



Bt maize effects on *Papilio machaon*

The effects of pollen consumption of transgenic Bt maize on the common swallowtail, *Papilio machaon* L. (Lepidoptera, Papilionidae)

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Side effects;
Risk assessment

Summary

Effects of exposure to maize pollen of event Bt176 (cultivar “Navares”) on the larvae of the European common swallowtail (*Papilio machaon* L.) were studied in the laboratory. First instar larvae were exposed to different pollen densities applied to leaf disks of *Pastinaca sativa* L. for 48 h. Pollen densities applied in this study were in the range recorded from the field. Larvae which were exposed to higher Bt maize pollen densities consumed more pollen and had a lower survival rate. The LD₅₀ with regard to larvae surviving to adulthood was 13.72 pollen grains consumed by first-instar larva. Uptake of Bt maize pollen led to a reduced plant consumption, to a lower body weight, and to a longer development time of larvae. Effects on pupal weight and duration of the pupal period were present but less pronounced and smaller than effects on larvae. Larvae having consumed Bt-maize pollen as first instars had a lower body weight as adult females and smaller forewings as adult males. We conclude that possible effects of Bt maize on European butterflies and moths must be evaluated more rigorously before Bt maize should be cultivated over large areas.

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Zusammenfassung

L1-Larven des Schwalbenschwanzes (*Papilio machaon* L.) wurden im Labor mit Pollen von Bt-Mais (Event 176, Sorte “Navares”) gefüttert. Die Larven waren 48 h lang verschiedenen Pollenkonzentrationen auf Futterpflanzenplättchen (Pastinak) ausgesetzt. Die im Laborversuch applizierten Pollendichten entsprachen bekannten Freilandwerten. Je höher die Pollendichte war desto mehr Pollen fraßen die Raupen,

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und eine höhere Konsumtion von Bt-Pollen führte zu geringeren Überlebensraten. Die LD₅₀ bezogen auf geschlüpfte Schmetterlinge betrug 13.72 von L1-Larven gefressene Pollenkörner. Fraß von Bt-Maispollen reduzierte die Fraßleistung und das Körpergewicht der Larven und verlängerte ihre Entwicklungszeit. Effekte auf Puppengewicht und Verpuppungsdauer waren weniger stark ausgeprägt. Die Konsumtion von Bt-Maispollen durch Larven führte zu einem geringeren Körpergewicht der adulten Weibchen und zu kleineren Vorderflügeln bei adulten Männchen. Es wird eine ausführlichere Risikobewertung vor einem flächendeckenden Anbau von Bt-Mais in Europa empfohlen.

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Introduction

Transgenic Bt maize varieties are used worldwide with an estimated area cropped of 9.1 million hectares (James, 2003). The Bt maize commercially available in Europe has been engineered with genes of the soil bacterium *Bacillus thuringiensis* Berliner (Bt) var. *kurstaki*, and expresses the insecticidal delta-endotoxin Cry1Ab specific against lepidopteran species like the European corn borer, *Ostrinia nubilalis* Hübner (Koziel et al., 1993). After oral uptake by a susceptible species, the microbial toxin binds to specific receptors in the mid-gut epithelium of the larvae causing cell lysis leading to the death of the insect (Whalon & Wingerd, 2003). Due to their selective impact on target organisms living in maize fields, Cry1Ab toxins of Bt maize are generally considered to be safe for most non-target organisms. However, the Bt toxin is not species-specific and may harm other species closely related to the target species, e.g. other moths and butterflies in the case of Bt var. *kurstaki* (Glare & O'Callaghan, 2000). Bt toxins are produced in most tissues of the Bt maize, and pollen with toxin may be transported by wind into adjacent areas, deposited on plants, and consumed by larvae of non-target species feeding on these plants. Initiated by the publication of Losey, Rayor, and Carter (1999), a series of studies were conducted and published in the USA to assess the potential side effects of Bt maize on non-target lepidopteran larvae which focused on the monarch butterfly, *Danaus plexippus* Linné (results summarized in Sears et al., 2001). Although a growing number of papers have been published recently, the available assessments of Bt maize pollen effects on butterflies (Papilionoidea and Hesperioidea) is based on only half a dozen species worldwide, in Europe only one non-target and three secondary pest species have been studied so far, and pupal and adult stages have mostly not been considered (Anderson, Hellmich, Sears, Sumnerford, & Lewis, 2004; Divly et al., 2004; Felke &

Langenbruch, 2001, 2003; Felke, Lorenz, & Langenbruch, 2002; Hansen Jesse, & Obrycki, 2000; Hellmich et al., 2001; Losey et al., 1999; Stanley-Horn et al., 2001; Wraight, Zangerl, Carroll, & Berenbaum, 2000; Zangerl et al., 2001). There is clearly an urgent need for testing further non-target butterflies as the effect of Bt can vary widely among different species (Felke et al., 2002). This is especially important in Europe where one of the two Bt maize events (i.e. Bt176 and MON810) registered by the European Community is the event 176, which produces high toxin amounts in pollen (Lang, Ludy, & Vojtech, 2004; Sears et al., 2001), and which is already cultivated in the field, e.g. on 32,000 ha in Spain in 2003 (Lumbierres, Albajes, & Pons, 2004).

In assessing potential Bt effects on Lepidoptera it is essential to consider components other than the immediate larval mortality. This has been accounted for by recording consequences of Bt pollen consumption on several sublethal parameters such as body weight, consumption rate or development time of the larvae. In order to assess possible impacts at the population level, however, information about components of relative fitness is significant, thus survival experiments should last at least one generation and include parameters of the adult stages (Andow & Hilbeck, 2004).

The European common swallowtail *Papilio machaon* Linné is a regular butterfly species occurring in agricultural land in central Europe, and frequently reproduces on host plants occurring on field margins (Ebert & Rennwald, 1991; Lang, 2004). Here, we report about laboratory experiments that quantify the effect of Bt maize pollen consumption on larvae of the common swallowtail. Larvae of *P. machaon* were fed with differing amounts of Bt176 maize pollen and the resulting effects analyzed with respect to the survival of the larvae, determining lethal doses and concentrations (LD and LC values), feeding and growth inhibition as well as development time of the larvae, pupal weight, and adult weight and size.

Material and methods

Insects

Eggs of *P. machaon* were obtained either by our own breeding culture or from commercial butterfly breeders (all larvae stemmed from adults caught in Bavaria or in Switzerland). Eggs were held in the laboratory under standardized conditions of 25 °C, 50–60% relative humidity and at a day–night cycle of 16:8 h. For the bioassays only first instar larvae were used within 15–28 h after hatching. After hatching, larvae were first kept on leaves of wild carrot (*Daucus carota* L.), and then starved 4–5 h prior to the bioassay in order to standardize their hunger level.

Bioassays

All experiments were carried out in the laboratory under the conditions described above, except for relative humidity which was close to 100% the first 48 h (see below). Bioassays involved exposure of swallowtail larvae to Bt maize pollen for 48 h, and were performed with 96-well plastic plates (125 × 82 mm, diameter of well = 7 mm). Leaf disks of wild parsnip (*Pastinaca sativa* L.) with differing densities of pollen deposited were placed in wells together with single first instar larvae.

Leaf disks used for the bioassay were cut from organically grown wild parsnip using a paper punch. The morning of the experiment, disks measuring 25 mm² were cut out, and one disk was put into each well of the plates with the upper leaf surface upwards.

Pollen was taken from transgenic Bt176 maize (cultivar “Navares” from Syngenta) planted on research farms of the Bavarian State Research Centre for Agriculture (Lang et al., in press). The toxin content (Cry1Ab) of Bt maize pollen was determined with an ELISA kit from Adgen®. The mean ($\pm 1SD$) toxin content of the Bt176 pollen was $2.59 \pm 0.40 \mu\text{g}$ Cry1Ab protein per g dry weight pollen (Lang et al., 2004). Collected maize pollen was sieved through a 1-mm mesh and stored at –20 °C for up to 12 months. Prior to the bioassay, the pollen was dried at 28 °C for 12 h and then sieved through a 0.1-mm mesh. Dried pollen was suspended in distilled water yielding the following dose suspensions (pollen per 10 ml water): 1, 2.5, 5, 7.5, 10, 20 and 30 mg. After that, 15 μl of the concerned stock suspension were pipetted onto each leaf disk. The control consisted of leaf disks with 15 μl distilled water. Leaf disks were dried for approximately 4 h, and the pollen deposited on disk

surfaces counted under a stereo-microscope (50 ×). Additionally, two treatments were carried out with conventional non-Bt maize pollen in order to check whether maize pollen itself affects larvae (see below).

A single larva was placed on a leaf disk into the wells of the plates. Filled plates were covered with glass and placed inside a plastic box with standing water to maintain humidity close to 100% in order to prevent leaf disk dehydration. The plates were controlled after 24 h and if a disk was completely eaten, it was replaced with a new untreated disk. After 24 and 48 h leaf consumption of larvae was recorded. The transparent plates were placed onto a 1-mm scale paper, and the number of empty squares counted, i.e. the squares that were no longer covered by leaf disk. This number was transformed into the proportion eaten of leaf surfaces (sum of day 1 and day 2 yielding nine categories: 0%, –12.5%, –25%, –37.5%, –50%, –62.5%, –75%, –87.5%, –100%). Then, the amount of consumed leaf area (mm²) was calculated from the proportion of leaf surface eaten. After 48 h the pollen left on leaf disks and in the wells was counted (larvae were checked for adhering pollen grains carefully), and the larvae of each treatment were transferred to an insect cage (length * width * height: 30 * 30 * 60 cm). Larvae which did not feed during the first 48 h were excluded from the experiment. Larvae were reared to adulthood and were supplied ad libitum with wild carrots (*D. carota*) from the greenhouse as host plants.

The mortality of larvae was recorded daily the first week, and subsequently every second day until pupation. In test series, where the control mortality was over 20% at day 7, the whole trial was excluded from analysis. The body weight of larvae was recorded before the experiment, after 48 h, 7 days, and 14 days, respectively. At the first and second recording larvae of each treatment were weighed in cohorts (7–10 larvae together), because body weights of single larvae were too low (< 0.1 mg). After 7 and 14 days larvae were weighed individually. Weight of pupae was determined approximately 24 h after pupation. The time from egg hatch until pupation as well as the time from pupation to emergence of the adult butterfly was recorded, and the adult butterflies were sexed and weighed 10–16 h after emergence prior to their first nectar meal, and the length and width of their forewings recorded in order to calculate wing area (length * width).

It is well known that conventional non-Bt maize pollen has no adverse effects on lepidopteran larvae (Felke & Langenbruch, 2001, 2003; Hansen et al., 2000; Hellmich et al., 2001; Wraight et al.,

2000). In addition, it was checked in a preliminary experiment that consumption of pollen from the near-isogenic maize cultivar (i.e. "Antares" from Syngenta) does not affect first instar larvae of the common swallowtail. The conventional maize pollen was also obtained from maize fields of the research farms. Following the procedure and methods described above, larvae were exposed to 15- μ l solutions of either 1 or 30 mg conventional pollen suspended in 10 ml water, and were compared with larvae which received no pollen at all (control). The conventional pollen treatments did not differ from the control in leaf consumption and weight gain after 48 h, and in mortality after 7 days (data not shown).

Statistical analysis

The survivorship curves of the different treatments were compared by Kaplan–Meier analysis. The lethal doses and concentrations (LD and LC values) of pollen were determined by probit regression analysis and using log-transformed data. LD/LC₃₀ and LD/LC₅₀ values were calculated for applied pollen suspensions, pollen densities on leaf disks, and consumed pollen grains with regard to different ages of larvae and adult butterflies. For the analysis of the Bt pollen effect on dependent sublethal variables a univariate ANCOVA was carried out with the body weight of larvae before pollen consumption as a covariate (except for the analysis of weight increase, where an ANOVA without the covariate was applied). Since most of the larvae in higher pollen dose treatments (> 7.5–10 mg/10 ml) died during the course of the experiment, high dose treatments were combined to create groups of sufficient sample size. For multiple post hoc tests among treatments Tam-

hane's T2-test for pairwise comparisons was applied. Also, simple GLM contrasts were used to test the different levels of the pollen treatments against the control group. Partial correlation analysis was applied to describe relationships between two variables while controlling for the effects of another variable. Variables were $\ln(x + 1)$ or arcsine transformed to meet the assumptions of homogeneity and normal distribution of the data and residuals. All average values are presented as arithmetic means \pm 1SD, and all analyses were conducted with SPSS, version 11.

The program nQuery Advisor[®], release 4.0, was used to calculate effect sizes (Cohen, 1988). The effect size (f) is calculated as the standard deviation of the observed treatment means (σ_m) divided by the within-group standard deviation ($f = \sigma_m/\sigma$). Thus, the effect size is an index of the separation among the observed means of the treatments.

Results

Lethal effects on larvae

The different Bt maize pollen suspensions led to distinct and significantly different pollen exposures on leaves (Table 1). Consequently, larvae exposed to higher pollen densities consumed more pollen (Table 1). The survival curves of first instar larvae differed significantly among treatments (Kaplan Meier, $p < 0.05$, Fig. 1). All treatments which received a dose higher than 2.5 mg/10 ml had a significantly lower survival rate than the control, and, in general, a higher dose was associated with lower survival (Fig. 1). LD/LC values are similar

Table 1. Feeding experiments with first instar *P. machaon* larvae with Bt maize pollen: experimental pollen exposition, and plant and pollen consumption of the larvae of the different treatments (Pollen applied = number of pollen grains applied to leaf disks; Pollen per surface = density of pollen grains per area leaf disk; Plant consumed = plant material consumed by larvae; Pollen consumed = number of pollen grains eaten by larvae). Values are means \pm 1SD. Values within a column showing different letters differ significantly ($p < 0.05$, Tamhane)

Treatment (Bt pollen/ 10 ml)	Pollen applied (n)	Pollen per surface (n/cm ²)	Plant consumed (mm ²)	Pollen consumed (n)
0 mg (n = 133)	0 a	0 a	40.93 \pm 12.25 a	0 a
1.0 mg (n = 31)	4.71 \pm 2.36 b	18.78 \pm 9.39 b	34.68 \pm 12.17 a	4.42 \pm 2.17 b
2.5 mg (n = 44)	9.86 \pm 1.83 c	39.34 \pm 7.28 c	22.44 \pm 12.67 b	8.86 \pm 2.14 c
5.0 mg (n = 44)	18.61 \pm 2.83 d	74.24 \pm 11.29 d	21.43 \pm 12.28 bc	16.02 \pm 4.10 d
7.5 mg (n = 47)	30.77 \pm 5.45 e	122.71 \pm 21.75 e	15.22 \pm 7.64 bcd	23.62 \pm 7.23 e
10 mg (n = 38)	39.89 \pm 6.68 f	159.12 \pm 26.65 f	12.33 \pm 6.76 de	27.61 \pm 11.47 e
20 mg (n = 35)	68.63 \pm 14.44 g	273.72 \pm 57.61 g	7.86 \pm 4.11 f	34.71 \pm 19.96 e
30 mg (n = 32)	107.84 \pm 28.44 h	430.14 \pm 113.41 h	9.18 \pm 5.94 ef	65.91 \pm 37.41 f

when calculated for 7–14 days after pollen consumption, but differ greatly in comparison to 2-day-old larvae, i.e. shortly after pollen consumption and shortly before pupation (Table 2). LD₃₀ and LC₃₀ values are approximately two times lower than LD₅₀/LC₅₀ values, and an average consumption of only 9.93 Bt pollen grains would kill 30% of the larvae after 14 days (Table 2).

Sublethal effects on larvae and pupae

The relative weight increase of the larvae differed among controls and Bt-pollen treatments (ANOVA, $p < 0.01$), and, generally, higher Bt pollen doses caused a reduced weight gain (Figs. 2A–C). However, the effect of pollen consumption on weight was less pronounced in later instars: effect size (f) for 2-day-old larvae was 0.93, while f was

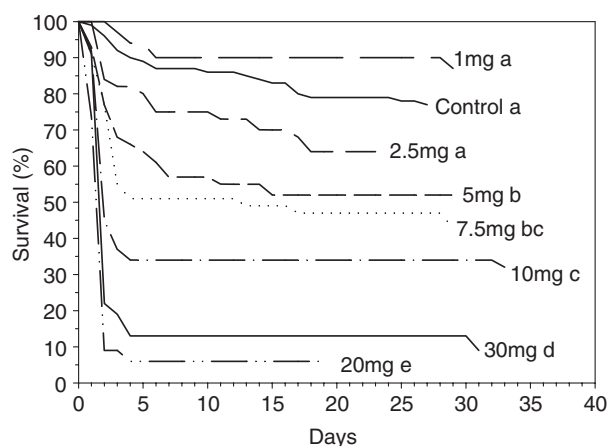


Figure 1. Survival curves for swallowtail larvae exposed to different doses of Bt maize pollen suspensions (mg pollen/10 ml water). Curves with different letters differ significantly ($p < 0.05$, Kaplan–Meier). Survival curves end when the last larvae of each treatment pupated or died.

0.32 for 7 and 0.30 for 14-day-old larvae. The body weights of the larvae of the different treatments before and after Bt pollen consumption can be obtained from Table 3.

The higher the density of Bt maize pollen on the leaf disks the more pollen the larvae ingested (Fig. 3A). The more pollen the larvae had consumed the less leaf material they would eat (Fig. 3B). Consequently, high pollen consumption was negatively correlated with body weight on day 2 (Fig. 4A), and larvae with low body weight needed a longer time for their development (Fig. 4B). Treatments differed in development time ($p < 0.001$, $f = 0.39$), pupation time ($p < 0.05$, $f = 0.14$), and in pupal weight ($p < 0.001$, $f = 0.26$, ANCOVA in all cases, Fig. 5). Some of the high-dose pollen treatments were associated with long development time, short pupation period and low pupal weight. The body weight of the larvae prior to pollen consumption had a significant influence on development time ($p < 0.001$), pupation period ($p < 0.01$) and pupal weight ($p < 0.001$, ANCOVA in all cases). Partial correlation analysis (controlling for larval weight prior to pollen consumption) revealed a significant correlation between larval weight on day 2 (i.e. after pollen consumption) and the dependent variables development time ($r_{\text{part}} = -0.51$, $p < 0.001$) and pupal weight ($r_{\text{part}} = 0.36$, $p < 0.001$), but not pupation period ($p > 0.05$).

Effects on adult butterflies

With respect to survival to the adult stage (after having consumed Bt maize pollen as first-instars), LD/LC values were always smaller than the calculated values during larval stages, but 95% confidence intervals overlapped wide (Table 2). Female butterflies differed in body weight, and higher pollen doses

Table 2. Lethal doses and lethal concentration estimates (95% confidence limits in parentheses) for the variables pollen suspension (LC), pollen density on leaf surface (LC), and the number of pollen grains consumed by the larvae (LD). First instar *P. machaon* larvae were fed Bt maize pollen, and estimates calculated for larvae at different times after pollen consumption (2, 7, 14 and 30 days), and for adult butterflies (adult)

LD values	Pollen suspension (mg Bt pollen/10 ml)	Pollen density (n/cm^2)	Pollen grains eaten (n)
LD/LC ₃₀ 2d	7.64 (4.20–12.24)	117.22 (62.69–190.68)	21.33 (10.30–35.32)
LD/LC ₃₀ 7d	3.10 (2.25–3.93)	50.13 (36.97–62.68)	10.58 (5.69–14.60)
LD/LC ₃₀ 14d	2.87 (2.03–3.69)	46.49 (33.42–58.93)	9.93 (4.92–14.01)
LD/LC ₃₀ adult	2.00 (1.26–2.73)	33.36 (21.54–44.64)	7.48 (5.09–9.61)
LD/LC ₅₀ 2d	14.91 (9.58–34.81)	222.48 (142.20–555.69)	36.3 (23.7–119.8)
LD/LC ₅₀ 7d	5.91 (4.76–7.26)	92.33 (75.17–112.28)	17.7 (12.4–24.2)
LD/LC ₅₀ 14d	5.62 (4.48–6.95)	88.07 (70.91–107.83)	17.0 (11.5–23.7)
LD/LC ₅₀ adult	4.28 (3.23–5.43)	68.30 (52.29–85.50)	13.72 (10.91–16.58)

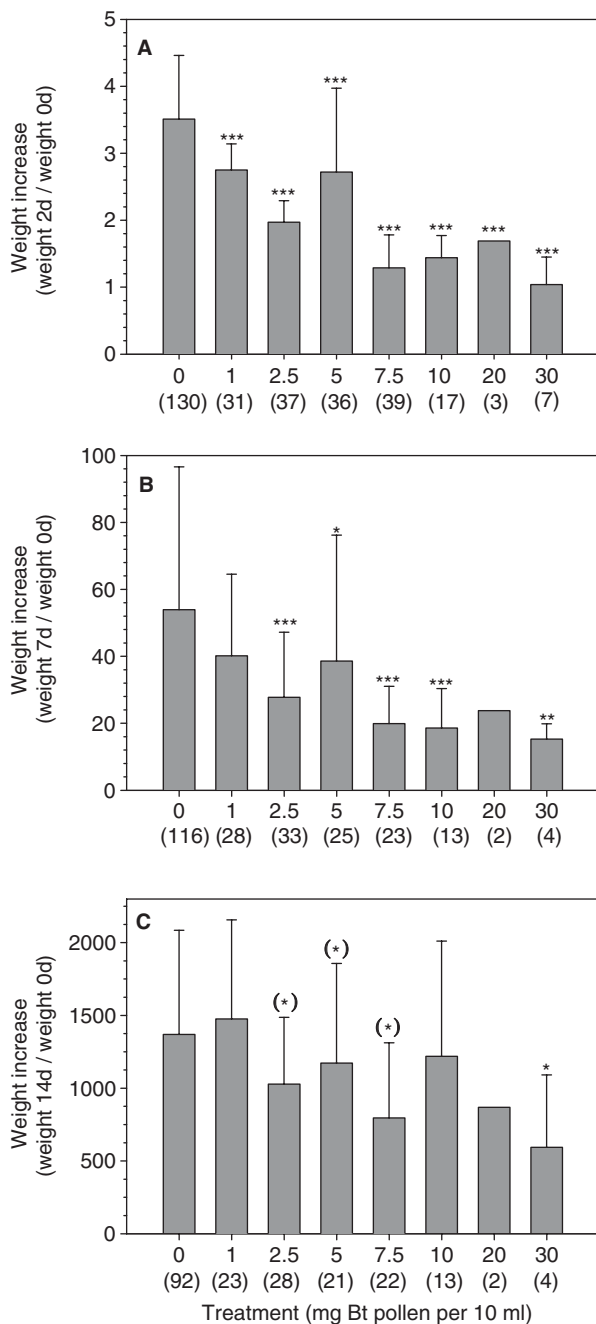


Figure 2. Relative weight increase of swallowtail larvae after exposure to different doses of Bt maize pollen suspensions (mg pollen/10 ml water). The body weight is given in relation to the weight on day 0 (i.e. prior to the start of the experiment) for different times of the experiment: day 2 (A), day 7 (B), and day 14 (C). Means + 1SD, number of larvae in parentheses. Asterisks denote significant differences of the pollen treatments when contrasted against the control: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, (*) $0.05 < p < 0.10$.

caused a lower weight (ANCOVA, $p < 0.001$, $f = 0.61$, Fig. 6A). Treatment had no effect on female forewing area (ANCOVA, $p = 0.14$, $f = 0.10$), but the contrasted

difference between the >7.5 -mg treatment and control was significant (Fig. 6B). There were no differences in male body weight ($p = 0.20$, $f = 0.26$, Fig. 6A). However, treatment had an effect on male forewing area (ANCOVA, $p = 0.01$, $f = 0.18$), and the 1-mg treatment and the >7.5 -mg treatment had smaller wings as compared to the control (Fig. 6B). Body weight of larvae prior to pollen consumption influenced male body weight ($p < 0.001$) and male wing area ($p < 0.06$, ANCOVA in both cases). Partial correlation analysis (controlling for larval weight prior to pollen consumption) revealed significant correlations between larval weight on day 2 (i.e. after pollen consumption) and the dependent variables male body weight ($r_{\text{part}} = 0.30$, $p < 0.01$), male forewing area ($r_{\text{part}} = 0.36$, $p < 0.001$), female body weight ($r_{\text{part}} = 0.42$, $p < 0.001$), and female forewing area ($r_{\text{part}} = 0.38$, $p < 0.001$).

Discussion

Consumption of Bt176-maize pollen had adverse effects on life history traits of the common swallowtail. *P. machaon* larvae fed with Bt pollen had a lower survival, a lower weight increase rate, a longer development time, and lower body weight and smaller wing size as adults, and these effects were significantly associated with Bt pollen density. So far, all previous studies quantified the exposure of lepidopteran larvae to Bt pollen, e.g. by measuring the concentration of applied pollen solutions, offered pollen numbers or the pollen densities on plant surfaces, rather than determining the actual numbers of pollen (and hence toxin amounts) taken up by the larvae. To our knowledge, no quantification of Bt pollen effects on lepidopteran larvae recording the exact number of pollen grains actually consumed by the larvae has been published. The LD₅₀ value showed that only 50% of L1-larvae having consumed 10.91–16.58 Bt maize pollen grains would survive to adulthood. Since a single Bt176 maize pollen grain contains on average 1.015 pg Cry1Ab protein (Lang et al., 2004), the number of pollen grains consumed can be directly related to the amount of Bt toxin ingested by the larvae. So far, LD and LC values for Bt pollen consumption have been calculated only for larvae ≤ 7 days of age (Felke & Langenbruch, 2001, 2003; Felke et al., 2002; Hellmich et al., 2001; Zangerl et al., 2001). Our results show that it is also important to calculate estimates for later life stages as LD/LC estimates tended to decrease during development probably due to long-term effects. The number of pollen consumed by larvae

Table 3. Body weights of first instar larvae (*P. machaon*) of different treatments, before Bt maize pollen consumption (Weight 0 d), and 2 days (Weight 2 d), 7 days (Weight 7 d) and 14 days (Weight 14 d) thereafter. Values are means \pm 1SD, and sample sizes are given in parentheses below. Values within a column showing different letters differ significantly ($p < 0.05$, Tamhane)

Treatment (Bt pollen/10 ml)	Weight 0 d (mg)	Weight 2 d (mg)	Weight 7 d (mg)	Weight 14 d (mg)
0 mg	1.02 \pm 0.32 abc (133)	3.41 \pm 0.77 a (130)	55.6 \pm 52.5 a (116)	1298 \pm 629 a (92)
1.0 mg	0.90 \pm 0.60 a (31)	2.46 \pm 0.23 b (31)	36.1 \pm 22.1 abc (28)	1320 \pm 623 a (23)
2.5 mg	1.19 \pm 0.34 b (44)	2.40 \pm 0.70 bc (37)	33.2 \pm 24.8 bc (33)	1205 \pm 556 a (28)
5.0 mg	0.91 \pm 0.28 acd (44)	2.14 \pm 0.50 c (36)	30.3 \pm 24.6 bc (25)	987 \pm 707 a (21)
7.5 mg	1.28 \pm 0.20 bc (47)	1.41 \pm 0.56 d (39)	22.7 \pm 14.8 bc (23)	937 \pm 691 a (22)
10 mg	1.05 \pm 0.17 bc (38)	1.54 \pm 0.50 d (17)	19.8 \pm 14.8 bc (13)	1266 \pm 792 a (13)
20 mg	0.79 \pm 0.14 d (35)	1.60 \pm 0.00 d (3)	22.5 ab (2)	821 a (2)
30 mg	0.92 \pm 0.17 ad (32)	1.04 \pm 0.35 d (7)	17.0 \pm 5.1 ab (4)	669 \pm 579 a (4)

could be predicted by pollen densities on leaf surfaces, and Bt pollen consumption was associated with reduced feeding. Such a feeding inhibition is a well-known effect of Bt (e.g. Anderson et al., 2004; Felke et al., 2002; Hellmich et al., 2001; Losey et al., 1999), and consequently resulted in a lower body weight of the swallowtail larvae. This sub-lethal effect could already be observed when larvae had consumed as few as 4.42 Bt pollen grains. Similar to the monarch butterfly (Stanley-Horn et al., 2001), effects on body weight were less pronounced in older larvae. This is likely due to the fact that 14-day-old larvae represent survivors only, whereas at an earlier state of the experiment the trials included individuals dying in high Bt pollen treatments. This suggests that the main Bt effect is on survival of young larvae. At the same time, the surviving swallowtail larvae may respond to Bt pollen consumption and concurrent feeding inhibition by a prolonged development time. Reserves acquired during the larval stages can be important for the butterflies' reproductive output as adults (Oberhauser, 1997), and an extended development time may compensate for food shortage experienced early in larval life. Indeed, swallowtail larvae of the Bt treatments took a longer period from egg hatch to pupation. For instance, according to the calculated regressions a consumption of 20 Bt pollen grains would reduce the body weight of 2-day-old swallowtail larvae by 31%, and delay their development by 1.4 days. Extension of development may have adverse consequences for *P. machaon* in the field as the

vulnerable larval life stages of common swallowtails are exposed for a longer time to predators, pathogens and other mortality factors, hence increasing generational mortality (Benrey & Denno, 1997; Clancy & Price, 1987; Dempster, King, & Lakhani, 1976; Nicholls & James, 1996).

Adult female swallowtails which were fed Bt maize pollen as first instar larvae had a lower body weight, and males had smaller wings. Partial correlation analyses indicated adverse effects on body weights and wing sizes of both males and females. To our knowledge, this study is the first published test of effects of Bt maize pollen consumption on adult European butterflies, and demonstrates adverse effects on adult stages as was also reported by a recent US study on the monarch butterfly (Dively et al., 2004). Generational relative fitness, i.e. relative lifetime survival and reproduction, is a particularly relevant experimental endpoint for risk assessment tests of genetically modified plants, because adverse effects of transgenic plants on non-target species would occur through some component of relative fitness (Andow & Hilbeck, 2004). Body mass of newly eclosed butterflies is strongly correlated with fat body containing nutrients and energy reserves for female egg production, and, therefore, lower body weight of swallowtail butterflies is likely to be associated with lower reproductive fitness (e.g. García-Barros, 2000; Karlsson & Wickman, 1990; Oberhauser, 1997; Wickman & Karlsson, 1989).

It is difficult to transfer laboratory results to the field, because the manifold and complex conditions

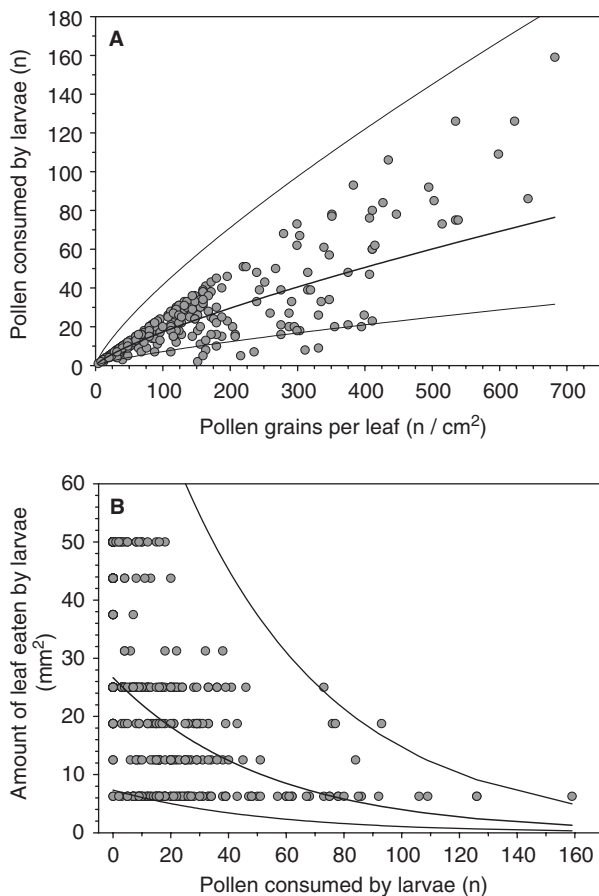


Figure 3. Number of Bt maize pollen consumed by swallowtail larvae related to pollen densities on leaves (A), and amount of leaf eaten (mm²) by larvae related to Bt maize pollen consumed (B). Regression curves are shown with 95% confidence intervals: (A) $y = 0.49 \times 0.77^x$, $R^2 = 0.75$, $p < 0.001$, $n = 271$; (B) $y = 26.67e^{-0.019x}$, $R^2 = 0.31$, $p < 0.001$, $n = 399$.

and relationships in the field can never be simulated adequately indoors. However, effective pollen densities applied in this study are in accordance with maize pollen densities found on swallowtail host plants in the field (Lang et al., 2004). Pollen densities in the field can even be much higher than reported by Lang et al. (2004), because their data refer only to 24-h counts and did not take into account maize pollen accumulation during the pollen shedding period. Pollen accumulation can build up to high amounts, e.g. up to 320 pollen grains per cm² on foliage of parsnip, *Pastinaca sativa*, another host plant of swallowtail larvae (Zangerl et al., 2001). The pollen shedding period of maize fields usually spans 5–8 days with a maximum of 14 days (Treu & Emberlin, 2000). Therefore, a longer and more realistic exposure time of butterfly larvae to Bt maize pollen than the 48 h of this study will result in stronger Bt maize

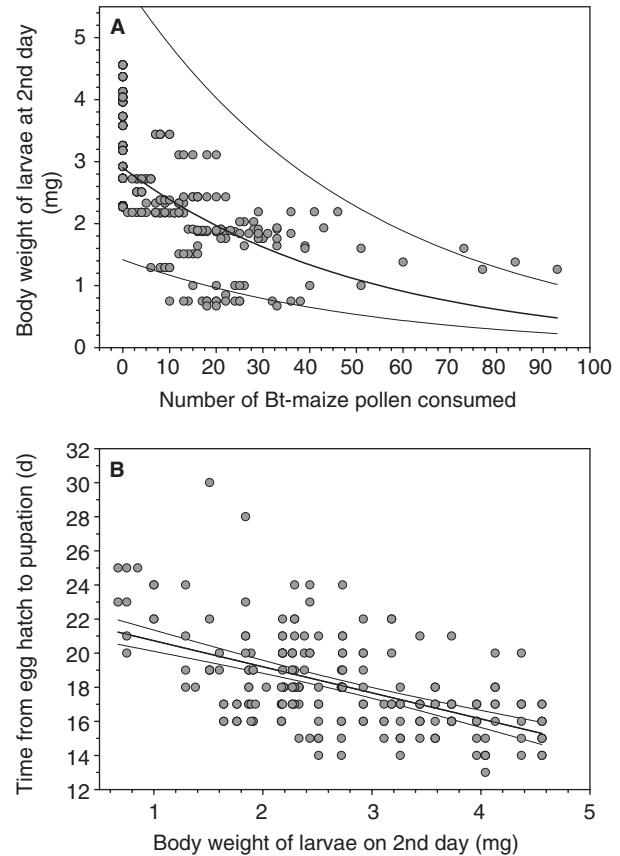


Figure 4. Body weight of swallowtail larvae (mg) 2 days after Bt maize pollen consumption related to number of pollen consumed (A), and development time of larvae (days) related to body weight after 2 days of Bt maize pollen consumption (B). Regression curves are shown with 95% confidence intervals: (A) $y = 2.89e^{-0.019x}$, $R^2 = 0.38$, $p < 0.001$, $n = 300$; (B) $y = 22.26 - 153x$, $R^2 = 0.30$, $p < 0.001$, $n = 218$.

effects, e.g. an exposure of more than 5 days as was demonstrated by Dively et al. (2004). Swallowtail larvae suffer regularly from infections (Clarke, 1977; Nicholls & James, 1996), and Bt consumption enhances the negative impact of bacterial infections on Lepidoptera larvae (e.g. Pierce, Solter, & Weinzierl, 2001), a factor not accounted for in our lab study. For instance, larvae of the European corn borer (Lepidoptera: Crambidae) which had been fed Cry1Ab-toxin were much less tolerant to *Nosema* infection than the control group resulting in higher mortality and stronger adverse sublethal effects (Reardon, Hellmich, Sumerford, & Lewis, 2004). Factors possibly reducing the Bt maize pollen effect in the field would include rain, which decreases maize pollen drift and washes off pollen deposited on plant leaves (Lang et al., 2004; Pleasants et al., 2001; Stanley-Horn et al., 2001; Zangerl et al., 2001). Larvae older than first instars

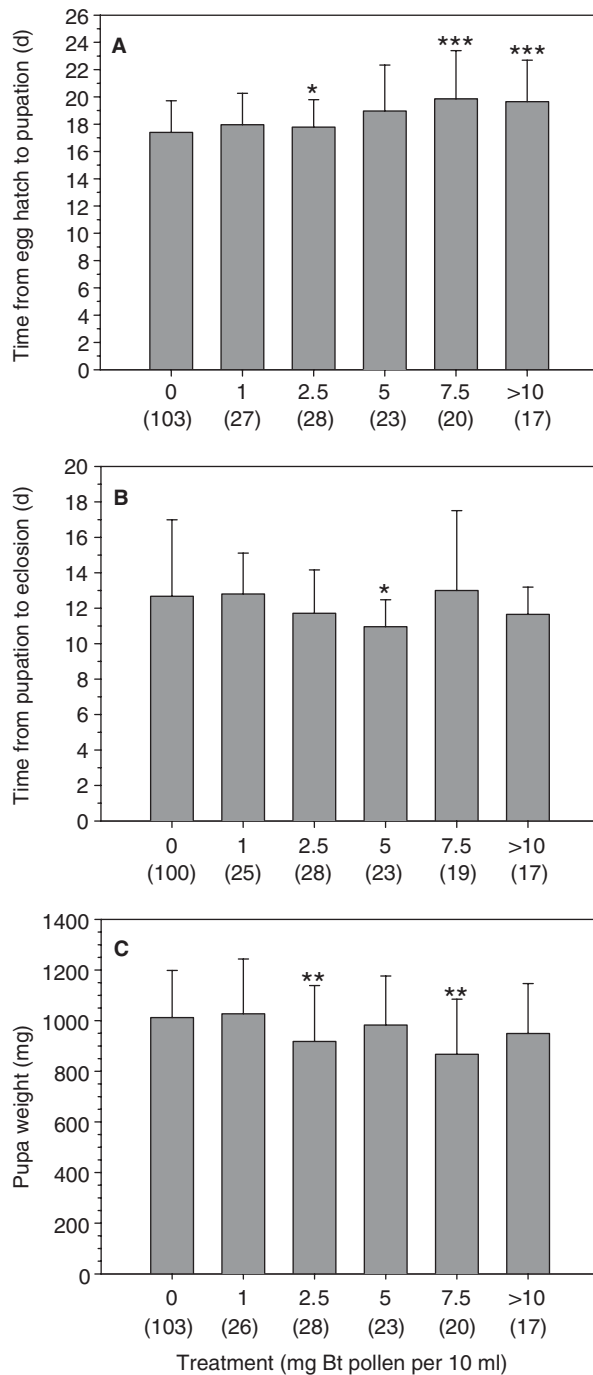


Figure 5. Larval development time (A), pupal time (B), and pupal weight (C) of swallowtail larvae after exposure to different doses of Bt maize pollen suspensions (mg pollen/10 ml water). Means + 1SD, number of larvae in parentheses. Asterisks denote significant differences of the pollen treatments when contrasted against the control: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

would be more tolerant to the consumption of Bt maize pollen (e.g. Felke & Langenbruch, 2003; Felke et al., 2002; Hellmich et al., 2001). Even though butterfly larvae may perhaps avoid maize

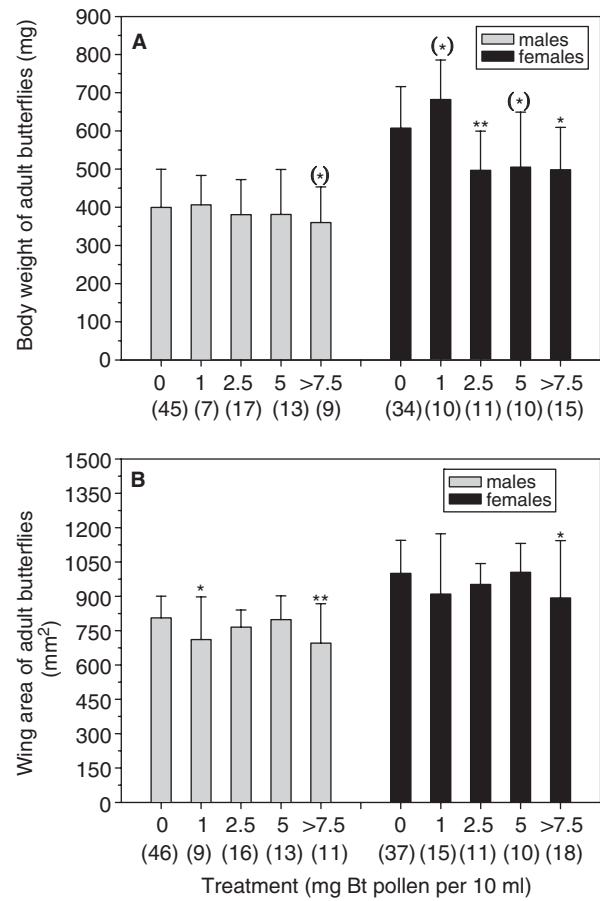


Figure 6. Body weight (A) and forewing wing area (B) of adult swallowtails after larval exposure to different doses of Bt maize pollen suspensions (mg pollen/10 ml water). Means + 1SD are given separately for males and females, number of butterflies are in parentheses. Asterisks denote significant differences of pollen treatments when contrasted against the control: ** $p < 0.01$, * $p < 0.05$, (*) $0.05 < p < 0.10$.

pollen consumption under field conditions by actively searching for leaves with low pollen densities, adverse effects of Bt176 maize on larvae of *P. polyxenes*, a close relative of *P. machaon*, have already been documented in a field setting (Zangerl et al., 2001).

This study demonstrated toxic effects of Bt176 maize pollen on *P. machaon*, however, additional data are indispensable for evaluating the actual risk for the common swallowtail associated with the cultivation of Bt maize. A complete risk assessment requires at least the following information (cf. Andow & Hilbeck, 2004; Sears et al., 2001; Wolt, Peterson, Bystrak, & Meade, 2003): (i) exposure of butterfly species to Bt maize pollen, which requires data about Bt toxin expression in pollen of the different events, pollen shedding periods (on a regional scale), dispersal and deposition of pollen

on host plants, the spatial and temporal occurrence and distribution of butterfly species and their host plants (regional and on a landscape scale), and oviposition preferences of butterflies, (ii) direct and indirect long-term effects of Bt maize pollen or Cry1Ab toxin in the laboratory and especially in the field, and (iii) information about (meta-)population dynamics and life history traits of the respective butterfly. A considerable effort was undertaken in the USA to carry out a risk assessment of Bt maize, in particular with regard to the monarch butterfly (e.g. Losey, Hufbauer, Hartzler, 2003; Sears et al., 2001; Wolt et al., 2003). However, results obtained for species occurring in North American agricultural landscapes cannot be transferred to Europe, and there is an urgent need for comprehensive risk assessments for European butterflies (e.g. Darvas, Csóti, et al., 2004). In Europe two Bt maize events are registered for cultivation, the Bt176 and the Mon810. This study and the papers of Felke et al. showed that the Bt176 maize has the potential to adversely affect larvae of European butterflies. Mon810 expresses much less toxin in pollen, and therefore, its effect on butterfly larvae was considered non-existent or negligible until recently. However, the study of Dively et al. (2004) demonstrated that realistic long-term exposure to Mon810 maize can cause negative effects on monarch larvae (as opposed to the short-term exposure of previous studies). Darvas, Lauber et al. (2004) also reported an adverse effect of Mon810 on a European butterfly species, and field experiments in Europe are missing so far. In consequence, the available database is too small to provide a well-founded basis to assess the risk for butterflies and moths associated with extensive cultivation of Bt maize in Europe in the future.

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