J. Appl. Ent. 126, 320–325 (2002)

ISSN 0931-2048

Laboratory studies on the effects of pollen from Bt-maize on larvae of some butterfly species

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Ms. received: February 8, 2001; accepted: August 6, 2001

Abstract: Three lepidopteran species were tested to determine their susceptibility against the ingestion of pollen from genetically modified maize plants. To prove the existence of dose–response relations between the applied amount of pollen (Bt-maize) and the damages on the tested larvae, a method was developed which makes it possible to feed caterpillars with defined amounts of pollen. If their food plants were contaminated with pollen of a cultivar of the Bt-176 maize-line *Pieris brassicae*, *Pieris rapae* and *Plutella xylostella*-larvae fed less, grew more slowly and showed a higher mortality than caterpillars of an untreated control group. The 50% lethality (LD_{50})-values were calculated for *P. xylostella* (L_4) with 19.2, for *P. rapae* (L_2) with 39.0 and also for *P. brassicae* (L_2) with 139.2 pollen of the transgenic maize Pactol CB. Studies with *P. brassicae*-caterpillars of different larval stages indicated, that older individuals showed a higher tolerance against pollen from Bt-maize than younger ones. It must be stated on the basis of the present studies, that ingestion of non-transgenic maize pollen has neither a positive nor a negative effect on caterpillars. It becomes clear from the information presented here that it is still difficult to make general statements about the endangering of butterflies, arising from cultivation of genetically modified maize lines. Further investigations on this issue are needed. Initially, the LD_{50}-values concerning the larvae of certain butterfly species have to be determined to anticipate the risks, and in addition the distances between habitats with caterpillar host plants and maize fields, and the abundance of these plants have to be considered.

1 Introduction

At present, the cultivation of transgenic crops such as cotton, maize, or potatoes and its effects on human health, or the environment, has led to controversial discussions. Maize, transformed with genetic material from the bacterium *Bacillus thuringiensis* (*B.t*), has been cultivated in the USA since 1995. This genetically transformed maize expresses a so-called *B.t.* toxin, which is harmful to certain lepidopteran larvae and protects the maize plants against the European corn borer (*Ostrinia nubilalis*), an important maize pest. For two different reasons, negative effects on non-target organisms had not been expected by the public at large in Europe. The first reason is the high specificity of *B.t.* toxin against butterflies. On the other hand, normally only larvae of *O. nubilalis* feed on maize plants in Germany. However, Losey et al. (1999) found in a laboratory assay that second and third instar larvae of the monarch butterfly (*Danaus plexippus*) came to harm when they were kept on milkweed leaves (*Asclepias curassavica*), which had been dusted with pollen from genetically modified maize (Bt-maize). These individuals consumed less, grew more slowly and suffered a higher mortality than larvae reared on leaves dusted with untransformed maize pollen or on leaves without any pollen. The pollen was applied on the forage plant by gently tapping a pollinating maize spatula over milkweed leaves that had been lightly misted with water. Due to this application method the amount of pollen on the leaves could not be defined. It was only stated that pollen density was set to visually match densities on milkweed leaves collected in maize fields. In addition, this study does not indicate how much pollen was eaten by the monarch larvae – if there was any pollen consumption at all. A further criticism is that pollen for this assay was collected from two different maize cultivars (N 4640 Bt-maize and an unrelated, untransformed hybrid). For these reasons, it is difficult to judge the risks for lepidopteran species under field conditions on the basis of the laboratory investigations of Losey et al. (1999).

For the present study it was necessary to develop a method to apply defined amounts of pollen on plants. Furthermore, it was necessary to clarify if lepidopteran larvae ingest pollen from Bt-maize at all, when feeding on leaves of their host plants. It seems possible, that pollen from transgenic maize causes avoidance reactions or stop of ingestion of the butterfly larvae. The existence of such behaviour could explain phenomena such as reduced ingestion, lesser growth rate, or higher mortality, as observed by Losey et al. (1999). If the low growth rate and also the increased mortality of monarch larvae, as observed by Losey et al. are really

 0931–2048/2002/2606–0320 $ 15.00/0

caused by ingestion of pollen from Bt-maize, there should be a correlation between the amount of pollen applied and these effects. One of the main aims of the study presented here was to find such cause–effect relationship and to calculate the LD_{50}-values for several butterfly species. In addition, the amount of pollen from transgenic maize that had to be consumed until sublethal effects, such as decreased growth rate, occurred was also investigated.

2 Materials and methods

Effects of transgenic maize pollen on butterfly larvae were tested on caterpillars of *Pieris brassicae*, *Pieris rapae* and *Plutella xylostella*. These three lepidopteran species are known as important cabbage pests and can be controlled by *Bacillus thuringiensis* strains belonging to pathotype A (Franz and Krieg, 1982). Some *B.t.* preparations are registered in Germany to control these butterflies. Pollen for the assay was taken from the transgenic Bt-176 maize hybrid Pactol CB and also from the isogenic, untransformed variety Pactol. Fresh pollen was only available for some bioassays. In most cases it was stored inside closed plastic boxes at −30°C for up to 2 months, before it was used for bioassays. Every bioassay consisted of an untreated control variant (no pollen applied on the leaf discs) and up to four variants with different amounts of pollen (from Pactol CB- or Pactol-maize).

Bioassays with second instar larvae of *P. brassicae* and *P. rapae*, and fourth instar larvae of *Plutella xylostella* were carried out in plastic boxes with the dimensions of 14 cm × 7 cm × 2 cm (length × width × height). These plastic boxes were filled up to a height of 1 cm with an Agar agar solution of 1.5% concentration. Then, a plastic grid of 50 square cells of 1.4 cm length was pressed into the liquid A. agar solution. After the agar had become solid, a fresh cabbage leaf disc (*Brassica oleracea* var. *acephala* subvar. *medullosa*), which measured 3 mm in diameter and fitted a surface of 7.07 mm^2, was placed into each cell with its upper leaf surface upward.

Finally, maize pollen was applied on the leaf discs. First, 2 or 3 mg of maize pollen were suspended in 2–10 ml of distilled water. After this, 1 μl of this defined solution was applied in the centre of a cabbage leaf disc by using an Eppendorf pipette. The surface of the leaf disc was dried by using a ventilator until all water had evaporated. This process lasted up to 4 h at room temperatures of between 22 and 25°C. At the end of this drying process about 20–30% of the surface of the leaf disc was covered with pollen. This application method guaranteed that the pollen stuck quite effective to the surface of the leaf. Finally, the precise amount of pollen was counted for every single cabbage leaf disc by using a microscope (magnification = 40 ×). After this, a caterpillar was placed into each cell. Larvae of the untreated control group were fed with cabbage leaf discs without any pollen. In order to prevent the escape of the larvae, the cells were covered with a piece of paper and a plastic plate, and then sealed by using two rubber bands. Previously, the mean weight of all larvae in the test had been determined. The first check took place 1 day after the start of the bioassay and the percentage of leaf area consumed during the last 24 h was determined. All of the individuals that had eaten the cabbage leaf disc totally, received a new untreated one. Larvae that had not eaten their food completely, did not receive a fresh cabbage leaf disc. After the next 24 h this procedure was repeated, so that the amount of the first leaf disc had been consumed by caterpillars within the first 48 h could be recorded.

Directly after this second check all surviving individuals belonging to the same bioassay variant were put together into a larger plastic box (18 cm × 12 cm × 7 cm) with a perforated lid, containing a whole untreated cabbage leaf. However, those caterpillars that had eaten less than 10% of the first cabbage leaf disc during the first 2 days of the bioassay, were removed, because in this case pollen consumption seemed very unlikely. One week after the bioassay had started, the mean weight of the surviving caterpillars was determined for each bioassay variant separately, so it was possible to determine the increase in larval weight in relation to the applied amount of pollen. The larvae were kept at a temperature of 25°C in a 16 h light : 8 h dark photoperiod.

The conditions for the bioassays with third and fourth instar larvae of *P. brassicae* and *P. rapae* were nearly the same as for smaller larvae, but in this case, plastic boxes which measured 31 cm × 22.5 cm were used. These boxes contained 25 holes that measured 4 cm in diameter and 3 cm in depth. The holes were filled with a 1.5% concentrated Agar agar solution. After the A. agar solution had become solid, a fresh cabbage leaf disc, measuring 16 mm in diameter (surface = 201 mm^2) was put into every cell. Finally, 100 μl of a pollen solution was applied to the middle of each leaf disc. Mostly, the applied amount of pollen was much higher than the value given to smaller caterpillars. In this case, it was not possible to count the exact number of pollen so the numbers had to be estimated.

For each bioassay variant the mean weight of all surviving larvae was calculated at the end of the experiment, so the mean increase in weight for these individuals could be determined. The mortality rate for each bioassay variant was determined from the number of larvae, which were still alive after 1 week. The mortalities were corrected for mortality in untreated control according to Abbott (1925). Only those bioassay variants that showed less than 20% mortality for the untreated individuals were used for calculation of mortality rates, increase in weight and probit-regression analysis. The dose–response relationship (LD_{50} and LD_{90}) were calculated by using the probit method of Finney (1971) and computed by means of the SPSS probit analysis program (SPSS Inc., Chicago, USA, release 8.0). Finally, all bioassay variants with similar amounts of Pactol CB- and Pactol-pollen were pooled, so the mortality rate and increase in weight always represents mean values. Mortality rates and increase in weight of the corresponding control groups were calculated in the same way. The mean mortality rate for a certain group was estimated by calculating the mortality rate for every single variant and was corrected for mortality in the corresponding untreated control according to Abbott (1925). It was not possible to include all the different variants in only one bioassay, because the number of larvae was limited. So, summarizing the bioassay variants with equal pollen amounts became necessary.

3 Results

3.1 Effects of pollen ingestion on larval food consumption, increase in weight and mortality

Figures 1, 2 and 3 show the consumption rates of *P. xylostella*, *P. rapae* and *P. brassicae* larvae which were fed with untreated leaf discs and leaf discs contaminated with variable amounts of pollen from Bt-maize.

Fourth instar larvae of *P. xylostella*, which served as untreated control groups had eaten their food nearly completely after the first 24 h. Caterpillars which were

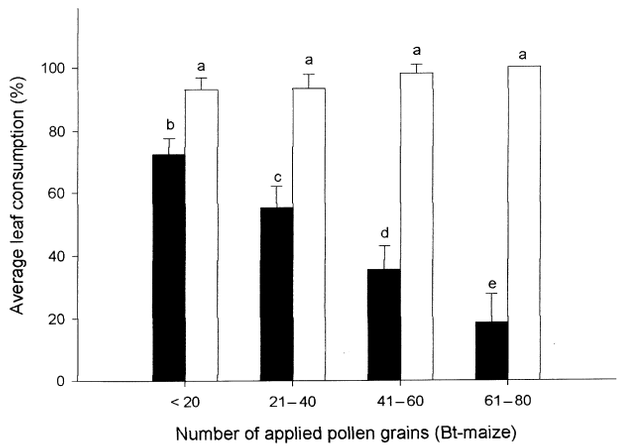

Fig. 1. *Mean consumption rates ± 95% confidence limits for fourth instar larvae of* Plutella xylostella *(in percent) during the first 24 h. Leaf discs covered with pollen (Bt-maize, ■) or without any pollen (□). Values followed by the same letter are not significantly different from each other at 5% level of significance, according to Duncan's multiple range test. Horizontal bars indicate 95% confidence limits*

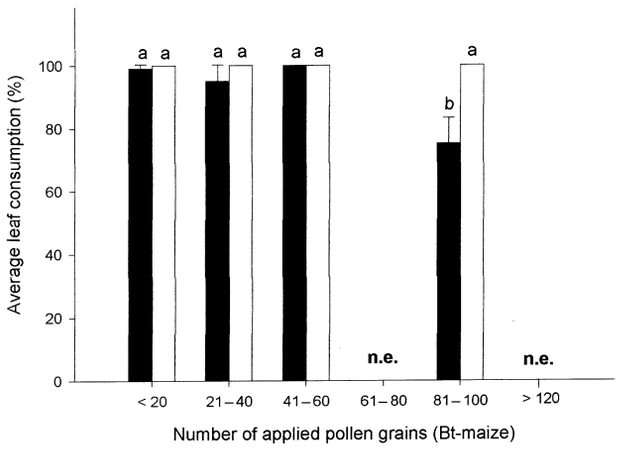

Fig. 3. *Mean consumption rates ± 95% confidence limits for second instar larvae of* Pieris brassicae *(in percent) during the first 24 h. Leaf discs covered with pollen (Bt-maize, ■) or without any pollen (□). Values followed by the same letter are not significantly different from each other at 5% level of significance, according to Duncan's multiple range test. Horizontal bars indicate 95% confidence limits. n.e. not evaluated*

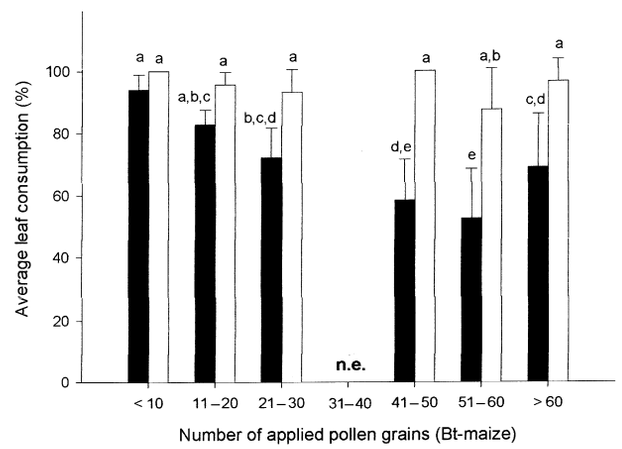

Fig. 2. *Mean consumption rates ± 95% confidence limits for second instar larvae of* Pieris rapae *(in percent) during the first 24 h. Leaf discs covered with pollen (Bt-maize, ■) or without any pollen (□). Values followed by the same letter are not significantly different from each other at 5% level of significance, according to Duncan's multiple range test. Horizontal bars indicate 95% confidence limits. n.e. not evaluated*

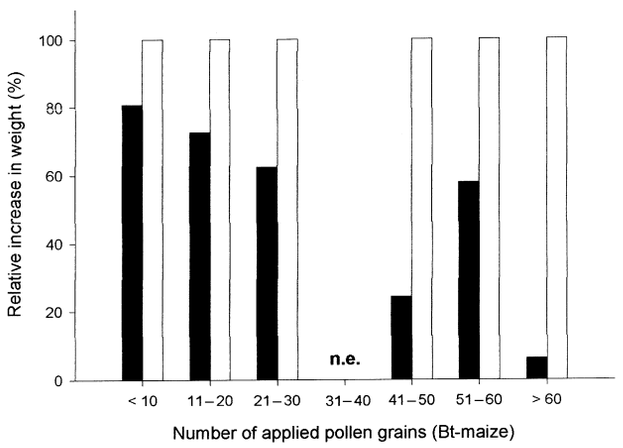

Fig. 4. *Increase in weight for second instar larvae of* Pieris rapae *in relation to different amounts of applied pollen (Bt-maize, ■). Increase in weight for individuals of the corresponding control group (no pollen, □) was set as 100%. 95% confidence limits and level of significance are not available. n.e. not evaluated*

forced to feed on leaf discs contaminated with Pactol CB pollen consumed significantly less than the untreated control larvae. Food consumption decreased dramatically with increasing numbers of applied pollen grains (fig. 1). Second instar larvae of *P. rapae* showed the same tendency for food consumption as the caterpillars of the diamondback moth (fig. 2). In contrast to caterpillars of the first two species second instar larvae of *P. brassicae* did not show such a clear relation between the amount of transformed maize pollen on their food source and consumption rates. Only individuals that were forced to eat leaf discs contaminated with approximately 90 pollen grains of Pactol CB maize consumed significantly less than the corresponding untreated larvae (fig. 3). Contamination of leaf discs with pollen of the non-transgenic maize hybrid Pactol had no significant effect on larval food consumption for all three species.

Figures 4, 5 and 6 show the dose–response-relations between larval increase in weight and the amount of pollen applied to their food source. Second instar larvae of *P. rapae* and *P. brassicae* obviously gained less weight, as they were fed with increasing amounts of pollen from Bt-maize (figs 4 and 5). On the other hand, third instar larvae of *P. brassicae*, which ingested such pollen, grew as fast as the untreated control (fig. 6). Pollen-consumption of the tested non-trans-

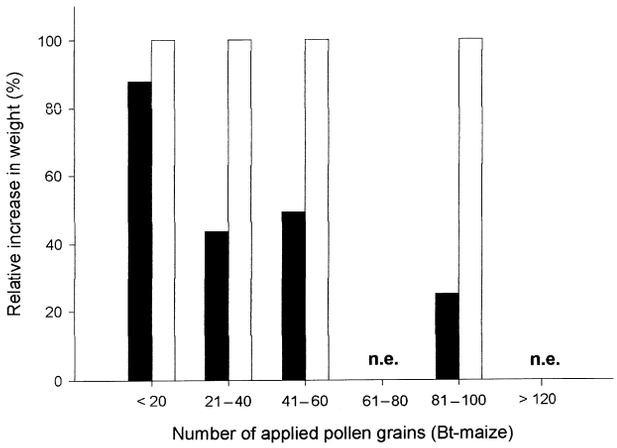

Fig. 5. *Increase in weight for second instar larvae of* Pieris brassicae *in relation to different amounts of applied pollen (Bt-maize, ■). Increase in weight for individuals of the corresponding control group (no pollen, □) was set as 100%. 95% confidence limits and level of significance are not available. n.e. not evaluated*

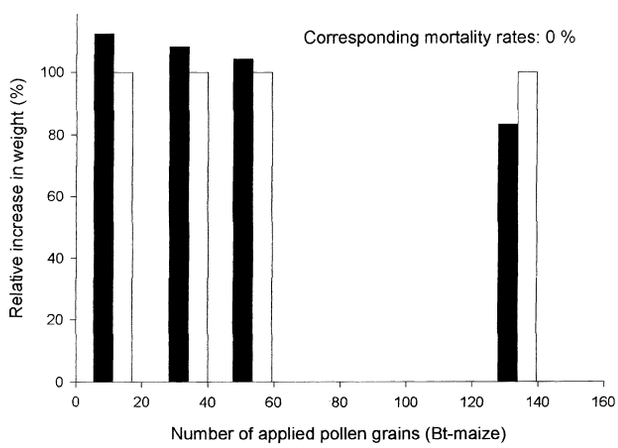

Fig. 6. *Increase in weight for third instar larvae of* Pieris brassicae *in relation to different amounts of applied pollen (Bt-maize, ■). Increase in weight for individuals of the corresponding control group (no pollen, □) was set as 100%. 95% confidence limits and level of significance are not available*

genic maize line seemed to have neither a positive nor a negative effect on larval development. The increase in weight of *P. xylostella* larvae could not be investigated, because most of the individuals pupated before the end of the bioassay and so only mortality rates could be calculated for this species. As fig. 7 indicates, there is a strong increase in mortality rate for fourth instar larvae of *P. xylostella* with increasing amounts of pollen from Bt-maize on the leaf discs.

This correlation between larval mortality and the amount of Pactol CB-pollen could also be detected for second instar larvae of *P. rapae* (fig. 8) and *P. brassicae* (fig. 9), although it was not so distinct.

However, third instar larvae of *P. brassicae* did not show any increased mortality when they were fed with pollen from Bt-maize. Even amounts of more than 130

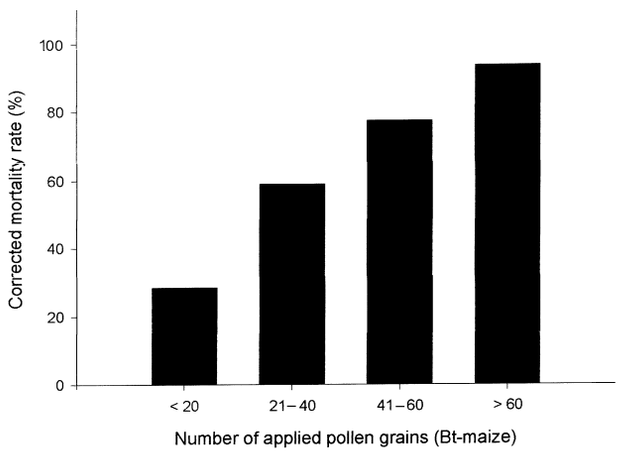

Fig. 7. *Mortality rate (corrected) for fourth instar larvae of* Plutella xylostella *in relation to different amounts of applied pollen (Bt-maize, ■). 95% confidence limits and level of significance are not available*

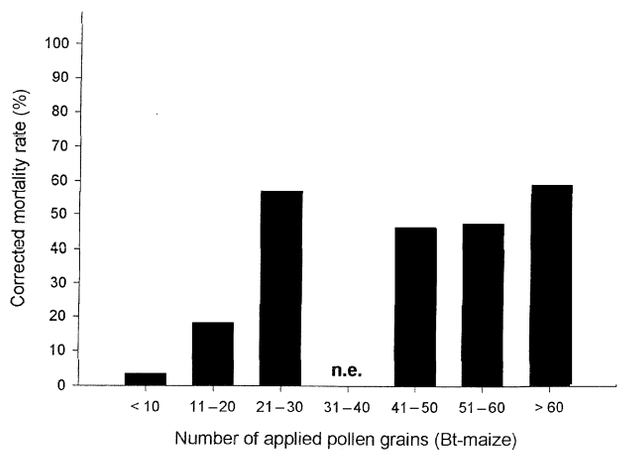

Fig. 8. *Mortality rate (corrected) for second instar larvae of* Pieris rapae *in relation to different amounts of applied pollen (Bt-maize, ■). 95% confidence limits and level of significance are not available. n.e. not evaluated*

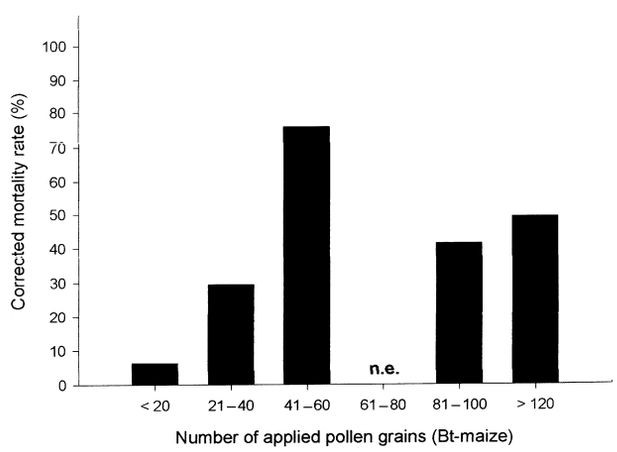

Fig. 9. *Mortality rate (corrected) for second instar larvae of* Pieris brassicae *in relation to different amounts of applied pollen (Bt-maize, ■). 95% confidence limits and level of significance are not available. n.e. not evaluated*

pollen grains per leaf disc did not lead to a higher mortality rate. Consumption of non-transgenic maize pollen did not cause an increased mortality in second instar larvae of *P. rapae* and *P. brassicae*; even an application of more than 50 pollen grains did not lead to increasing mortality rates. In contrast, the application of a similar amount of Pactol CB pollen caused a corrected mortality rate of 47.8% on second instar larvae of *P. rapae* in a certain bioassay.

3.2 Evaluation of LD_{50}- and LD_{90}-values

The LD_{50}- and LD_{90}-values were determined for fourth instar larvae of *P. xylostella* and for second instar larvae of *P. rapae* and *P. brassicae* (table 1). The number of bioassay variants and number of larvae which were used to calculate LD_{50}- and LD_{90}-values do not include untreated control variants.

4 Discussion

The present results are the first quantitative data about effects of pollen from Bt-176 maize on larvae of these three lepidopteran species. They have to be verified by later bioassays and some details still remain open. To prove the existence of dose–response relations between the applied amount of pollen from Bt-maize and the damage to the tested larvae, a method was developed which makes it possible to feed caterpillars with defined amounts of pollen. Using this method, the pollen stuck relatively tightly to the surface of the leaves, so it could not easily be removed by the larvae. By isolating the caterpillars for 2 days they were forced to eat most of the pollen, because they could not switch to another food source that was not contaminated with pollen from genetically modified maize. It should be investigated under field conditions, how effectively the pollen can stick on certain host plants of butterflies. It seems to be possible that dewfall in the morning, or rainy weather conditions during the time of pollen dispersion, can lead to similar situations as those developed under artificial laboratory conditions. About 10% of the pollen grains burst during the application process. Later bioassays showed, that this phenomenon had no significant effect on the results mentioned here.

Our studies reveal that there is a relation between the amount of pollen from Bt-maize applied to the host plants and larval food consumption, increase in weight and mortality. As figs 1–6 show, food consumption and increase in weight of the caterpillars decreased as pollen amounts increased and simultaneously, the mortality increased (figs 7–9). Often the larvae did not eat all of the pollen grains that had been applied to the leaves and so the LD_{50}-values given here always refer to the applied amount of pollen. It seems to be very likely that the LD_{50}-values related to larval pollen ingestion will be significantly lower than the LD_{50}-values presented here, because the food consumption of butterfly larvae decreased as pollen amounts increased. Among the three butterfly species tested here, second instar larvae of *P. brassicae* showed the highest, whereas fourth instar larvae of *P. xylostella* presented the lowest food consumption rate. Thus it can be assumed that the differences between LD_{50}-values presented here and LD_{50}-values related to larval pollen ingestion will be much higher for fourth instar larvae of *P. xylostella* than for second instar larvae of *P. brassicae*. The results of the study presented here indicate that tolerance against *B.t.* toxin differs markedly between caterpillars of different butterfly species. Thus the LD_{50}-values were 19.2 pollen grains of the transgenic maize Pactol CB for fourth instar larvae of *P. xylostella*, 39.0 for second instar larvae of *P. rapae* and 139.2 for second instar larvae of *P. brassicae*. In particular, the difference between both *Pieris* species seems to be very interesting, because they are closely related phylogenetically. The mean values of the caterpillars' weight did not differ significantly, so there must be other reasons for the different LD_{50}-values. For instance, these distinctions could be based upon differences in gut proteases or *B.t.*-toxin-receptors. Until now, we have just investigated two members of the family Pieridae and one species of the family Plutellidae. So, evaluation of LD_{50}-values would be very interesting, concerning other groups of Lepidoptera, as for example Satyridae, Nymphalidae or Papilionidae.

Investigations with larvae of *P. rapae*, and *P. brassicae* of different stages indicate that the susceptibility of caterpillars to *B.t.* toxin also depends on the size of the larvae. Second instar larvae of *P. brassicae* showed an obvious increase in mortality if more than 30 grains of pollen from Bt-maize had been applied to their food source (fig. 9). On the other hand, no third instar larva of *P. brassicae* died when it was forced to eat a cabbage leaf disc that contained more than 130 Pactol CB pollen grains. The mean weight of

Table 1. *LD_{50}- and LD_{90}-values concerning the applied number of Pactol CB pollen grains for larvae of* P. xylostella, P. rapae *and* P. brassicae

Species (larval stage)	Number of bioassay variants	Number of larvae	LD_{50}-values (95% confidence limits)	LD_{90}-values (95% confidence limits)
Plutella xylostella (L_4)	7	206	19.2 (9.2–29.8)	186.5 (58.5–405.5)
Pieris rapae (L_2)	19	359	39 25.7–122.7	186.5 (77–6065)
Pieris brassicae (L_2)	12	174	139.2 (54.6–845 862)	3.123 (383–28 270 618)

these last-named individuals had been five times higher than the mean value of the tested second instar larvae.

Generally, substances are also harmful to organisms in concentrations that are distinctly lower than the given LD_{50}-values. Certain bioassays of our study showed, that the application of less than five pollen grains from transgenic maize can harm fourth instar larvae of *P. xylostella*. Less than 15 Pactol CB pollen grains can cause sublethal effects among second instar larvae of *P. rapae*, and less than 20 among second instar larvae of *P. brassicae*. Ingestion of pollen from Bt-maize not only led to a slow down in larval development, reduced food consumption and increased mortality, but also caused lethargic reactions of the caterpillars. Beyond this, *P. rapae* larvae showed some changes in their behavioural patterns after they had eaten this pollen. Normally, caterpillars of this species feed on the underside of the leaves of their host plant. This could be interpreted as protection behaviour against some predators such as birds. After feeding on pollen from Bt-maize the larvae often stayed on the upper side. It can be assumed that these changes in behavioural patterns, as well as the lethargic reactions, will reduce the ability of the caterpillars to survive under natural conditions. Thus it seems to be possible that even consumption of low pollen amounts from transgenic maize can cause negative effects on lepidopteran larvae.

Losey et al. (1999) determined that larvae of the monarch butterfly (*Danaus plexippus*) grew more slowly than caterpillars of an untreated control group if the leaves of their host plant were dusted with pollen of an untransformed maize hybrid. We can not confirm these results on the basis of our previous studies. Feeding bioassays with non-transgenic pollen did not cause increased mortality rates for second instar larvae of *P. rapae*. In one of two bioassays these caterpillars grew even faster than individuals that were fed on cabbage leaf discs without pollen. This result does not seem very surprising, taking into account that pollen contains high amounts of protein and thus provides very good nutrition. Nevertheless more studies on this topic are needed.

Our study indicates very clearly that even consumption of small amounts of pollen from Bt-maize can have a negative impact on caterpillars, although it seems to be quite difficult to draw any conclusions from these laboratory results that might apply to the situation under field conditions. There are several further pieces of information needed in order to ascertain the endangering of butterfly populations from cultivation of transgenic maize lines. First, LD_{50}-values must be determined for more lepidopteran species, but it is also very important to know how much pollen can be digested by caterpillars under natural conditions. Information about the dispersion of pollen around maize fields varies widely. Raynor et al. (1972) stated that maize pollen can be dispersed over at least 60 m by wind. On the other hand, Hansen and Obrycki (1999) could not find any pollen on the leaves of milkweed plants located 5 m away from the edge of a blooming maize field. Furthermore, the question if, and to which extent, the amount of pollen on plants decreases due to rain and wind requires resolution. It is also likely that the toxicity of pollen from Bt-maize decreases after some time. For example, effects of UV-radiation seem to be possible, as reported by Pusztai et al. (1991) for purified δ-endotoxin crystals from *B.t.* subspecies HD-1 and HD-73.

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