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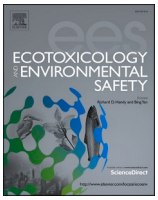

Addressing the challenges of non-target feeding studies with genetically engineered plant material – stacked *Bt* maize and *Daphnia magna*

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ABSTRACT

Previous studies reported adverse effects of genetically engineered maize that produces insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*) on the water flea *Daphnia magna*. In the current study, effects of flour, leaves, or pollen from stacked *Bt* maize that contains six *Bt* proteins (SmartStax) in two plant backgrounds on life table parameters of *D. magna* were investigated. Adverse effects were observed for *Bt* maize flour, originating from different production fields and years, but not for leaves or pollen, produced from plants grown concurrently in a glasshouse. Because leaves contained eight to ten times more Cry protein than flour, the effects of the flour were probably not caused by the Cry proteins, but by compositional differences between the plant backgrounds. Furthermore, considering the natural range of variation in the response of *D. magna* to conventional maize lines, the observed effects of *Bt* maize flour were unlikely to be of biological relevance. Our study demonstrates how Cry protein effects can be separated from plant background effects in non-target studies using *Bt* plant material as the test substance and how detected effects can be judged for their biological relevance.

1. Introduction

The development of genetically engineered (GE) plants is a major achievement in modern plant breeding. A range of crops has been transformed with genes from the bacterium *Bacillus thuringiensis* (*Bt*). They produce insecticidal *Bt* Cry or VIP proteins to control Lepidoptera or Coleoptera pests. This often allows reduced applications of insecticides and thus benefits human and environmental health (Klümper and Qaim, 2014; NASEM, 2016; Smyth, 2020).

One concern with *Bt* crops is that the produced insecticidal proteins may affect beneficial non-target species with potential consequences for biodiversity and ecosystem services, such as pollination, biological control, decomposition, and nutrient cycling (Mendelson et al., 2003; EFSA, 2010; Romeis et al., 2008; NASEM, 2016). GE crops are regulated worldwide and have to pass a risk assessment before being released commercially. For regulatory purposes, potential risks for non-target species are commonly assessed by exposing selected species to high doses of purified insecticidal proteins via artificial diet. In some cases, however, non-target species may also be exposed directly or indirectly (via prey or hosts) to plant material from GE crops. Such studies are conducted if risks cannot be excluded by purified protein studies, if no suitable test systems with artificial diet are available, or if specifically required by legislation (Rose, 2007; EFSA, 2010; Romeis et al., 2011). In addition to regulatory studies commissioned by the applicants, scientific non-target studies with GE plant material as the test substance have been published.

Previous research on non-target effects of *Bt* crops has mainly focused on terrestrial ecosystems with herbivores, natural enemies, pollinators, or decomposers as non-target organisms (Naranjo, 2009; Romeis et al., 2019; Krogh et al., 2020), while studies on aquatic ecosystems are less common (Venter and Bøhn, 2016). Low levels of *Bt* protein from transgenic crops can enter water bodies through post-harvest crop residues, pollen deposition, rhizosphere secretion, and other forms of diffusion (Carstens et al., 2012; Chen et al., 2013; Venter and Bøhn, 2016). *Bt* maize in particular can contribute a substantial input of pollen and residues to streams that drain agricultural fields, especially when shredded plants remain in the field (Rosi-Marshall et al., 2007; Jensen et al., 2010; Tank et al., 2010; Carstens et al., 2012). Although maize detritus can persist and release *Bt* proteins for several months, exposure for aquatic organisms is in the ng/L range and therefore rather low (Shogren et al., 2019; Tank et al., 2010).

The water flea *Daphnia magna* (Diplostraca: Cladocera) is widely used as a surrogate test species in environmental risk assessments for various stressors including *Bt* crops. No effects on *D. magna* were

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reported in studies with purified Cry1C protein (Chen et al., 2018a), maize pollen containing Cry1F or Cry1Ab (Mendelson et al., 2003), rice flour containing Cry1Ab/c (Zhang et al., 2016), maize flour containing Cry1Ab (Zhang et al., 2018), medium from submerged rice straw containing Cry1C (Chen et al., 2018b), and water collected from *Bt* rice paddies containing Cry1Ab/Ac and Cry2A (Li et al., 2014). In contrast, adverse effects on *D. magna* were reported in studies with purified Cry1Ab, Cry2Aa, or a combination of both (Bøhn et al., 2016), purified VIP3A (Raybould and Vlachos, 2011), maize leaves containing Cry1Ab (Holderbaum et al., 2015), and maize flour containing Cry1Ab (Bøhn et al., 2008, 2010). One reason for conflicting results may be a lack of standardized protocols for assessing effects of orally active insecticidal proteins or plant tissue on *D. magna*. Experiments were often not replicated in time, and results have generally not been corroborated by other research groups, which increases the likelihood of reporting artefacts. For example, adverse effects of VIP3A on *D. magna* reported by Raybould and Vlachos (2011) were artefacts, as confirmed by the authors in a subsequent study using the non-toxic bovin serum albumin (Raybould et al., 2014). A major problem of non-target studies with plant material is that they commonly comprised only one *Bt* line and one non-*Bt* control. Even if the non-*Bt* control is the nearest comparator to the *Bt* line, changes in plant composition and physiology introduced by the transformation process and the subsequent regeneration and breeding steps may affect the performance of organisms feeding on the transformed plants compared to the untransformed plants (Ladics et al., 2015; Schnell et al., 2015). In such simple test systems with plant material, it is almost impossible to separate effects of the *Bt* proteins from effects caused by other compounds in the plant. In the current study with *D. magna*, we attempted to disentangle *Bt* protein effects from plant background effects by testing the same transgenic *Bt* trait in two different plant backgrounds (Exp 258 and Exp 262) and by using maize materials with different concentrations of *Bt* proteins (flour, leaves, and pollen). We used SmartStax maize that expresses six insecticidal Cry proteins and two herbicide tolerance genes and that is commercialized in the USA (ISAAA, 2019). SmartStax maize currently represents the GE plant that exposes non-target organisms to the largest amounts of insecticidal Cry proteins. While stacked GE plants producing multiple transgenes are increasingly grown in the field, most previous non-target studies on aquatic organisms were conducted with *Bt* crops producing only one insecticidal protein.

If statistically significant differences between a GE plant and its comparator are observed, it is important to evaluate their biological relevance. For this evaluation, it is necessary to know the range of variation among conventional maize lines. Such data, however, are rarely available. In a recent study with maize flour, leaves, and pollen, we determined the range of variation for five diverse non-*Bt* maize lines on *D. magna* performance (Chen et al., 2021). The results of the current study are discussed in the context of an in-study natural range of variation based on three non-GE maize lines and the natural range of variation reported by Chen et al. (2021).

2. Materials and methods

2.1. Maize materials

Five maize lines were used: 1) EXP 258; 2) SmartStax (event MON89034 × TC1507 × MON88017 × DAS-59122–7, expressing *Bt* genes *cry1A.105*, *cry2Ab2*, and *cry1F* for Lepidoptera-resistance, *cry3Bb1*, *cry34Ab1*, and *cry35Ab1* for Coleoptera-resistance, herbicide tolerance genes *pat* for glufosinate-tolerance and *epsps* for glyphosate-tolerance, genetic background EXP 258); 3) EXP 262; 4) SmartStax+RR (MON87427 × SmartStax, expressing the same transgenes as SmartStax plus one additional tissue specific *epsps* gene at low levels in pollen, genetic background EXP 262); and 5) Rheintaler (Swiss landrace, population maize). All maize lines were planted on 23 April 2019 in a glasshouse and maize materials (flour, leaves, pollen) were prepared and stored according to Chen et al. (2021). In brief, leaves were collected from 7-week-old plants and lyophilized. Pollen was collected in cellulose bags and dried under ambient conditions. Grains were used directly from the batches received from the producer. All maize materials were pulverized in a bead mill and passed through a 75-μm sieve. The sieved powders were suspended in Aachener Daphnien Medium (ADAM), at 3 mg/mL and stored at −20 °C (Ebert et al., 1998). Those food suspensions were used for the feeding experiment.

For non-target studies with *Bt* plant material it is important that *Bt* protein levels in the used tissues and their stability during the experimental period are characterized. Concentrations of Cry1A.105, Cry1F, Cry2Ab2, Cry3Bb1, and Cry34Ab1 were measured in pulverized maize materials, in maize materials suspended in ADAM medium at different time points, and in *D. magna* using commercial enzyme-linked immunosorbent assays (ELISA). For details on protocols and results see Supplemental Material A. ELISAs of maize foods from SmartStax and SmartStax+RR revealed that total Cry protein concentration was eight to ten times higher in leaves than in flour and was intermediate in pollen (Table 1, Supplemental Material A).

2.2. Chronic effects of stacked Bt maize on D. magna

The experiments were conducted with *Daphnia magna* (strain GB-EL75–69) that were originally obtained from Dieter Ebert, Zoological Institute of Evolutionary Biology, University of Basel (Switzerland). The water fleas were cultured in ADAM medium at 20 °C, 70% relative humidity, and a 16 h light / 8 h dark cycle.

Newly hatched *D. magna* (6–24 h old) were kept individually in 100-mL glass beakers containing 50 mL of ADAM medium. On each day, each animal was fed 100 μL maize food suspension (containing ca. 0.15 mg carbon). There were 15 treatment combinations: three maize materials (flour, leaves, pollen) × five maize lines (EXP 258, SmartStax, EXP 262, SmartStax+RR, Rheintaler). Each treatment was represented by 10 replicate beakers (one *D. magna* per beaker) and the experiment was conducted three times; a total of 450 *D. magna* were used.

Every other day, *D. magna* were moved to new beakers with ADAM medium to ensure that the medium quality remained stable. The beakers were stored in a climate chamber at 20 °C, 70% relative humidity, and a 16 h light / 8 h dark cycle. Every day the following parameters were recorded: number of surviving *D. magna*, molts, and released offspring. After day 28 the specimens were checked every second day, but food was provided daily during the whole experimental period. Offspring were removed from the beakers. Body size (length and width) was recorded on days 7, 14, 28, and 42 according to Chen et al. (2021). Under the stereo microscope (Keyence VHX 6000, Mechelen, Belgium) we could clearly see the respective maize tissues in the gut of *D. magna* (Supplemental Material A, Figure A.1), which confirmed the uptake of maize food. The experiment was terminated on day 50 when all surviving individuals were washed with fresh ADAM medium, dried on a paper towel, gently transferred to a 2-mL centrifuge tube, and weighed on an electronic microbalance (MX5, Mettler Toledo, Mettler-Toledo AG, Greifensee, Switzerland). All individuals were then stored at −70 °C until subsequent determination of Cry protein content using ELISA (Supplemental Material A).

Medium quality in the experiment was measured in each of the three repetitions according to OECD211 (OECD, 2012). The requirements specified in the guideline were fulfilled: pH between 6 and 9, dissolved oxygen concentration >3 mg/L, and total hardness > 140 mg/L (Supplemental Material B, Table B.1). See Chen et al. (2021) for details on materials and procedures.

2.3. Data analysis

Statistical analysis were conducted in R, version 4.0.2 (The R Foundation for Statistical Computing, Vienna, Austria). The measured parameters of *D. magna* were analysed separately for flour, leaves, and

Table 1
Cry protein concentrations (μg/g dry weight) in flour, leaves, and pollen from two SmartStax hybrids. Data are presented as median ± 95CI for each hybrid (n = 11 for SmartStax and 5 for SmartStax+RR).

Cry protein	Flour		Leaves		Pollen	
	SmartStax	SmartStax+RR	SmartStax	SmartStax+RR	SmartStax	SmartStax+RR
Cry1A.105	2.5 (2.0; 2.8)	4.5 (2.7; 5.2)	85.5 (61.3; 85.1)	155.8 (87.4; 190.3)	1.3 (1.1; 1.7)	1.0 (0.7; 1.3)
Cry1F	4.9 (4.1; 5.5)	8.7 (7.5; 9.6)	14.2 (12.6; 20.6)	28.1 (18.7; 37.3)	15.0 (13.2; 17.0)	17.0 (9.8; 21.0)
Cry2Ab2	2.5 (2.0; 2.9)	2.7 (2.2; 3.1)	69.9 (64.0; 105.5)	75.4 (52.2; 88.8)	0.3 (0.2; 0.5)	0.3 (0.1; 0.5)
Cry3Bb1	13.2 (12.1; 16.5)	11.1 (8.0; 12.7)	105.7 (76.0; 134.8)	154.0 (100.3; 185.1)	7.4 (6.8; 9.1)	8.4 (5.7; 10.0)
Cry34Ab1	22.2 (20.3; 25.2)	23.2 (21.3; 28.8)	88.9 (79.4; 108.4)	96.9 (71.1; 111.5)	58.3 (45.2; 70.7)	52.5 (41.2; 56.9)
Total	45.3	50.2	364.2	510.2	82.3	79.2

pollen.

Survival probability was analysed by Kaplan-Meier estimates and log-rank tests (survival package). Other parameters were analysed with full factorial linear mixed effects models (LMER) or generalized linear mixed effects models (GLMER) with plant background (EXP 258, EXP 262) and *Bt* (Bt^+, Bt^-) as fixed factors, and experimental repetition as random factor (lme4 package) according to Chen et al. (2021). When interactions between the factors plant background and *Bt* were significant, separate analyses for both factors were conducted.

The in-study range of variation (IRV) was calculated from the three non-*Bt* lines (i.e., EXP 258, EXP 262, Rheintaler) tested in parallel with the two *Bt* lines. A second range, the external range of variation (ERV), was established from the data of five conventional non-GE maize lines (EXP 258, Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx) of a previous study (Chen et al., 2021). For both ranges, the lowest and the highest value of the 95CIs were used for the means of each parameter.

3. Results

3.1. Performance of D. magna on maize foods

After *D. magna* were fed exclusively flour, pulverized leaves, or pollen from two SmartStax *Bt* maize lines ("SmartStax" and "SmartStax+RR"), two non-*Bt* nearest comparator lines ("EXP 258″ and "EXP 262″, respectively), and one unrelated non-*Bt* maize line ("Rheintaler") for 50 days, life table parameters of *D. magna* fed *Bt* lines or their comparators were assessed statistically.

The survival probability of *D. magna* on EXP 258, SmartStax, EXP 262, and SmartStax+RR differed for all maize materials (Kaplan-Meier procedure and log-rank test, flour: $\chi^2 = 23.2$, $p < 0.0001$; leaves: $\chi^2 = 8.3$, $p = 0.04$; pollen: $\chi^2 = 9.3$, $p = 0.03$) (Fig. 1). Survival probability was higher when *D. magna* were fed SmartStax flour rather than SmartStax+RR flour (plant background effect: $\chi^2 = 24.4$, $p < 0.0001$) or EXP 258 flour (*Bt* effect: $\chi^2 = 7.6$, $p = 0.006$); when fed EXP 262 or SmartStax leaves rather than EXP 258 leaves (plant background effect: $\chi^2 = 5.6$, $p = 0.02$; *Bt* effect: $\chi^2 = 5.9$, $p = 0.02$); or when fed SmartStax pollen rather than SmartStax+RR pollen (plant background effect: $\chi^2 = 7.0$, $p = 0.008$) or EXP 258 pollen (*Bt* effect: $\chi^2 = 7.7$, $p = 0.005$). Other comparisons were not significant (Fig. 1).

Mean values, SEs, and the 95% confidence intervals (95CIs) of the parameters presented in the following paragraphs are available in the supplemental online material (Supplemental Material B, Table B.2, Table B.3). In addition, details of the statistical analyses are available for flour (Table B.4), leaves (Table B.5), and pollen (Table B.6).

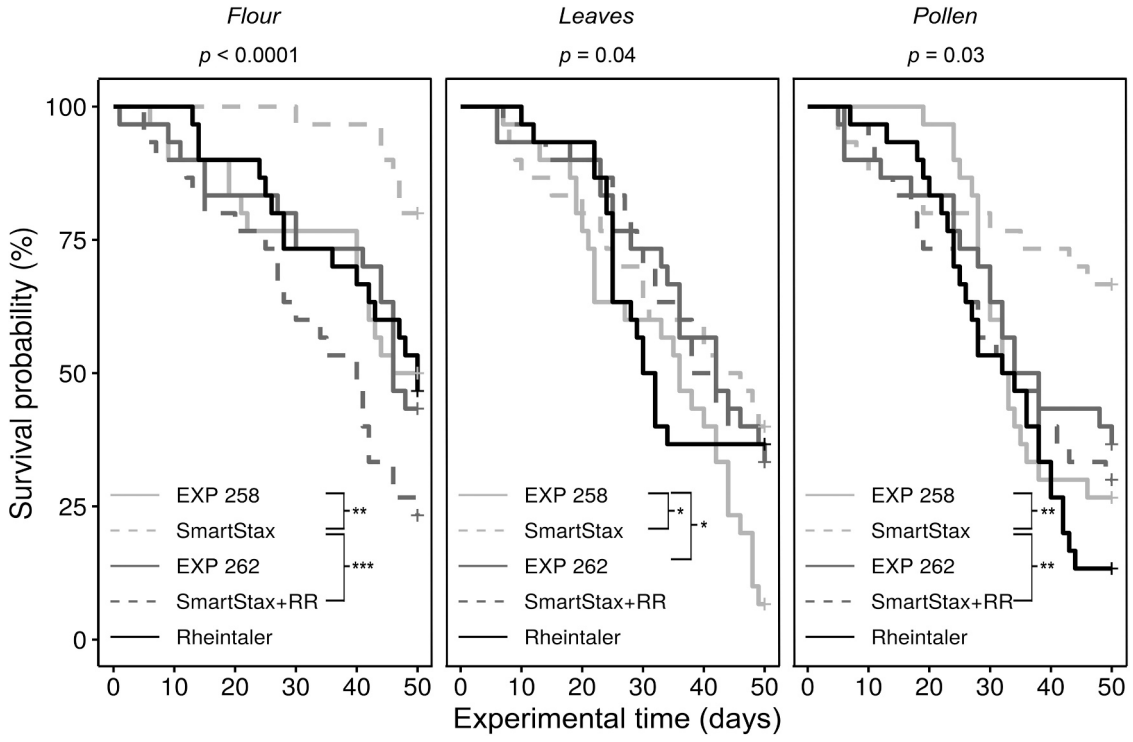

Fig. 1. Survival of *Daphnia magna* fed flour, leaves, or pollen from five maize lines (n = 30). Data from EXP 258, SmartStax, EXP 262, and SmartStax+RR were separately analysed for each food source using Kaplan-Meier estimates and log-rank tests. Asterisks indicate significant differences (* $p \leq 0.05$,** $p \leq 0.01$, *** $p \leq 0.001$). Rheintaler was tested as a conventional check but was not included in the statistical analyses.

The body length and body width of *D. magna* fed maize materials increased significantly over time (Fig. 2). *D. magna* fed non-*Bt* maize flour (EXP 258, EXP 262) had significantly greater body length and width than those fed the corresponding *Bt* lines (SmartStax, SmartStax+RR). For maize leaf treatments, there were no significant differences among maize lines. When fed pollen, *D. magna* body length and width were significantly affected by plant background but not by *Bt*. *D. magna* fed pollen from lines with EXP 258 background (EXP 258 and SmartStax) were smaller than those fed pollen from EXP 262 background (EXP 262 and SmartStax+RR).

The number of molts to first offspring release was not affected by the factors plant background or *Bt* for any of the maize materials (Fig. 3A). For maize flour treatments, the time to first offspring release was significantly affected by *Bt* but not by plant background (Fig. 3B). First offspring were released significantly later with the two *Bt* lines than with the non-*Bt* comparators. For leaf or pollen treatments, time to first offspring release was not affected by *Bt* or plant background. The number of offspring in the first clutch was significantly affected by plant background and *Bt* for flour treatments (Fig. 3C), i.e., individuals produced more offspring in the first clutch if fed EXP 262 rather than EXP 258 flour (plant background effect) or SmartStax+RR flour (*Bt* effect). In addition, *D. magna* fed SmartStax flour had more offspring in the first clutch than those fed SmartStax+RR flour (plant background effect). There were no significant differences in this parameter for leaf or pollen treatments.

The total number of clutches produced by *D. magna* was affected by both plant background and *Bt* (Fig. 3D). *D. magna* fed EXP 262 flour produced more clutches than those fed EXP 258 (plant background effect) or SmartStax+RR (*Bt* effect) flour. For leaf treatments, *D. magna* produced fewer clutches when fed EXP 258 than those fed EXP 262 (plant background effect) or those fed SmartStax (*Bt* effect). *D. magna* fed SmartStax pollen produced more clutches than those fed SmartStax+RR (plant background effect) or EXP 258 (*Bt* effect) pollen.

For flour treatments, the total number of offspring was affected by

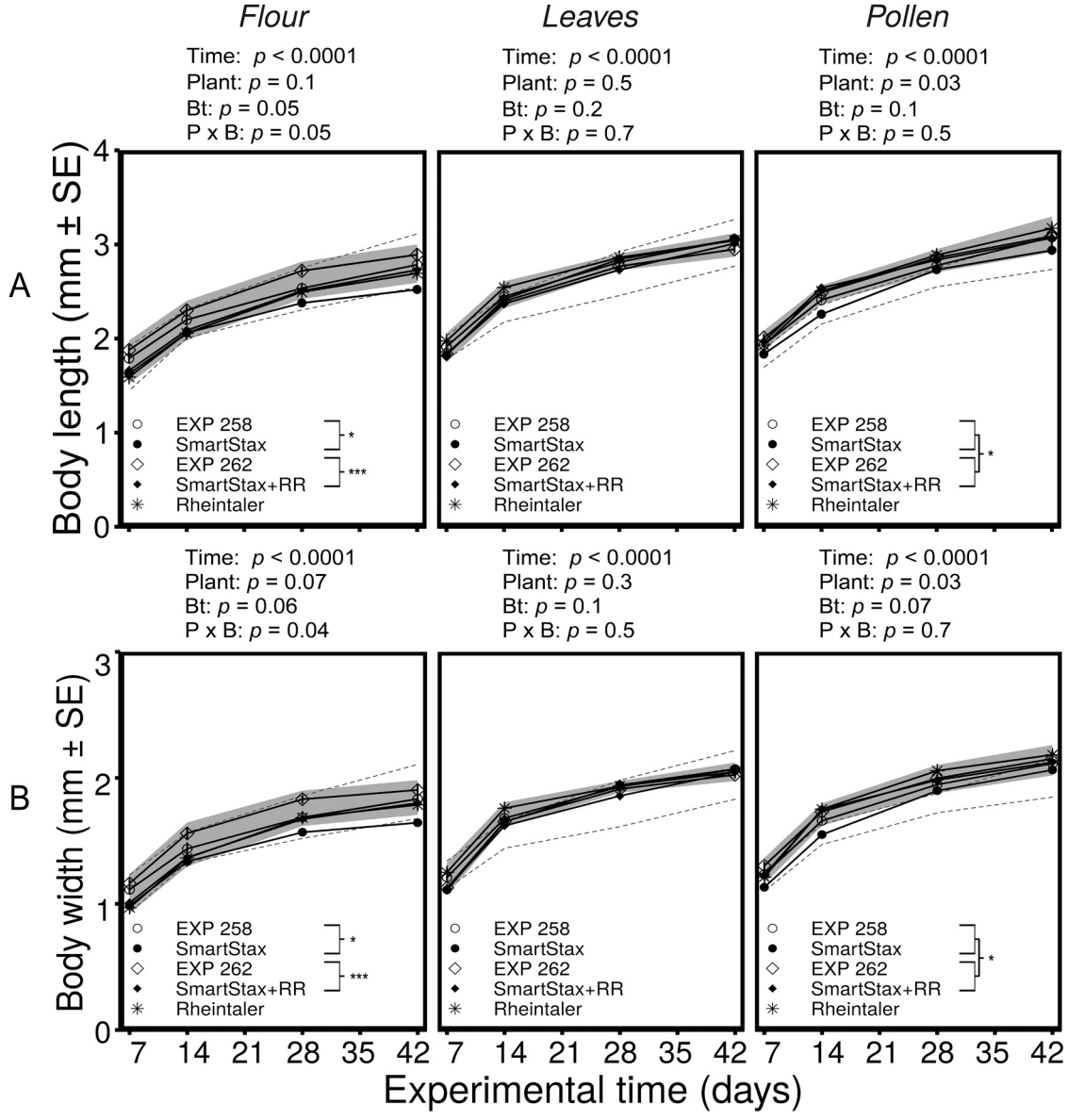

Fig. 2. Length (A) and width (B) of *Daphnia magna* fed flour, leaves, or pollen from five maize lines (n = 6–30). Measurements were taken at day 7, 14, 28, and 42. Data from EXP 258, SmartStax, EXP 262, and SmartStax+RR were analyzed using full factorial linear mixed effects models (LMER) with the fixed factors plant background (EXP 258, EXP 262), *Bt* (Bt^+, Bt^-), and time (days of measurements), and with specimen (individual *D. magna*) as a random factor. Asterisks indicate significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Grey bands and dashed lines indicate the in-study range of variation (IRV) and the external range of variation (ERV), respectively.

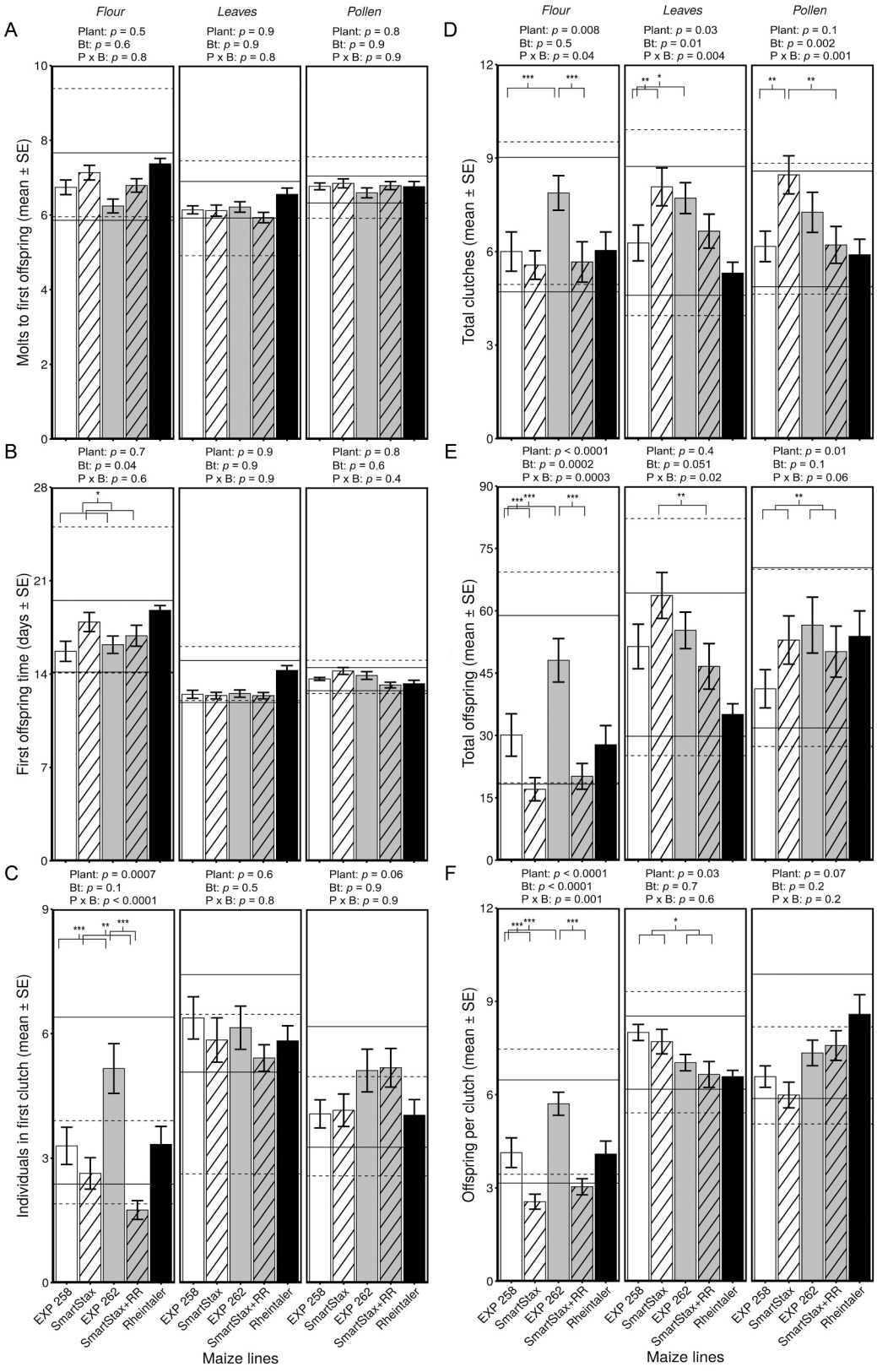

Fig. 3. Number of molts to first offspring release (A), time to first offspring release (B), number of individuals in the first clutch (C), total number of clutches (D), total number of offspring (E), and number of offspring per clutch (F) of *Daphnia magna* fed flour, leaves, or pollen from five maize lines. Data from EXP 258, SmartStax, EXP 262, and SmartStax+RR were analyzed using GLMER with Poisson distribution (A-D) or LMER (E-F) with plant background (EXP 258, EXP 262) and *Bt* (Bt^+, Bt^-) as fixed factors, and experimental repetition as random factor. Asterisks indicate significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). (n = 24–30). Solid lines and dashed lines indicate the in-study range of variation (IRV) and the external range of variation (ERV), respectively.

plant background and *Bt* (Fig. 3E). *D. magna* fed EXP 262 had more total offspring than those fed EXP 258 (plant background effect) or SmartStax+RR (*Bt* effect). In addition, *D. magna* fed EXP 258 had more offspring than those fed SmartStax (*Bt* effect). For leaf treatments, *D. magna* fed SmartStax had more offspring than those fed SmartStax+RR (plant background effect). For pollen treatments, total offspring was affected by plant background; values were lower for the EXP 258 background (EXP 258 and SmartStax) than for the EXP 262 background (EXP 262 and SmartStax+RR).

The number of offspring per clutch in the flour treatments was affected by plant background and *Bt* (Fig. 3F); the number was greater with EXP 262 than with EXP 258 (plant background effect) or SmartStax+RR (*Bt* effect), and was greater with EXP 258 than with SmartStax (*Bt* effect). For leaf treatments, the number of offspring per clutch was affected by plant background but not by *Bt*; the number was higher with EXP 258 background (EXP 258 and SmartStax) than with EXP 262 background (EXP 262 and SmartStax+RR). There were no differences among maize lines when *D. magna* fed pollen.

To assess how the mean values of the various measured parameters compare with the natural range of variation of conventional maize lines, we calculated an in-study range of variation (IRV) and an external range of variation (ERV) based on the 95CIs. Means were generally within both ranges or at least within one of the ranges with the following exceptions: with SmartStax flour, *D. magna* body length and width on day 42, total offspring, and offspring per clutch and with SmartStax+RR flour, the number of offspring in the first clutch and the number of offspring per clutch were below both ranges of variation.

4. Discussion

Consistent with Chen et al. (2021), *D. magna* were able to survive, grow, and reproduce when feeding exclusively on maize flour, leaves, or pollen. No evidence was found for adverse effects caused by the presence of the *Bt* Cry proteins in the two SmartStax maize lines, but *D. magna* life table parameters were affected by unidentified factors in the maize plant background.

4.1. Differences between SmartStax maize lines and their controls

Most of the significant differences in *D. magna* life table parameters were observed between the two *Bt* maize lines and their respective non-*Bt* comparators (SmartStax vs. EXP 258; SmartStax+RR vs. EXP 262) when flour rather than leaf or pollen material was provided. Individuals fed SmartStax flour lived longer than those fed EXP 258 flour, but they were smaller, needed longer for first offspring release, and produced fewer total offspring and fewer offspring per clutch. Similarly, *D. magna* fed SmartStax+RR flour were smaller than those fed EXP 262 flour, required more time for first offspring release, and had fewer offspring in the first clutch, fewer clutches, fewer total offspring, and fewer offspring per clutch. These parameters, however, are not independent from each other. For example, slower growth will lead to delayed reproduction, smaller size, and reduced fecundity.

In contrast to flour, only a few differences between the *Bt* lines and their controls were observed for *D. magna* fed pollen or leaf material. When fed either material from SmartStax, *D. magna* survived longer than on material from EXP 258 and produced more clutches during their lifetime.

ELISA measurements revealed that concentrations of all Cry proteins were eight to ten-times higher in leaf powder than in flour (Table 1). That no adverse effects on *D. magna* were observed in the leaf treatments suggests that the effects observed in the flour treatments were not caused by the Cry proteins in the *Bt* maize materials. This is supported by the finding from the treatments with pollen, which also contained higher total amounts of Cry protein than flour.

The most probable explanation for the observed effects is in the way the different *Bt* and non-*Bt* maize materials were produced. While leaves and pollen were harvested from plants that were grown together in the same glasshouse, flour was produced from the original grains obtained from the breeding company. Those grains were produced in the field likely in different locations and years, and with different management. It is thus possible that differences in cultivation led to differences in the nutritional quality of the flour for *D. magna*. An alternative explanation could be a tissue-specific interaction of the *Bt* proteins with plant factors that lead to toxicity in flour, but not in pollen or leaves. This, however, is unlikely because of the known mode of action of the Cry proteins used in the current *Bt* maize hybrids (NASEM, 2016; Naranjo, 2009; Romeis et al., 2019).

To assess whether observed differences in the performance of *D. magna* between *Bt* and control lines indicate potential harm, it is informative to compare the results with a range of conventional maize lines, because such lines are generally considered safe for the environment (Chen et al., 2021). In our research, we have therefore included an in-study range of variation (IRV) of the three non-transformed maize lines and an external range of variation (ERV) calculated from the study of Chen et al. (2021) that included five conventional maize lines. Both ranges together indicate how variable the respective *D. magna* parameters are among conventional maize lines. A similar approach is applied in compositional equivalence studies that support the food/feed safety assessment of GE plants (Anderson et al., 2019, 2020). In our study, most of the measured *D. magna* parameters were within the IRV and the ERV, except that some *D. magna* parameters were below these ranges for SmartStax and SmartStax+RR flour.

Assessments of laboratory feeding studies should also link experimental exposure levels to realistic exposure levels in the field. The aim of the present study was to create realistic worst-case exposure conditions by the following means: 1) SmartStax contains the most Cry proteins and the highest total concentrations among the currently available GE constructs; 2) leaves were collected from green plants, lyophilized, and mixed in ADAM medium to obtain food suspensions; 3) *D. magna* were fed exclusively with maize materials; and 4) new maize material was provided as food every 24 h. That *D. magna* actually consumed the different maize materials was confirmed by the fact that the materials were clearly visible in the guts of the organism under the microscope (Supplemental Material A, Figure A.1). Despite this fact, no *Bt* protein was detected in the *D. magna* specimens collected at the end of the feeding experiment and in *D. magna* collected after 7 days feeding on different maize materials (Supplemental Material A). Several factors might have led to *Bt* protein concentrations below the limit of detection in *D. magna*. First, measured Cry protein concentrations in the food suspensions were lower than estimated based on the concentrations in lyophilized maize materials and decreased further between feeding events. Second, in addition to degradation processes in the ADAM medium, *Bt* proteins are likely further digested in the *D. magna* gut and finally excreted. And third, the proportion of food material including *Bt* proteins in the gut of *D. magna* compared to the whole body (from which *Bt* proteins were extracted) is small. Compared to the worst-case exposure conditions in our laboratory experiment, exposure of water fleas in the field can be expected to be much lower. Maize debris degrades over time, as shown by Tank et al. (2010), who measured 100–1000 times less Cry1Ab in maize debris collected in and around streams 6 months after harvest compared to fresh maize tissue. Furthermore, the natural food spectrum of *D. magna* likely contains low amounts of maize materials.

Several studies have investigated the effects of *Bt* maize flour on *D. magna*. Zhang et al. (2018) fed *D. magna* for 28 days with flour from seeds containing Cry1Ab. In that study, *D. magna* survival, body size, and reproduction did not significantly differ between the *Bt* and the parental non-*Bt* maize treatments, but the authors did not describe how their maize materials were produced. In contrast, Bøhn et al. (2008, 2010) reported that *D. magna* fed flour from Cry1Ab-containing, field-produced *Bt* maize had reduced longevity, a lower proportion of females reaching sexual maturation, and lower overall egg production than

those fed non-*Bt* maize. In the latter studies however, the relatedness of the *Bt* maize to the non-*Bt* maize was unclear because the two maize lines were produced by different farmers in different fields, and field conditions and management likely differed. This suggests that differences in the plant material and in the way it was produced may have influenced the study results, as observed in the current study. Holderbaum et al. (2015) fed *D. magna* for 42 days with maize leaf powder from Cry1Ab-producing *Bt* maize and its near-isogenic non-*Bt* maize cultivated in growth chambers under comparable conditions; when fed *Bt* maize, *D. magna* were smaller and produced more ephippia and fewer juveniles. This is in contrast to our study, where *D. magna* performance was similar or slightly better when the animals were fed SmartStax or SmartStax+RR leaves. Mendelson et al. (2003) reported no treatment-related acute toxicity when *D. magna* was fed for 48 h with maize pollen containing Cry1Ab or Cry1F, but how the test material was produced was not indicated.

Non-target studies with *Bt* plant material have the problem in that it is difficult to establish an optimal control. The transformation process and the following regeneration and breeding steps are likely to change plant composition and physiology, which may further affect the life table parameters of organisms feeding on the transformed plants, even if the non-*Bt* maize is the nearest available comparator to the *Bt* line (Ladics et al., 2015; Schnell et al., 2015). In all previous studies with *D. magna* and *Bt* maize, only one *Bt* maize hybrid was compared to one non-*Bt* maize line. Indirect, plant-related effects can easily occur in such systems. Furthermore, effects may be particularly pronounced when the water fleas are reared on suboptimal food, such as maize materials (Chen et al., 2021). Therefore, it is possible that the previously published adverse effects on *D. magna* were plant background-related effects in combination with nutritional stress, rather than *Bt* protein effects (Romeis et al., 2013).

In our study with SmartStax, we addressed those shortcomings by using the transgenic traits in two different plant backgrounds and by using three food materials with different *Bt* protein concentrations. Our study did not reveal consistent adverse effects of SmartStax on *D. magna*. This is despite the fact that the total amount of *Bt* proteins was higher in the stacked plants in our study than in the single-toxin plants used in previous studies. This confirms 1) that the spectrum of activity of the Cry proteins used in current *Bt* crops is narrow, and 2) that the combination of multiple *Bt* proteins does not result in unexpected, synergistic effects on non-target species exceeding those of single protein plants, as demonstrated by a recent systematic literature search (Romeis and Meissle, 2020).

4.2. Influence of plant backgrounds

To differentiate between the effects of *Bt* proteins and those of plant backgrounds, we included the SmartStax traits in two plant backgrounds: EXP 258 (plant background for SmartStax) and EXP 262 (plant background for SmartStax+RR). Our results demonstrate several plant background effects. These effects were consistent in some cases, e.g., offspring per clutch in the leaf treatments was higher for EXP 258 and SmartStax than for EXP 262 and SmartStax+RR. In most cases, however, differences were only observed in one plant pair. In addition, some observed plant background effects differed in direction (positive or negative). An example is the offspring in the first clutch, which was higher with flour of EXP 262 than EXP 258 but was lower with flour of SmartStax+RR than SmartStax.

Few non-target studies have included various plant backgrounds that enabled the researchers to separate plant background and *Bt*-related effects. This includes studies on soil nematodes and microbial community structures, isopods, and aquatic Diptera (Clark et al., 2006; Griffiths et al., 2007; Jensen et al., 2010). All three studies revealed that observed effects were caused by differences in the plant backgrounds rather than by the *Bt* proteins.

4.3. Implications for risk assessment

In the environmental risk assessment of GE crops, potential effects on non-target organisms are generally assessed in a tiered way (Garcia-Alonso et al., 2006; Romeis et al., 2008). Early-tier studies are represented by highly controlled feeding assays in the laboratory (Rose, 2007; Romeis et al., 2011). Typically, purified insecticidal proteins are provided to non-target species in an artificial diet. Such studies have the advantage that the test organism can be exposed to high doses of the insecticidal compound and that any effects observed can be directly linked to the insecticidal protein. In certain situations, however, bioassays in which GE plant material is fed to non-target species are warranted. Such assays may be a regulatory requirement (e.g., EFSA, 2010), may have been indicated from early-tier risk assessment, or may be necessary when assays with artificial diet and purified insecticidal protein are not available or practicable (Rose, 2007; Romeis et al., 2011).

The current study was not conducted to support the regulatory risk assessment of SmartStax maize, but as a case study that demonstrates how to address challenges with laboratory feeding studies that use plant material as a test substance. Ideally, the GE and non-GE plant material should be produced together under identical conditions (location, climate, management) to avoid confounding effects as observed in the flour treatments in our experiment. If possible, the test materials should be of high nutritional value for the test species to avoid nutritional stress, which may lead to confounding effects (Chen et al., 2021). The selection of appropriate treatments and controls is important so that obtained results can be interpreted, in particular if suboptimal food is involved.

5. Conclusions

Our study with stacked *Bt* maize in two plant backgrounds and three food materials did not reveal consistent adverse effects on *D. magna*, which indicates the absence of *Bt* protein effects on this species under realistic worst-case exposure conditions.

For future risk assessment studies with GE plant material and non-target species, we recommend to first test the GE plant against its nearest non-GE control. However, feeding assays with plant material always bear the risk that observed effects were caused by the plant background and not by the insecticidal compound of concern. Thus in the case that adverse effects of the GE plant compared to the nearest non-GE control are observed, we recommend to conduct additional experiments to separate plant background effects from effects caused by the insecticidal proteins under assessment by:

1) Including the GE event in several plant backgrounds. If effects between the GE and the comparator lines are inconsistent, plant background effects are likely. An alternative is to include several different GE events with the same trait and their control lines, e.g., *Bt* 11 and MON810, which both produce Cry1Ab.
2) Including multiple food materials from the same plants. Effects of insecticidal proteins should be consistent and should correspond to the concentrations in the different tissues. Some basic dose-response relationships should be evident when the food materials contain different levels of *Bt* proteins, the nutritional value of the different tissues is comparable, and no tissue-specific compounds affect the toxicity of the *Bt* proteins.

Finally, to assess the biological relevance of differences detected between a particular GE plant and a non-GE control, data from multiple unrelated conventional varieties are valuable. Such data allow the definition of a range of natural variation, while assuming that the conventional lines pose no environmental harm. This can be done by discussing historical data and/or by including additional conventional lines in the experiments.

CRediT authorship contribution statement

Yi Chen: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Jörg Romeis:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **Michael Meissle:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112721.

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Supplemental Online Material A: Cry protein quantification: methods, results, and discussion

Addressing the challenges of non-target feeding studies with genetically engineered plant material – SmartStax maize and *Daphnia magna*

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1. Quantification of Cry Proteins

Cry protein content was analysed in the pulverized maize materials, in ADAM medium containing maize materials, and in *D. magna* using enzyme-linked immunosorbent assays (ELISA).

1.1. Maize materials

When leaves and pollen samples were collected in the glasshouse, material from 4 plants was combined into one storage vessel, resulting in 4 vessels for each material and maize line. For ELISA of the *Bt* maize lines, 1-3 technical samples were taken out of each vessel to obtain 11 samples for SmartStax and 5 samples for SmartStax+RR. For *Bt* maize flour, all analyzed samples were taken from the same pool for each maize line. For the feeding experiments with *D. magna*, pooled maize material from all the plants was used.

1.2. Stability of Cry proteins

To study the presence and stability of Cry proteins in ADAM medium, a test was conducted under the same experimental conditions as the chronic *D. magna* experiments. A 3 mL volume of food suspension (3 mg of maize flour, leaves, or pollen per mL) from SmartStax maize were added to 30 mL of ADAM medium. This represents 50 times more than the daily feeding dose to *D. magna* in the chronic feeding experiment. At 6 time points (0, 3, 6, 12, 24, and 48 h), 6 technical samples of 700 µL each were taken from the maize food treatment. All samples were centrifuged at 13,000 × *g* for 5 min at 4 °C. The supernatants (650 µL each) were then collected and frozen in new tubes (referred to as “medium samples”) at -70°C for subsequent determination of Cry protein content by ELISA. The remaining pellet was also frozen (“pellet samples”).

1.3. Daphnia magna

For *D. magna*, analyses were conducted on the individuals that were still alive after 50 days in the chronic feeding experiment ("50-day individuals"). In addition, a separate experiment was conducted to measure the Cry protein content in *D. magna* after a shorter feeding period. A 7-day test was conducted under similar experimental conditions as the chronic experiment. Juvenile *D. magna* (within 7 days of hatching) were randomly assigned to groups of 5 individuals and were kept in 350-mL glass beakers containing 250 mL of ADAM medium. Each group was fed 500 µL of a food suspension (flour, leaves, or pollen) from 4 maize lines (EXP 258, SmartStax, EXP 262, SmartStax+RR) per group per day. Each of the maize lines had three replications. On day 7, all living individuals ("7-day individuals") of each group were washed with fresh ADAM medium, dried, weighed, and stored at −70 °C.

1.4. ELISA measurements

Cry protein (Cry1A.105, Cry1F, Cry2Ab2, Cry3Bb1, and Cry34Ab1) contents in the pulverized maize materials (flour, leaves, pollen), in the medium (medium samples, pellet samples), and in *D. magna* (7-day and 50-day individuals) were measured by ELISA using the corresponding detection kits (PathoScreen *Bt*-Cry1Ab/Ac for Cry1A.105; *Bt*-Cry1F, *Bt*-Cry2A, *Bt*-Cry3Bb1, *Bt*-Cry34Ab1, Agdia Inc., Elkhart, USA). Cry34Ab1 and Cry35Ab1 function as a binary toxin complex and a commercial detection kit is only available for Cry34Ab1. Samples of maize materials, pellet samples, and *D. magna* samples were suspended in 650 µL of PBST extraction buffer along with a 3-mm-diameter tungsten carbide ball. Protein was extracted twice with a Tissue Lyser II (Qiagen, Hombrechtikon, Switzerland) at 30 Hertz for 30 sec. Samples were then centrifuged at 13,000 × *g* for 5 min at 4 °C, and the supernatant (600 µL) was collected. Some of the SmartStax and SmartStax+RR samples required dilution (pollen: Cry1F and Cry3Bb1 20 ×, Cry34Ab1 200 ×; leaves: Cry1A.105 and Cry1F 20 x, Cry3Bb1 100 ×, Cry2Ab2 and Cry34Ab1 200 x; flour: Cry1F 5 x, Cry2Ab2 and Cry3Bb1 20 x, and Cry34Ab1 200 x). Some medium samples also required dilution (pollen: Cry34Ab1 20 ×; leaves: Cry3Bb1 and Cry34Ab1 20). Samples of non-*Bt* maize, samples of *D. magna*, and pellet samples remained undiluted. Purified Cry1A.105, Cry2Ab2, and Cry3Bb1 of certified quality were supplied by Bayer Crop Science (St Louis, USA), and Cry1F and Cry34Ab1 were provided by Corteva Agriscience (Wilmington, USA). Appropriate dilutions of each protein served as standards for the ELISA (7 concentrations loaded twice on each plate). In addition, at least 4 PBST-only blanks were loaded per plate.

All samples, standards, and blanks were loaded on the respective 96-well ELISA plates pre-coated with enzyme conjugate, and the plates were incubated over night at 4°C. On the next day, the plates were washed 7 times with PBST before TMB substrate was added.

Optical density was read after 20 min at 620 nm with a plate reader (infinite® 200, Tecan Group Ltd., Männedorf, Switzerland).

1.5. Data analysis

Standard curves were established based on a single rectangular hyperbola model. The concentrations of each Cry protein in samples were calculated on the basis of the corresponding standard curve. For the ELISA limit of detection (LOD) of each Cry protein, the standard deviation of all blank values from five ELISA plates was calculated. Three times this standard deviation was then considered the LOD, and corresponding LOD concentrations (µg/g) were calculated for each plate and sample using the corresponding standard curve.

Values for the medium (centrifuged ADAM supernatant) and pellet (resuspended and extracted in PBST) were added for the statistical analyses. ELISA data are presented as median concentrations with 95CIs. Differences were considered significant for non-overlapping 95CIs.

2. Results & Discussion

2.1. ELISA of maize materials

The ELISA assay with maize foods from SmartStax and SmartStax+RR revealed that the concentrations of *Bt* proteins were highest in leaf powder, followed by pollen powder and flour. The total concentration was approximately 8 to 10 times higher in leaves than in flour. The concentration in pollen was intermediate (Table 1 in the main manuscript). In leaves, the concentrations were highest for Cry3Bb1 and Cry1A.105, and lowest for Cry1F. In flour and pollen, the concentrations were highest for Cry34Ab1 and lowest for Cry2Ab2 and Cry1A.105. To some extent, Cry protein concentrations also varied among the two *Bt* maize lines. SmartStax+RR flour contained significantly higher concentrations of Cry1F protein than SmartStax flour, and SmartStax+RR leaves contained significantly higher concentrations of Cry1A.105 protein than SmartStax leaves (Table 1). No differences between the two *Bt* maize lines were evident for the other Cry protein/food-source comparisons. No Cry proteins were detected in EXP 258 or EXP 262 maize foods.

In summary, Cry protein concentrations mainly varied among the maize materials with concentrations higher in leaves than in pollen or flour. For leaves and pollen, this confirms previous findings (Svobodová *et al.*, 2017).

2.2. Stability of Cry proteins over time

Concentrations of Cry proteins from SmartStax in ADAM medium after 0, 3, 6, 12, 24, and 48 h generally decreased (Table A.1). Cry protein concentrations were highest in ADAM medium containing maize leaves. In medium with leaves, concentrations were highest for Cry34Ab1 and lowest for Cry1F. In ADAM medium containing flour, concentrations were highest for Cry34Ab1 and lowest for Cry2Ab2, while the concentrations of Cry1A.105 at any time point were below the LOD of the ELISA (0.4 ng/mL). In ADAM medium containing pollen, concentrations were highest for Cry34Ab1 and lowest for Cry3Bb1, while the concentrations of Cry1A.105 and Cry2Ab2 proteins at any time point were below the LOD of the ELISA (0.4 and 0.02 ng/mL for Cry1A.105 and Cry2Ab2, respectively).

Table A.1. Cry protein concentrations in ADAM medium containing SmartStax maize flour, leaves, or pollen at different time points (pooled medium and pellet samples, ng/mL food suspension). Values are medians ± 95CI for each time point (n = 6). Values below the limit of detection (LOD) are presented as < 0.4 for Cry1A.105 and < 0.02 ng/mL for Cry2Ab2.

	Time (h)	Flour	Leaves	Pollen
Cry1A.105	0	< 0.4	3.4 (2.6; 4.7)	< 0.4
	3	< 0.4	3.7 (2.6; 4.6)	< 0.4
	6	< 0.4	3.4 (2.4; 3.8)	< 0.4
	12	< 0.4	3.4 (2.9; 4.0)	< 0.4
	24	< 0.4	3.7 (2.3; 4.8)	< 0.4
	48	< 0.4	2.4 (2.1; 2.8)	< 0.4
Cry1F	0	0.3 (0.3; 0.4)	1.1 (1.0; 1.2)	0.9 (0.5; 1.2)
	3	0.2 (0.2; 0.3)	0.6 (0.6; 0.7)	0.8 (0.7; 0.9)
	6	0.2 (0.2; 0.3)	0.5 (0.2; 0.6)	0.7 (0.5; 0.9)
	12	0.2 (0.1; 0.2)	0.4 (0.3; 0.4)	0.4 (0.2; 0.6)
	24	0.1 (0.1; 0.2)	0.3 (0.2; 0.4)	0.4 (0.4; 0.5)
	48	0.1 (0.1; 0.1)	0.2 (0.2; 0.3)	0.4 (0.3; 0.5)
Cry2Ab2	0	0.1 (0.1; 0.2)	10.5 (9.2; 11.7)	< 0.02
	3	0.1 (0.1; 0.1)	6.5 (5.2; 7.1)	< 0.02
	6	0.1 (0.1; 0.1)	5.5 (4.7; 6.0)	< 0.02
	12	0.1 (0.1; 0.1)	4.4 (3.5; 5.6)	< 0.02
	24	0.04 (0.03; 0.05)	2.2 (1.6; 3.4)	< 0.02
	48	0.03 (0.03; 0.04)	1.4 (1.2; 1.8)	< 0.02
Cry3Bb1	0	0.7 (0.5; 1.0)	15.5 (10.9; 18.5)	0.4 (0.2; 0.6)
	3	0.5 (0.5; 0.6)	10.4 (9.0; 12.8)	0.4 (0.3; 0.5)
	6	0.5 (0.4; 0.5)	8.3 (6.1; 12.7)	0.4 (0.3; 0.5)
	12	0.4 (0.3; 0.4)	7.5 (5.0; 11.9)	0.3 (0.2; 0.4)
	24	0.3 (0.2; 0.3)	7.0 (5.0; 10.7)	0.2 (0.1; 0.5)
	48	0.1 (0.0; 0.3)	6.3 (4.7; 8.1)	0.3 (0.2; 0.5)
Cry34Ab1	0	3.5 (3.1; 4.0)	17.1 (7.0; 27.0)	8.4 (4.7; 13.5)
	3	3.9 (3.4; 4.3)	18.4 (7.1; 26.4)	8.9 (5.0; 12.6)
	6	3.5 (3.0; 3.8)	16.2 (6.8; 20.1)	11.3 (5.3; 16.1)
	12	3.5 (3.1; 4.0)	17.9 (7.3; 22.5)	10.2 (5.1; 15.3)
	24	3.3 (3.1; 3.6)	13.0 (7.4; 17.2)	9.4 (5.0; 13.0)
	48	3.5 (3.1; 4.0)	12.3 (7.9; 15.5)	8.7 (5.3; 10.5)

At time point 0, the content of measured Cry protein in the medium expressed as a percentage of the expected concentration ranged from 14% (Cry2Ab2 in the flour treatment) and to 71% (Cry34Ab1 in the leaf treatment), while Cry1A.105 was not detected in the flour and pollen treatments, and Cry2Ab2 was not detected in the pollen treatment (Table A.1). Cry34Ab1 was the most stable *Bt* protein in all food sources (53–71%). This suggests that the experimental procedure led to a loss of Cry proteins. In this procedure, dry food material

was first suspended in the medium, frozen for storage, and then added to medium in beakers just before the experiment. This procedure was the same as in the feeding experiment with *D. magna*. For the ELISA measurements, a sample of the medium was taken, centrifuged, frozen, and thawed again, and the concentrations in the ADAM supernatant and pellet were measured and the values were combined for analysis.

Table A.2. Expected concentrations (ng/mL), measured concentrations (ng/mL), and measured concentrations expressed as a percentage of expected concentrations of Cry proteins in ADAM medium containing SmartStax maize materials. Expected concentrations were calculated based on the ELISA results with SmartStax maize materials (n = 11); measured concentrations were the values of ELISA results in ADAM medium at time point 0 h (n = 6).

	Cry1A.105	Cry1F	Cry2Ab2	Cry3Bb1	Cry34Ab1
Flour					
Expected (ng/mL)	0.7	1.3	0.7	3.6	6.1
Measured (ng/mL)	<0.4	0.3	0.1	0.7	3.5
Measured / expected	<57.1%	23.10%	14.30%	19.40%	57.40%
Leaves					
Expected (ng/mL)	23.3	3.9	19.1	28.8	24.2
Measured (ng/mL)	3.4	1.1	10.5	15.5	17.1
Measured / expected	14.60%	28.20%	55.00%	53.80%	70.70%
Pollen					
Expected (ng/mL)	0.4	4.1	0.08	2	15.9
Measured (ng/mL)	<0.4	0.9	<0.02	0.4	8.4
Measured / expected	<100.0%	22.00%	<25.0%	20.00%	52.80%

Throughout the 48 h exposure period, the concentrations of most *Bt* proteins decreased (Table A.2). The decrease was highest for Cry2Ab2 protein in medium containing SmartStax leaves and was lowest for Cry34Ab1 in medium containing SmartStax flour. Other studies also reported a rapid degradation of Cry proteins in aquatic ecosystems, such as Cry1Ab protein (Böttger *et al*., 2014; Griffiths *et al*., 2017; Pott *et al*., 2020), Cry1C protein (Chen *et al*., 2018), and Cry3Bb1 protein (Prihoda and Coats, 2009). In our experiment, new food was provided every 24 h to ensure that *D. magna* was exposed to Cry proteins for the whole experimental time, but concentrations were lower than expected and decreased between feeding events.

2.3. ELISA of Daphnia magna

The median concentrations of Cry proteins in *D. magna* fed flour, leaves, or pollen from SmartStax or SmartStax+RR for 7 days or for 50 days were all below the LOD of the ELISA assay. The LODs for each Cry protein were as follows: 0.03–0.10 µg/g for Cry1A.105; 0.007–0.020 µg/g for Cry1F; 0.003–0.007 µg/g for Cry2Ab2; 0.007–0.010 µg/g for Cry3Bb1; and 0.002–0.006 µg/g for Cry34Ab1. However, individual measurements were above the LOD (7-day-individuals, SmartStax, flour, Cry34Ab1: 0.006 µg/g; SmartStax+RR, flour, Cry3Bb1: 0.01 µg/g; 0.01 µg/g; Cry34Ab1: 0.007 µg/g; 0.008 µg/g; pollen, Cry3Bb1: 0.01 µg/g; 50-day-

individuals, SmartStax, flour, Cry34Ab1: 0.006 µg/g, 0.007 µg/g; SmartStax+RR, pollen: Cry1F, 0.01 µg/g).

It is well established that Cry proteins ingested by arthropods are further diluted, digested in the gut, and excreted (Svobodová *et al.*, 2017; Meissle *et al.*, 2021; Meissle and Romeis, 2018; Zhang *et al*., 2017; Zhao *et al*., 2016). The final concentrations in the *D. magna* in our experiment were too low to be detected. Nevertheless, *D. magna* clearly ingested all maize materials as evident from the photographs (Figure A.1).

In summary, our measurements demonstrated that the food ingested by *D. magna* contained Cry protein, but that exposure levels were low as is typical for aquatic environments (Carstens *et al.*, 2012).

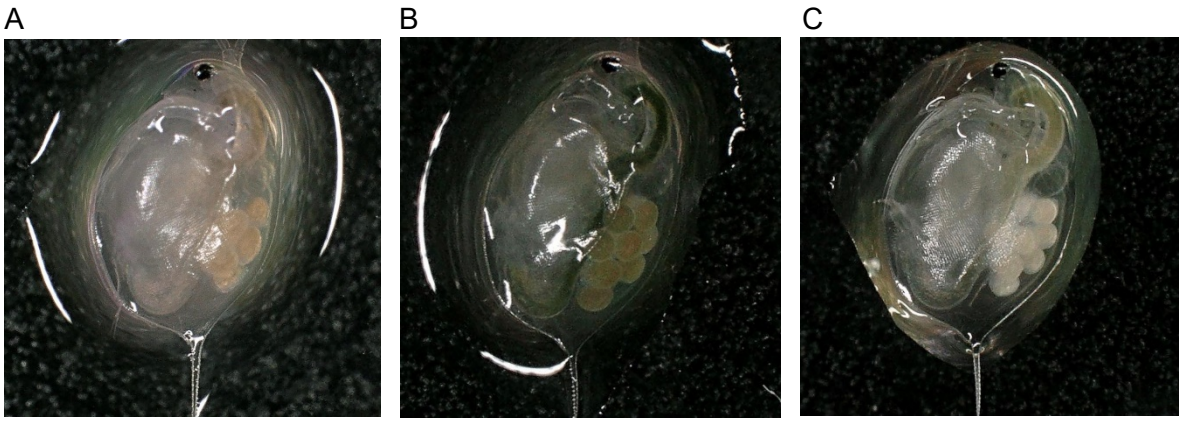

Figure A.1. Photographs of *D. magna* after feeding on SmartStax maize A) flour, B) leaves, or C) pollen. Note the different color of the gut for the different maize materials.

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Supplemental Online Material B: *D. magna* life table parameters and statistical analysis

Addressing the challenges of non-target feeding studies with genetically engineered plant material – SmartStax maize and *Daphnia magna*

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Table B.1. Medium quality parameters: pH value; dissolved oxygen concentration (DOC); hardness of ADAM medium containing maize materials from five maize lines. W0: pure ADAM medium; W1: ADAM medium containing food; W2: W1 after 24 h, containing 1 *Daphnia magna* per beaker; W3: W2 with added food, including *D. magna*; W4: W3 after 24 h; n = 3. Values are means ± SE.

Maize material and line	**pH**				**DOC (mg/L)**				**Hardness (mg/L)**			
	W0: 7.8 ± 0.05				W0: 5.7 ± 0.32				W0: 225 ± 2.9			
Flour	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4
EXP 258	7.9 ± 0.03	7.9 ± 0.03	7.8 ± 0.06	7.9 ± 0.07	5.0 ± 0.29	5.0 ± 0.29	4.9 ± 0.38	5.0 ± 0.27	237 ± 7.3	263 ± 4.4	253 ± 4.4	278 ± 12.0
SmartStax	7.8 ± 0.02	7.9 ± 0.03	7.8 ± 0.04	7.9 ± 0.02	5.0 ± 0.25	4.8 ± 0.49	5.0 ± 0.18	4.7 ± 0.36	237 ± 9.3	253 ± 1.7	260 ± 2.9	277 ± 9.3
EXP 262	7.8 ± 0.05	7.8 ± 0.07	7.9 ± 0.04	7.8 ± 0.02	5.1 ± 0.20	5.2 ± 0.26	4.6 ± 0.27	4.9 ± 0.09	237 ± 3.3	250 ± 7.6	252 ± 3.3	283 ± 8.8
SmartStax+RR	7.8 ± 0.05	7.9 ± 0.00	7.8 ± 0.02	7.9 ± 0.08	4.6 ± 0.45	5.1 ± 0.20	4.6 ± 0.19	5.0 ± 0.14	235 ± 5.8	247 ± 12.0	252 ± 1.7	280 ± 10.4
Rheintaler	7.8 ± 0.04	7.8 ± 0.04	7.8 ± 0.03	7.9 ± 0.03	4.9 ± 0.38	5.0 ± 0.26	4.8 ± 0.18	4.9 ± 0.20	233 ± 4.4	242 ± 6.0	257 ± 4.4	293 ± 4.4
Leaves												
EXP 258	7.9 ± 0.03	7.9 ± 0.06	7.9 ± 0.03	7.9 ± 0.02	4.9 ± 0.08	5.1 ± 0.27	5.0 ± 0.30	4.8 ± 0.12	233 ± 3.3	252 ± 6.0	255 ± 5.8	273 ± 10.1
SmartStax	7.9 ± 0.06	7.8 ± 0.02	7.9 ± 0.02	7.9 ± 0.05	5.0 ± 0.09	5.1 ± 0.23	5.0 ± 0.36	4.7 ± 0.28	230 ± 5.8	245 ± 7.6	250 ± 0.0	270 ± 7.6
EXP 262	7.8 ± 0.05	7.8 ± 0.02	7.9 ± 0.03	7.8 ± 0.02	4.7 ± 0.09	4.9 ± 0.16	4.6 ± 0.29	4.8 ± 0.21	240 ± 2.9	248 ± 3.3	248 ± 3.3	268 ± 3.3
SmartStax+RR	7.8 ± 0.07	7.8 ± 0.02	7.8 ± 0.01	7.9 ± 0.08	5.0 ± 0.17	5.3 ± 0.13	4.6 ± 0.15	4.6 ± 0.09	232 ± 4.4	248 ± 13.6	247 ± 4.4	265 ± 2.9
Rheintaler	7.8 ± 0.01	7.9 ± 0.01	7.8 ± 0.03	7.9 ± 0.05	5.0 ± 0.24	4.8 ± 0.13	4.6 ± 0.04	4.7 ± 0.10	235 ± 2.9	245 ± 5.0	252 ± 7.3	287 ± 1.7
Pollen												
EXP 258	7.8 ± 0.07	7.9 ± 0.08	7.8 ± 0.06	7.9 ± 0.04	5.3 ± 0.25	4.9 ± 0.24	4.9 ± 0.21	5.1 ± 0.06	237 ± 3.4	255 ± 2.9	257 ± 3.3	273 ± 3.3
SmartStax	7.9 ± 0.05	7.8 ± 0.01	7.9 ± 0.01	7.8 ± 0.07	4.6 ± 0.10	5.1 ± 0.21	5.2 ± 0.26	5.0 ± 0.21	240 ± 5.8	255 ± 8.7	253 ± 4.4	282 ± 13.0
EXP 262	7.8 ± 0.05	7.8 ± 0.03	7.9 ± 0.03	7.9 ± 0.06	5.1 ± 0.10	5.0 ± 0.28	4.9 ± 0.15	4.8 ± 0.13	240 ± 5.0	250 ± 5.8	252 ± 1.7	273 ± 6.0
SmartStax+RR	7.8 ± 0.06	7.8 ± 0.03	7.8 ± 0.02	7.9 ± 0.05	5.2 ± 0.13	5.2 ± 0.25	5.0 ± 0.23	4.8 ± 0.19	232 ± 7.3	243 ± 13.3	243 ± 3.3	265 ± 7.6
Rheintaler	7.8 ± 0.06	7.8 ± 0.04	7.9 ± 0.04	7.9 ± 0.03	4.7 ± 0.15	4.7 ± 0.30	4.7 ± 0.06	4.9 ± 0.12	233 ± 8.3	253 ± 4.4	252 ± 4.4	292 ± 6.0

Table B.2. Body length (mm) of *Daphnia magna* fed flour, leaves, or pollen from 5 maize lines. Values are means ± SE. The 95CIs are presented in parenthesis. The lowest and highest boundary values of the non-*Bt* maize lines EXP 258, EXP 262, and Rheintaler (bold) represent the in-study range of variation (IRV). The external range of variation (ERV) was calculated in a similar way for 5 non-*Bt* maize lines from data by Chen *et al.* (2021).

Day	Maize line	N	Body length (mm)	ERV	Body width (mm)	ERV
Flour						
7	EXP 258	29	1.79 ± 0.049 (1.69; 1.89)	(1.44; 1.92)	1.11 ± 0.035 (1.04; 1.18)	(0.93; 1.23)
	SmartStax	30	1.63 ± 0.050 (1.52; 1.73)		0.99 ± 0.035 (0.92; 1.06)	
	EXP 262	29	1.88 ± 0.050 (1.78; **1.98**)		1.16 ± 0.036 (1.09; **1.24**)	
	SmartStax+RR	27	1.66 ± 0.041 (1.58; 1.75)		1.01 ± 0.029 (0.95; 1.07)	
	Rheintaler	30	1.59 ± 0.035 (**1.52**; 1.66)		0.97 ± 0.024 (**0.92**; 1.02)	
14	EXP 258	27	2.20 ± 0.054 (2.09; 2.31)	(2.01; 2.31)	1.44 ± 0.047 (1.34; 1.53)	(1.33; 1.57)
	SmartStax	30	2.06 ± 0.043 (1.97; 2.15)		1.34 ± 0.037 (1.26; 1.41)	
	EXP 262	27	2.30 ± 0.051 (2.19; **2.40**)		1.56 ± 0.043 (1.47; **1.65**)	
	SmartStax+RR	25	2.09 ± 0.036 (2.01; 2.16)		1.37 ± 0.032 (1.30; 1.43)	
	Rheintaler	27	2.06 ± 0.037 (**1.98**; 2.13)		1.36 ± 0.032 (**1.30**; 1.43)	
28	EXP 258	23	2.54 ± 0.051 (2.43; 2.64)	(2.30; 2.75)	1.69 ± 0.032 (1.62; 1.75)	(1.52; 1.86)
	SmartStax	30	2.38 ± 0.032 (2.31; 2.44)		1.57 ± 0.021 (1.53; 1.61)	
	EXP 262	24	2.72 ± 0.047 (2.62; **2.82**)		1.83 ± 0.033 (1.76; **1.90**)	
	SmartStax+RR	19	2.51 ± 0.020 (2.47; 2.55)		1.67 ± 0.015 (1.64; 1.70)	
	Rheintaler	22	2.50 ± 0.035 (**2.42**; 2.57)		1.69 ± 0.032 (**1.62**; 1.75)	
42	EXP 258	18	2.78 ± 0.049 (2.68; 2.89)	(2.55; 3.11)	1.83 ± 0.035 (1.76; 1.91)	(1.68; 2.11)
	SmartStax	29	2.52 ± 0.032 (2.46; 2.59)		1.64 ± 0.023 (1.60; 1.69)	
	EXP 262	21	2.89 ± 0.053 (2.78; **3.00**)		1.90 ± 0.037 (1.83; **1.98**)	
	SmartStax+RR	10	2.73 ± 0.035 (2.65; 2.81)		1.80 ± 0.020 (1.76; 1.85)	
	Rheintaler	19	2.69 ± 0.048 (**2.59**; 2.79)		1.79 ± 0.038 (**1.71**; 1.87)	
Leaves						
7	EXP 258	29	1.91 ± 0.044 (1.82; 2.01)	(1.78; 2.03)	1.21 ± 0.035 (1.14; 1.28)	(1.12; 1.34)
	SmartStax	29	1.82 ± 0.029 (1.76; 1.88)		1.11 ± 0.026 (1.06; 1.16)	
	EXP 262	28	1.84 ± 0.044 (**1.75**; 1.93)		1.13 ± 0.028 (**1.07**; 1.19)	
	SmartStax+RR	29	1.83 ± 0.027 (1.78; 1.89)		1.12 ± 0.020 (1.08; 1.16)	
	Rheintaler	30	1.97 ± 0.041 (1.89; **2.06**)		1.25 ± 0.038 (1.17; **1.33**)	
14	EXP 258	27	2.44 ± 0.033 (2.38; 2.51)	(2.18; 2.44)	1.68 ± 0.025 (1.63; 1.73)	(1.44; 1.66)
	SmartStax	26	2.42 ± 0.015 (2.39; 2.45)		1.65 ± 0.014 (1.62; 1.68)	
	EXP 262	28	2.40 ± 0.036 (**2.32**; 2.47)		1.65 ± 0.021 (**1.61**; 1.70)	
	SmartStax+RR	27	2.37 ± 0.030 (2.31; 2.43)		1.62 ± 0.024 (1.60; 1.67)	
	Rheintaler	28	2.54 ± 0.032 (2.48; **2.61**)		1.76 ± 0.024 (1.71; **1.81**)	
28	EXP 258	18	2.81 ± 0.028 (2.75; 2.87)	(2.46; 2.92)	1.93 ± 0.020 (1.89; 1.97)	(1.61; 1.98)
	SmartStax	21	2.84 ± 0.020 (2.80; 2.89)		1.94 ± 0.016 (1.91; 1.98)	
	EXP 262	22	2.77 ± 0.020 (**2.72**; 2.81)		1.91 ± 0.014 (**1.88**; 1.94)	
	SmartStax+RR	23	2.73 ± 0.037 (2.65; 2.81)		1.86 ± 0.032 (1.79; 1.92)	
	Rheintaler	19	2.86 ± 0.018 (2.82; **2.90**)		1.93 ± 0.020 (1.89; **1.98**)	
42	EXP 258	10	3.05 ± 0.022 (3.00; 3.10)	(2.77; 3.27)	2.07 ± 0.024 (2.01; **2.12**)	(1.83; 2.22)
	SmartStax	15	3.05 ± 0.020 (3.01; 3.09)		2.07 ± 0.016 (2.03; 2.11)	
	EXP 262	14	2.95 ± 0.038 (**2.87**; 3.03)		2.02 ± 0.023 (**1.98**; 2.07)	
	SmartStax+RR	13	3.01 ± 0.041 (2.92; 3.10)		2.05 ± 0.028 (1.99; 2.11)	
	Rheintaler	11	3.04 ± 0.035 (2.96; **3.12**)		2.05 ± 0.017 (2.01; 2.09)	
Pollen						
7	EXP 258	30	1.93 ± 0.026 (**1.88**; 1.99)	(1.69; 1.89)	1.23 ± 0.022 (1.19; 1.28)	(1.10; 1.25)
	SmartStax	28	1.83 ± 0.022 (1.79; 1.88)		1.13 ± 0.013 (1.10; 1.16)	
	EXP 262	27	2.01 ± 0.037 (1.93; **2.08**)		1.30 ± 0.030 (1.24; **1.37**)	
	SmartStax+RR	29	1.97 ± 0.027 (1.91; 2.02)		1.23 ± 0.024 (1.18; 1.28)	
	Rheintaler	29	1.93 ± 0.027 (1.88; 1.99)		1.21 ± 0.027 (**1.16**; 1.27)	
14	EXP 258	30	2.41 ± 0.021 (**2.37**; 2.45)	(2.16; 2.36)	1.66 ± 0.019 (**1.62**; 1.70)	(1.47; 1.63)
	SmartStax	25	2.26 ± 0.033 (2.19; 2.33)		1.55 ± 0.025 (1.50; 1.60)	
	EXP 262	26	2.49 ± 0.025 (2.44; 2.54)		1.73 ± 0.022 (1.69; 1.78)	
	SmartStax+RR	25	2.54 ± 0.035 (2.46; 2.61)		1.75 ± 0.025 (1.70; 1.80)	
	Rheintaler	28	2.50 ± 0.030 (2.44; **2.57**)		1.75 ± 0.023 (1.71; **1.80**)	
28	EXP 258	21	2.78 ± 0.034 (**2.70**; 2.85)	(2.55; 2.75)	1.95 ± 0.032 (**1.88**; 2.01)	(1.72; 1.88)
	SmartStax	24	2.73 ± 0.033 (2.66; 2.80)		1.90 ± 0.025 (1.85; 1.95)	
	EXP 262	21	2.86 ± 0.029 (2.80; 2.92)		2.00 ± 0.029 (1.94; 2.06)	
	SmartStax+RR	17	2.84 ± 0.057 (2.72; 2.96)		1.98 ± 0.041 (1.90; 2.07)	
	Rheintaler	16	2.89 ± 0.030 (2.82; **2.95**)		2.06 ± 0.021 (2.01; **2.10**)	
42	EXP 258	9	3.08 ± 0.070 (**2.92**; 3.24)	(2.73; 3.11)	2.11 ± 0.041 (**2.02**; 2.21)	(1.85; 2.15)
	SmartStax	22	2.94 ± 0.041 (2.85; 3.02)		2.06 ± 0.031 (2.00; 2.13)	
	EXP 262	13	3.09 ± 0.043 (2.99; 3.18)		2.15 ± 0.037 (2.07; 2.23)	
	SmartStax+RR	11	3.07 ± 0.094 (2.86; 3.28)		2.12 ± 0.074 (1.96; 2.29)	
	Rheintaler	6	3.17 ± 0.049 (3.05; **3.30**)		2.18 ± 0.030 (2.11; **2.26**)	

Table B.3. Life table parameters of *Daphnia magna* fed flour, leaves, or pollen from five maize lines. Values are means ± SE. The 95CIs are presented in parenthesis. The lowest and highest boundary values of the non-*Bt* maize lines EXP 258, EXP 262, and Rheintaler (bold) represent the in-study range of variation (IRV). The external range of variation (ERV) was calculated in a similar way for 5 non-*Bt* maize lines from data by Chen *et al.* (2021).

Maize line	Maize material					
	Flour	**N**	**Leaves**	**N**	**Pollen**	**N**
Molts to first offspring (#)						
EXP 258	6.7 ± 0.20 (6.3; 7.1)	27	6.1 ± 0.11 (5.9; 6.4)	29	6.8 ± 0.09 (6.6; 7.0)	30
SmartStax	7.1 ± 0.20 (6.7; 7.5)	30	6.1 ± 0.15 (5.8; 6.4)	26	6.9 ± 0.12 (6.6; **7.1**)	26
EXP 262	6.2 ± 0.19 (**5.9**; 6.6)	25	6.2 ± 0.14 (5.9; 6.5)	28	6.6 ± 0.13 (**6.3**; 6.9)	27
SmartStax+RR	6.8 ± 0.18 (6.4; 7.2)	24	5.9 ± 0.14 (**5.6**; 6.2)	29	6.8 ± 0.11 (6.6; 7.0)	28
Rheintaler	7.4 ± 0.14 (7.1; **7.7**)	27	6.6 ± 0.17 (6.2; **6.9**)	29	6.8 ± 0.14 (6.5; 7.0)	29
ERV	*(6.0; 9.4)*		*(4.9; 7.5)*		*(5.9; 7.6)*	
First offspring time (d)						
EXP 258	15.7 ± 0.76 (**14.1**; 17.3)	27	12.5 ± 0.30 (**11.9**; 13.1)	29	13.6 ± 0.11 (13.4; 13.9)	30
SmartStax	17.9 ± 0.72 (16.4; 19.4)	30	12.4 ± 0.25 (11.9; 12.9)	26	14.2 ± 0.26 (13.7; 14.8)	26
EXP 262	16.2 ± 0.65 (14.9; 17.5)	25	12.5 ± 0.27 (12.0; 13.1)	28	13.9 ± 0.29 (13.3; **14.5**)	27
SmartStax+RR	16.9 ± 0.79 (15.2; 18.5)	24	12.4 ± 0.24 (11.9; 12.9)	29	13.2 ± 0.21 (12.7; 13.6)	28
Rheintaler	18.8 ± 0.36 (18.0; **19.5**)	27	14.3 ± 0.36 (13.5; **15.0**)	29	13.3 ± 0.26 (**12.7**; 13.8)	29
ERV	*(14.1; 25.0)*		*(12.0; 16.1)*		*(12.5; 15.0)*	
Individuals in first clutch (#)						
EXP 258	3.3 ± 0.45 (**2.4**; 4.2)	27	6.4 ± 0.51 (5.3; 7.4)	29	4.1 ± 0.34 (3.4; 4.8)	30
SmartStax	2.6 ± 0.38 (1.9; 3.4)	30	5.8 ± 0.53 (4.7; 6.9)	26	4.2 ± 0.39 (3.3; 5.0)	26
EXP 262	5.2 ± 0.60 (3.9; **6.4**)	25	6.1 ± 0.52 (5.1; **7.2**)	28	5.1 ± 0.52 (4.1; **6.2**)	27
SmartStax+RR	1.8 ± 0.23 (1.3; 2.2)	24	5.4 ± 0.32 (4.8; 6.1)	29	5.2 ± 0.47 (4.2; 6.1)	28
Rheintaler	3.3 ± 0.43 (2.4; 4.2)	27	5.8 ± 0.36 (**5.1**; 6.6)	29	4.0 ± 0.38 (**3.3**; 4.8)	29
ERV	*(1.9; 3.9)*		*(2.6; 6.5)*		*(2.6; 5.0)*	
Total clutches (#)						
EXP 258	6.0 ± 0.63 (**4.7**; 7.3)	27	6.3 ± 0.57 (5.1; 7.5)	29	6.2 ± 0.49 (5.2; 7.2)	30
SmartStax	5.6 ± 0.46 (4.6; 6.5)	30	8.1 ± 0.61 (6.8; 9.3)	26	8.5 ± 0.61 (7.2; 9.7)	26
EXP 262	7.9 ± 0.56 (6.7; **9.0**)	25	7.7 ± 0.50 (6.7; **8.7**)	28	7.3 ± 0.64 (5.9; **8.6**)	27
SmartStax+RR	5.7 ± 0.65 (4.3; 7.0)	24	6.7 ± 0.54 (5.5; 7.8)	29	6.2 ± 0.59 (5.0; 7.4)	28
Rheintaler	6.0 ± 0.59 (4.8; 7.3)	27	5.3 ± 0.35 (**4.6**; 6.0)	29	5.9 ± 0.50 (**4.9**; 6.9)	29
ERV	*(5.0; 9.5)*		*(4.0; 9.9)*		*(4.6; 8.8)*	
Total offspring (#)						
EXP 258	30.1 ± 5.10 (19.6; 40.6)	27	51.4 ± 5.36 (40.4; 62.4)	29	41.2 ± 4.61 (**31.8**; 50.7)	30
SmartStax	17.1 ± 2.78 (11.4; 22.7)	30	63.7 ± 5.52 (52.3; 75.1)	26	53.0 ± 5.81 (41.0; 64.9)	26
EXP 262	48.1 ± 5.23 (37.3; **58.9**)	25	55.3 ± 4.38 (46.3; **64.3**)	28	56.6 ± 6.72 (42.7; **70.4**)	27
SmartStax+RR	20.2 ± 3.10 (13.7; 26.6)	24	46.6 ± 5.47 (35.4; 57.8)	29	50.2 ± 6.14 (37.6; 62.8)	28
Rheintaler	27.8 ± 4.62 (**18.3**; 37.3)	27	35.1 ± 2.56 (**29.8**; 40.3)	29	53.9 ± 6.12 (41.3; 66.4)	29
ERV	*(18.6; 69.3)*		*(25.2; 82.2)*		*(27.3; 70.0)*	
Offspring per clutch (#)						
EXP 258	4.1 ± 0.48 (3.2; 5.1)	27	8.0 ± 0.26 (7.5; **8.5**)	29	6.6 ± 0.35 (**5.9**; 7.3)	30
SmartStax	2.6 ± 0.24 (2.1; 3.1)	30	7.7 ± 0.39 (6.9; 8.5)	26	6.0 ± 0.41 (5.1; 6.8)	26
EXP 262	5.7 ± 0.37 (4.9; **6.5**)	25	7.0 ± 0.26 (6.5; 7.6)	28	7.3 ± 0.41 (6.5; 8.2)	27
SmartStax+RR	3.0 ± 0.26 (2.5; 3.6)	24	6.7 ± 0.42 (5.8; 7.5)	29	7.6 ± 0.48 (6.6; 8.6)	28
Rheintaler	4.1 ± 0.42 (**3.2**; 4.9)	27	6.6 ± 0.20 (**6.2**; 7.0)	29	8.6 ± 0.63 (7.3; **9.9**)	29
ERV	*(3.4; 7.5)*		*(5.4; 9.3)*		*(5.1; 8.2)*	

Table B.4. Statistics of life table parameters of *Daphnia magna* fed flour from four maize lines, i.e., SmartStax and Smartstax+RR and the corresponding non-*Bt* EXP 258 and EXP 262. P × B stands for plant background × *Bt* interaction. For significant interactions in the primary statistical analysis, separate analyses were conducted for these two factors (secondary statistical analyses). For the plant background factor, Bt^-, means the comparison between EXP 258 and EXP 262; Bt^+ means the comparison between SmartStax and SmartStax+RR. For the factor *Bt*, EXP 258 means the comparison between EXP 258 and SmartStax; EXP 262 means the comparison between EXP 262 and SmartStax+RR.

Parameter	**Primary statistical analysis**	**Secondary statistical analysis**	
		Plant background	***Bt***
Body length (mm)	Time: $\chi^2 = 433.6$, $p < 0.0001$	Bt^-: $\chi^2 = 2.8$, $p = 0.1$	EXP 258: $\chi^2 = 4.0$, $p = 0.04$
(LMER)	Plant: $\chi^2 = 2.7$, $p = 0.1$	Bt^+: $\chi^2 = 0.9$, $p = 0.3$	EXP 262: $\chi^2 = 22.8$, $p < 0.0001$
	Bt: $\chi^2 = 27.7$, $p = 0.052$		
	P x B: $\chi^2 = 3.9$, $p = 0.05$		
Body width (mm)	Time: $\chi^2 = 270.4$, $p < 0.0001$	Bt^-: $\chi^2 = 3.1$, $p = 0.08$	EXP 258: $\chi^2 = 4.5$, $p = 0.03$
(LMER)	Plant: $\chi^2 = 3.4$, $p = 0.07$	Bt^+: $\chi^2 = 0.9$, $p = 0.3$	EXP 262: $\chi^2 = 20.8$, $p < 0.0001$
	Bt: $\chi^2 = 3.7$, $p = 0.06$		
	P x B: $\chi^2 = 4.1$, $p = 0.04$		
Molts to first offspring (#)	Plant: $\chi^2 = 0.5$, $p = 0.5$		
(GLMER)	*Bt*: $\chi^2 = 0.3$, $p = 0.6$		
	P x B: $\chi^2 = 0.06$, $p = 0.8$		
First Offspring Time (d)	Plant: $\chi^2 = 0.1$, $p = 0.7$		
(GLMER)	*Bt*: $\chi^2 = 4.1$, $p = 0.04$		
	P x B: $\chi^2 = 0.3$, $p = 0.6$		
Individuals in first clutch (#)	Plant: $\chi^2 = 11.5$, $p = 0.0007$	Bt^-: $\chi^2 = 11.6$, $p = 0.0007$	EXP 258: $\chi^2 = 2.1$, $p = 0.1$
(GLMER)	*Bt*: $\chi^2 = 2.1$, $p = 0.1$	Bt^+: $\chi^2 = 6.6$, $p = 0.01$	EXP 262: $\chi^2 = 43.0$, $p < 0.0001$
	P x B: $\chi^2 = 16.8$, $p < 0.0001$		
Total clutches (#)	Plant: $\chi^2 = 7.1$, $p = 0.008$	Bt^-: $\chi^2 = 7.1$, $p = 0.0008$	EXP 258: $\chi^2 = 0.5$, $p = 0.5$
(GLMER)	*Bt*: $\chi^2 = 0.5$, $p = 0.5$	Bt^+: $\chi^2 = 0.1$, $p = 0.7$	EXP 262: $\chi^2 = 11.5$, $p = 0.0007$
	P x B: $\chi^2 = 4.1$, $p = 0.04$		
Total offspring (#)	Plant: $\chi^2 = 26.2$, $p < 0.0001$	Bt^-: $\chi^2 = 19.2$, $p < 0.0001$	EXP 258: $\chi^2 = 23.8$, $p < 0.0001$
(LMER)	*Bt*: $\chi^2 = 14.1$, $p = 0.0002$	Bt^+: $\chi^2 = 0.2$, $p = 0.7$	EXP 262: $\chi^2 = 57.3$, $p < 0.0001$
	P x B: $\chi^2 = 13.4$, $p = 0.0003$		
Offspring per clutch (#)	Plant: $\chi^2 = 27.0$, $p < 0.0001$	Bt^-: $\chi^2 = 21.2$, $p < 0.0001$	EXP 258: $\chi^2 = 37.1$, $p < 0.0001$
(LMER)	*Bt*: $\chi^2 = 27.7$, $p < 0.0001$	Bt^+: $\chi^2 = 1.9$, $p = 0.2$	EXP 262: $\chi^2 = 91.3$, $p < 0.0001$
	P x B: $\chi^2 = 10.3$, $p = 0.001$		

Table B.5. Statistics of life table parameters of *Daphnia magna* fed leaves from four maize lines, i.e., SmartStax and Smartstax+RR and the corresponding non-*Bt* EXP 258 and EXP 262. P × B stands for plant background × *Bt* interaction. For significant interactions in the primary statistical analysis, separate analyses were conducted for these two factors (secondary statistical analyses). For the plant background factor, Bt^-, means the comparison between EXP 258 and EXP 262; Bt^+ means the comparison between SmartStax and SmartStax+RR. For the factor *Bt*, EXP 258 means the comparison between EXP 258 and SmartStax; EXP 262 means the comparison between EXP 262 and SmartStax+RR.

Parameter	**Primary statistical analysis**	**Secondary statistical analysis**	
		Plant background	***Bt***
Body length (mm)	Time: $\chi^2 = 292.8$, $p < 0.0001$		
(LMER)	Plant: $\chi^2 = 0.5$, $p = 0.5$		
	Bt: $\chi^2 = 1.6$, $p = 0.2$		
	P x B: $\chi^2 = 0.2$, $p = 0.7$		
Body width (mm)	Time: $\chi^2 = 216.4$, $p < 0.0001$		
(LMER)	Plant: $\chi^2 = 0.9$, $p = 0.3$		
	Bt: $\chi^2 = 2.5$, $p = 0.1$		
	P x B: $\chi^2 = 0.4$, $p = 0.5$		
Molts to first offspring (#)	Plant: $\chi^2 = 0.01$, $p = 0.9$		
(GLMER)	*Bt*: $\chi^2 = 0.001$, $p = 0.9$		
	P x B: $\chi^2 = 0.08$, $p = 0.8$		
First Offspring Time (d)	Plant: $\chi^2 = 0.01$, $p = 0.9$		
(GLMER)	*Bt*: $\chi^2 = 0.005$, $p = 0.9$		
	P x B: $\chi^2 = 0.007$, $p = 0.9$		
Individuals in first clutch (#)	Plant: $\chi^2 = 0.2$, $p = 0.6$		
(GLMER)	*Bt*: $\chi^2 = 0.5$, $p = 0.5$		
	P x B: $\chi^2 = 0.06$, $p = 0.8$		
Total clutches (#)	Plant: $\chi^2 = 4.5$, $p = 0.03$	Bt^-: $\chi^2 = 4.3$, $p = 0.04$	EXP 258: $\chi^2 = 6.2$, $p = 0.01$
(GLMER)	*Bt*: $\chi^2 = 6.2$, $p = 0.01$	Bt^+: $\chi^2 = 3.7$, $p = 0.06$	EXP 262: $\chi^2 = 2.4$, $p = 0.1$
	P x B: $\chi^2 = 8.1$, $p = 0.004$		
Total offspring (#)	Plant: $\chi^2 = 0.6$, $p = 0.4$	Bt^-: $\chi^2 = 0.6$, $p = 0.4$	EXP 258: $\chi^2 = 3.2$, $p = 0.08$
(LMER)	*Bt*: $\chi^2 = 3.8$, $p = 0.051$	Bt^+: $\chi^2 = 6.0$, $p = 0.01$	EXP 262: $\chi^2 = 2.4$, $p = 0.1$
	P x B: $\chi^2 = 5.6$, $p = 0.02$		
Offspring per clutch (#)	Plant: $\chi^2 = 4.6$, $p = 0.03$		
(LMER)	*Bt*: $\chi^2 = 0.1$, $p = 0.7$		
	P x B: $\chi^2 = 0.2$, $p = 0.6$		

Table B.6. Statistics of life table parameters of *Daphnia magna* fed pollen from four maize lines, i.e., SmartStax and Smartstax+RR and the corresponding non-*Bt* EXP 258 and EXP 262. P × B stands for plant background × *Bt* interaction. For significant interactions in the primary statistical analysis, separate analyses were conducted for these two factors (secondary statistical analyses). For the plant background factor, Bt^-, means the comparison between EXP 258 and EXP 262; Bt^+ means the comparison between SmartStax and SmartStax+RR. For the factor *Bt*, EXP 258 means the comparison between EXP 258 and SmartStax; EXP 262 means the comparison between EXP 262 and SmartStax+RR.

Parameter	Primary statistical analysis	Secondary statistical analysis	
		Plant background	***Bt***
Body length (mm)	Time: $\chi^2 = 315.0$, $p < 0.0001$		
(LMER)	Plant: $\chi^2 = 5.0$, $p = 0.03$		
	Bt: $\chi^2 = 2.3$, $p = 0.1$		
	P x B: $\chi^2 = 0.5$, $p = 0.5$		
Body width (mm)	Time: $\chi^2 = 240.7$, $p < 0.0001$		
(LMER)	Plant: $\chi^2 = 4.6$, $p = 0.03$		
	Bt: $\chi^2 = 3.3$, $p = 0.07$		
	P x B: $\chi^2 = 0.1$, $p = 0.7$		
Molts to first offspring (#)	Plant: $\chi^2 = 0.06$, $p = 0.8$		
(GLMER)	*Bt*: $\chi^2 = 0.01$, $p = 0.9$		
	P x B: $\chi^2 = 0.081$ $p = 0.9$		
First Offspring Time (d)	Plant: $\chi^2 = 0.07$, $p = 0.8$		
(GLMER)	*Bt*: $\chi^2 = 0.4$, $p = 0.6$		
	P x B: $\chi^2 = 0.9$, $p = 0.4$		
Individuals in first clutch (#)	Plant: $\chi^2 = 3.5$, $p = 0.06$		
(GLMER)	*Bt*: $\chi^2 = 0.02$, $p = 0.9$		
	P x B: $\chi^2 = 0.002$, $p = 0.9$		
Total clutches (#)	Plant: $\chi^2 = 2.7$, $p = 0.1$	Bt^-: $\chi^2 = 2.6$, $p = 0.1$	EXP 258: $\chi^2 = 9.4$, $p = 0.002$
(GLMER)	*Bt*: $\chi^2 = 9.5$, $p = 0.002$	Bt^+: $\chi^2 = 8.2$, $p = 0.004$	EXP 262: $\chi^2 = 2.4$, $p = 0.1$
	P x B: $\chi^2 = 10.2$, $p = 0.001$		
Total offspring (#)	Plant: $\chi^2 = 6.0$, $p = 0.01$		
(LMER)	*Bt*: $\chi^2 = 2.7$, $p = 0.1$		
	P x B: $\chi^2 = 3.4$, $p = 0.06$		
Offspring per clutch (#)	Plant: $\chi^2 = 3.2$, $p = 0.07$		
(LMER)	*Bt*: $\chi^2 = 1.9$, $p = 0.2$		
	P x B: $\chi^2 = 1.7$, $p = 0.2$		