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Effect of Bt-176 maize pollen on first instar larvae of the Peacock butterfly (*Inachis io*) (Lepidoptera; Nymphalidae)

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More than 10 years after registration of the first Bt maize cultivar in Europe, there still exists a remarkable lack of data on effects on Lepidoptera which would be necessary for a complete and comprehensive environmental risk assessment. So far only very few European butterfly species have been tested in this aspect. In our study the effect of transgenic *Bacillus thuringiensis* (Bt) maize pollen (event Bt-176) on the development and survival of neonate larvae of the Peacock butterfly, *Inachis io* (L.) was for the first time shown. The results of our study suggest that the Peacock butterfly may serve as a model organism for assessing potential side effects of new developed transgenic Bt crops on non-target butterflies in a GMO environmental risk assessment. The study was done under laboratory conditions by exposing larvae of the Peacock butterfly to various pollen doses of transgenic maize event Bt-176 (cv. PACTOL CB) or the conventional isogenic maize (cv. PACTOL) using a no-choice test. Larvae feeding for 48 h on nettle plants (*Urtica dioica*) that were contaminated with higher pollen concentrations from Bt-176 maize (205 and 388 applied pollen.cm^{-2}) suffered a significantly higher mortality rate (68 and 85% respectively) compared to larvae feeding on leaves with no pollen (11%), or feeding on leaves with pollen from conventional maize (6 to 25%). At lower Bt maize pollen doses (23–104 applied pollen.cm^{-2}), mortality ranged from 11–25% and there were no apparent differences among treatments. The corresponding LC$_{50}$-and LC$_{90}$-values for neonate larvae of the Peacock butterfly were 187 and 448 applied pollen grains.cm^{-2} of Bt-176, respectively. Weight of larvae surviving consumption of Bt-176 maize pollen declined between 10 and 81% with increased pollen doses ($r = -0.95$). The highest weight reduction (81%) corresponded to the highest pollen concentration (388 pollen grains applied.cm^{-2}). Ingestion of pollen from the conventional maize hybrid did not have negative effects on larval weight gain or survival rate.

Keywords: Peacock butterfly / Cry1Ab-toxin / transgenic crops / Bt-176 / environmental risk assessment

INTRODUCTION

For the last decade there has been a steady increase in the global hectares of transgenic field crops grown. In 1998 it was estimated that there were about 28 million ha of transgenic crops grown worldwide, excluding China (James, 1998). In 2008 the global area of genetically modified crops reached 125 million hectares and worldwide 13.3 million farmers from 25 countries planted transgenic crops (James, 2008). Insect resistance, based on *Bacillus thuringiensis* Berliner (Bt) delta-endotoxins is still the second most widely used trait (after herbicide resistance) in genetically modified (GM) crops (James, 2003, 2008). Transgenic maize expressing Cry1Ab protein, derived from the soil bacterium *B. thuringiensis kurstaki* was developed to control the European corn borer, *Ostrinia nubilalis* Hübner, which is one of the major maize pests in the USA and many other regions of the world (Bagrintseva et al., 2004; Ivezic and Raspudic, 2004; Lisowicz, 2003; Rice and Ostlie, 1997; Velasco et al., 2007). Bt maize expresses the insecticidal δ endotoxin Cry1Ab in various plant tissues. This Cry protein acts selectively against larvae of some families in Lepidoptera (for example Pieridae and Nymphalidae). Because of this selectivity the effects on non-target organisms were not considered at the early stages of GM maize development (Pilcher and Rice, 1998). However, the work of (Losey et al., 1999) demonstrated that pollen from the transgenic maize hybrid N 4640 (event Bt-11)

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could be hazardous to the larvae of the monarch butterfly, *Danaus plexippus* (L.). Subsequent field and laboratory studies confirmed the possibility that pollen from Bt maize could have adverse effects on caterpillars of other butterfly species as well (Felke and Langenbruch, 2001; Hansen and Obrycki, 2000; Hellmich et al., 2001; Lang and Vojtech, 2006; Zangerl et al., 2001). But so far effects of Bt maize pollen have been tested only on very few butterfly species and even more than 10 years after cultivation of the first Bt maize cultivar, there still exists a remarkable lack of data on effects on Lepidoptera which would be necessary for a complete and comprehensive environmental risk assessment (Lang and Otto, 2010). Therefore studies on sensitivity of more butterfly species on Bt maize pollen are essential.

Although the steady growth in global planting of GM plants attests to their usefulness for many farmers and their acceptance in many markets, the imposition of the moratoria in several countries reflects the scepticism and public concern about a range of issues around GM crops, including potential impact on the environment, ecosystem and human health (Braun, 2001; Dale, 1999; Liu et al., 2005; Wolfenbarger and Phifer, 2000). Considering these public concerns, the European Parliament prescribed a risk assessment for GM crops prior to commercial cultivation (EC, 2001, 2003). The principles regulating the deliberate release into the environment of GMOs are laid down in Council Directive 2001/18/EC (EC, 2001). This Directive puts in place a step-by-step approval process made on a case-by-case assessment of the risk to human health, non-target organisms and the environment before any GMOs can be released into the environment, or placed on the market as, or in, products. An assessment of the possible immediate and/or delayed environmental impact resulting from direct and indirect interactions of the GMO with non-target organisms, including impact on population levels of competitors, herbivores, symbionts, predators, parasites and pathogens is required (EFSA, 2006). According to the (EFSA, 2006) guidance document assessors should use a tiered approach to this environmental risk assessment, first identifying potential hazards in controlled tests and then evaluating exposure in the field in order to estimate potential risks. If first tier tests do not identify sensitivity in exposed species then second and third tier test may not be required. In this context our laboratory studies with the Peacock butterfly have to be considered as first tier tests. The Peacock butterfly is common in central Europe and caterpillars are abundant during the shedding of maize pollen (Settele et al., 2005). The nettle, *Urtica dioica* (L.) is their only host plant and is commonly found in agricultural habitats (Ebert and Rennwald, 1991). Because of the species life cycle and habitat preferences, exposure of Peacock butterfly larvae to Bt maize pollen is possible under field conditions. Therefore, assessing the impact of Bt-maize cultivation on *Inachis io* is indispensable. We assessed, for the first time, the impact of transgenic maize pollen (event Bt-176) on the development and survival of neonate larvae of the Peacock butterfly in the laboratory. This transgenic maize event expresses a high level of Cry1Ab toxin in pollen grains (Lang et al., 2004; Stanley-Horn et al., 2001). In 2003 it was planted for example on 32 000 ha in Spain (Lumbierres et al., 2004). In Europe Bt-176 maize was registered by the European Community from January 1st 1997 to April 18th 2007. Nowadays Bt-176 maize is no longer cultivated in Europe nor in the rest of the world.

Previously, we demonstrated the susceptibility of older stages of Peacock butterfly larvae to Bt-176 maize pollen (Felke and Langenbruch, 2003). Therefore, the aim of the current study focuses to assess the effect of Bt-176 maize pollen and the isogenic, non-transgenic cultivar on mortality and growth rate of neonate larvae of the Peacock butterfly. We included the isogenic cultivar to rule out the possibility that this cultivar had adverse effects. The possibilities of using the Peacock butterfly as a model organism in a European GMO risk assessment are discussed.

The following objectives should be tested in this study:

- Effect of Bt-176 maize pollen on mortality and growth rate of neonate *I. io* larvae;
- Effect of maize pollen of the isogenic, non-transgenic cultivar on mortality and growth rate of neonate Peacock butterfly larvae to rule out the possibility that this cultivar had adverse effects;
- How is the susceptibility of neonate Peacock butterfly larvae to Bt-176 maize pollen compared to older larval stages of this species?
- Can the Peacock butterfly serve as a model organism in a European GMO risk assessment?

RESULTS

Differences between treatments with respect to mortality rate of neonate Peacock butterfly larvae ($F = 11.35$; df = 10; $P < 0.0001$) and larval weight gain ($F = 17.26$; df = 10; $P < 0.0001$) were significant. Mortality of Peacock butterfly larvae increased when exposed or fed 205 and 388 applied pollen grains.cm^{-2} (Fig. 1). However, mortality from these concentrations was not significantly different from each other but differed significantly from cv. PACTOL pollen grain concentrations and the untreated control (Fig. 1). The mortality of larvae that received between 23 and 104 applied pollen grains.cm^{-2} from Bt-176 maize ranged from 11.3 to 25 % and did not differ significantly from the untreated

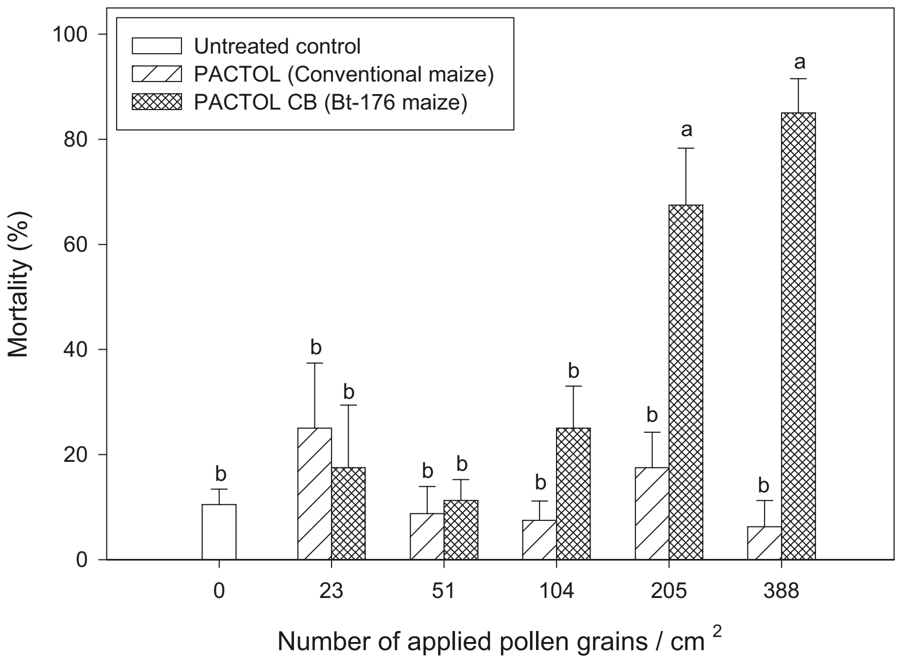

Figure 1. Average mortality (%) for 1st instar larvae of the Peacock butterfly exposed to various pollen grain concentrations of transgenic and non transgenic maize after 7 d. Bars with the same letter are not statistically different (One-way ANOVA, $F = 17.26$; df = 10; $P < 0.0001$, Student Newman Keuls test). Error bars represent standard error of the mean.

control (mortality = 10.5%) and from the conventional maize pollen (mortality ranged from 6.3 to 25%). The parameters of the probit analysis of Bt-176 maize pollen applied.cm^{-2} and mortality of neonate Peacock butterfly larvae are presented in Table 1. The LC$_{50}$- and LC$_{90}$-values for larvae were 186.8 (95% CL = 159.8–215.08) and 448 (95% CL = 361.68–633.04) applied Bt pollen grains.cm^{-2}, respectively. Pollen from Bt-176 maize had a significant influence on weight gain ($Y = -0.006x + 2.638$; $r = -0.95$; $R^2 = 0.9$; $P = 0.0038$) (Figs. 2 and 3). Ingestion of transgenic maize pollen reduced larval weight 10 to 81% depending on the amount of pollen used. The highest weight reduction corresponded to the highest pollen concentrations applied (Figs. 2 and 3). The weight reduction that occurred at the lowest concentration (23 Bt-176 applied pollen grains.cm^{-2}) was not different from the control and from the conventional maize pollen treatments. Moreover, there were no significant differences between the five different conventional maize pollen concentrations and the untreated control with respect to larval weight gain (Fig. 2).

DISCUSSION

Our study demonstrated for the first time the impact of transgenic Bt maize pollen on the development and survival of 1st instar larvae of the Peacock butterfly. Results demonstrated that growth and survival of the larvae was influenced by Bt-176 maize pollen consumption. A dose response relationship study revealed that with increasing pollen doses, larval survival rate and weight gain decreased significantly. The ingestion of pollen from a conventional maize hybrid obviously did not have a negative effect on larval development. Similar results have been reported from studies on other Lepidoptera species (Felke et al., 2002; Hellmich et al., 2001; Losey et al., 1999; Wraight et al., 2000). Our study shows, that the negative impacts on caterpillars after consumption of pollen from transgenic Bt maize is not caused by the occurrence of foreign material on their food plants (pollen), but from the Cry1Ab toxin contained in this pollen.

The estimated LC$_{50}$- and LC$_{90}$-values reported refer to the number of applied pollen grains.cm^{-2} leaf disc and not to the number of ingested pollen grains.larva^{-1}. It is possible with our methodology that in the same replicate some individuals consumed more pollen grains than others. Previous attempts to provide a small leaf disk covered evenly with pollen for single larvae failed, so one replicate always consisted of a group of 10 individuals feeding from a larger leaflet. Ingestion rate of caterpillars decreased with increasing pollen doses and in treatments with higher pollen doses the applied pollen was not consumed completely. This may indicate that the LC$_{50}$-value

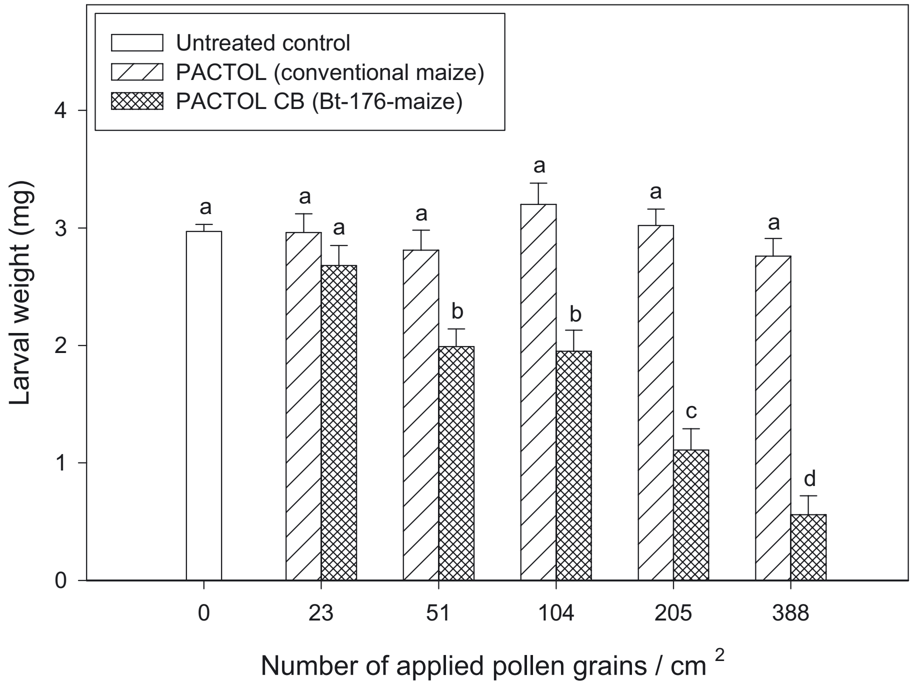

Figure 2. Average weight (mg) for 1st instar larvae of the Peacock butterfly exposed to various pollen grain concentrations of transgenic and non transgenic maize after 7 d. Bars with the same letter are not statistically different (One-way ANOVA, $F = 11.35$; df = 10; $P < 0.0001$, Student Newman Keuls test). Error bar represents standard error of the mean.

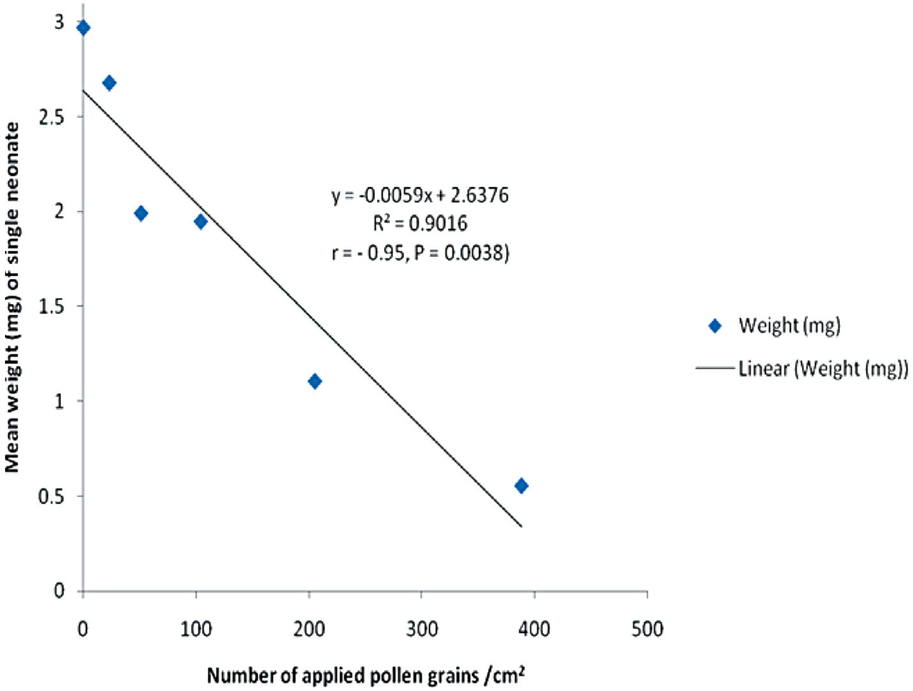

Figure 3. Relationship between Bt-176 maize pollen applied.cm^{-2} and mean weight (mg) of neonate Peacock butterfly larvae after 7 d. Each value is mean of 12 to 358 surviving larvae depending on pollen doses ($N = 6$; $F = 36.64$; $P = 0.0038$).

Table 1. Pollen dose and mortality relationship of Peacock butterfly larvae exposed in groups of 10 individuals for 48 h to various pollen grain concentrations of Bt-176 maize. Values are based on $\log_{10}$ of pollen grain concentration applied.cm^{-2}.

Treatment	*n*	Slope	LC_{50} (95% CL)	LC_{90} (95% CL)	Intercept	*P*	χ^2
Bt-176 maize	400	5.789 ± 0.83	2.27 (2.20–2.33)	2.65 (2.56–2.80)	–13.149 ± 1.92	< 0.0001	48.92

Table 2. LC_{50} values (number of applied Bt-176 maize pollen grains per cm^2) for neonate larvae of different butterfly species.

Species	**LC_{50}**	**Cultivar**	**Reference**
Papilio machaon	92.3 (17.7)^a	NAVARES	Lang and Vojtech (2006)
Inachis io	186.8	PACTOL CB	this study
Danaus plexippus	389	Maximizer 357	Sears and Stanley-Horn (2000)
Papilio polyxenes	613	Max 454	Zangerl et al. (2001)

^{a} LD_{50}-value in brackets refers to the number of ingested pollen.individual^{-1}.

based on ingested pollen numbers will be lower than the given number of 186.8 applied pollen grains.cm^{-2}. Lang and Vojtech (2006) compared LC_{50} values for *Papilio machaon* L. based on applied and ingested pollen numbers. Their findings demonstrated that LC_{50}-values calculated for the number of applied pollen grains.cm^{-2} was about five times higher than the LC_{50}-values based on the number of ingested pollen grains.

It could be proved in our study that 50 applied pollen grains.cm^{-2} cause significant sublethal effects on 1st instar larvae of the Peacock butterfly. Because application of 200 or more pollen grains.cm^{-2} led to a significant increase in mortality, Peacock butterfly larvae seem to be less sensitive to pollen from Bt-176 maize than 1st instar larvae of *P. machaon* (Lang and Vojtech, 2006) but more susceptible than neonate caterpillars of *Danaus plexippus* (Sears and Stanley-Horn, 2000) or *Papilio polyxenes* Fabricius, 1775 (Zangerl et al., 2001). A summery of LC_{50}-values of neonate larvae from various butterfly species related to Bt-176 maize pollen consumption is presented in Table 3. Lang and Vojtech (2006) estimated LC_{50}-values for neonate larvae of *Papilio machaon* based on pollen density.cm^{-2} and on the number of ingested pollen grains.individual^{-1} (see Tab. 2).

The results reported in the current study are based on a 48 h acute exposure of neonate *I. io* larvae to pollen numbers that would otherwise occur inside or in the vicinity of a blooming maize field (Senior Author, personal observation). Due to the nature of the experimental arena used in the current study, keeping the same leaf disc more than 48 h would result in increased fungal contamination which may confound the results. However, since in the field maize usually sheds pollen for about seven days in average, future studies should consider to address the effect of chronic exposure on larval mortality and weight gain, adult fitness and fecundity or generational fitness of the Peacock butterfly.

In comparison to older Peacock butterfly larvae, neonates are more susceptible to Bt-176 maize pollen. The LD_{50}-value for 2nd instar Peacock butterfly larvae was 61.4 pollen grains of the Bt-176 hybrid PACTOL CB applied per individual which corresponds to a pollen density of about 241 pollen grains.cm^{-2} (Felke and Langenbruch, 2003). Fourth instar were even less sensitive and estimation of the LD_{50}-value was not possible. Ingestion of 80 pollen grains.individual^{-1} did not cause increased mortality (Felke and Langenbruch, 2003). Generally, within the same lepidopteran species older larvae are less sensitive to Bt toxins than younger ones (Krieg and Langenbruch, 1981). Differences in susceptibility to Bt pollen between younger and older larvae have been reported for *Danaus plexippus* (Hellmich et al., 2001), *Pieris rapae* (L.) and *P. brassicae* (L.) (Felke and Langenbruch, 2001).

Several aspects have to be considered to assess the possible impact of Bt maize on nontarget butterfly species. The acute toxic effect of Bt maize pollen on caterpillars can be determined during laboratory assays. Another crucial point is the degree to which larvae would be exposed to toxic amounts of Bt pollen. In central Europe larvae of the Peacock butterfly can be found mainly in July and August (Ebert and Rennwald, 1991). This means that occurrence of larvae of the second generation is overlapping with maize pollen shed, which usually starts in July in Germany (Lang et al., 2004). Caterpillars of the Peacock butterfly are feeding exclusively on the common nettle plant (*U. dioica*) which is quite common in agriculturally used areas. Larval habitats can be situated close to maize fields because sun-exposed groups of nettle plants are known to be the typical larval habitat of this butterfly species (Ebert and Rennwald, 1991; Korneck and Pretscher, 2001; Lang et al., 2004).

Nettle are effective maize pollen collectors (own observations). Maize pollen can be dispersed very effectively by wind, but references regarding pollen dispersion vary. Generally pollen deposition decreases with increasing distance to the field's edge. Pleasants et al. (2001) found 63.1 maize pollen.cm^{-2} on potted milkweeds which had been placed directly on the edge of a blooming maize field. Inside the field pollen concentration was much higher matching on average 170.6 pollen grains.cm^{-2}. Wraight et al. (2000) collected pollen grains by using sticky slides and found an average of up to 210 pollen grains in direct vicinity (half a meter) from the edge of a blooming maize field. Using the same method, Lang et al. (2004) estimated the maximum pollen.cm^{-2} at various distances from the edge of maize fields (Bt-176, cv. NAVARES). Four meters inside a blooming maize field the maximum pollen number.cm^{-2} was 429. Lang et al. (2004) also recorded pollen numbers from the field's edge ($\cong$ 270) and at a distance of 1 m ($\cong$ 160), 3 m ($\cong$ 160), 5 m ($\cong$ 60), 6 m ($\cong$ 150) and 10 m ($\cong$ 80) to the maize field margin. These field observations on maize pollen dispersion show that neonate larvae of the Peacock butterfly can be confronted with lethal pollen doses if they are feeding on nettle growing inside or in direct vicinity of a blooming Bt-176 maize field. Sublethal effects like reduced weight gain could be expected up to a distance of at least 10 m from the edge of a blooming maize field.

Bt-176 maize was registered by the European Community from January 1st 1997 to April 4th 2007. Nowadays Bt-176 maize is no longer cultivated in Europe nor in the rest of the world. Nevertheless it is important from our point of view to present the results from this study as effects of Bt maize pollen on caterpillars have so far been reported only for a handful of species. Based on the few existing data on effects on Lepidoptera a complete and comprehensive environmental risk assessment is still not possible (Lang and Otto, 2010). One major goal of this study was to complete the sensitivity study of *Peacock butterfly* to Bt-176 maize pollen. Previously, Felke and Langenbruch (2003) studied susceptibility of 2nd and 4th instar larvae to Bt maize pollen. Our results suggest that the Peacock butterfly could be used as a model organism for an European environmental risk assessment (ERA) to investigate potential side effects of new Bt-maize variants on non-target butterflies in the laboratory as well as in the field because of the following aspects:

- Neonate larvae of the Peacock butterfly are highly susceptible to the Cry1Ab toxin which is produced in pollen of different Bt-maize events.
- The Peacock butterfly (*Inachis io*) is common in Europe.
- The Peacock butterfly is quite common in agricultural settings.
- The biology of this species is well known.
- Larvae of the Peacock butterfly feed on only one host plant (*Urtica dioica*).
- Identification and recording of adults and larvae is simple.
- The species has distinct and non-overlapping generations.
- Maize pollen exposure to Peacock butterfly larvae is very likely for many parts of Europe.
- Rearing of the Peacock butterfly under semi-natural conditions is simple.

MATERIAL AND METHODS

Insect sources

A laboratory colony of the Peacock butterfly was established from larvae collected near Darmstadt (state of Hesse, Germany) in May 2002. Adults were placed in mating cages measuring 2 × 2 × 2 m, containing potted nettle plants (*U. dioica*). The butterflies were provided with a 10% honey solution and laid their eggs on nettle plants (Felke, 2003). The clusters of eggs were kept in the same cage for hatching; however, in some cases parts of the nettle plants containing the eggs were transferred to a climate chamber (15 °C) to slow down their development. First instars (< 24 h old) were used for bioassays.

Pollen sources

Pollen from PACTOL CB (Bt-176 maize) and the conventional isogenic maize cultivar PACTOL were used. Both cultivars were produced and distributed by SYNGENTA SEEDS (Germany). The transgenic maize event Bt-176 contains a maize pollen specific promoter and a phosphoenolpyruvate carboxylase promoter. Cry1Ab-toxin is expressed in pollen and photosynthetic tissues (Koziel et al., 1993). Transgenic and non-transgenic pollen grains were collected from greenhouse-grown maize by gently tapping the spatula of a blooming plant. Immediately after collection pollen was sieved through a 0.1 mm mesh to remove contaminants.

Only fresh pollen was used for the bioassays. Toxin content of the PACTOL CB pollen grains used for this study was not determined. The Bt-176 maize cultivar NAVARES contains 2.59 ± 0.40 mg Cry1Ab protein per g dry weight pollen (Lang et al., 2004). Sears and Stanley-Horn (2000) report that Bt-176 maize pollen contains on average between 1.4 and 2.3 Cry1Ab protein.g^{-1} pollen.

Table 3. Average number of pollen grains (± SE) counted per 0.5 μL drop of different pollen suspensions and pollen numbers applied per cm^2 leaf disk. (N = 5, SE: standard error of the mean.)

Pollen (mg) per 1 mL of tap water	Number of pollen grains (± SE) per 0.5 μL[a]	Cm^{2b}
2.5	4.7 ± 0.5	23.3 ± 2.5
5	10.2 ± 1.6	50.8 ± 8.0
10	20.8 ± 1.6	104.2 ± 7.9
20	41.0 ± 2.8	205.0 ± 14.0
40	77.7 ± 3.1	388.3 ± 15.7

[a] Values are average of five observations.

[b] Values are estimated from the number of drops (10 × 0.5 μL) applied on 2 cm^2 leaf surface.

Different amount of pollen was suspended in 1 mL of tap water and mixed by gently shaking the tubes. Subsequently, 10 drops each with 0.5 μL of a defined pollen solution was applied evenly on leaf discs (2 cm^2) of *U. dioica*. The number of pollen grains per 0.5 μL pollen solution was assessed at the beginning of the study by taking five random samples. The resulting pollen numbers after application on the leaf varied on average from 23 (lowest pollen concentration) to 388 (highest pollen concentration) applied pollen grains.cm^{-2} (Tab. 3).

Pollen contaminated leaves were then placed individually in a Petri-dish (3-cm ϕ) filled with 2% agar medium to keep the leaves fresh. After the leaves were dry, 10 larvae were placed on each leaf disc by using a fine brush. Petri dishes were covered with towel paper and incubated at 25 °C/15 °C (day/night) and L:D 16/8 h for 7 d. Controls had *U. dioica* leaf discs without pollen. Insects were monitored daily and if the leaf discs were consumed within the first 24 h they were replaced with fresh untreated ones. After 48 h, remains of pollen treated leaves in all treatments were removed and the insects were provided *ad libitum* with fresh and untreated discs. Mortality was recorded daily and surviving larvae were weighed 7 d post-treatment. The bioassay consisted of three treatments: conventional maize (cv. PACTOL) and Bt-maize (cv. PACTOL CB) each with five different pollen concentrations and the control (5 replications with 10 larvae each). The experiment was repeated eight times between 22nd of July and 1st of August 2002.

Statistical analysis

Percentage mortality and weight gain were arcsine transformed and subjected to one-way analysis of variance using the general linear model procedure (PROC GLM). Similarly, larval weight (mg) of all surviving individuals (measured separately) were subjected to one-way ANOVA (PROC GLM) (SAS Institute, 2003). In all cases, means were separated using a Student Newman Keuls test (SAS Institute, 2003). Subsequently, correlation analyses were performed for the mean weight (mg) of the neonates and the Bt-176 maize pollen doses. Observed mortality was corrected for the corresponding control mortality (Abbott, 1925) and probit analysis (PROC PROBIT) was used to estimate the LC$_{50}$- and LC$_{90}$-values with 95% confidence limits (SAS Institute, 2003).

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