maize and Cry toxins on larvae of non-target butterflies and moths in the laboratory (*see citations in references). The overwhelming majority of the data has been recorded in the USA, and a small part in Western Europe (Germany). Three papers

alone (Jesse & Obrycki, 2000; Hellmich et al., 2001; Anderson et al., 2005) contributed a large amount of data, representing 56% of all experiments and 55% of all observations. There was one paper each from China (Li et al., 2005) and Japan (Shirai & Takahashi, 2005). No studies were found from other parts of Europe and Asia, South America, Africa, or Australia/Oceania (Table 1). Studies performed included the larvae of 11 lepidopteran species from nine families (Table 1). Four of these species can be regarded as secondary pest species [*Plutella xylostella* (L.), *Galleria mellonella* (L.), *Pieris rapae* (L.), and *Pieris brassicae* (L.)], i.e., these species are not targeted by Bt maize and are not pests of maize but of other crops (*P. xylostella*, *P. rapae*, and *P. brassicae*) or of bee hives (*G. mellonella*). Day-active butterflies of the superfamily Papilionoidea dominated the tested species. Only one species from each of four other families were tested: *Euchaetes egle* Drury (Noctuidae, owlet moths), *Antheraea pernyi* Guérin-Meneville (Saturniidae, silk moths), *G. mellonella* (Pyrilidae, pyralid moths), and *P. xylostella* (Plutellidae, diamondback moths). Over two-thirds of all experiments and observations (Table 1) were performed on *Danaus plexippus* (L.), the Monarch butterfly. A variety of transgenic maize events and Cry proteins have been studied with events MON810, Bt11, and Bt176, all containing the Cry1Ab, being the most frequent ones (Table 1). In most experiments, Bt-maize pollen was offered to lepidopteran larvae for consumption. Other approaches included the provision of Bt-maize anthers (Anderson et al., 2004; Prasifka et al., 2007), a combination of maize anthers and pollen (Anderson et al., 2005), or purified Cry toxins (Hellmich et al., 2001; Table 1). The tested material was either mixed into an artificial diet (Hellmich et al., 2001; Hanley et al., 2003), applied to small leaf discs (Lang & Vojtech, 2006) or whole leaves (Mattila et al., 2005) of the larval host plants, or the whole host plant itself was used (Anderson et al., 2005; Table 1). The majority of the trials were carried out with first instars (Table 1). These larvae were exposed to the Bt material and subsequent effects recorded for varying periods: in 53% of the experiments, the larvae were exposed for 2 days (mean \pm SD = 4.24 ± 3.01 days, range 2–11 days), and 60% of the experiments were terminated within 7 days (9.82 ± 7.49 days, range 2–29 days).

Fresh pollen or anthers were used in five studies which represented 18% of all experiments and 30% of all observations; the remainder used pollen/anthers sampled some time before the study and stored frozen until the test trials. Nine publications, representing 62% of the experiments and 63% of the observations, did not measure or report the amount of Bt toxin in the plant material (pollen and anthers) used for the tests. In five publications, dose–effect

relationships were calculated, such as lethal doses (LD), lethal concentrations (LC), or effective concentrations (EC). These dose–effect relationships were recorded for various Cry toxins (Hellmich et al., 2001) and for the pollen of the Bt176 maize event (Zangerl et al., 2001; Felke et al., 2002; Felke & Langenbruch, 2003; Lang & Vojtech, 2006), using seven lepidopteran species: *Papilio machaon* L., *Papilio polyxenes* Fabricius, *P. rapae*, *P. brassicae*, *Inachis io* (L.), *D. plexippus*, and *P. xylostella*. Only one publication quantified the actual dose taken up by the larvae (Lang & Vojtech, 2006). All other studies estimated intake in a relative and indirect way, such as number of pollen grains applied per larva (Felke & Langenbruch, 2003), density of pollen grains on leaf discs (Jesse & Obrycki, 2000), or concentration of Cry toxin in artificial diet (Hellmich et al., 2001).

For our analysis, we classified the observations (response variables) into 10 categories (Table 2). As a result of the lepidopteran-toxic nature of the tested Bt-maize products and Cry proteins, significant effects were adverse. Most observations were recorded for survival/mortality and body mass of the tested larvae, and in about half of all observations, adverse effects on lepidopteran larvae were found. Adverse effects on larvae were observed in every category, albeit in different proportions. Larval behaviour and all parameters for adult stages can be affected adversely, but these variables were rarely measured. For example, larvae of the Monarch butterfly exposed to Bt-maize anthers behaved differently and were more likely to move off host plant leaves than larvae exposed to non-Bt anthers (Prasifka et al., 2007). Three Cry1Ab maize events were predominant in the tests and overall, the transgenic events Bt176 and Bt11 yielded more negative observations than MON810 (Table 3). For all events, the number of observations was rather low for parameters of the adult stage, with no observation for MON810 (Table 3).

In general, laboratory experiments that detected adverse effects had more replicates per treatment, studied more larvae both per treatment and overall, larvae were exposed for a longer time to Bt, and the experiments lasted longer (Table 4). However, compared with experiments recording no effect, these differences were not significant (Mann–Whitney U-test: $P > 0.05$), except for a trend regarding exposure time ($P = 0.06$). Sample sizes for the analysis of association of effects were generally low. Effects on pupal mass were most often positively associated with the effects on three other parameters: the duration of the pupal period ($C = 0.58$, $P = 0.08$; $n = 6$), adult mass ($C = 0.70$, $P = 0.04$; $n = 4$), and adult size ($C = 0.71$, $P = 0.08$; $n = 3$). Effects on larval developmental time were associated with effects on duration of the pupal

Table 1 Summary of laboratory-based studies used in this review. Listed are the numbers of publications, experiments, and observations per country, species, Bt event/toxin, material tested, larval food, and instars tested (% in parentheses)

	No. publications	No. experiments	No. observations
Country			
USA	11 (68.8)	37 (82.2)	121 (84.0)
Germany	3 (18.8)	6 (13.3)	17 (11.8)
China	1 (6.2)	1 (2.2)	5 (3.5)
Japan	1 (6.2)	1 (2.2)	1 (0.7)
Species			
<i>Danaus plexippus</i> (Danaiidae)	7 (38.9)	32 (71.1)	114 (79.2)
<i>Papilio polyxenes</i> (Papilionidae)	2 (11.1)	2 (4.4)	3 (2.1)
<i>Papilio machaon</i> (Papilionidae)	1 (5.6)	1 (2.2)	7 (4.9)
<i>Pieris rapae</i> (Pieridae)	1 (5.6)	1 (2.2)	3 (2.1)
<i>Pieris brassicae</i> (Pieridae)	1 (5.6)	1 (2.2)	2 (1.4)
<i>Pseudozizeeria maha</i> (Lycaenidae)	1 (5.6)	1 (2.2)	1 (0.7)
<i>Inachis io</i> (Nymphalidae)	1 (5.6)	2 (4.4)	3 (2.1)
<i>Euchaetes egle</i> (Noctuidae)	1 (5.6)	1 (2.2)	2 (1.4)
<i>Antheraea pernyi</i> (Saturniidae)	1 (5.6)	1 (2.2)	5 (3.5)
<i>Plutella xylostella</i> (Plutellidae)	1 (5.6)	1 (2.2)	2 (1.4)
<i>Galleria mellonella</i> (Pyralidae)	1 (5.6)	2 (4.4)	2 (1.4)
Event/toxin			
MON810 event (Cry1Ab)	5 (16.7)	7 (12.5)	50 (34.7)
Bt176 event (Cry1Ab)	9 (30.0)	17 (30.4)	34 (23.6)
Bt11 event (Cry1Ab)	6 (20.0)	19 (33.9)	35 (24.3)
MON863 event (Cry3Bb1)	1 (3.3)	1 (1.8)	4 (2.8)
Cry1Ac event	1 (3.3)	1 (1.8)	1 (0.7)
Cry1F event	2 (6.7)	2 (3.6)	2 (1.4)
Cry9C event	1 (3.3)	2 (3.6)	2 (1.4)
Cry1Ab/Cry2Ab2 event	1 (3.3)	1 (1.8)	4 (2.8)
Cry1Ab toxin	1 (3.3)	3 (5.4)	6 (4.2)
Cry1Ac toxin	1 (3.3)	1 (1.8)	2 (1.4)
Cry1F toxin	1 (3.3)	1 (1.8)	2 (1.4)
Cry9C toxin	1 (3.3)	1 (1.8)	2 (1.4)
Material tested			
Purified toxin	1 (5.0)	6 (11.8)	12 (8.3)
Pollen	14 (70.0)	33 (64.7)	84 (58.3)
Anthers	3 (15.0)	7 (13.7)	34 (23.6)
Pollen and anthers	2 (10.0)	5 (9.8)	14 (9.7)
Larval food			
Artificial diet	2 (10.5)	8 (17.8)	14 (9.7)
Leaf discs	11 (57.9)	24 (53.3)	81 (56.3)
Whole leaves	5 (26.3)	11 (24.4)	33 (22.9)
Whole plant	1 (5.3)	2 (4.4)	16 (11.1)
Instar			
L1	12 (44.4)	31 (56.4)	115 (66.5)
L2	7 (25.9)	12 (21.8)	32 (18.5)
L3	4 (14.8)	8 (14.5)	21 (12.1)
L4	3 (11.1)	2 (3.6)	3 (1.7)
Unknown	1 (3.7)	2 (3.6)	2 (1.2)

period ($C = 0.59$, $P = 0.05$; $n = 7$), and effects on adult mass were correlated with effects on adult size ($C = 0.70$, $P = 0.08$; $n = 3$).

Field studies

Overall, seven peer-reviewed publications were identified that included field trials about the toxic effect of Bt maize

Table 2 Summary of laboratory- and field-based effects on lepidopteran larvae. Listed are the numbers of observations for variables recorded in the experiments analysed in this review (% in parentheses); –, not studied.

Parameter	Laboratory		Field	
	Adverse effect	No effect	Adverse effect	No effect
Survival	23 (65.7)	12 (34.3)	5 (19.2)	21 (80.8)
Food consumption	11 (68.75)	5 (31.25)	0 (0.0)	1 (100.0)
Larval body mass	22 (64.7)	12 (35.3)	3 (27.3)	8 (72.7)
Larval developmental time	10 (50.0)	10 (50.0)	3 (42.9)	4 (57.1)
Larval behaviour	1 (20.0)	4 (80.0)	–	–
Pupal mass	2 (14.3)	12 (85.7)	2 (25.0)	6 (75.0)
Length of the pupal period	5 (50.0)	5 (50.0)	2 (40.0)	3 (60.0)
Adult body mass	1 (16.7)	5 (83.3)	1 (14.3)	6 (85.7)
Adult size	1 (20.0)	4 (80.0)	0 (0.0)	6 (100.0)
Adult sex ratio	–	–	0 (0.0)	3 (100.0)

Table 3 Laboratory-based effects of three Bt-maize events on lepidopteran larvae. Listed are the numbers of observations (% in parentheses) for variables recorded in the experiments analysed in this review; –, not studied.

Parameter	MON810 (n = 48)		Bt176 (n = 36)		Bt11 (n = 34)	
	Adverse effect	No effect	Adverse effect	No effect	Adverse effect	No effect
Survival	2 (33.3)	4 (66.7)	12 (85.7)	2 (14.3)	4 (66.7)	2 (33.3)
Food consumption	3 (42.9)	4 (57.1)	4 (100.0)	0 (0.0)	3 (100.0)	0 (0.0)
Larval body mass	4 (44.4)	5 (55.6)	4 (80.0)	1 (20.0)	6 (75.0)	2 (25.0)
Larval developmental time	5 (55.6)	4 (44.4)	2 (50.0)	2 (50.0)	2 (40.0)	3 (60.0)
Larval behaviour	1 (20.0)	4 (80.0)	–	–	–	–
Pupal mass	1 (16.7)	5 (83.3)	1 (33.3)	2 (66.7)	0 (0.0)	5 (100.0)
Length of the pupal period	3 (50.0)	3 (50.0)	1 (100.0)	0 (0.0)	1 (33.3)	2 (66.7)
Adult body mass	–	–	1 (33.3)	2 (66.7)	0 (0.0)	3 (100.0)
Adult size	–	–	1 (33.3)	2 (66.7)	0 (0.0)	2 (100.0)
Adult sex ratio	–	–	–	–	–	–

Table 4 Comparison of mean (\pm SD) sample sizes, exposure times, and duration of experiments which recorded adverse effects vs. no effect of Bt

	No effect	Adverse effect	Mann–Whitney U-test
Laboratory experiments	n = 14	n = 30–31	
Replicates per treatment	2.11 \pm 1.36	3.90 \pm 4.47	
Larvae per treatment	36.50 \pm 27.35	51.23 \pm 49.75	
Overall number of larvae	211.86 \pm 209.03	348.83 \pm 504.76	
Exposure time to Bt (days)	3.07 \pm 2.06	4.77 \pm 3.24	0.05 < P < 0.10
Duration of experiment (days)	7.29 \pm 3.67	10.97 \pm 8.49	
Field experiments	n = 15–17	n = 7	
Replicates per treatment	8.27 \pm 3.52	19.43 \pm 16.99	
Larvae per treatment	78.20 \pm 62.68	112.86 \pm 88.06	
Overall number of larvae	340.00 \pm 232.03	590.29 \pm 315.54	0.05 < P < 0.10
Exposure time to Bt (days)	7.06 \pm 2.44	4.00 \pm 1.41	P < 0.05
Duration of experiment (days)	16.25 \pm 11.28	15.71 \pm 10.34	

on larvae of non-target butterflies (Table 5). Six of the field studies were carried out in the USA and one in Germany (Gathmann et al., 2006b). Two papers (Stanley-Horn

et al., 2001; Dively et al., 2004) dominated the analysed dataset: they contributed 46% of all experiments and 71% of all observations. Field studies were performed with

Table 5 Summary of field-based studies used in this review. Listed are the numbers of publications, experiments, and observations per country, species, Bt event/toxin, experimental approach, experimental treatments, and instars tested (% in parentheses)

	No. publications	No. experiments	No. observations
Country			
USA	6 (85.7)	18 (75.0)	69 (92.0)
Germany	1 (14.3)	6 (25.0)	6 (8.0)
Species			
<i>Danaus plexippus</i> (Danaiidae)	5 (50.0)	14 (60.9)	63 (84.0)
<i>Papilio polyxenes</i> (Papilionidae)	2 (20.0)	3 (13.0)	6 (8.0)
<i>Pieris rapae</i> (Pieridae)	1 (10.0)	3 (13.0)	3 (4.0)
<i>Plutella xylostella</i> (Plutellidae)	2 (20.0)	3 (13.0)	3 (4.0)
Event (toxin)			
MON810 event (Cry1Ab)	5 (50.0)	12 (50.0)	29 (38.7)
Bt176 event (Cry1Ab)	2 (20.0)	4 (16.7)	8 (10.7)
Bt11 event (Cry1Ab)	3 (30.0)	8 (33.3)	38 (50.7)
Experimental method			
Unfenced host plants	5 (50.0)	14 (56.0)	20 (26.7)
Field cages (open)	3 (30.0)	8 (32.0)	44 (58.7)
Field cages (closed)	1 (10.0)	1 (4.0)	9 (12.0)
Natural situation	1 (10.0)	2 (8.0)	2 (2.7)
Treatment			
Location (Bt and control)	4 (57.1)	19 (79.2)	61 (81.3)
Transect (distance to Bt field)	2 (28.6)	4 (16.7)	8 (10.7)
Manipulation	1 (14.3)	1 (4.2)	6 (8.0)
Instar			
L1	6 (33.3)	22 (40.0)	65 (43.3)
L2	4 (22.2)	12 (21.8)	35 (23.3)
L3	5 (27.8)	13 (23.6)	42 (28.0)
L4	3 (16.7)	8 (14.5)	8 (5.3)

larvae of four non-target lepidopteran species from four families (Table 5); of these, two species (*P. xylostella* and *P. rapae*) are secondary pests. The majority of data came from studies of the Monarch butterfly (84% of all observations; Table 5). Three transgenic Cry1Ab maize events have been studied in the field; studies on events with other Cry proteins have not been published (Table 5). Methods applied varied among the field studies. In addition to observations in a natural situation, suitable host plants for larvae were sowed/planted in the field (unfenced), or host plants were installed within cages (open and closed) (Table 5). The experiments were run during the pollen-shedding period of maize, and host plants were placed in, alongside, or at various distances from Bt-maize fields so that host plants were dusted naturally with different concentrations of Bt-maize pollen and anthers (and non-transgenic control pollen and anthers respectively). Only in one study Bt-maize anthers were applied manually to the host plants (Anderson et al., 2004). Some studies included insecticide-treated control plots, and some of the studies used a combination of the experimental approaches described above. In those cases, several larvae

(range 3–24 per plant) were placed onto experimental host plants and subsequent effects were recorded. One study did not follow the manipulative experimental setup as detailed above, but monitored the naturally occurring abundance of eggs and larvae in the field during two seasons (Jesse & Obrycki, 2003). Gathmann et al. (2006b) counted naturally occurring larvae in weed strips experimentally sown with host plants. All studies recorded and reported pollen and/or anther densities on host plants, but few quantified the actual Bt concentrations in pollen and/or anthers (one exception is Wraight et al., 2000).

Younger larval stages, especially first instars, predominated in the experiments (Table 5). Many experiments started with the beginning of maize anthesis (38%) or 1 day thereafter (14%). Experiments were either terminated following (experimental) exposure, or larvae were transferred after field exposure to the laboratory and kept there for an additional period. Duration of exposure of the larvae and of the entire experiment was not always stated in the publications, but it was sometimes possible to estimate the approximate times. In general, larvae in about 58% of the experiments were exposed for less than 1 week

(estimated mean \pm SD = 6.1 ± 2.7 days). The average duration of the experiments varied between 4 and 42 days (15.9 ± 10.8 days), the study lasting 42 days (Jesse & Obrycki, 2003) being the one in which the abundance of naturally occurring eggs and larvae was monitored over two field seasons.

An adverse effect on lepidopteran larvae was recorded in 21% of all observations (Table 2). Adverse effects were found in all data categories with the exception of larval consumption rate, adult size, and adult sex ratio (for adult sex ratio, a male-biased value was considered detrimental), but absence of effects could have been a result of low sample sizes (Tables 2 and 4). The data categories survival/mortality, body mass, and some aspect of developmental time of larvae were recorded most frequently (64% of all observations; Table 2). The behaviour of larvae was not studied in any field experiment. Observations summarized separately for the three Cry1Ab maize events did not yield additional insights, because of their small sample sizes.

Field experiments detecting adverse effects included more replicates per treatment ($P > 0.05$), and involved more larvae per treatment ($P > 0.05$), as well as a higher overall number of larvae ($P = 0.08$) (Mann–Whitney U-tests; Table 4). Surprisingly, in field experiments which recorded no effects, the larvae were exposed ($P = 0.03$) and studied ($P > 0.05$) for a longer time period (Table 4); this was mainly because of two publications contributing a large share (33%) of experiments with long exposure times and study durations but recording no effects (Jesse & Obrycki, 2003; Gathmann et al., 2006b).

Discussion

This review of the effects of transgenic Bt maize on non-target butterflies and moths has revealed important knowledge gaps. First, only 20 papers were published since 1999, with a decreasing number since 2007. The small number of publications contrasts with the fact that Lepidoptera are the non-target group most at risk from current Bt-maize events that produce lepidopteran-specific toxins. Aspects of biological control, pollination, or soil fertility appear to have received more attention than adverse effects on (lepidopteran) biodiversity in arable land and on protected (lepidopteran) species. More papers were published about the impact of GM plants on other taxonomic or functional groups, such as Coleoptera or natural enemies (Marvier et al., 2007; Lövei et al., 2009; Naranjo, 2009), although that database is also not very species-rich (Lövei et al., 2009). The domination of studies and lepidopteran species from the USA was most striking: 81% of all observations (laboratory and field pooled) referred to only one species,

the Monarch butterfly. Moreover, only a handful of USA-originated papers contributed more than 50% of the entire published dataset (Jesse & Obrycki, 2000; Hellmich et al., 2001; Stanley-Horn et al., 2001; Dively et al., 2004; Anderson et al., 2005). Studies focused on day-active butterflies, while species outside the Papilionoidea were hardly considered (including Hesperioidea, the skipper butterflies), although members of these other families represent the greatest part of the known lepidopteran fauna, including many species occurring in agricultural landscapes (Ebert, 1994–2003; Losey et al., 2003). Available studies so far are also too scant to allow assessment of the risk for rare or protected Lepidoptera, which may be exposed to pollen during the commercial cultivation of Bt maize. The total of 11 studied non-target species (four of them secondary pests) is very limited in view of the 160 000 described or half a million estimated Lepidoptera species worldwide (Kristensen et al., 2007). There are no published studies from the southern hemisphere, e.g., from Africa. Likewise, the six European species studied (four of them pests) represent only a minute part of Europe's lepidopteran fauna consisting of approximately 10 000 species (Karsholt & Razowski, 1996). Evidently, only a portion of the lepidopteran fauna would be exposed to Bt-maize cultivation; Losey et al. (2003) estimated that 229 butterfly and moth species are associated with maize cultivation areas in the USA (see also below the discussion on exposure).

Results of the current analysis clearly indicate adverse effects, both lethal and sublethal, of Bt toxins and Bt maize on larvae of non-target Lepidoptera (cf. Dolezel et al., 2005). Most observations focused on mortality, although mortality is considered a less sensitive and/or meaningful indicator in current pesticide testing than sublethal parameters (Hilbeck et al., 2008). Adverse effects were reported for all main current Bt events (MON810, Bt11, and Bt176) both in the laboratory and the field (see Tables 2 and 3), and for pollen/anther densities that can occur under natural conditions (Stanley-Horn et al., 2001; Dively et al., 2004; Lang et al., 2004; Anderson et al., 2005; Lang & Vojtech, 2006). However, Bt176 maize, the event with the highest recorded Cry1Ab expression in pollen, is no longer commercialized. Although young instars are most susceptible to Bt-pollen, effects were also demonstrated for older instars (Felke et al., 2002). Anther fractions should be checked in laboratory experiments, as accidental mixing of pollen with anther fractions may impact the results of pollen experiments (Hellmich et al., 2001).

Some of the recorded variables may be inter-correlated and it has been argued that the reported amount of adverse effects could be inflated if non-independent traits measured within one study on a certain species are all taken into account (Naranjo, 2009). In contrast, Lövei et al.

(2009) argued that individual instar responses provide more relevant information than summary statistics, if different developmental stages differ in their tolerance to a given toxin. Indeed, stage-specific considerations should not always be discarded. For instance, early lepidopteran instars can suffer high mortality by invertebrate predators (Dempster et al., 1976), and quickly reaching later and larger instars releases them from this size-dependent predation (Nicholls & James, 1996). Therefore, pooling developmental time from egg hatch to eclosion potentially masks qualities and knock-down effects of developmental time for the various instars. In some cases, it may be justified to count only the value of the last instar, e.g., for body mass (as we did in our analysis), because decreased body mass of earlier instars may indicate an inferior condition of larvae that die later on, and which is recorded as survival rate.

Parameters clearly underrepresented were effects on the adult stages caused by exposure of larvae to Cry toxins, because experiments were mostly terminated before eclosion of the pupae. Adverse effects on adults have been reported (Dively et al., 2004; Lang & Vojtech, 2006), and especially body mass is correlated with the fat body of adult Lepidoptera and concurrent fecundity (Miller, 2005). Notably, no study recorded a direct parameter of fecundity such as number of eggs produced by individual females. Effects on fecundity parameters of adults are important, because they would represent generational relative fitness. Relative lifetime survival and reproduction are particularly relevant experimental endpoints for risk-assessment tests of genetically modified plants, because adverse effects of transgenic plants on non-target species would occur through some component of fitness (Andow & Hilbeck, 2004).

To date, standardized values for dose–response relationships, such as LD_{50} , LC_{50} , and EC_{50} have been derived for seven lepidopteran species fed on Bt176 maize and various Cry proteins (Hellmich et al., 2001; Zangerl et al., 2001; Felke et al., 2002; Felke & Langenbruch, 2003; Lang & Vojtech, 2006), but none for pollen of MON810 and Bt11 maize, which are the very events that prevail on the market. But even LC_{50}/LD_{50} values often do not allow an unequivocal comparison among studies and species. In many studies, the toxin concentration and activity of the Bt material tested has not been quantified, whereas information about the amount applied and bioactivity of toxins would be paramount in assessing the observed toxic effects. Approaches applied in the majority of laboratory experiments, such as using frozen and subsequently thawed pollen or chemical treatment of host plant leaves before the experiments, may influence the toxicity of the pollen (Haas & Scriber, 1998; Jesse & Obrycki, 2000; Shirai & Takahashi, 2005). Nguyen

& Jehle (2009), for example, showed that long-term storage at -20 and 4 °C significantly reduced the bioactivity of the Cry1Ab protein. Another issue is that it is difficult, if not impossible, to translate LC/EC values referring to number of pollen grains applied per (area) host plant and/or per larva (Zangerl et al., 2001; Felke et al., 2002) to a Bt concentration in an artificial diet (Hellmich et al., 2001). To do so, the actual amount of Bt toxin ingested by a single larvae should be known (Lang & Vojtech, 2006), but this information is mostly missing. The timing of the determination of these values is also crucial. Most of the values were recorded for larvae ≤ 7 days old. But larvae continue to die because of the Bt impact during following life stages, thus LC/EC values are overestimated if this delayed effect is not recorded (Lang & Vojtech, 2006). In other words, susceptibility of larvae to Bt is generally higher than indicated by LC/EC values if they are only recorded for short experimental periods. This is also in accordance with the fact that more adverse effects were recorded in longer-lasting laboratory experiments than in short ones (Table 4).

As with laboratory experiments on natural enemies (Lövei & Arpaia, 2005), conditions of laboratory tests with Lepidoptera did not reflect adequately the natural situation in important ecological parameters, thus creating favourable conditions for the tested specimens (Lang et al., 2007). For example, in most experiments only healthy larvae were studied and larvae in bad condition excluded (e.g., Felke et al., 2002; Lang & Vojtech, 2006; Prasifka et al., 2007), larvae did not experience food shortages, as ample food was provided (e.g., Wraight et al., 2000; Felke & Langenbruch, 2003), adverse climatic conditions such as low temperature and rainfall were not accounted for (only Mattila et al., 2005; applied a day–night cycle in air temperature), and larvae often received high and acute Bt doses instead of a natural, chronic exposure (e.g., Jesse & Obrycki, 2000; Zangerl et al., 2001). Any additional stressor has the potential to exacerbate the adverse effect of a toxin such as Bt. In particular, caterpillars often suffer from bacterial infections (Nicholls & James, 1996), and these infections amplify the susceptibility to Bt, a well-known fact that is applied in the control of lepidopteran pests (Pierce et al., 2001; Reardon et al., 2004; Jung & Kim, 2007). A large part of the laboratory experiments (53%) was performed with small discs cut out of the host plants, and only few tests used whole leaves or the whole host plant. The former approach may have wounding effects and affect inducible defences of the host plant. Host plants of Lepidoptera often produce and contain compounds for chemical defence against herbivores, and cutting leaf discs for experiments may reduce (or increase) these substances, thus modifying a stressor normally

encountered by the larvae in the field (Arteel & Lindroth, 1992; Hwang et al., 1995; Zalucki & Malcolm, 1999; Zalucki et al., 2001). Host plant species or quality could also mediate a Bt effect on lepidopteran larvae (Hwang et al., 1995; Kouassi et al., 2001), which was only accounted for in one study (Anderson et al., 2005). In many laboratory experiments, larvae were exposed to pollen or anthers no longer than 2 days, which is markedly shorter than the average 5–8 days of pollen shedding in maize fields according to Treu & Emberlin (2000); indeed, a longer experimental exposure time was more often associated with the detection of adverse effects (Table 4).

So far, the number of field tests appears to be relatively low (only seven papers contributed field-based tests) presumably reflecting the large amount of work and effort necessary for such studies. Observations of negative effects were less frequent in field than in laboratory studies, which may indicate a smaller Bt effect under natural conditions. However, the general problems inherent to field experiments and the resulting decrease of statistical power suggest that this is most likely because of the difficulty of detecting a Bt effect under field conditions. Field studies pose several methodological challenges, such as high larval mortality, the need for large numbers of larvae at a specific date (requiring permanent laboratory stocks of test specimens), and the temporal matching of maize pollen shedding with the breeding of larvae. Field mortality of the larvae commonly reaches >80% because of predation and pathogens (Stanley-Horn et al., 2001; Zangerl et al., 2001; Dively et al., 2004; Lang, 2006). Depending on the original number of larvae, this can severely decrease sample size, thus reducing statistical power, a fact that is sometimes acknowledged by the authors (e.g., Wraight et al., 2000). In field studies, larvae were exposed for the full length of a pollen-shedding period in only 42% of experiments and 63% of observations. All experiments were accompanied by measurements of pollen and/or anther deposition, but Bt concentrations in maize pollen and/or anthers were usually not quantified. Sometimes, authors acknowledged that rainfall reduced maize pollen densities during the experiments (Wraight et al., 2000; Stanley-Horn et al., 2001; Zangerl et al., 2001). Although rainfall may reflect the natural situation, it can cause an underestimation of the Bt effect if the experiments are run shorter than the entire pollen-shedding period, which was usually the case.

In conclusion, the available information clearly indicates a potential hazard for non-target lepidopteran larvae living in and near Bt-maize fields, i.e., possible adverse effects (lethally and sub-lethally) because of feeding on pollen of lepidopteran-specific Bt maize. However, a comprehensive assessment of the possible risk would require the following data, in addition to the hazard characteriza-

tion (Sears et al., 2001; Wolt et al., 2003; Andow & Hilbeck, 2004; Dively et al., 2004; Lang & Vojtech, 2006): (i) extent of the exposure of butterfly and moth species to Bt-maize pollen in the field; this means that it is essential to collect data about Bt toxin expression in pollen of the different events and varieties, pollen-shedding periods (on a regional scale), the area covered by Bt-maize fields in a given landscape, dispersal and deposition of pollen on host plants, the spatial and temporal occurrence and distribution of Lepidoptera and their host plants (regional and on a landscape scale), and oviposition preferences of butterflies and moths and (ii) knowledge of (meta)population size and dynamics, and information about life-history traits of the species. Point (ii) would determine if a substantial impact and resulting risk is likely for a given species or population following the existence of adverse effects and exposure.

Worldwide, a quantitative environmental risk assessment for Lepidoptera including the essential steps and contents previously mentioned has been carried out only for the Monarch butterfly (Sears et al., 2001; Dively et al., 2004), and partly for the pale grass blue butterfly, *Pseudozeeria maha* Kollar (Wolt et al., 2005). Short-term exposure of larvae of the Monarch butterfly to Bt11 and MON810 maize indicated no significant adverse effects (Sears et al., 2001; Stanley-Horn et al., 2001), whereas continuous exposure in the field revealed adverse effects with regard to survivorship and developmental time of larvae, as well as body weights of pupae and adults (Dively et al., 2004). Risk-assessment models were developed based on an extensive data collection of toxic effects to Monarch larvae, host plant distribution, distribution of maize fields and adoption rates of Bt-maize, maize pollen drift, and temporal and spatial overlap of butterfly populations and shedding Bt-maize fields (Sears et al., 2001; Dively et al., 2004). Generally, Dively et al. (2004) estimated that 50% of the breeding population of the Monarch in North America occurred within the US maize cropping area; of these only 2.4% might suffer from adverse effects on survivorship and reproduction by Bt-maize cropping. Thus, Dively et al. (2004) concluded that the overall risk to the Monarch butterfly would be low as a result of low exposure of the breeding population.

Similar risk assessments including exposure studies have not been carried out outside the USA, and only limited information has been published on number of Lepidoptera possibly exposed to Bt-maize cultivation. In southern Germany, Lang (2004) recorded 33 species of adult butterflies occurring near maize fields, whereas Felke & Langenbruch (2005) reported 158 species (33 butterflies and 125 moths). Of the latter, about half would be likely to be exposed to maize pollen as larvae. In a theoretical

assessment, Schmitz et al. (2003) estimated that 96 species of butterflies and moths typically occur in some agricultural habitats in Germany and may get into contact with maize pollen. However, in the studies of Lang (2004), Felke & Langenbruch (2005), and Schmitz et al. (2003) the number of exposed species were underestimated, because these studies included only some of the habitat types found in agricultural landscapes, and Schmitz et al. (2003) limited their assessment to those species typically occurring in arable land (therefore, excluding species present but usually having other habitat preferences). In a more comprehensive evaluation, Traxler et al. (2005) assessed that of 215 butterfly species of Austria, 152 appear in agricultural landscapes, of which eight had no temporal overlap of their larval phase with maize pollen-shedding period, whereas the remainder would be exposed to maize pollen drift to varying degrees (ranging from 8% to 100% overlap with pollen-shed period).

We emphasize that even for an essential and basic step of a risk assessment, i.e., the identification of adverse effects, the existing information is too erratic to allow for a comprehensive hazard characterization. The current lack of knowledge is mainly caused by the generally low numbers of studies, the limitations of both laboratory and field experiments, and the focus on too few species. From a nature-conservation viewpoint, secondary pest species (36% of all species tested) may not be equally valuable as other (protected) non-target Lepidoptera in an environmental risk assessment of Bt maize. At present, USA and day-active species are over-represented and do not allow us to estimate either the exposure or the susceptibility of species in other parts of the world. Susceptibility to Bt-maize pollen can vary greatly among butterfly species, e.g., 10-fold between close relatives within a genus (Felke et al., 2002). Large (intra-generic) variability in susceptibility to Bt among lepidopteran species and even among developmental stages within one species are also known from studies with Bt sprays, the various species and stages ranging from insensitive to highly susceptible, making a prediction of the susceptibility to Bt for any given species difficult (Peacock et al., 1998). For example, no published eco-toxicological laboratory study with MON810 maize exists outside the USA, despite its broad approval globally. The only non-US study with MON810, a field study from Europe (Gathmann et al., 2006b), was conclusive for only two secondary pest species and focused on the abundance (counted as survival) of less-susceptible late instars for a 2-week period during which there was a limited overlap of the studied larvae with maize pollen shed. In addition to an improved eco-toxicological protocol and a larger number of lepidopteran test species, a complete and comprehensive risk assessment would also require a robust

exposure analysis for populations of butterflies and moths (cf. Sears et al., 2001), not only of common species in agro-ecosystems but also of rare and protected species. Furthermore, the majority of studies focused on Cry1Ab, neglecting other lepidopteran-specific Cry toxins or interactions between different toxins. Stacked GM events expressing several different Cry toxins will lead to a higher exposure and have been shown to affect target Lepidoptera by a synergistic action of Cry proteins (Lee et al., 1996; I-bargutxi et al., 2008). A synergistic action between Cry1Ab and Cry2Ab2 has also been discussed in the only published study (to date) on the effects of a stacked Bt maize on the Monarch butterfly (Mattila et al., 2005).

The findings of this review suggest that a greater number of more realistic, ecologically meaningful as well as detailed experiments and analyses are required to fill the considerable knowledge gaps regarding possible effects and concomitant risks of Bt-maize cultivation for Lepidoptera. Moreover, the results indicate that an increase in sample size of the experiments and exposure time of the larvae would support the identification of adverse effects. Future work should also emphasize the impact of long-term and large-scale cultivation of Bt maize on populations, including different biogeographical regions. Identification of unresolved issues, and subsequent systematic studies, will accumulate empirical knowledge and serve an improved and more robust risk assessment in the future.

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(*Citations included in the meta-analysis)

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