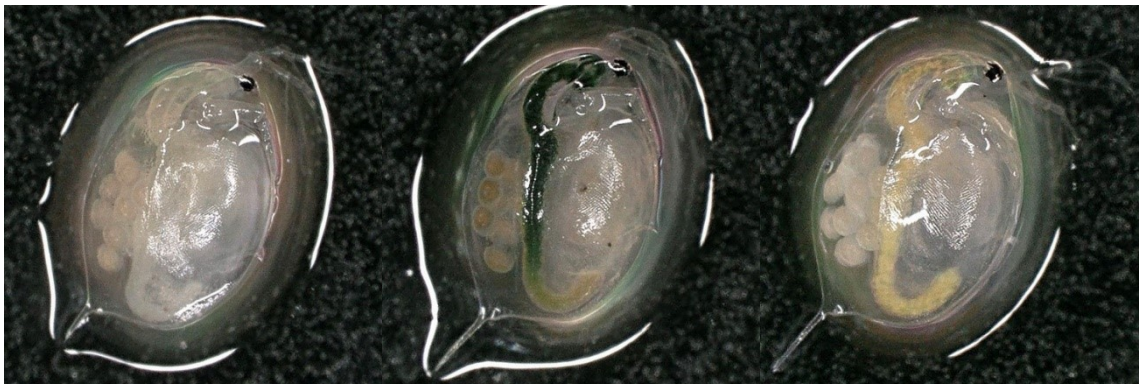
Impact of stacked *Bt* maize on aquatic non-target arthropods



PhD Thesis
Yi Chen
Agroscope

Impact of stacked *Bt* maize on aquatic non-target arthropods

Inaugural dissertation
of the Faculty of Science,
University of Bern

presented by
Yi Chen
from China

Supervisor of the doctoral thesis:

PD Dr. Jörg Romeis
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The Dean
Prof. Dr. Zoltan Balogh

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ABSTRACT

Material from genetically engineered (GE) maize that produces insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*) may enter aquatic ecosystems and affect non-target organisms. In this thesis, two aquatic arthropods, the water flea *Daphnia magna* (Crustacea, filter-feeder) and the midge *Chironomus riparius* (Insecta, collector-gatherer), were selected as surrogates to assess the potential environmental risk of stacked GE maize (SmartStax). This GE maize produces a total of six Cry proteins and thus provides a worst-case exposure condition for non-target organisms.

Previous studies about effects of *Bt* plants on the life table parameters of *D. magna* reported ambiguous results. In the first part of this thesis, the suitability of three different maize materials, i.e., flour, leaf and pollen, from five diverse conventional maize lines, as exclusive food for *D. magna,* was tested. The experiments revealed that maize material is a suboptimal food for *D. magna* causing nutritional stress. By calculating the 95% confidence interval for all measured parameters of *D. magna* performance for each maize line, the natural range of variation was captured, which can be informative for future risk assessment studies.

Flour, leaves, and pollen of SmartStax maize in two different plant backgrounds (SmartStax; SmartStax+RR) were used for the second part of the thesis. 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 when fed flour, but not for leaf or pollen material. Due to the fact that flour was made directly from original grains that had been produced in different locations, years, and with potentially different management, observed effects could be caused by the way of production rather than by the *Bt* trait. An in-study natural range of variation (IRV) and an external range of variation (ERV) based on the first part of this thesis were applied to interpret differences between *Bt* and non-*Bt* comparators in the context of differences among conventional maize lines. Most of the measured *D. magna* parameters in SmartStax hybrids were within the IRV and the ERV. Furthermore, when fed leaves, which contained the highest amounts of Cry protein, no significant adverse effects on *D. magna* compared with their respective non-*Bt* comparators were observed. This indicates that *D. magna* is not sensitive to the six Cry proteins produced by SmartStax maize under realistic worst-case exposure conditions.

Experiments with SmartStax leaves and *C. riparius* were conducted in the third part of this thesis. A significant difference in *C. riparius* performance was only observed for the female development time when fed with the two *Bt* maize lines compared to their

respective non-*Bt* controls. Female *C. riparius* fed with SmartStax or SmartStax+RR maize leaves showed significantly shorter development time than those fed with the two non-*Bt* comparator maize lines, which is not considered an adverse effect. All measured *C. riparius* parameters in the two SmartStax maize lines were within the natural range of variation, which indicates no effects of SmartStax maize leaves on *C. riparius*.

This thesis emphasized the importance of using the natural range of variation for interpretating observed effects between *Bt* and non-*Bt* comparators, and using different plant materials with different plant background to disentangle potential plant background effects from *Bt* effects in the environmental safety assessment for aquatic ecosystems.

CHAPTER I

General Introduction and Thesis Outline

Cultivation of genetically engineered crops

With the rapid development of gene technology, transgenic crops have been created and widely promoted, and their emergence was one of the most important achievements in modern plant breeding. Genetically engineered (GE) crops are created by transferring exogenous genes into crops for recruiting traits that cannot be achieved by conventional breeding (NASEM, 2016). At present, the main species of transgenic crops include soybean, maize, cotton, canola, alfalfa, sugar beet, papaya, squash, potato, eggplant and apple (ISAAA, 2019). In the 24th year of commercialization in 2019, 29 countries grew 190.4 million hectares of GE (ISAAA, 2019). GE crops have contributed to the sustainable development of agriculture, such as increasing crop productivity, conserving biodiversity, reducing the use of pesticides, providing a better environment, mitigating climate change, and helping alleviate poverty and hunger (ISAAA, 2019). Among the GE crops, *Bt* crops produce crystal proteins (Cry proteins) from the bacterium *Bacillus thuringiensis* (*Bt*) to effectively control damaging Lepidoptera or Coleoptera pests. In target pests, the Cry proteins bind to specific receptors in the midgut, perforate the membranes, and eventually cause death (Vachon *et al*., 2012; Jurat-Fuentes and Crickmore, 2017). This affects the feeding behaviour of the insect, thus preventing damage and controlling the target pest (Schnepf *et al.*, 1998; Hannay and Fitz, 1955). Transgenic insect-resistant crops can effectively control pest damage, and also bring benefits to the environment and economy, such as reducing the application of chemical pesticides, reducing environmental pollution, improving crop quality and yield (Brookes and Barfoot, 2018 a,b).

Importance of studying aquatic ecosystems

Potential drawbacks of *Bt* crops include the evolution of resistance in target pests, adverse effects on non-target organisms, and potential outcrossing of the *Bt* trait to related plant species, and the evoliution of weeds that are more difficult to control (Li *et al.*, 2016). The research on non-target effects of *Bt* crops mainly focused on plant-feeding

insects, natural enemies, pollinating insects, economically important insects and soil microorganisms in terrestrial ecosystems (Yang *et al.*, 2015; Yuan *et al.*, 2013; Qi *et al.*, 2015; Wang *et al.*, 2016; Yang *et al.*, 2014; Li *et al.*, 2015; Li and Romeis, 2010; Duan *et al.*, 2008; Yao *et al.*, 2008; Vaufleurya *et al.*, 2007). Studies on species in aquatic ecosystems are scarce because the amount of *Bt* protein that is released into the stream draining agricultural fileds has long been believed to be negligible.

The concentration of soluble Cry1Ab protein in the water of a river downstream of transgenic maize fields was reported to be up to 21 ng/L (Tank *et al.*, 2010). Douville *et al.* (2007, 2009) detected the presence of the *cry1Ab* gene in surface water, sediment and freshwater mussel of an adjacent transgenic *Bt* maize field using real-time fluorescent quantitative PCR (RT-PCR). Bai *et al*. (2004) showed that even after 100 days, plant-derived *Bt* protein can be detected in water. These studies indicate that the *Bt* protein released from remnants of *Bt* plant tissue remain in water for quite some time.

The *Bt* protein from transgenic crops can get into water through the pollen, rhizosphere secretion, post-harvest crop residues and other forms of diffusion, so that organisms in aquatic ecosystems are principally exposed to *Bt* protein (Carstens *et al.*, 2012; Chen *et al.*, 2013). The *Bt* protein can potentially aquatic organisms when they are susceptible to the protein at the encountered concentrations. Therefore, when evaluating the environmental safety of transgenic crops, one needs to consider whether it will pose a risk to aquatic ecosystems.

Non-target studies with aquatic species and insecticidal *Bt* crops

Despite the fact that aquatic ecosystems are less studied in the context of *Bt* crops than terrestrial ecosystems, several studies have been conducted to evaluate potential impacts of *Bt* crops on aquatic arthropods belonging to different arthropod classes and orders (Venter and Bøhn, 2016; Devos *et al*., 2012; De Schrijver *et al.*, 2016; Pott *et al*., 2018). Key studies are briefly summarized below.

Crustaceans

Cladocera

Most of the studies on the impact of *Bt* crops on non-target aquatic organisms were conducted with the water flea *Daphnia magna* (Cladocera: Pulicidae), but the reported results are often ambiguous.

Purified Cry1C did not cause detrimental effects, while biological activity and ingestion

of the Cry protein were confirmed (Chen *et al*., 2018a). In contrast, when *D. magna* was exposed to high concentrations of purified Cry1Ab, Cry2Aa, or a combination of both, negative effects on mortality, body size and reproduction were observed in an assay covering the whole life of the water flea (Bøhn *et al*., 2016). Similarly, purified VIP3A, a vegetative insecticidal protein from *Bt*, reduced the body size, but no effects on mortality and fecundity were observed in an assay lasting for 10 days at a high exposure concentration of 752.6 µg/L (Raybould and Vlachos, 2011). A follow-up study, however, showed that this result was a laboratory artefact as similar adverse effects on body size were observed when a comparable concentration of a non-toxic protein, i.e. bovine serum albumin, was used (Raybould *et al.*, 2014).

Regarding the impact of *Bt* maize on *D. magna*, no effects were observed when Cry1F or Cry1Ab containing maize pollen were fed for 48 hours to *D. magna* (Mendelsohn *et al*., 2003). However, *D. magna* may be exposed to *Bt* proteins for several days/weeks, thus the exposure duration in this study is rather short and the results are thus of little relevance. Pulverized Cry1Ab-containing leaf material (from the climate chamber) resulted in negative effects on growth and fecundity (Holderbaum *et al*., 2015). This study, however, was conducted under a 24 h photoperiod which may have significantly affect the growth and fecundity of *D. magna*. Flour from Cry1Ab-containing, field-produced *Bt* maize also caused negative effects on survival and reproduction compared to non-*Bt* maize material (Bøhn *et al*., 2008, 2010). However, the relationship between the *Bt* and non-*Bt* maize in this study remained unclear, and it is likely that the results were due to the variances of plant materials or different cultivation methods under which they were grown. Zhang *et al*. (2018) fed maize flour containing *cry1Ab* and *epsps* gene to *D. magna* and found no significant difference compared with non-GE flour in survival, body length and reproduction, but the authors did not describe how their maize materials were produced.

For the impact of *Bt* rice on *D. magna*, Zhang *et al*. (2016) fed rice flour containing Cry1Ab/c to *D. magna* and reported comparable survival, body mass and reproduction compared to non-*Bt* rice flour. But the authours didn’t go into details of how the seeds and the rice flour was produced. Own research (Chen *et al*., 2018b) revealed that Cry1C-containing rice straw submerged in the *D. magna* medium did not affect survival, growth and reproduction of the water flea as compared to non-*Bt* rice straw. Similarly, an experiment with water collected from *Bt* and conventional rice paddies managed according to agricultural practice showed that the *Bt* rice was safer to *D. magna* than the conventionally managed rice (Li *et al*., 2014). In this study, however, non-*Bt* rice fileds were sprayed 5 times with insecticides to control non-lepidopteran pests, while *Bt* rice fileds were sprayed only 2 times. As a consequence, the water collected from the *Bt* rice

fields contained higher pesticide residues than water from non-*Bt* rice fields which could have affected the results.

In a study using *Bt* rice expressing Cry1Ab/1Ac straw as food source for *Daphnia hyalina* (Cladocera: Pulicidae), the density of *D. hyalina* did not differ between *Bt* rice treatments and non-*Bt* rice treatments. This laboratory experiment found that purified *Bt* toxins Cry1Ab and Cry1Ac had no toxic effect on *D. hyalina* even in the treatment in which the *Bt* toxin concentration was as high as 2500 ng/ml (Wang *et al*., 2013).

Amphipoda

Acute test (10 d duration) with the amphipod *Hyalella azteca* (Amphipoda: Hyalellidae) revealed some toxic effects of cotton seed extract when large amounts were added to the sediment (Li *et al*., 2013). Unfortunately, the study lack a propper control (extract from non-*Bt* cotton seeds) and thus the effects observed cannot be linked to the Cry1Ac protein. Chambers *et al*. (2010) ran *H. azteca* trials for 7-10 days and no effects were observed when fed with *Bt* (Cry1Ab) maize leaves compared to the non-*Bt* control. However, the physical and chemical characteristics of the streams where the *Bt* and non-*Bt* maize material was collected were different, which may influence the results. Whiting and Lydy (2015) conducted a site-specific ecological risk assessment to examine the simultaneous use of Cry1Ab maize with the insecticides clothianidin and tefluthrin. They conducted an acute toxicity bioassay in which *H. azteca* was exposed to single insecticides as well as the mixture of the three. GE maize insecticidal proteins and clothianidin were not found exceeding benchmark values for ecological effects at environmental concentrations (Whiting and Lydy, 2015).

Decapoda

In the case of the crayfish *Orconectes rusticus* (Decapoda: Cambaridae), Linn and Moore (2014) found that after exposured to Cry1Ab maize for 8 weeks, survivorship was 31% lower in the *Bt* treatment compared with the near-isogenic treatments. Interestingly, growth was not affected which would have been expected if the Cry protein had a toxic effect on the crayfish. Furthermore, when the maize material was offered together with American sycamore (*Platanus occidentalis*), no *Bt* effect was observed. West and Moore (2019) reported that juvenile *Faxonius rusticus* (Decapoda: Cambaridae) fed with *Bt* maize varieties (SmartStax: expressed 6 Cry proteins plus 2 herbicide proteins; VT Triple Pro: expressed 3 Cry proteins plus 1 herbicide protein) exhibited a significantly lower growth rate than those fed with the corresponding non-*Bt* varieties. The authors concluded that certain *Bt* varieties may lead to negative effects on the growth and survivorship of

juvenile crayfish.

Isopoda

Jensen *et al*. (2010) found that the isopod *Caecidotea communis* (Isopoda: Asellidae) exposed to Cry1Ab maize leaves was shorter and lighter, and suffered a higher mortality compared to those fed leaves from the non-*Bt* near-isoline. However, no such effects were evident for stacked maize containing Cry1Ab+Cry3Bb1. Thus, the adverse effects observed for Cry1Ab maize were unlikely caused by the Cry protein.

Insects

Diptera

Reduced survival of *Chironomus dilutes* (Diptera: Chironomidae), a benthic detritus feeding midge, was observed when food slurry was spiked with Cry3Bb1 containing *Bt* maize root extract at nominal concentrations of 30 and 48 ng/mL compared to 17 ng/mL and a water-only control, while growth was not affected (Prihoda and Coats, 2008). However, it remains unclear whether the observed adverse effects were caused by the Cry3Bb1 protein or other compounds from the maize root extract since the study did not contain a control based on root extract from non-*Bt* maize. In an acute study for *C. dilutes* spiked with Cry1Ac-containing *Bt* cotton seed extract, the median lethal concentration (LC_{50}) was 155 ng/g dry weight for sediment and 201 ng/mL for water only (Li *et al*., 2013). But again, this study did not contain a proper non-*Bt* control and it can thus not be ruled out that the observed effects were artefacts caused by other unknown compounds in the extraction.

Larvae of the fly *Tipula abdominalis* (Diptera: Tipulidae) were fed with three maize lines (non-*Bt* maize, Cry1Ab maize, stacked Cry1Ab+Cry3Bb1 maize) for 30 days. *T. abdominalis* fed with Cry1Ab maize grew slower compared to the non-*Bt* near isoline, but not when fed Cry1Ab+Cry3Bb1 stacked maize (Jensen *et al*., 2010). Thus, the adverse effects observed for Cry1Ab maize were unlikely caused by the Cry protein.

Trichoptera

Larvae of the caddisfly *Lepidostoma liba* (Trichoptera: Lepidostomatidae) were fed with Cry1Ab-containing *Bt* maize leaves for 29 days. Larvae had a slower growth compared to leaves of an unrelated conventional variety that was selected based on similar lignin content and C/N ratio (Rosi-Marshall *et al.*, 2007; Chambers *et al*., 2010). In both studies, the used *Bt* and non-*Bt* maize varieties were clearly unrelated. Moreover, the

physical and chemical characteristics of the streams where the *Bt* maize and non-*Bt* maize collected were different, which may have bias the results. In contrast, Jensen *et al*. (2010) observed no difference for the parameters of head capsule growth and dry mass between *Bt* maize producing Cry1Ab or Cry1Ab+Cry3Bb1 and a near isoline on growth parameters of *Lepidostoma* spp (Trichoptera: Lepidostomatidae).

Another caddisfly, *Pycnopsyche scabripennis* (Trichoptera: Limnephilidae), showed higher final dry mass when fed with stacked *Bt* maize compared to either non-*Bt* maize or Cry1Ab producing maize while no differences in survival were observed (Jensen *et al*., 2010).

The filter feeding caddisfly *Helicopsyche borealis* (Trichoptera: Helicopsychidae) showed similar survival when fed with a mean realistic dose of *Bt* and non-*Bt* maize pollen for 18 days, but a higher mortality was observed in the *Bt* treatment when pollen was applied at the concentration two or three times exceeding the highest concentration observed in the field (Rosi-Marshall *et al*., 2007). But as stated above, the *Bt* and non-*Bt* maize varieties used were not related.

Study system

Cultivation of GE crops often involves plants with multiple transgenes providing similar or different traits stacked into one plant. One product that has become commercially available (in the USA) is SmartStax maize expressing 2 herbicide tolerance genes and 6 insecticidal *cry* genes from *Bt* (Head *et al*., 2013), which thus represents the worst-case exposure of non-target arthropods to transgene products in a single GE plant variety. It allows the testing of several transgenes (and their products) as well as their interactions simultaneously. Stacking of multiple insecticidal genes increases the total load of foreign protein released into the agro-environment. Despite the narrow spectrum of activity of each of the single genes, mainly restricted to Lepidoptera or Coleoptera, concerns have been raised that the combination of multiple Cry proteins may result in synergistic effects that may lead to unexpected and unintended adverse effects on non-target organisms (Hilbeck and Otto, 2015). In regard to aquatic ecosystems, GE maize may contribute to a high input of crop detritus because it has the highest biomass among the available annual crops (Rosi-Marshall *et al.*, 2007; Jensen *et al.*, 2010; Tank *et al.*, 2010), especially when only cobs are harvested and the residues are shredded and left on the field. In addition, maize is open pollinated and releases high amounts of pollen during anthesis, which contains Cry proteins and is likely to enter small streams. Moreover, the presence of Cry-proteins in streams may be also from the exudates from roots and

decaying biomass (Rosi-Marshall *et al.*, 2007; Carstens *et al.*, 2012).

The review above summarizes the key non-target studies with aquatic species and insecticidal *Bt* crops and revealed that some studies apparently reported laboratory artefacts due to bad study design. This included in particular the lacked of proper non-*Bt* controls in a number of studies that makes it likely that plant background effects are mistaken for toxic effects of *Bt* Cry proteins. Good study design is thus of major importance when conducting non-target studies to support the environmental risk assessment of GE crops. This thesis used two aquatic arthropods, the water flea *D. magna* and the midge *C. riparius* as surrogates to demonstrate how non-target studies can be conducted to unambiguously assess non-target effects of the plant produced Cry proteins.

D. magna (Crustacea: Cladocera) is a filter feeder, which is relatively easy to obtain and culture. There are several standardized testing protocols for *D. magna* including short term tests (24 or 48 hours) for the acute toxicity of chemicals, water samples, and sediments (ISO, 2012; ASTM, 2005; OECD, 2004) and long term tests for more subtle, chronic toxicity including effects on reproduction (ASTM, 2005; OECD, 2012). Such protocols are not designed for orally active substances (Cry proteins), so they cannot be used directly for assessing the potential impacts of *Bt* plant materials on *D. magna*. Consequently, it is necessary to do research for adapting these protocols. Previously published studies reported conflicting results that may have been caused by problems in study design. Thus, developing reliable protocols for testing GE plant material on *D. magna* that allow to minimize laboratory artefacts is of importance.

Chironomid larvae are widely distributed in water bodies and are very typical benthic invertebrates; their biomass can reach about 70% - 80% of the total benthic biomass (Ferrington, 2008). Due to the wide variety and the sensitivity to changes in water quality, *Chironomus* spp. have been an important indicator of water quality. There are several standardized testing protocols: ASTM (ASTM, 2005) (*Chironomus riparius, Chironomus dilutus*), EPA (EPA, 2000) (*Chironomus dilutus*) and OECD (OECD, 2010) (*Chironomus riparius, Chironomus dilutus, Chironomus yoshimatsui*) that have determined Chironomid larvae as recommended species for detecting the toxicity of sediments or chemicals in ecotoxicology. In recent years, they have been widely used as an indicator to evaluate the toxicity of pollutants in water.

Thesis outline

For this PhD project, two aquatic non-target arthropods, *Daphnia magna* and *Chironomus riparius*, were selected as surrogates to assess the potential non-target effects of stacked *Bt* maize (SmartStax) expressing six Cry proteins.

In Chapter II an experiment is presented that tests the suitability of three different maize materials, i.e., flour, leaves and pollen, from five diverse non-GE maize lines as exclusive food for *D. magna*. The recorded parameters included survival, sublethal endpoints, and population measures. 95% confidence intervals were used for the means of the five maize lines for all measured parameters of *D. magna* performance in the study to capture the natural range of variation. This information is useful for the interpretation of observed differences in *D. magna* performance between a GE plant and its non-GE comparator as it helps judging whether observed effects are likely to be of biological relevance.

For Chapter III a study was conducted with stacked transgenic SmartStax maize in two plant backgrounds as exlusive food for *D. magna*. Aim of the study was to demonstrate how Cry protein effects can be separated from plant background effects in non-target studies of *Bt* plant material as the test substance and how effects that are detected can be judged for their biological relevance. This research contributes to the debate whether transgenic *Bt* maize will affect *D. magna*, and to some extent it promotes sound methods for research on the safety of aquatic environments and safety for aquatic organisms.

In Chapter IV a test is presented to investigate effects of SmartStax maize leaves producing six different Cry proteins in two plant backgrounds on life table parameters of the non-biting midge *C. riparius*. 95% confidence intervals for the means of the six in-study conventional maize lines for all measured parameters of *C. riparius* performance were used to capture the natural range of variation, which is useful for judging if observed effects between GE and non-GE maize are biologically relevant.

In Chapter V the experimental findings from this thesis are discussed and general conclusions are drawn. Implications for the risk assessment of stacked *Bt* maize on aquatic non-target arthropods are also discussed.

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CHAPTER II

Performance of *Daphnia magna* on flour, leaves, and pollen from different maize lines: Implications for risk assessment of genetically engineered crops

Abstract: Non-target effects of genetically engineered (GE) plants on aquatic *Daphnia magna* have been studied by feeding the species with different maize materials containing insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*). The results of those studies were often difficult to interpret, because only one GE plant was compared to one related non-GE control. In such a setting, effects of the Cry proteins cannot be distinguished from plant background effects, in particular when the test species is nutritionally stressed. In the present study, we tested the suitability of three different maize materials, i.e., flour, leaves and pollen, from five diverse non-GE maize lines (including EXP 258, a breeding line that is closely related to a SmartStax *Bt* maize) as exclusive food sources for *D. magna*. The parameters recorded included survival, sublethal endpoints such as body size, number of moltings to first offspring, time to first offspring, number of individuals in first clutch, total number of clutches, total number of offspring, average number of offspring per clutch, and population measures such as net reproductive rate R_0, generation time T and intrinsic rate of increase r_m. The results showed that *D. magna* can survive, grow and reproduce when fed only with maize materials, although the performance was poorer than when fed with algae, which indicates nutritional stress. Large differences in life table and population parameters of *D. magna* were observed among the different maize lines. Our results suggest that comfounding effects caused by nutritional stress and plant background might explain some of the conflicting results previously published on the effects of *Bt* crops on *D. magna*. Using 95% confidence intervals for the means of the five maize lines for all measured parameters of *D. magna* performance in our study, we captured the natural range of variation. This information is useful for the interpretation of observed differences in *D. magna* performance between a GE plant and its non-GE comparator as it helps judging whether observed effects are of biological relevance. If differences between a GE and comparator line are observed and their biological relevance needs to be assessed in future risk assessments of GE maize, 1) the data on natural variation of the different parameters generated by previous studies

can be informative (e.g. data from our study for maize fed *D. magna*); 2) for additional experiments the inclusion of multiple unrelated non-GE comparators should be considered. In addition, it should be taken into account that nutritional stress can affect the outcome of the study.

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1. Introduction

Aquatic and terrestrial environments are interlinked and influenced by human activity, such as agriculture, mining, landfills, industrial and urban wastewater, as well as natural geogenic releases (Schwarzenbach *et al*., 2010). Pollutants include heavy metals, hormonally active substances, micro plastic, and chemicals. Agriculture, which releases several million tons of fertilizers and pesticides each year, is an important source of pollutants (Bockstaller *et al.*, 2009). With the rapid development of gene technology, genetically engineered (GE) crops are grown on steadily increasing areas worldwide (ISAAA, 2018). GE crops can reduce the need for pesticides (Brookes and Barfoot, 2018). On the other hand, the currently grown insect-resistant GE crops produce high amounts of insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*) that can also enter terrestrial and aquatic ecosystems and pose a risk to non-target organisms (Carstens *et al*., 2012; Tank *et al.,* 2010; Viktorov, 2011). The Cry proteins that are produced by *Bt* crops have an oral mode of action. After ingestion and activation in the gut, they bind to specific gut receptors of sensitive insects, where they lead to pore formation, unbalanced ion fluxes and ultimately death (Bravo *et al*., 2012; Jurat-Fuentes and Crickmore, 2017).

Zooplankton is an essential part of the aquatic food chain. It is the main consumer of bacteria, small algae, and organic detritus, and at the same time, a major food source for higher trophic levels. Changes in abundance, diversity, and distribution of zooplankton may thus have cascading effects throughout a water ecosystem (Gannon and Stemberger, 1978). Moreover, zooplankton is very sensitive to many contaminants and thus used as an indicator to monitor changes in water quality (McNaught, 1992). The Cladocera species *Daphnia magna* (Diplostraca: Cladocera) is one representative of zooplankton, and widely used in environmental toxicology because of its rapid life cycle, a predominantly asexual mode of reproduction, minimal genetic variation, and high sensitivity to environmental contaminants (Brausch and Salice, 2011; Meyer *et al*., 2015). There are several standardized testing protocols for *D. magna* including short term tests (24 or 48 hours) for the acute toxicity of chemicals, water samples, and sediments (ASTM, 2005; ISO, 2012; OECD, 2004) and longer term tests for more subtle, chronic toxicity including effects on reproduction (ASTM, 2005; OECD, 2012). Those ecotoxicological tests with *D. magna* have mainly been used for testing industry pollutants (Alkimin *et al*., 2020; Galhano *et al*., 2020; Liu *et al*., 2020; Zimmermann *et al.* 2020), medical pollutants (Pan *et al*., 2019; Grzesiuk *et al*., 2020; Sarapultseva *et al*., 2017), and agricultural pollutants (Aksakal and Arslan, 2020; Knapik and Ramsdorf, 2020; Wyn *et al*., 2007).

D. magna may be exposed to plant-produced Cry proteins through ingestion of pollen,

plant residues or root exudates that enter aquatic environments (Carstens *et al*., 2012; Viktorov, 2011). Maize in particular has a high biomass and detritus, such as shredded plant remains after harvest, can enter small streams draining the fields. In addition, maize is open pollinated and releases high amounts of pollen, which can also enter waterbodies (Carstens *et al*., 2012; Douville *et al*., 2007; Jensen *et al*., 2010; Rosi-Marshall *et al*., 2007; Tank *et al*., 2010). Even though maize might not be a natural food for *D. magna*, exposure to maize material in agricultural landscapes is likely. For the environmental risk assessment of GE crops, *D. magna* has been used in non-target studies as one representative species for aquatic environments. Studies were conducted with *Bt* rice (Zhang *et al*., 2016) and *Bt* maize (summarized in Pott *et al*., 2018). For *Bt* maize, several studies have investigated the impact on *D. magna* using pollen (Mendelson *et al*., 2003), pulverized leaves (Holderbaum *et al*., 2015), or flour (Bøhn *et al*., 2008, 2010; Zhang *et al*., 2018) as test material. Maize flour is a less realistic route of exposure for *D. magna*, but can serve as a model material to expose the test animals to Cry proteins. The aim of such feeding studies is to create worst case exposure scenarios, where the test animals ingest large amounts of *Bt* maize (and the insecticidal Cry proteins contained therein). In contrast to chemicals in water or sediment, the GE plant material that contains the orally active test substances (e.g. insecticidal Cry protein) also serves as food for the test species (e.g. *D. magna*) and ideally there is no need for additional food supply (e.g., green algae). Consequently, *D. magna* has been fed exclusively with *Bt* maize material to achieve high exposure. For suitable test protocols, however, it is essential that the plant materials containing the insecticidal proteins can be ingested by *D. magna* (appropriate particle size) and that they supply enough nutrients for survival, growth and reproduction so that the organisms are not under nutritional stress. The standardized ASTM, ISO and OECD test protocols mentioned above include validity criteria for the tests. However, they are not designed for orally active substances (Bundschuh *et al*., 2019). Researchers thus had to adapt the protocols for assessing potential impacts of *Bt* plant materials, but those have usually not been validated or ring tested in different laboratories. Consequently, the published studies conducted with different maize materials resulted in unconfirmed and sometimes conflicting results on the effects of *Bt* crops on *D. magna* (Pott *et al*., 2018).

One problem with most previous studies with *Bt* maize is that only one *Bt* maize hybrid was compared to one non-*Bt* maize hybrid. Even if the non-*Bt* maize is the nearest comparator line to the *Bt* line, the transformation process and several breeding steps may lead to subtle changes in plant composition and physiology, which may translate into differences in performance of organisms feeding on those plants (Ladics *et al*., 2015). There is the possibility that adverse effects seen in some of the *Bt* maize studies might

have been caused by such plant background-related effects rather than the *Bt* protein itself (Romeis *et al*., 2011, 2019). Another problem of studies using maize materials to feed *D. magna* is the possibility that nutritional stress might have led to effects in addition to those caused by the plant background and the *Bt* proteins, which could impede the interpretation of the study results.

It is evident that there is a large variation in various compositional analytes including nutrients and antinutrients in conventional maize lines that are grown commercially and have a history of safe use (Cong *et al.*, 2015; Hong *et al*., 2014), but it is difficult to link the composition of those compounds to the performance of *D. magna*. As long as the mechanistic relationship between plant components and *D. magna* performance remains unknown, the relevance of differences between particular lines can be judged if the natural variation among different conventional maize lines is known.

In the present study, we tested the suitability of three different maize materials, i.e., flour, leaves and pollen, as exclusive food sources for *D. magna*. We used those materials from five diverse conventional maize lines, including one breeding line that is closely related to a SmartStax *Bt* maize. The following objectives were addressed:
1) How suitable are maize flour, leaves, and pollen as exclusive food sources for *D. magna* to sustain growth and reproduction compared to green algae?
2) How do life table and population parameters of *D. magna* differ among non-GE maize lines and what is the potential natural range of variation?

The data generated in our study with non-GE maize lines is useful for the interpretation of observed differences in *D. magna* performance between GE plants and their non-GE comparators in the context of future risk assessments of GE maize.

2. Materials and methods

2.1 Plant materials

Five lines of conventional maize were used for all experiments: Rheintaler, a Swiss landrace and population maize, Tasty Sweet, a sweet maize, ES-Eurojet and Planoxx, two commercial varieties used in Switzerland (ES-Eurojet is early maturing durum maize while Planoxx is late maturing dent maize), and EXP 258, the nearest conventional hybrid to one SmartStax *Bt* line. All maize lines were planted on May 14th, 2018 in two heated glasshouse cabins, set to 21°C during the day and 17°C at night and additional light to ensure a minimum day length of 16 h. Plants were grown individually in 12 L pots filled with soil. Ca. 40 g long-term fertilizer (Manna Cote 4M Wilhelm Haug GmbH, Ammerbuch, Germany) were added per pot. Pots were arranged in a block design (each block

containing each maize line) to account for differences in light and climatic conditions within the glasshouse cabins. After plants were 4 weeks old, they were fertilized with liquid fertilizer (0.2%) Manna (Wilhelm Haug GmbH) once per week.

Seven weeks after planting (highest maize plants had 15-16 leaves), the 10th true leaf counted from the bottom of each plant was cut and the middle vein was removed. The leaves were cut into pieces and stored in paper envelopes at -70 °C. Later, leaf-pieces were lyophilized and ground with a coffee mill for 5 min. Subsequently, a finer powder was generated with a mixer mill (MM400, Retsch, Haan, Germany) set to a frequency of 25 Hz and a grinding time of 30 sec., with a 20 mm diameter tungsten carbide ball. Finally, the powder was sieved through a 75 µm metal sieve and stored at -70 °C.

Maize tassels were placed in air-permeable cellulose bags (Celloclair, Liestal, Switzerland) and pollen was collected every second day. The collected pollen was poured through a 200 µm gauze to remove anthers into a 12 cm glass Petri dish, where it was left for 24 h for drying at room temperature. After that, the pollen was stored in screw-cap glass tubes at -70°C. Plants were discarded when pollen shedding stopped. Because pollen grains, which have a diameter of 80 - 90 µm (Meissle *et al.,* 2014), are too large for *D. magna* as food (Burns, 1968), pollen was also ground with the mixer mill (MM400), at 25 Hz for 30 sec., sieved through a 75 µm mesh, and stored at -70 °C.

Finally, maize grains were also ground with the coffee grinder (5 min), and Mixer Mill (MM400) at 30 Hz for 150 sec., sieved through a 75 µm mesh, and stored at -70 °C. In contrast to leaves and pollen, which were collected from plants uniformly grown in our glasshouse, maize flour was produced from the original batch of (untreated) seeds obtained from the breeders. This implies that the plants from the different maize lines were raised in the field under different conditions.

For the feeding assays, the sieved maize materials were used to make suspensions with a concentration of 3 mg/mL using Aachener Daphnien Medium (ADAM) (Klüttgen *et al*., 1994, medium composition modified after Ebert *et al*., 1998), which were stored in 2 ml aliquots at -20 °C.

2.2 Algae and D. magna

Algae (*Acutodesmus obliquus*) that served as optimal food for *D. magna* and a monoclonal strain of *Daphnia magna* (strain GB-EL75-69) were obtained from Dieter Ebert, Zoological Institute of Evolutionary Biology, University of Basel (Switzerland).

D. magna were cultured in ADAM medium in a climate chamber (20 °C, 70% RH, 16 h light / 8 h dark cycle). The medium was prepared and stirred at room temperature for at least 12 hours before use. *D. magna* of the culture were transferred to new medium every

two weeks, using Pasteur pipettes. The cultured *D. magna* showed no signs of stress, i.e., presence of males or ephippia, discolored animals or high mortality.

The culture medium for the green algae was prepared according to the description by D. Ebert (Web-guide to *Daphnia* parasites, http://www.evolution.unibas.ch/ebert/lab/algae.htm) and autoclaved in 1 L baffled flasks. When the medium cooled down, ca. 2 mL algae suspension were added and each flask was closed with a sterilized PTFE membrane cap. The bottles were incubated on a platform shaker in a climate cabinet (20 °C) with lights from three directions and a 23 h light / 1 h dark cycle. When the color of the algae suspension was dark green, the bottles were stored at 4 °C. Before feeding to *D. magna*, the algae were centrifuged (4500 × *g*, 15 min) in 50 mL centrifuge tubes, the supernatant was discarded and the pellet was resuspended in ca. 25 mL ADAM medium by shaking the tubes.

The carbon concentration of algae was measured in a Euro EA300 elemental analyser (HEKAtech GmbH, Wegberg, Germany) and calculated with CallidusH 2E3 (HEKAtech, Germany). The carbon content of the algae was about 55% of the dry weight and 10 million algal cells had a dry weight of ca. 0.28 mg. Algae were counted in a Thoma chamber (http://www.evolution.unibas.ch/ebert/lab/counting.htm#4).

2.3 Effects of maize materials on D. magna

Newly hatched *D. magna* (within 6-24 h of hatching) from the culture were kept individually in 100 mL glass beakers containing 50 mL ADAM medium, and fed with 100 µL suspension of maize materials from one of the five maize lines per animal per day. According to guideline OECD211, the amount of supplied diet should be based on organic carbon and the recommended feeding ration per *D. magna* per day is between 0.1 and 0.2 mg C (OECD, 2012). Assuming a carbon content of ca. 50% in maize materials (Hart *et al.*, 2007, unpublished raw data of Meissle *et al.*, 2011), 100 µL of the 3 mg/mL suspension prepared for the different maize materials contained ca. 0.15 mg C. The suitability of this feeding dose had been confirmed in a preliminary experiment using a different clone of *D. magna* (Table S4).

The experiment had two repetitions and ten *D. magna* per maize material (flour, leaves, pollen) and maize line (Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258) were tested in each repetition (in total 20 replicates). Thus the total number of *D. magna* fed with maize material in this experiment was 300.

As a control treatment, 10 additional *D. magna* in each experimental repetition were fed daily with 10 million algae, which equals ca. 0.15 mg C. *D. magna* were transferred to new medium every two days to ensure high medium quality throughout the experiment.

The experiment was conducted in a climate chamber (20 °C, 70% RH) under a 16 h light / 8 h dark cycle. The number of *D. magna* surviving, the number of molts, and the number of released offspring were recorded daily until day 28, and then every two days. Food was provided daily throughout the experiment. All offspring were removed after counting, so it was not possible to determine the sex of the offspring. The body length (distance from the top of the head to the base of the caudal spine) and body width (distance between back and front) was measured on day 7, day 14, and then every 14 days. Individual *D. magna* were removed from the rearing containers, photographed with a photomicroscope (Keyence VHX 6000, Mechelen, Belgium), and returned to the medium as soon as possible. Body length and body width were subsequently measured with ImageJ (ImageJ-win64, version 1.8.0, National Institutes of Health, USA). Ingestion of the different food materials was evident by the color of the gut under the stereo-microscope (Fig. S1). The experiment ended when all individuals had died.

2.4 Medium quality analyses

The quality of the ADAM medium was measured at different time points during the experiment described previously to make sure the values were within the recommend range of guideline OECD211 (OECD, 2012):

W0: pure ADAM medium; W1: ADAM medium after adding food (flour, leaves, pollen or algae); W2: ADAM medium 24 h after adding food (including one *D. magna* per container); W3: W2 after adding another food dose for one day; W4: W3 after another 24h.

For the first repetition of the feeding experiment, the medium quality W1-W4 in one randomly chosen replicate of each treatment was measured once within the first week of the feeding experiment, when *D. magna* were juveniles, and once when *D. magna* were adults. For the second experimental repetition, the medium quality of all treatments was checked randomly three times throughout the experiment.

The following parameters were analysed: pH value (FiveEasy™ FiveGO™ pH meter FE20, Mettler-Toledo AG, Greifensee, Switzerland), total hardness (MColortest™ Total Hardness Test, Merck KGaA, Darmstadt, Germany) and dissolved oxygen concentration (DOC) (FiveGo™ F4 portable meter, Mettler-Toledo AG, Greifensee, Switzerland).

2.5 Data analysis

Data were analysed using R, version 3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria). All data are presented as mean ± standard error (SE), unless otherwise indicated. Data were compared among the different maize treatments (lines and

materials). Data from the control treatment (*D. magna* fed exclusively with algae) were not included in the analyses. The data used for statistical analysis are available in the supplemental material.

The survival probability of *D. magna* was analysed for each food source separately using Kaplan-Meier estimates and log-rank test (survival package). Total offspring and offspring per clutch were analysed using full factorial linear mixed effects models (LMER) with maize (five maize lines) and food (flour, leaves, pollen) as fixed factors, and experimental repetition as random factor (lme4 package). The time when first offspring was released (days), the number of moltings to first offspring, the total number of clutches, and the number of individuals in the first clutch were analysed by generalized linear mixed effects models (GLMER) assuming Poisson distribution with the same factors (lme4 package). Comparisons among treatments were analysed with Anova function using type III sum of squares (car package). Body length and body width were analysed using full factorial LMER with the fixed factors maize, food and time (days when measurements were taken) and individual (each *D. magna*) as random factor. In all models, factor contrasts were set to orthogonal. Differences were considered significant at $p < 0.05$. When interactions between food and maize were significant in the overall analyses, we conducted separate analyses for each food type. The net reproductive rate (R_0), generation time (T) and intrinsic rate of increase (r_m) of *D. magna* were calculated based on the theory of age-stage, two-sex life table (Chi and Liu, 1985; Chi, 1988) using bootstrap method (Akca *et al*., 2015) with 10’000 bootstrap replicates. The differences among maize lines were analysed with paired bootstrap tests (Hesterberg *et al*., 2010; Smucker *et al*., 2007) for each food type separately. Those lifetable analysis were performed using TWOSEX-MSChart program (TWOSEX-MSChart-B100000, version 2020.05.28, National Chung Hsing University; Chi H).

To illustrate the variability among different maize lines, we calculated the 95% confidence interval (CI) for the mean of each parameter for each maize line. The range of variation was then defined as the interval from the highest upper to the lowest lower CI boundary of all maize lines. We also calculated the ratio between the highest and the lowest mean of each parameter and the ratio between the highest upper and the lowest lower CI boundary (highest / lowest).

3. Results

3.1 Medium quality

All pH values of the ADAM medium were between 7.7 and 8.1 (Table S1). The value was lowest 24 hours after adding food (W2) and then increased slightly. All DOC values were between 4.0 mg/L and 6.3 mg/L. The hardness gradually increased with time, and all values were between 210 mg/L and 305 mg/L. All values for the water quality (pH, DOC, hardness) were within the range demanded in OECD211 (OECD, 2012), i.e., pH 6 - 9, DOC > 3 mg/L and hardness > 140 mg/L.

3.2 Performance of D. magna in the control treatment

After 21 days (the time when the test recommended by OECD211 ends), mortality in the control treatment (algae, *A. obliquus*, as food) was 0%, *D. magna* moulted 5.1 ± 0.05 times to first offspring release, which occurred after 9.2 ± 0.09 days. The mean number of individuals in the first clutch was 15 ± 0.4. *D. magna* produced 4 ± 0.0 clutches, the mean total number of offspring produced was 101 ± 2.0, and each clutch consisted of 25 ± 0.5 offspring.

D. magna in the control treatment survived for a maximum of 123 days. The *D. magna* started to die at day 32, and reached a mortality of 20% at day 69. The mean longevity was 93 ± 5.6 days. In total, *D. magna* produced 23 ± 1.4 clutches and the mean total number of offspring produced during the whole life time was 665 ± 39. Each clutch consisted of 30 ± 0.60 offspring. The net reproductive rate R_0 was 665 ± 38, the generation time T was 18 ± 0.20 days, and the intrinsic rate of increase r_m was 0.35 ± 0.0024 day^{-1}. The body length and body width of *D. magna* in the control treatment increased from day 7 (n=20) to day 112 (n=4), from 2.7 ± 0.02 mm to 4.7 ± 0.04 mm length and 1.8 ± 0.02 mm to 3.1 ± 0.03 mm width.

3.3 Mortality

There was no statistically significant difference in *D. magna* survival among the five maize lines for each of the food sources (all $p \geq 0.1$) (Table 1, Fig. 1). When fed with maize flour, *D. magna* lived longest, i.e., a mean of 54 - 77 days, depending on maize line (Table S2, Fig. S2). The ratio between the highest and the lowest mean was 1.4 (Table S3).In the maize leaves treatments, mean longevity was 27 - 38 days (ratio 1.4), and when fed maize pollen, mean longevity was 35 - 42 days (ratio 1.2). The last *D. magna* in the flour treatment died between day 99 (ES-Eurojet) and day 105 (Tasty Sweet); in the leaves treatment between day 60 (Rheintaler) and day 78 (Tasty Sweet); and in the pollen

treatment between day 58 (ES-Eurojet) and day 87 (Tasty Sweet).

The 95% confidence interval (CI) of the mean longevity of *D. magna* fed flour from any of the five maize lines ranged between 40 and 90 days (ratio 2.3), for maize leaves the CI was between 19 and 49 days (ratio 2.6) and for maize pollen between 28 and 52 days (ratio 1.9) (Table S2 and S3, Fig. S2).

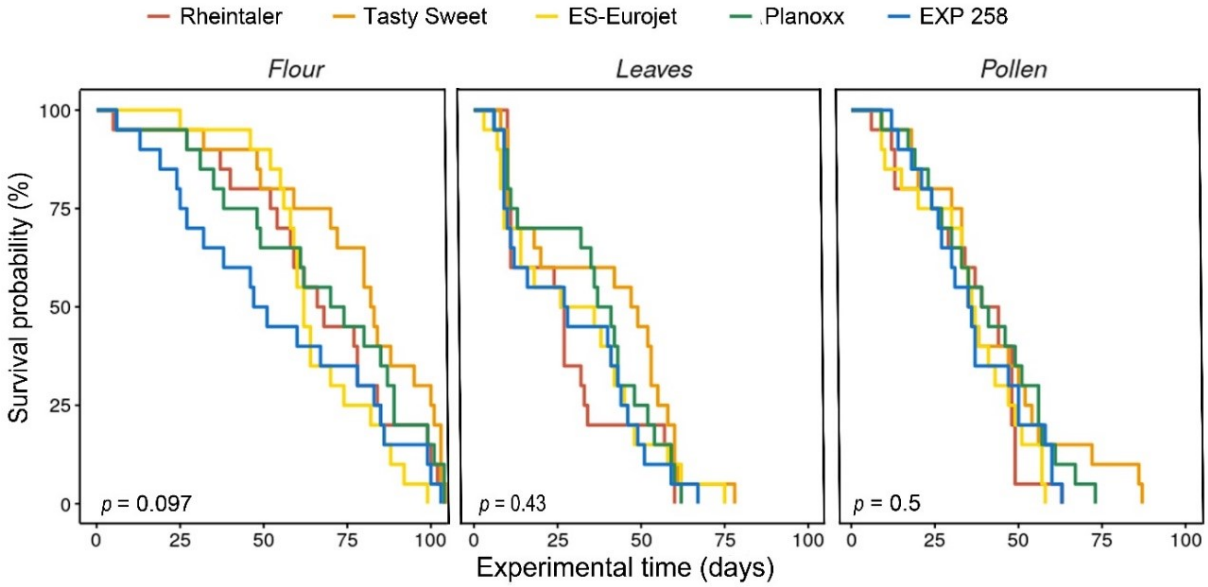

Fig. 1. Survival probability (%) of *Daphnia magna* fed with flour, leaves, or pollen from five maize lines (n=20). Data were analyzed for each food source separately using the Kaplan-Meier procedure with log-rank test.

Table 1. Statistics of life table parameters of *Daphnia magna* fed with flour, leaves, or pollen from five maize lines during their whole life time. N = 20 per maize material and line.

Parameter	Statistics main analysis[a]	Statistics separate analysis for maize materials		
		Flour	Leaves	Pollen
Longevity (Kaplan-Meier with log rank)		$\chi^2 = 7.9$, $p = 0.1$	$\chi^2 = 3.9$, $p = 0.4$	$\chi^2 = 3.4$, $p = 0.5$
Moltings to first offspring (GLMER)	Food: $\chi^2 = 4.6$, $p = 0.1$ Plant: $\chi^2 = 4.5$, $p = 0.3$ F x P: $\chi^2 = 6.7$, $p = 0.6$			
First offspring time (GLMER)	Food: $\chi^2 = 36.1$, $p < 0.0001$ Plant: $\chi^2 = 20.1$, $p = 0.0005$ F x P: $\chi^2 = 21.7$, $p = 0.006$	$\chi^2 = 47.3$, $p < 0.0001$	$\chi^2 = 4.6$, $p = 0.3$	$\chi^2 = 0.9$, $p = 0.9$
Individuals in first clutch (GLMER)	Food: $\chi^2 = 34.8$, $p < 0.0001$ Plant: $\chi^2 = 4.9$, $p = 0.3$ F x P: $\chi^2 = 6.3$, $p = 0.6$			
Total clutches (GLMER)	Food: $\chi^2 = 137.5$, $p < 0.0001$ Plant: $\chi^2 = 33.7$, $p < 0.0001$ F x P: $\chi^2 = 38.2$, $p < 0.0001$	$\chi^2 = 33.6$, $p < 0.0001$	$\chi^2 = 33.6$, $p < 0.0001$	$\chi^2 = 4.3$, $p = 0.4$
Total offspring (LMER)	Food: $\chi^2 = 38.0$, $p < 0.0001$ Plant: $\chi^2 = 31.0$, $p < 0.0001$ F x P: $\chi^2 = 43.9$, $p < 0.0001$	$\chi^2 = 36.7$, $p < 0.0001$	$\chi^2 = 24.5$, $p < 0.0001$	$\chi^2 = 6.6$, $p = 0.2$
Offspring per clutch (LMER)	Food: $\chi^2 = 38.9$, $p < 0.0001$ Plant: $\chi^2 = 38.5$, $p < 0.0001$ F x P: $\chi^2 = 32.9$, $p < 0.0001$	$\chi^2 = 65.8$, $p < 0.0001$	$\chi^2 = 16.0$, $p = 0.003$	$\chi^2 = 10.3$, $p = 0.04$

[a] F × P stands for food × plant interaction. In case of significant interactions in the main analysis, separate analysis were conducted for each maize material.

3.4 Growth parameters

The body length and body width of *D. magna* feeding on maize materials increased over time (body length: $\chi^2 = 753.6$, $p < 0.0001$; body width: $\chi^2 = 498.1$, $p < 0.0001$) (Fig. 2). Size differed significantly among the five maize lines (body length: $\chi^2 = 37.9$, $p < 0.0001$; body width: $\chi^2 = 24.3$, $p < 0.0001$) and the three food sources (body length: $\chi^2 = 7.5$, $p = 0.02$; body width: $\chi^2 = 7.9$, $p = 0.02$). Because the interaction of the factors time, food source and maize line was also significant (body length: $\chi^2 = 24.5$, $p = 0.002$; body width: $\chi^2 = 20.6$, $p = 0.008$), separate analyses were conducted for each food source. For maize flour treatments, *D. magna* fed with Rheintaler had significantly lower length and width compared with the other maize lines, EXP 258 had lower length and width than Planoxx, Tasty Sweet, and ES-Eurojet, and individuals fed with ES-Eurojet had higher length and width compared with the other lines (length: maize line: $\chi^2 = 33.4$, $p < 0.0001$; interaction maize line × time: $\chi^2 = 28.4$, $p < 0.0001$; width: maize line: $\chi^2 = 22.2$, $p = 0.0002$; interaction maize line × time: $\chi^2 = 18.2$, $p = 0.001$). When fed Rheintaler leaves, *D. magna* had significantly lower length (maize line: $\chi^2 = 8.9$, $p < 0.0001$; interaction maize line × time: $\chi^2 = 2.3$, $p = 0.7$) and width (maize line: $\chi^2 = 12.8$, $p < 0.0001$; interaction maize line × time: $\chi^2 = 2.7$, $p = 0.6$) than when fed maize from the other lines. For pollen treatments, there were no differences among the maize lines in length (maize line: $\chi^2 = 10.1$, $p = 0.8$; interaction maize line × time: $\chi^2 = 15.6$, $p = 0.004$) and width (maize line: $\chi^2 = 7.6$, $p = 0.7$; interaction maize line × time: $\chi^2 = 15.5$, $p = 0.004$).

There were no significant differences in the number of moltings to first offspring release for *D. magna* feeding on the three food sources from the five maize lines (food, maize lines, and interaction, all $p \geq 0.1$, Table 1, Fig. 3A). The ratios of the highest to the lowest means were 1.4, 1.2, and 1.1 for flour, leaves, and pollen, respectively. The 95% CI for the mean number of moltings to first offspring release ranged between 6.0 - 9.4 for maize flour (ratio 1.6), 4.9 - 7.4 for maize leaves (ratio 1.5) and 5.9 - 7.6 for pollen (ratio 1.3) (Fig. 3A, Table S2 and S3).

3.5 Reproduction parameters

For the time to first offspring release, significant differences were identified among the three food sources ($p < 0.0001$) and the five maize lines ($p = 0.0005$) (Table 1). Since the interaction of food source and maize line was also significant ($p = 0.006$), separate analyses were conducted for each food source. *D. magna* feeding on Rheintaler maize flour needed longer to reproduce than those feeding on flour of the other four lines of maize ($p < 0.0001$). For maize pollen or leave treatments, there were no significant differences among maize lines (all $p \geq 0.3$, Table 1, Fig. 3B). The ratios of the highest to

the lowest means were 1.5, 1.2, and 1.1 for flour, leaves, and pollen, respectively. The 95% CI for the mean time of first offspring release of *D. magna* fed with flour of the five maize lines was 14 - 25 days (ratio 1.8), for maize leaves 12 - 16 days (ratio 1.3) and for maize pollen 13 - 15 days (ratio 1.2) (Fig. 3B, Table S2 and S3).

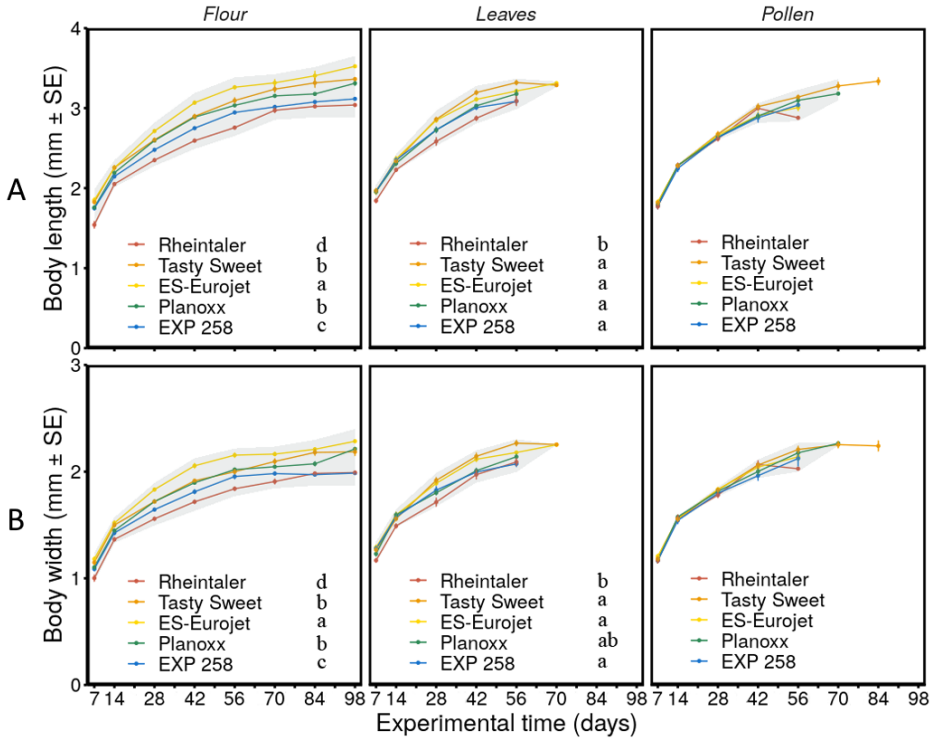

Fig. 2. Body length (A) and body width (B) of *Daphnia magna* fed with flour, leaves, or pollen from five maize lines (n=20). Measurements were taken at day 7, day 14, and then every 14 days. Data were analyzed using full factorial linear mixed effects models (LMER) with the fixed factors maize line, food and days of measurements, individual (each *D. magna*) as random factor. Different letters indicate significant differences ($p < 0.05$). Grey bands illustrate the highest and lowest value of the 95% confidence intervals of each maize line.

The number of offspring in the first clutch of *D. magna* was significantly different among the three food sources ($p < 0.0001$), but not among the five maize lines ($p = 0.3$), and no interaction between the two factors was present ($p = 0.6$) (Table 1). *D. magna* feeding on maize leaves produced more offspring in the first clutch than those feeding on pollen or flour, and *D. magna* feeding on pollen produced more than on flour ($p < 0.0001$, Table 1, Fig. 3C). The ratios of the highest to the lowest means were 1.4, 1.4, and 1.3 for flour, leaves, and pollen, respectively. The 95% CI for the mean number of individuals in the first clutch was 1.9 - 3.9 for flour (ratio 2.1), 2.6 - 6.5 for leaves (ratio 2.5) and 2.6 - 5.0 for pollen (ratio 1.9) (Fig. 3C, Table S2 and S3).

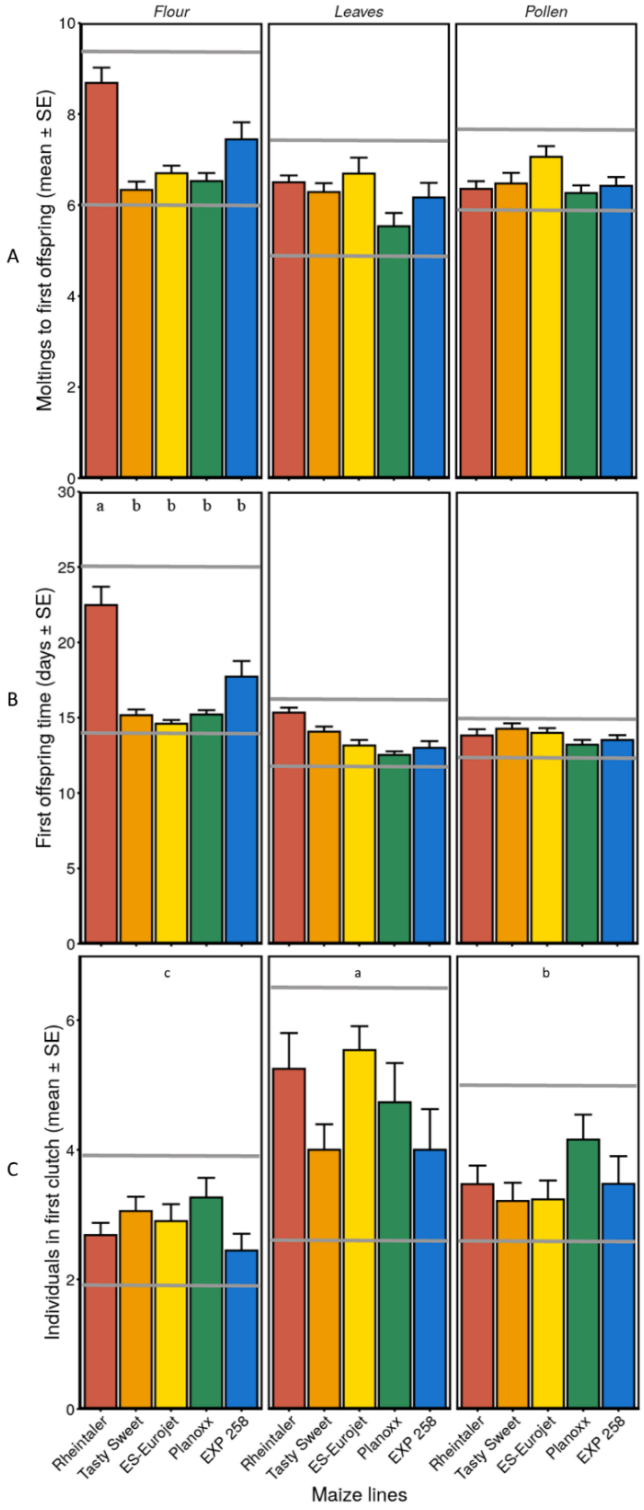

Fig. 3. Moltings to first offspring (A), time to first offspring (B), and individuals in first clutch (C) of *D. magna* feeding on flour, leaves, or pollen from five maize lines. Data were analysed using GLMER with maize (five lines) and food (flour, leaves, pollen) as fixed factor, experimental repetition as random factor. Different letters indicate significant differences ($p < 0.05$). Bars represent means ± SE for each maize line (n=20). Grey lines illustrate the highest and lowest value of the 95% confidence intervals over all maize lines.

The total number of clutches of *D. magna* differed significantly among the three food sources and among the five maize lines with the interaction of food source and maize line also being significant (all $p < 0.0001$, Table 1). Thus, separate analyses were conducted for each food source. The total number of clutches of *D. magna* feeding on Rheintaler or EXP 258 maize flour was lower than for the other three maize lines ($p < 0.0001$). *D. magna* feeding on Rheintaler or ES-Eurojet leaves produced significantly less clutches than individuals feeding on Tasty Sweet or Planoxx leaves and individuals feeding on EXP 258 had fewer clutches than those on Tasty Sweet ($p < 0.0001$). There were no significant differences for clutch number among maize lines in the pollen treatment ($p = 0.4$, Table 1, Fig. 4A). The ratios of the highest to the lowest means were 1.6, 2.1, and 1.2 for flour, leaves, and pollen, respectively. The 95% CI for the mean number of clutches was 6.5 - 17 for maize flour (ratio 2.5), 4.0 - 12 for leaves (ratio 3.0) and 5.0 - 9.5 for pollen (ratio 1.9) (Fig. 4A, Table S2 and S3).

The total number of offspring of *D. magna* differed significantly among the three food sources and the five lines of maize with a significant interaction (all $p < 0.0001$, Table 1). Separate analysis for each food source revealed that *D. magna* feeding on Rheintaler or EXP 258 flour produced significantly less total offspring than those feeding on Tasty Sweet or ES-Eurojet flour ($p < 0.0001$). *D. magna* feeding on Rheintaler maize leaves produced significantly less total offspring than those on Tasty Sweet or Planoxx leaves and those feeding on Eurojet produced less than those on Tasty Sweet ($p < 0.0001$). There were no significant differences among maize lines in the pollen treatment ($p = 0.2$, Table 1, Fig. 4B). The ratios of the highest to the lowest means were 2.1, 2.5, and 1.4 for flour, leaves, and pollen, respectively. The 95% CI for the mean total number of offspring of *D. magna* fed with five maize lines was 30 - 116 for maize flour (ratio 3.8), 26 - 99 for maize leaves (ratio 3.9) and 30 - 71 for pollen (ratio 2.3) (Fig. 4B, Table S2 and S3).

Similar to the total number of offspring, also the number of offspring per clutch of *D. magna* differed for the three food sources and the five lines of maize with a significant interaction (all $p < 0.0001$). *D. magna* feeding on EXP 258 maize flour produced significantly less offspring per clutch than those feeding on other maize lines except for Rheintaler, those feeding on Rheintaler produced significantly less offspring per clutch than those feeding on Tasty Sweet or ES-Eurojet, and those feeding on Planoxx or Tasty Sweet produced significantly less offspring per clutch than those feeding on ES-Eurojet ($p < 0.0001$). *D. magna* feeding on Planoxx or EXP 258 maize leaves produced significantly less offspring per clutch than those feeding on ES-Eurojet leaves ($p = 0.003$). *D. magna* fed with Rheintaler pollen had significantly more offspring per clutch than those fed with EXP 258 pollen ($p = 0.04$, Table 1, Fig. 4C). The ratios of the highest to the lowest means

were 1.6, 1.3, and 1.3 for flour, leaves, and pollen, respectively. The 95% CI for the mean number of offspring per clutch of *D. magna* fed with five maize lines was 4.0 - 8.0 for maize flour (ratio 2.0), 5.4 - 9.3 for maize leaves (ratio 1.7) and 5.1 - 8.2 for pollen (ratio 1.6) (Fig. 4C, Table S2 and S3).

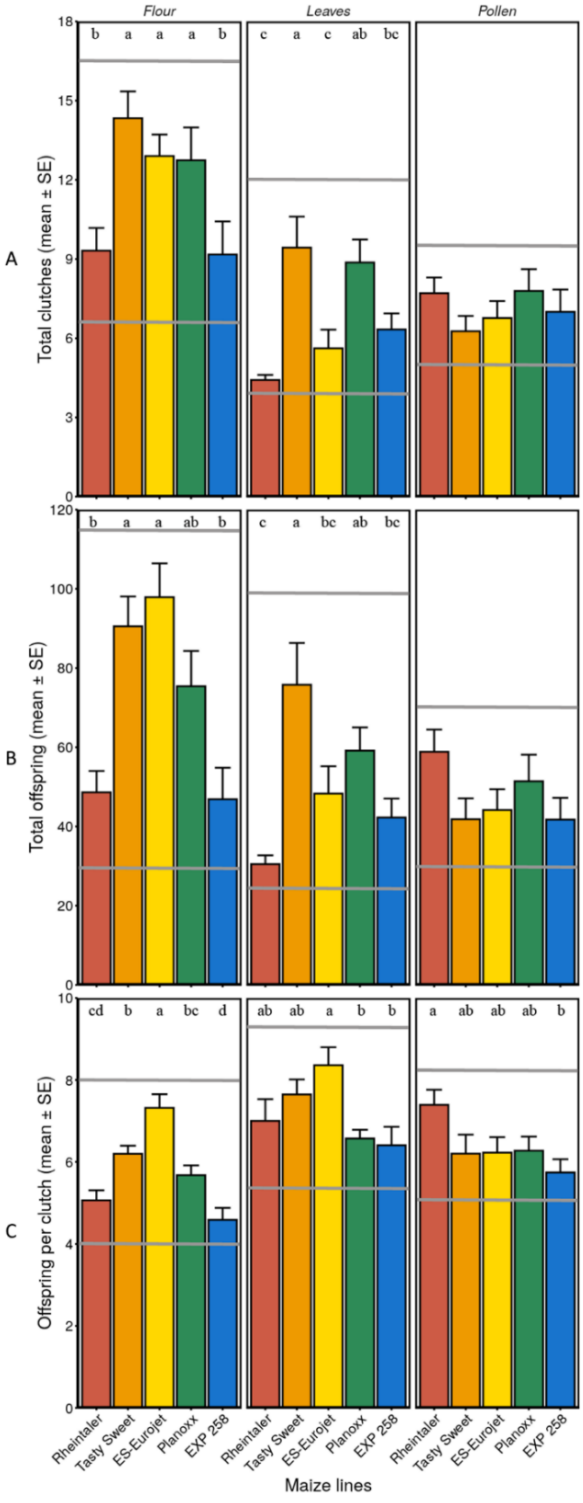

Fig. 4. Total number of clutches (A), total number of offspring (B), and number of offspring per clutch (C) of *D. magna* feeding on flour, leaves, or pollen from five maize lines. Data were analyzed using GLMER with maize (five lines) and food (flour, leaves, pollen) as fixed factor, experimental repetition as random factor. Different letters indicate significant differences ($p < 0.05$). Bars represent means ± SE for each maize line (n=20). Grey lines illustrate the highest and lowest value of the 95% confidence intervals over all maize lines.

3.6 Age-stage life table parameters

The net reproductive rate (R_0) of *D. magna* fed with ES-Eurojet or Tasty Sweet maize flour was significantly higher than that of *D. magna* fed with flour from Rheintaler and EXP 258 maize lines (ES-Eurojet with Rheintaler, $p < 0.0001$, adj. $\alpha = 0.005$; with EXP 258, $p < 0.0001$, adj. $\alpha = 0.005$; Tasty Sweet with Rheintaler, $p = 0.0008$, adj. $\alpha = 0.006$; with EXP 258, $p = 0.0009$, adj. $\alpha = 0.007$). R_0 of *D. magna* fed with Tasty Sweet or Planoxx maize leaves was significantly higher than that of *D. magna* fed with Rheintaler leaves ($p = 0.002$, adj. $\alpha = 0.005$; $p = 0.0008$, adj. $\alpha = 0.006$, respectively). The R_0 of *D. magna* fed with pollen was not affected by the different maize lines (all $p >$ adj. $\alpha = 0.005$) (Fig. 5A). The ratios of the highest to the lowest means were 2.3, 2.9, and 1.3 for flour, leaves, and pollen, respectively. The 95% CI for R_0 of *D. magna* fed with five maize lines was 27 - 114 for maize flour (ratio 4.2), 11 - 74 for maize leaves (ratio 6.5) and 27 - 63 for pollen (ratio 2.3) (Fig. 5A, Table S2 and S3).

The generation time (T) of *D. magna* fed with Rheintaler maize flour was significantly higher than those fed with flour from other maize lines except for EXP 258 (differences with ES-Eurojet, $p = 0.0006$, adj. $\alpha = 0.005$; with Planoxx, $p = 0.002$, adj. $\alpha = 0.006$; with Tasty Sweet, $p = 0.005$, adj. $\alpha = 0.006$). T of *D. magna* fed with Tasty Sweet maize leaves was significantly higher than those fed with leaves from other maize lines (differences with EXP 258, $p = 0.0001$, adj. $\alpha = 0.005$; with ES-Eurojet, $p = 0.0001$, adj. $\alpha = 0.005$; with Planoxx, $p = 0.0004$, adj. $\alpha = 0.006$; with Rheintaler, $p = 0.001$, adj. $\alpha = 0.007$) and T of *D. magna* fed with pollen was not affected by the different maize lines (all $p >$ adj. $\alpha = 0.005$) (Fig. 5B). The ratios of the highest to the lowest means were 1.2, 1.3, and 1.1 for flour, leaves, and pollen, respectively. The 95% CI for T of *D. magna* fed with five maize lines was 28 - 42 days for maize flour (ratio 1.5), 20 - 28 days for maize leaves (ratio 1.5) and 21 - 26 days for pollen (ratio 1.2) (Fig. 5B, Table S2 and S3).

The intrinsic rate of increase (r_m) of *D. magna* fed with ES-Eurojet maize flour was significantly higher than that of *D. magna* fed with flour from the other maize lines except for Tasty Sweet maize line (differences with EXP 258, $p < 0.0001$, adj. $\alpha = 0.005$; with Rheintaler, $p < 0.0001$, adj. $\alpha = 0.005$; with Planoxx, $p = 0.007$, adj. $\alpha = 0.008$); r_m of *D. magna* fed with Tasty Sweet or Planoxx maize flour was significantly higher than those fed with Rheintaler maize flour (all $p < 0.0001$, adj. $\alpha = 0.005$). The r_m of *D. magna* fed with pollen or leaves was not affected by the different maize lines (all $p >$ adj. $\alpha = 0.005$) (Fig. 5C). The ratios of the highest to the lowest means were 1.5, 142, and 1.1 for flour, leaves, and pollen, respectively. The 95% CI for r_m of *D. magna* fed with five maize lines was 0.09 - 0.15 day^{-1} for maize flour (ratio 1.6), 0.11 - 0.20 day^{-1} for maize leaves (ratio 1.8) and 0.14 - 0.19 day^{-1} for pollen (ratio 1.4) (Fig. 5C, Table S2 and S3).

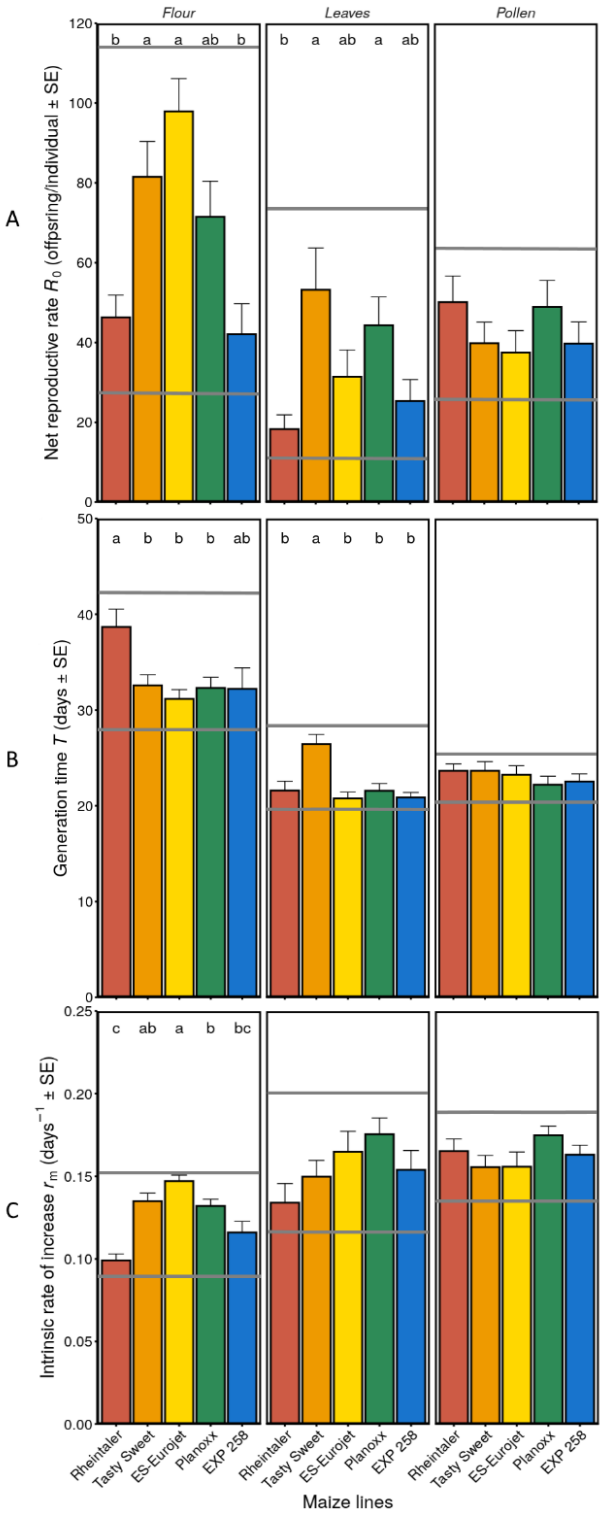

Fig. 5. Age-stage life table parameters of *D. magna* feeding on flour, leaves, or pollen from five maize lines: net reproductive rate R_0 (A), generation time T (B) and intrinsic rate of increase r_m (C). Data were analyzed by paired bootstrap test with the TWOSEX-MSChart software. Bars represent means ± standard error (SE) calculated with 10'000 bootstrap replicates. Different letters within the same column indicate significant difference (p < adj. α). Grey lines illustrate the highest and lowest value of the 95% confidence intervals over all maize lines.

4. Discussion

4.1 Experimental conditions

The experimental conditions in our study were adjusted according to the guideline OECD211 (OECD, 2012), and all values for the quality of the ADAM medium (pH, DOC, hardness) were within the demanded range. According to the OECD guideline standardized M4 or M7 medium is recommended, but other media are accepted if the performance of *D. magna* is shown to meet the validity criteria of the test. Several studies used ADAM medium, which is well suited for culturing *D. magna* (Ebert *et al*., 1998; Ho *et al*., 2019; Martin-Creuzburg *et al.*, 2019). We also conducted a preliminary experiment when *D. magna* were fed with algae for 21 days to compare ADAM medium with M4 medium. No individuals died in either media, and no significant difference was observed for growth or reproduction parameters (Table S4). Therefore, we decided to use ADAM medium, which is less complex and easier to prepare. At day 21 of the present study, *D. magna* fed with algae showed 0% mortality and the cumulative fecundity was 101, which is in accordance with the validity criteria of the OECD211 test, i.e., mortality after 21 days < 20% and mean number of living offspring produced per parent animal ≥ 60 (OECD, 2012). This indicates that the specimens used for our experiment were healthy and the experimental conditions suitable. At day 69, the mortality of *D. magna* reached 20%, at which time *D. magna* had been measured for body size for five times. This indicates that the handling necessary for recording body measurements did not impair *D. magna* performance.

4.2 Suitability of maize materials as exclusive food for D. magna

D. magna can survive, grow and reproduce when fed only maize material and all three materials tested proved to sustain survival, growth and reproduction. At day 21, the mortality of *D. magna* fed with maize flour was 0 - 15%, when fed with leaves it was 30 - 45%, and when fed with pollen it was 15 - 20% depending on the maize line. Mortality was thus exceeding the maximum of 20% set as a validity criterion in the OECD standard in the leaf treatments. In addition, the mean total number of offspring produced by *D. magna* fed with maize material within the first 21 days remained below the minimum of 60 offspring set by the OECD for all maize materials and lines (varying between 3.3 and 19 depending on maize material and line).

For the full life-cycle, *D. magna* fed with maize flour survived longer than those fed with pollen or leaves, but had a higher generation time *T* and a reduced body length and body width. In addition, *D. magna* fed with flour produced more offspring and more

clutches during their life time and had a higher net reproductive rate R_0, than those fed with pollen or leaves, but they needed more time to release the first offspring, and they had a lower intrinsic rate of increase r_m. This demonstrates that the maize materials have a different nutritional quality for *D. magna*, and also the allocation of the different nutrients to survival, growth and reproduction may differ. *D. magna* fed with maize flour tended to allocate nutrients to survive first, followed by growth and reproduction. Compared with the algae treatment, however, *D. magna* fed with maize flour, leaves or pollen showed smaller body size, lag in the first time of reproduction, a reduction in the total number of offspring, a reduction of the net reproductive rate R_0, an increase in generation time T and a reduction of the intrinsic rate of increase r_m.

Previous studies have shown effects of low quality food on *D. magna* performance. Stige *et al*. (2004) reported that when *D. magna* were exposed to nutritional stress by reduced food (green algae *Selenastrum capricornutum*) quantity and/or quality (phosphorus-limitation) they showed reduced growth and reproduction. Bouchnak and Steinberg (2010) reported that fertility was decreased in *D. magna* fed with low quality food (baker's yeast compared to green algae *Pseudokirchneriella subcapitata*). In addition, food stress has also been reported to initiate diapause (Han *et al.*, 2011) and increase the production of male offspring (Hobaek and Larson, 1990; Kleiven *et al*., 1992).

Some previous studies to assess GE plant effects on *D. magna* used maize materials as food. When Zhang *et al.* (2018) fed *D. magna* with maize flour for 28 days, the mean time of first offspring release was 12.5 day and similar results were reported by Bøhn *et al*. (2010) (13 days). Thus the values of both studies were slightly lower than the range of the five maize lines of the present study (14 - 25 days). The reported mean body length in Zhang *et al.* (2018) at day 28 was 2.54 mm, in the studies of Bøhn *et al*. (2008, 2010) between 2.5 - 3.0 mm at day 42. The means of five maize lines in our study cover those values with body length of *D. magna* at day 28 between 2.3 - 2.7 mm and at day 42 between 2.6 - 3.1 mm. Bøhn *et al*. (2008) fed *D. magna* with maize flour at a similar feeding dose as in our study for 42 days, and the mean number of offspring per clutch was 5.1, which was within the range of our results (4.0 - 8.0). While their study showed that not all individuals in the experiments reached maturation, in our experiments, all the individuals in both experimental repetitions reached maturation before 42 days. Holderbaum *et al.* (2015) fed *D. magna* with maize leaves at a similar dose than in our study for 42 days, and the median time of first offspring release was 12 days, which was within the range of the five maize lines in our study (12 - 16 days). However, Holderbaum *et al.* (2015) observed the production of ephippia (protective structures enclosing two dormant eggs), while no ephippia were produced in our study. These differences are likely

due to the different *D. magna* clones and a different photoperiod used in the different studies. Holderbaum *et al*. (2015) and Bøhn *et al*. (2008, 2010) used an arctic clone and a photoperiod of 24 h daylight. Photoperiod can change the life cycle of zooplankton and significantly affect the development and proliferation. Ferrari and Hebert (1982) found that arctic clones of *D. magna* with 24 h daylight tend to produce ephippia and males, which is part of the survivorship and reproductive behavior adapted to extreme conditions, i.e., populations must produce males and bisexual eggs to survive the periods when ponds are frozen. Furthermore, Gao *et al*. (2006) reported that *D. magna* has a reduced feeding rate under 24 h daylight. The clone we selected for our study produced only female and no ephippia under our experimental conditions.

In summary, *D. magna* can survive, grow, and reproduce on different maize materials, but performance is reduced compared to optimal food, such as green algae. This has also been acknowledged in previous studies (Bøhn *et al*., 2008; Holderbaum *et al*., 2015; Zhang *et al*., 2018). The fact that the OECD validity criteria for chronic exposure tests with *D. magna* are not met indicates nutritional stress. This bears the risk of confounding effects, which may generally limit the reliability of studies.

4.3 Differences among maize lines

In this study, five very different maize lines were used. Rheintaler is a Swiss landrace and population maize (no hybrid), with different breeding goals and obvious phenotypically differences to commercial field maize. Tasty Sweet is a sweet maize with different grain composition than field maize (very little starch in the grains), and bred for human consumption. ES-Eurojet and Planoxx are two commercial varieties used in Switzerland with different maturation times and different grain characteristics (dent maize and durum maize), and EXP 258 is a breeding line from the USA and the nearest conventional hybrid to one SmartStax *Bt* line.

In the flour treatments of our study, *D. magna* fed with Rheintaler showed a smaller body size, longer time to first offspring release, less clutches, less total offspring, higher generation time T, lower net reproductive rate R_0, and lower intrinsic rate of increase r_m than those fed with other maize lines. Similarly, *D. magna* fed with Rheintaler leaves had the smallest body size, least total clutches, least total offspring, least net reproductive rate R_0 and least intrinsic rate of increase r_m. In contrast, in the pollen treatments, *D. magna* fed with Rheintaler produced more offspring per clutch than those fed with EXP 258 maize pollen. Differences of EXP 258 maize to the other hybrid maize lines were less pronounced. In the flour treatments, however, *D. magna* fed EXP 258 were smaller, had less offspring, and reduced R_0 and r_m than at least one of the three commercial hybrids. In

addition, some differences between EXP 258 and other hybrid lines were also observed when fed leaves.

These results illustrate that different maize materials and lines differed in their nutritional quality for *D. magna*. In the maize flour and leaf treatments, more significant differences and higher variability for the life table parameters of *D. magna* were observed than in the pollen treatments. Reproductive parameters showed a relatively high variability among the different maize lines, such as the total number of clutches, total offspring, and R_0 for flour and leaf treatments and offspring per clutch for flour (ratios of highest to lowest mean values between 1.6 and 2.9). Other parameters in the flour and leaf treatments and all parameters in the pollen treatments were less variable with ratios between 1.1 and 1.5.

By calculating the 95% CI around each parameter mean for each maize material and line, we provide estimates in which ranges the true means would lie. We defined the interval between the highest value and the lowest value of those 95% CI boundaries over all maize lines as the natural range of variation and the ratio of the highest value divided by the lowest value provides an impression how variable the individual parameters can be among different maize lines. Naturally, those ratios of the highest and lowest confidence limits are higher than the ratios of the actual means. Once more, the highest ratios were evident for total number of clutches, total offspring, and R_0 (ratios between 1.9 and 6.5), while other parameters had lower ratios (1.2 – 1.9). When we take the total number of offspring as an example, those ratios indicate that the true mean of one maize line might be around 4 times higher than that of another maize line. This is relevant since the commercialized non-GE maize lines are generally considered to cause no unacceptable harm to the environment.

That life-table parameters or food consumption of non-target insects can strongly vary among different maize hybrids has previously been reported in laboratory feeding studies for different terrestrial species, including *Porcellio scaber* (Isopoda: Oniscidea) (Wandeler *et al*., 2002), *Drosophila melanogaster* (Diptera: Drosophilidae) (Knecht and Nentwig, 2010), *Megaselia scalaris* (Diptera: Phoridae) (Knecht and Nentwig, 2010), *Coleomegilla maculata* (Coleoptera: Coccinellidae (Pilorget *et al*., 2010), *Oulema melanopus* (Coleoptera: Chrysomelidae) (Meissle *et al*., 2012), and *Chrysoperla carnea* (Neuroptera: Chrysopidae) (Meissle *et al*., 2014).

4.4 Implications for risk assessment of GE plants

Previous scientific studies to assess the impact of *Bt* maize on *D. magna* usually compared tissue from one *Bt* maize line to that of a non-*Bt* line. This carries the risk that adverse effects seen in some studies might have been caused by differences in the plant

background rather than the *Bt* protein itself (Romeis *et al*., 2011, 2013), especially since maize material is clearly a suboptimal food for *D. magna* causing nutritional stress. Even if the closest related conventional counterparts to a given GE plant is chosen as a comparator, the transformation process, the production of the new GE trait, and the breeding steps after the transformation may lead to differences in plant composition. It is thus very difficult to control for plant background effects, especially because knowledge about the effects of all the different nutrients and antinutrients in plant material on *D. magna* (and other species used for ecotoxicological testing) is limited. To address this, Chambers *et al*. (2010) selected *Bt* and non-*Bt* maize lines for testing different stream macroinvertebrates based on C:N ratios and lignin content. However, this selection seems arbitrary because there might be many other plant compounds that potentially influence invertebrate performance.

The natural variation among conventional maize lines can be used to interpret statistical differences detected when comparing a particular GE line with its non-GE comparator and to define whether they might be of biological relevance. In the GE crop risk assessment this approach is commonly applied in the comparative food/feed safety assessment where substantial equivalence analyses are conducted to assess whether foods and feed derived from the GE crop are as safe as their conventional counterparts (Anderson *et al*., 2019, 2020; EFSA, 2010; Hong *et al*., 2014). In our study, the natural variation for our maize lines, based on the ratios of the highest to the lowest confidence limit, ranged between a factor of 1.2 (first offspring time of D. magna when fed pollen) to 6.5 (R_0 when fed leaves).

We acknowledge, however, that our subsample of five maize lines is unlikely to represent the population of all possible maize lines, so the natural range of variation for all potential maize lines is likely to be much broader.

For example, Bøhn *et al*. (2008) reported that *D. magna* fed flour of a *Bt* maize showed a 37% reduction in longevity compared to a non-*Bt* line (ratio 1.6). Despite the fact that this reduction was statistically significant, it might not be of high biological relevance given the fact that the maximum mean difference in longevity among the various non-GE maize lines in our study was also around 30% (ratio 1.4) and the potential difference based on the 95% CI was estimated to be 56% (ratio 2.3).

Better than interpreting the values of the current study would be if future studies with plant material from GE and non-GE maize would include multiple conventional lines to capture the natural range of variation in that particular context. This, however, would increase the complexity (and costs) of non-target studies and would only be helpful if differences between the GE and non-GE comparator would actually be detected. One

solution would be to first conduct a study with only the GE and non-GE comparator and only if adverse effects of the GE line are observed, repeat the study with multiple conventional comparators, 1) to confirm the observed effects between the GE and non-GE comparator, and 2) to interpret this effect in the context of natural variation of conventional lines.

In the case of *D. magna* even feeding studies with a range of maize lines as additional comparators need to be interpreted with caution given the fact that maize material overall is of low nutritional quality for *D. magna*. In the environment the organisms will have access to a range of different food items and maize material is likely to represent only a small fraction of their diet. One might thus question if *D. magna* is a suitable surrogate test organism for crop residues in aquatic ecosystems or if there are other species that perform better when fed maize materials. In fact, other aquatic species have been used for feeding assays with *Bt* maize, e.g. other crustaceans, such as isopods (Jensen *et al*., 2010) or amphipods (Li *et al*., 2013; Chambers *et al*., 2010), caddisflies (Rosi-Marshall *et al*., 2007; Chambers *et al*., 2010; Jensen *et al*., 2010), or fly larvae, such as Tipulidae (Jensen *et al*., 2010) and Chironomidae (Prihoda and Coats, 2008; Li *et al*., 2013). Similar to *D. magna*, however, the nutritional quality of maize material as exclusive food for those species is also likely to be suboptimal and standardized test protocols for oral toxicity are also lacking.

5. Conclusions

To our knowledge, this is the first study, which compared different food types (flour, leaves and pollen) from a number of non-GE maize lines throughout the complete *D. magna* life cycle. The species can survive, grow, and reproduce on all three maize materials. Performance of *D. magna* fed maize, however, was reduced compared to high quality food (green algae) and some of the validity criteria formulated by the OECD standard (OECD, 2012) were not met. It is thus apparent that *D. magna* provided only with maize as food are nutritionally stressed. This implies that confounding effects of poor food quality might have influenced previously published results on the effects of *Bt* maize on *D. magna*. In our study, large differences in life table and population parameters of *D. magna* were observed among the five different maize lines. The natural range of variation based on 95% CI showed that in particular reproductive parameters may vary up to a factor of 6, while other parameters, such as time to first offspring release, were less variable (factor 1.2 - 1.8).

If differences between a GE and comparator line are observed and their biological

relevance needs to be assessed in future risk assessments of GE maize, 1) the data on natural variation of the different parameters generated by previous studies can be informative (e.g. data from our study for maize fed D. magna); 2) for additional experiments the inclusion of multiple unrelated non-GE comparators should be considered. In addition, it should be taken into account that nutritional stress can affect the outcome of the study.

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Table S1. Medium quality parameters (pH value; dissolved oxygen concentration (DOC); hardness) of ADAM medium containing algae (*Acutodesmus obliquus*) or flour, leaves, or pollen from five conventional maize lines. Values represent means ± SE.

Food	Variety	pH vaule					DOC (mg/L)					Hardness (mg/L)				
		W0[a]	W1[b]	W2[c]	W3[d]	W4[e]	W0[a]	W1[b]	W2[c]	W3[d]	W4[e]	W0[a]	W1[b]	W2[c]	W3[d]	W4[e]
Algae	*Acutodesmus obliquus*	7.9 ± 0.04	8.0 ± 0.05	8.0 ± 0.05	8.0 ± 0.02	8.0 ± 0.03	5.1 ± 0.64	4.9 ± 0.17	5.2 ± 0.17	5.1 ± 0.22	5.5 ± 0.12	228 ± 1.7	232 ± 2.6	253 ± 6.3	257 ± 6.8	278 ± 7.5
Flour	Rheintaler		8.0 ± 0.05	8.0 ± 0.05	8.0 ± 0.02	8.0 ± 0.03		4.9 ± 0.17	5.2 ± 0.17	5.1 ± 0.22	5.5 ± 0.12		232 ± 2.6	253 ± 6.3	257 ± 6.8	278 ± 7.5
	Tasty Sweet		7.9 ± 0.01	7.9 ± 0.02	7.9 ± 0.02	8.0 ± 0.06		4.8 ± 0.17	4.9 ± 0.07	5.1 ± 0.26	5.2 ± 0.07		225 ± 2.7	233 ± 3.0	245 ± 3.5	281 ± 7.5
	ES-Eurojet		8.0 ± 0.04	7.8 ± 0.04	7.8 ± 0.02	7.9 ± 0.02		4.8 ± 0.10	4.8 ± 0.14	5.0 ± 0.20	4.9 ± 0.19		226 ± 4.3	241 ± 4.3	252 ± 4.1	286 ± 5.3
	Planoxx		7.9 ± 0.04	7.8 ± 0.01	7.9 ± 0.02	7.9 ± 0.04		4.9 ± 0.13	4.7 ± 0.12	5.1 ± 0.25	4.8 ± 0.19		225 ± 2.2	235 ± 1.6	242 ± 2.6	267 ± 1.2
	EXP 258		7.9 ± 0.05	7.8 ± 0.01	7.9 ± 0.01	7.8 ± 0.02		4.7 ± 0.29	4.9 ± 0.19	4.7 ± 0.14	4.8 ± 0.09		227 ± 1.2	241 ± 5.1	247 ± 3.0	267 ± 2.6
Leaves	Rheintaler		7.9 ± 0.02	7.9 ± 0.01	7.9 ± 0.05	8.0 ± 0.05		5.1 ± 0.29	5.2 ± 0.24	4.8 ± 0.21	4.8 ± 0.11		227 ± 3.4	232 ± 3.7	241 ± 2.5	282 ± 2.6
	Tasty Sweet		7.9 ± 0.02	7.8 ± 0.01	7.9 ± 0.01	7.9 ± 0.04		5.0 ± 0.05	4.9 ± 0.25	5.1 ± 0.27	4.6 ± 0.12		227 ± 3.0	238 ± 3.4	245 ± 2.2	276 ± 3.3
	ES-Eurojet		8.0 ± 0.02	7.8 ± 0.02	7.9 ± 0.01	7.9 ± 0.04		4.7 ± 0.06	4.7 ± 0.11	5.2 ± 0.17	4.8 ± 0.11		233 ± 4.1	244 ± 1.9	248 ± 2.6	263 ± 2.6
	Planoxx		7.9 ± 0.04	7.8 ± 0.01	7.9 ± 0.01	7.8 ± 0.02		5.0 ± 0.14	5.1 ± 0.25	4.5 ± 0.21	4.6 ± 0.10		230 ± 1.6	237 ± 4.4	245 ± 4.7	263 ± 2.0
	EXP 258		8.0 ± 0.03	7.8 ± 0.02	7.9 ± 0.01	7.9 ± 0.02		4.8 ± 0.16	5.1 ± 0.27	4.6 ± 0.10	4.9 ± 0.10		223 ± 2.6	237 ± 2.0	240 ± 1.6	262 ± 4.6
Pollen	Rheintaler		7.9 ± 0.01	7.9 ± 0.03	7.9 ± 0.04	8.0 ± 0.04		4.9 ± 0.10	4.8 ± 0.16	5.3 ± 0.30	5.1 ± 0.07		220 ± 2.7	238 ± 5.2	244 ± 1.9	286 ± 5.3
	Tasty Sweet		7.9 ± 0.01	7.8 ± 0.01	7.9 ± 0.02	7.9 ± 0.05		4.8 ± 0.06	4.8 ± 0.12	4.9 ± 0.15	5.0 ± 0.10		224 ± 2.9	245 ± 4.2	248 ± 4.6	290 ± 6.9
	ES-Eurojet		8.0 ± 0.02	7.8 ± 0.01	7.9 ± 0.01	7.9 ± 0.04		5.0 ± 0.16	5.3 ± 0.18	4.8 ± 0.03	4.8 ± 0.17		234 ± 1.9	238 ± 1.2	249 ± 1.9	281 ± 1.0
	Planoxx		7.9 ± 0.04	7.8 ± 0.01	7.9 ± 0.01	7.9 ± 0.03		5.1 ± 0.08	5.0 ± 0.27	4.6 ± 0.02	4.6 ± 0.17		227 ± 2.6	236 ± 3.7	242 ± 4.6	263 ± 2.0
	EXP 258		7.9 ± 0.05	7.8 ± 0.02	7.9 ± 0.02	7.9 ± 0.02		4.7 ± 0.16	5.1 ± 0.31	4.7 ± 0.12	4.8 ± 0.14		227 ± 3.0	241 ± 3.3	244 ± 2.9	265 ± 1.6

[a] W0: pure ADAM medium, n= 3.
[b] W1: pure ADAM medium containing food, n=5.
[c] W2: W1 after 24 hours, n=5.
[d] W3: W2 with added food, n=5.
[e] W4: W3 after 24 hours, n=5.

Table S2. Life table parameters of *D. magna* (strain GB-EL75-69) feeding on flour, leaves, or pollen from five lines of maize during their whole life time. Values represent means ± SE, n=20, the 95% confidence intervals are presented in parenthesis.

Parameters	Maize lines	Maize materials		
		Flour*	Leaves*	Pollen*
Longevity (#)	Rheintaler	66.8 ± 5.92 (54.4; 79.1)	27.5 ± 4.11 (18.8; 36.1)	36.2 ± 3.54 (28.8; 43.6)
	Tasty Sweet	77.2 ± 5.98 (64.6; 89.7)	38.2 ± 5.18 (27.4; 49.0)	42.2 ± 4.76 (32.2; 52.1)
	ES-Eurojet	65.7 ± 3.88 (57.6; 73.8)	30.3 ± 4.81 (20.3; 40.4)	35.4 ± 3.57 (27.9; 42.9)
	Planoxx	67.0 ± 6.50 (53.3; 80.6)	35.2 ± 4.23 (26.3; 44.0)	40.7 ± 4.01 (32.3; 49.0)
	EXP 258	54.5 ± 7.01 (39.8; 69.1)	29.3 ± 4.44 (20.0; 38.6)	36.8 ± 3.58 (29.3; 44.3)
Moltings to first offspring (#)	Rheintaler	8.7 ± 0.33 (8.0; 9.4)	6.5 ± 0.15 (6.2; 6.8)	6.4 ± 0.17 (6.0; 6.7)
	Tasty Sweet	6.3 ± 0.18 (6.0; 6.7)	6.3 ± 0.19 (5.9; 6.7)	6.5 ± 0.23 (6.0; 7.0)
	ES-Eurojet	6.7 ± 0.16 (6.4; 7.0)	6.7 ± 0.35 (5.9; 7.4)	7.1 ± 0.23 (6.6; 7.6)
	Planoxx	6.5 ± 0.18 (6.2; 6.9)	5.5 ± 0.29 (4.9; 6.2)	6.3 ± 0.17 (5.9; 6.6)
	EXP 258	7.4 ± 0.37 (6.7; 8.2)	6.2 ± 0.32 (5.5; 6.9)	6.4 ± 0.19 (6.0; 6.8)
First offspring time (d)	Rheintaler	22.5 ± 1.22 (19.9; 25.0)	15.3 ± 0.33 (14.6; 16.1)	13.8 ± 0.40 (13.0; 14.7)
	Tasty Sweet	15.2 ± 0.38 (14.4; 16.0)	14.1 ± 0.34 (13.3; 14.8)	14.3 ± 0.37 (13.5; 15.0)
	ES-Eurojet	14.6 ± 0.25 (14.1; 15.1)	13.2 ± 0.37 (12.3; 14.0)	14.0 ± 0.31 (13.3; 14.7)
	Planoxx	15.2 ± 0.29 (14.6; 15.8)	12.5 ± 0.24 (12.0; 13.0)	13.2 ± 0.32 (12.5; 13.9)
	EXP 258	17.7 ± 1.04 (15.5; 19.9)	13.0 ± 0.44 (12.0; 14.0)	13.5 ± 0.32 (12.9; 14.2)
Individuals in first clutch (#)	Rheintaler	2.7 ± 0.19 (2.3; 3.1)	5.3 ± 0.55 (4.0; 6.5)	3.5 ± 0.29 (2.9; 4.1)
	Tasty Sweet	3.1 ± 0.22 (2.6; 3.5)	4.0 ± 0.39 (3.2; 4.8)	3.2 ± 0.28 (2.6; 3.8)
	ES-Eurojet	2.9 ± 0.26 (2.4; 3.4)	5.5 ± 0.37 (4.7; 6.3)	3.2 ± 0.29 (2.6; 3.9)
	Planoxx	3.3 ± 0.30 (2.6; 3.9)	4.7 ± 0.61 (3.4; 6.0)	4.2 ± 0.38 (3.4; 5.0)
	EXP 258	2.4 ± 0.26 (1.9; 3.0)	4.0 ± 0.63 (2.6; 5.4)	3.5 ± 0.43 (2.6; 4.4)
Total clutches (#)	Rheintaler	9.3 ± 0.86 (7.5; 11.1)	4.4 ± 0.19 (4.0; 4.8)	7.7 ± 0.59 (6.4; 9.0)
	Tasty Sweet	14.3 ± 1.02 (12.2; 16.5)	9.4 ± 1.18 (6.9; 12.0)	6.3 ± 0.58 (5.0; 7.5)
	ES-Eurojet	12.9 ± 0.81 (11.2; 14.6)	5.6 ± 0.71 (4.1; 7.2)	6.8 ± 0.64 (5.4; 8.1)
	Planoxx	12.7 ± 1.25 (10.1; 15.4)	8.9 ± 0.87 (7.0; 10.7)	7.8 ± 0.83 (6.1; 9.5)
	EXP 258	9.2 ± 1.23 (6.5; 11.8)	6.3 ± 0.61 (5.0; 7.7)	7.0 ± 0.84 (5.2; 8.8)
Total offspring (#)	Rheintaler	48.6 ± 5.36 (37.4; 59.9)	30.5 ± 2.22 (25.6; 35.4)	58.9 ± 5.61 (47.0; 70.8)
	Tasty Sweet	90.6 ± 7.53 (74.7; 106.4)	75.8 ± 10.55 (53.0; 98.6)	41.8 ± 5.27 (30.8; 52.9)
	ES-Eurojet	97.9 ± 8.52 (80.1; 115.7)	48.3 ± 6.90 (33.3; 63.3)	44.2 ± 5.24 (33.1; 55.3)
	Planoxx	75.4 ± 8.90 (56.7; 94.1)	59.1 ± 5.89 (46.5; 71.8)	51.4 ± 6.70 (37.3; 65.5)
	EXP 258	46.9 ± 7.95 (30.1; 63.7)	42.3 ± 4.81 (31.7; 52.8)	41.7 ± 5.49 (30.2; 53.3)
Offspring per clutch (#)	Rheintaler	5.1 ± 0.24 (4.6; 5.6)	7.0 ± 0.53 (5.8; 8.2)	7.4 ± 0.37 (6.6; 8.2)
	Tasty Sweet	6.2 ± 0.20 (5.8; 6.6)	7.7 ± 0.37 (6.9; 8.4)	6.2 ± 0.46 (5.2; 7.2)
	ES-Eurojet	7.3 ± 0.33 (6.6; 8.0)	8.4 ± 0.44 (7.4; 9.3)	6.2 ± 0.38 (5.4; 7.0)
	Planoxx	5.7 ± 0.24 (5.2; 6.2)	6.6 ± 0.21 (6.1; 7.0)	6.3 ± 0.35 (5.5; 7.0)
	EXP 258	4.6 ± 0.29 (4.0; 5.2)	6.4 ± 0.45 (5.4; 7.4)	5.7 ± 0.32 (5.1; 6.4)
R_0 (offspring / individual)[a]	Rheintaler	46.2 ± 5.57 (35.3; 57.1)	18.3 ± 3.57 (11.3; 25.3)	50.1 ± 6.56 (37.2; 62.9)
	Tasty Sweet	81.5 ± 8.88 (64.1; 98.9)	53.1 ± 10.48 (32.5; 73.6)	39.8 ± 5.30 (29.4; 50.1)
	ES-Eurojet	97.9 ± 8.23 (81.8; 114.0)	31.4 ± 6.74 (18.2; 44.6)	37.6 ± 5.47 (26.8; 48.3)
	Planoxx	71.7 ± 8.92 (54.2; 89.1)	44.4 ± 7.11 (30.4; 58.3)	48.9 ± 6.65 (35.8; 61.9)
	EXP 258	42.2 ± 7.62 (27.3; 57.1)	25.4 ± 5.40 (14.8; 35.9)	39.7 ± 5.44 (29.0; 50.3)
T (d)[a]	Rheintaler	38.6 ± 1.87 (35.0; 42.3)	21.5 ± 0.97 (19.6; 23.4)	23.6 ± 0.72 (22.2; 25.0)
	Tasty Sweet	32.6 ± 1.10 (30.4; 34.7)	26.4 ± 1.02 (24.4; 28.4)	23.6 ± 0.96 (21.7; 25.5)
	ES-Eurojet	31.1 ± 0.97 (29.2; 33.1)	20.7 ± 0.65 (19.5; 22.0)	23.2 ± 0.95 (21.3; 25.0)

	Planoxx	32.3 ± 1.11 (30.2; 34.5)	21.5 ± 0.75 (20.1; 23.0)	22.2 ± 0.89 (20.5; 24.0)
	EXP 258	32.2 ± 2.20 (27.9; 36.6)	20.9 ± 0.53 (19.8; 21.9)	22.5 ± 0.80 (21.0; 24.1)
r_m (d^{-1})[a]	Rheintaler	0.10 ± 0.0039 (0.092; 0.11)	0.13 ± 0.012 (0.11; 0.16)	0.17 ± 0.0074 (0.15; 0.18)
	Tasty Sweet	0.14 ± 0.0048 (0.13; 0.14)	0.15 ± 0.0098 (0.13; 0.17)	0.16 ± 0.0071 (0.14; 0.17)
	ES-Eurojet	0.15 ± 0.0036 (0.14; 0.15)	0.17 ± 0.012 (0.14; 0.19)	0.16 ± 0.0089 (0.14; 0.17)
	Planoxx	0.13 ± 0.0041 (0.12; 0.14)	0.18 ± 0.0099 (0.16; 0.20)	0.18 ± 0.0055 (0.16; 0.19)
	EXP 258	0.11 ± 0.0067 (0.10; 0.13)	0.16 ± 0.012 (0.13; 0.18)	0.16 ± 0.0058 (0.15; 0.17)

[a] SEs and CIs calculated based on n=10'000 bootstrap replicates.

Table S3. Life table parameters of *D. magna* (strain HU-HO-2) feeding on different feeding dose of algae (*Acutodesmus obliquus*) in ADAM and M4 medium for 21 days. For this preliminary experiment a different clone was used than in the main experiment. Total offspring and offspring per clutch were analysed using linear mixed effects models (LMER) with medium as fixed factor and feeding dose as random factor (lme4 package). Moltings to first offspring, first offspring time, total clutches, and individuals in first clutch were analysed by generalized linear mixed effects models (GLMER) assuming Poisson distribution with the same factors. Comparisons were analysed with Anova function using type III sum of squares (car package).

Medium	**Feeding dose** (mg Carbon)	**Moltings to first offspring** (#)	**First offspring time** (d)	**Individuals in first clutch** (#)	**Total clutches** (#)	**Total offspring** (#)	**Offspring per clutch** (#)
ADAM	**0.15**	6.4 ± 0.22	11.3 ± 0.18	5.2 ± 0.27	3.9 ± 0.08	23.4 ± 1.00	6.0 ± 0.21
	0.3	6.3 ± 0.16	11.4 ± 0.26	6.4 ± 0.35	4.4 ± 0.13	39.7 ± 2.11	9.0 ± 0.28
	0.6	5.6 ± 0.13	10.9 ± 0.10	7.5 ± 0.40	4.2 ± 0.08	36.8 ± 1.28	8.9 ± 0.26
M4	**0.15**	6.2 ± 0.25	11.2 ± 0.27	5.3 ± 0.56	3.9 ± 0.11	19.8 ± 1.31	5.2 ± 0.38
	0.3	6.1 ± 0.05	11.0 ± 0.00	6.7 ± 0.40	4.0 ± 0.00	33.8 ± 1.19	8.5 ± 0.30
	0.6	4.9 ± 0.16	11.1 ± 0.23	6.4 ± 0.68	4.3 ± 0.13	39.6 ± 1.55	9.2 ± 0.27
Statistics		GLMER $\chi^2 = 0.7$, $p = 0.4$	GLMER $\chi^2 = 0.02$, $p = 0.9$	GLMER $\chi^2 = 0.3$, $p = 0.6$	GLMER $\chi^2 = 0.03$, $p = 0.9$	LMER $\chi^2 = 3.3$, $p = 0.07$	LMER $\chi^2 = 2.0$, $p = 0.2$

Fig. S1. Photographs of *D. magna* after feeding on A) maize flour, B) maize leaves, or C) maize pollen. Note the different color of the gut for the different maize materials.

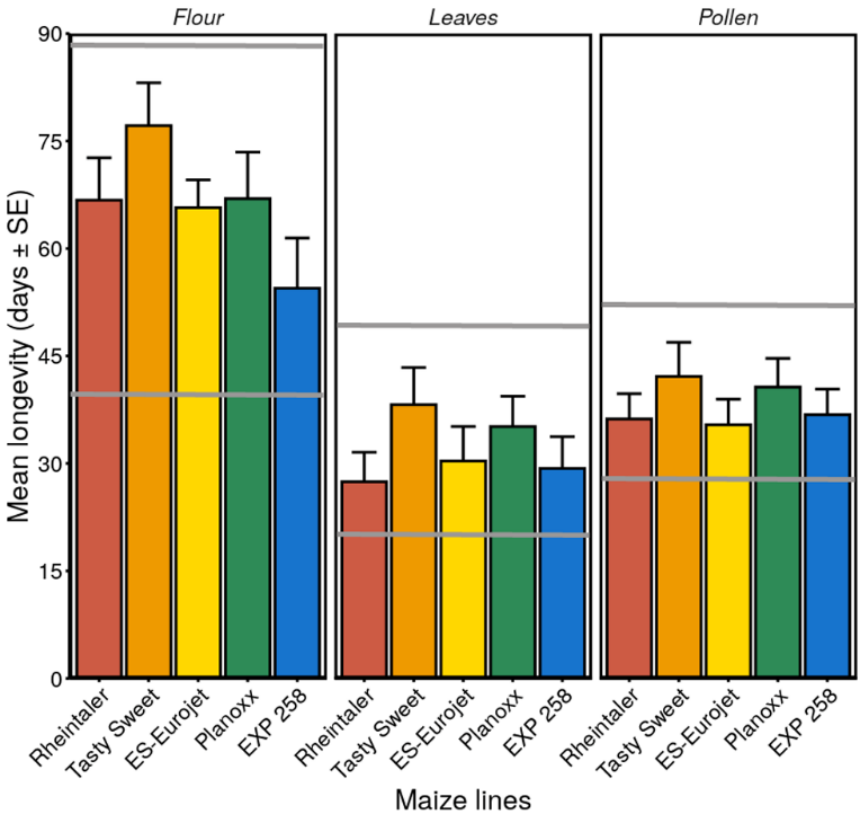

Fig. S2. Mean longevity of *D. magna* feeding on flour, leaves, or pollen from five lines of maize during their whole life time. Data were analysed for each food source separately using the Kaplan-Meier procedure with log-rank test. Differences among maize lines were not significant ($p \geq 0.1$). Bars represent means ± SE for each maize line (n=20). Grey lines illustrate the highest and lowest value of the 95% confidence intervals over all maize lines.

CHAPTER III

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

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 SmartStax maize that contains six *Bt* proteins 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.

Based on: Yi Chen, Jörg Romeis, Michael Meissle. Addressing the challenges of non-target feeding studies with genetically engineered plant material – SmartStax maize and *Daphnia magna*. Manuscript submitted.

1. Introduction

The development of genetically engineered (GE) crops is a major achievement in modern plant breeding. Among GE crops, *Bt* crops produce insecticidal Cry or VIP proteins from the bacterium *Bacillus thuringiensis* (*Bt*) 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).

Potential risks associated with *Bt* crops include adverse effects on biodiversity and ecosystem services (Mendelsohn *et al*., 2003; EFSA, 2010; Romeis *et al*., 2008; NASEM, 2016). For regulatory purposes, such risks are commonly assessed by exposing selected species to high doses of purified insecticidal proteins via artificial diet. Studies with plant material may also be conducted, however, 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 have 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), but 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 is 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 reported in studies with purified Cry1C protein (Chen *et al*., 2018a), maize pollen containing Cry1F or Cry1Ab (Mendelsohn *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*. In addition, 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). Another problem of non-target studies with plant material is that often only one *Bt* line and one non-*Bt* control were used. In such systems, it is impossible to separate effects of the *Bt* proteins from effects caused by other components in the plant background.

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 therefore determined the range of variation for five diverse non-*Bt* maize lines on *D. magna* performance (Chen *et al*., 2021).

While most previous non-target studies on aquatic organisms were conducted with *Bt* crops producing one insecticidal protein, stacked GE plants with multiple genes providing similar or different traits are increasingly grown in the field. The latest product that has become commercially available in the USA is SmartStax maize that expresses six insecticidal Cry proteins and two herbicide tolerance genes (ISAAA, 2018). SmartStax maize currently represents the GE plant that exposes non-target organisms to the largest amounts of insecticidal Cry proteins.

In the present study, we used the experimental protocol of Chen *et al*. (2021) to investigate the effects of SmartStax maize on the life table parameters of *D. magna*. By testing the SmartStax traits in two different plant backgrounds (EXP 258, EXP 262) and by using maize materials with different concentrations of *Bt* proteins (flour, leaves, pollen), we attempted to disentangle plant background effects from effects of the *Bt* proteins. The results are discussed in the context of the natural range of variation.

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 *cry1A.105*, *cry2Ab2*, *cry1F*, *cry3Bb1*, *cry34Ab1*, and *cry35Ab1*, genetic background EXP 258); 3) EXP 262; 4) SmartStax+RR (MON87427×SmartStax, expressing the same insecticidal proteins as SmartStax plus the herbicide-tolerance gene *epsps*, genetic background EXP 262); and 5) Rheintaler (Swiss landrace, population maize). All maize lines were planted on 23 April 2019 in a glasshouse according to Chen *et al*. (2021).

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).

ELISAs of maize foods from SmartStax and SmartStax+RR revealed that total Cry protein concentration was 8 to 10 times higher in leaves than in flour and was intermediate in pollen (Table 1, Supplemental Material B).

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

2.2 Chronic effects of maize materials on D. magna

The experiments were conducted with *Daphnia magna* (strain GB-EL75-69) that was originally obtained from Dieter Ebert, Zoological Institute of Evolutionary Biology, University of Basel (Switzerland). The species was 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 food suspension (ca. 0.15 mg of 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, and the experiment was conducted three times; a total of 450 *D. magna* were used.

Every other day, *D. magna* were moved to a new beaker 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 (RH), 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). In the stereo microscope, we could see the respective maize tissues in the gut of *D. magna* (Supplemental Material B, Fig. B.1). The experiment was terminated on day 50, when each individual was 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 for subsequent determination of Cry protein content using ELISA (Supplemental Material B).

Medium quality in the experiment was measured in each of the three repetitions (see Chen *et al*., 2021) 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 A, Table A.1).

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 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 value of the 95CIs and the highest value was used for the means of each parameter.

3. Results

Performance of D. magna on maize foods

D. magna was 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. Enzyme linked immunosorbent assays (ELISA) revealed much higher Cry protein amounts in leaves than in pollen or flour (Table 1).

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* 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).

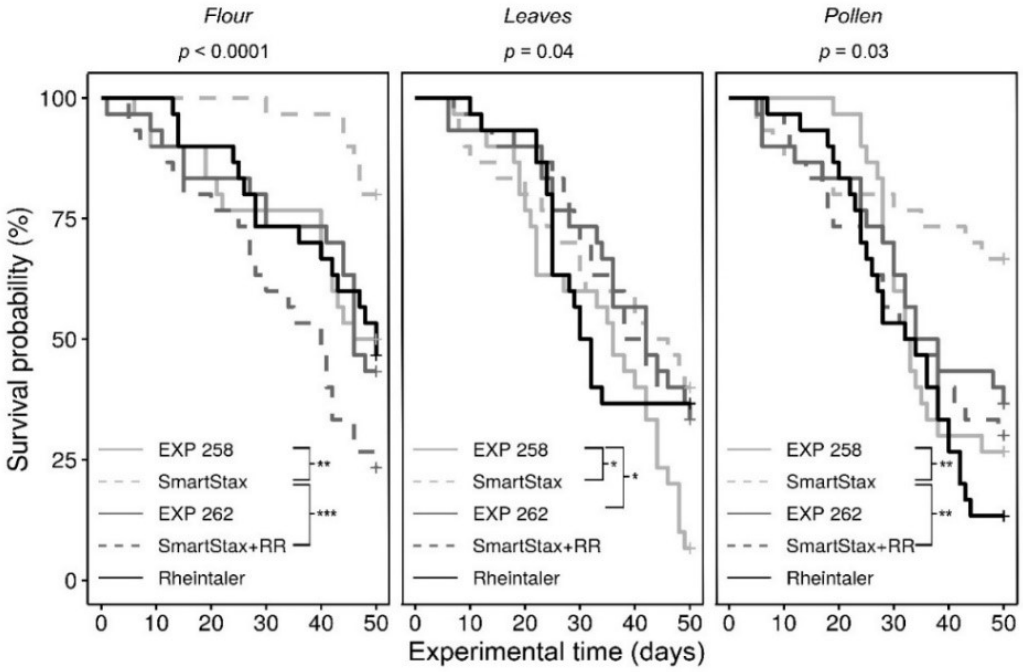

Fig. 1. Survival of *Daphnia magna* fed flour, leaves, or pollen from five maize lines (n = 30). Data from EXP 258, SmartStax, EXP 262, and SmartStax+RR were separately analysed for each food source using Kaplan-Meier estimates and log-rank tests. Asterisks indicate significant differences (* $p \leq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

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 A, Table A.2, Table A.3). In addition, details of the statistical analyses are available for flour (Table A.4), leaves (Table A.5), and pollen (Table A.6).

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, SmarStax+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).

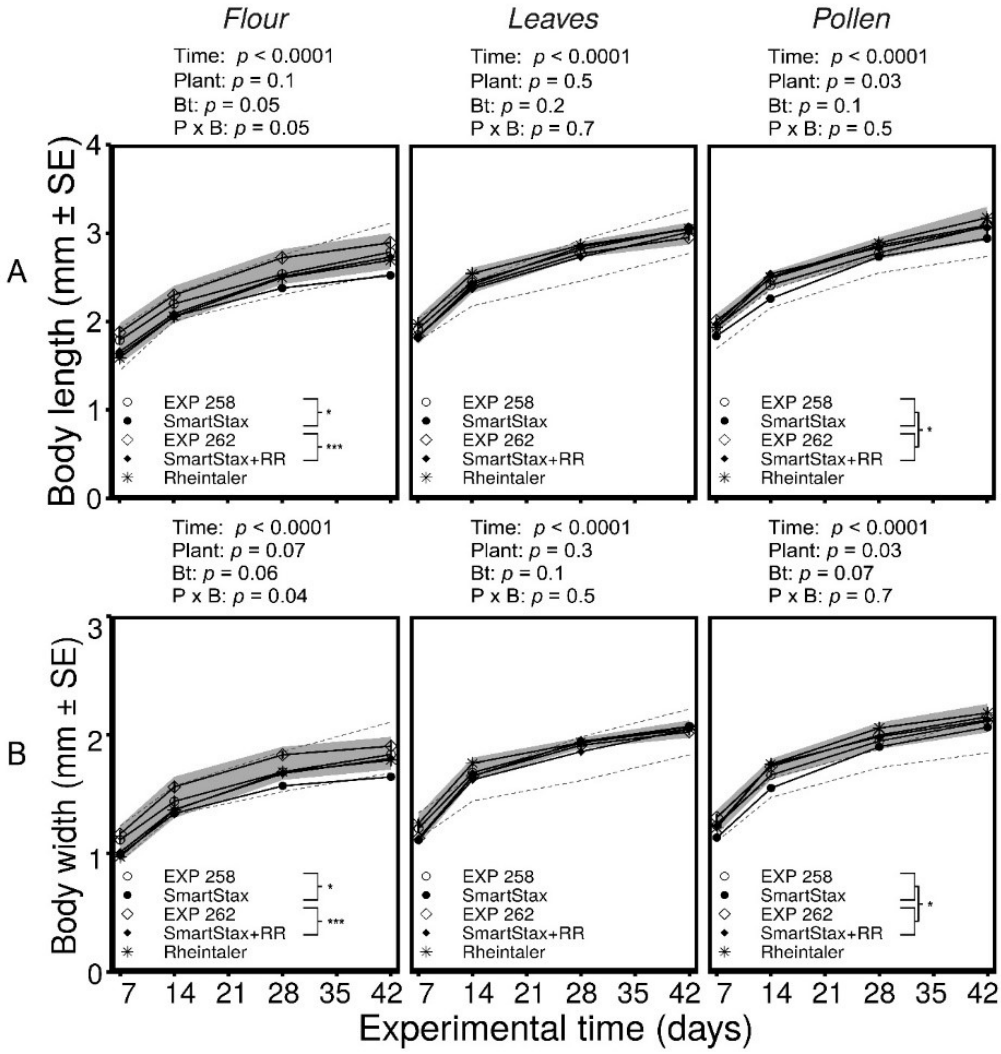

Fig. 2. Length (A) and width (B) of *Daphnia magna* fed flour, leaves, or pollen from five maize lines (n = 6 - 30). Measurements were taken at day 7, 14, 28, and 42. Data from EXP 258, SmartStax, EXP 262, and SmartStax+RR were analyzed using full factorial linear mixed effects models (LMER) with the 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.

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 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, the 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 were below both ranges of variation; 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.

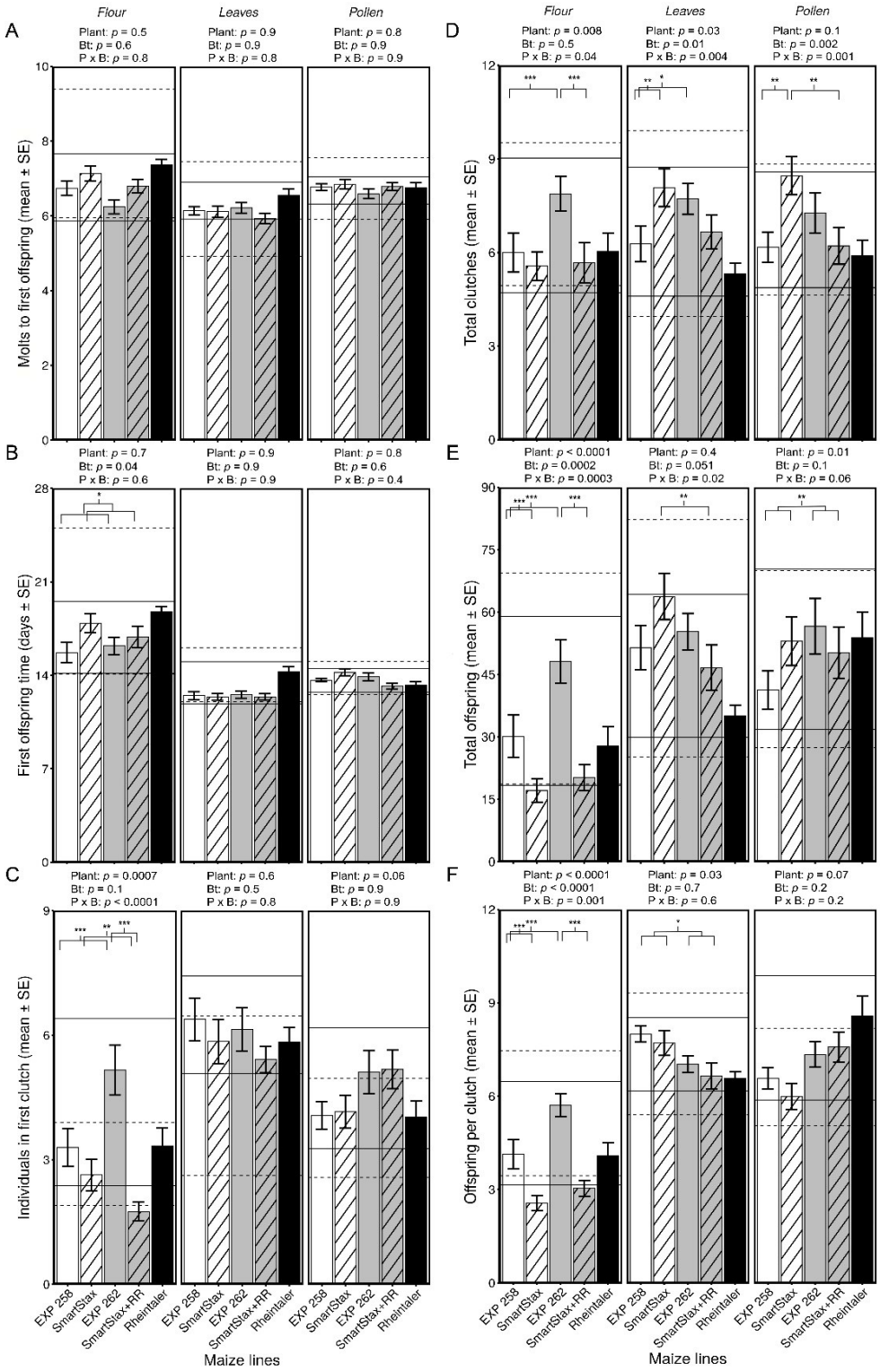

Fig. 3. Number of molts to first offspring release (A), time to first offspring release (B), number of individuals in the first clutch (C), total number of clutches (D), total number of offspring (E), and number of offspring per clutch (F) of *Daphnia magna* fed flour, leaves, or pollen from five maize 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.

4. Discussion

Consistent with a previous study (Chen *et al*., 2021), *D. magna* was 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 8- to 10-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 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 in our study 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 and 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 the Cry proteins used in the current *Bt* maize hybrids are known to be specific to the target orders of Lepidoptera and Coleoptera and direct toxic effects on *D. magna* are unexpected based on the known mode of action (Krogh *et al*., 2020; 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 data of a previous study that included five conventional maize lines (Chen *et al.*, 2021). 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 worst case exposure conditions. Although measured Cry protein concentrations in the food suspensions were lower than expected (based on the concentrations in lyophilized maize materials) and decreased further between feeding events (Supplemental Online Material B), we are confident that the leaf treatments in our study represent a worst case exposure situation for *D. magna* to Cry proteins. Because 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 processed directly into food suspensions, while maize debris in the field degrades over time, as shown by Tank *et al*. (2010), who measured 100 to 1000 times less Cry1Ab in maize debris collected in and around streams 6 months after harvest compared to fresh maize tissue; 3) *D. magna* was fed exclusively with maize materials, while the natural food spectrum likely contains low amounts of maize materials; and 4) new maize material was provided as food every 24 h.

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. Mendelsohn *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 that it is difficult to establish an optimal control. The transformation process and the following 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 was 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 in experiments in which the organisms are reared on suboptimal food, as in the present system with *D. magna* fed 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 summary, our study with the SmartStax traits in two plant backgrounds did not reveal consistent adverse effects of multiple *Bt* proteins 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 GE 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 demonstrated 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. The test materials should also be of high nutritional value for the test species to avoid nutritional stress, which may lead to confounding effects. As evident from our previous study (Chen *et al*., 2021), maize materials are not optimal food for *D. magna*.

5. Conclusion

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. If the GE plant and its comparator have different effects on a non-target species, plant background effects could be disentangled 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 and allow the definition of a range of natural variation, 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.

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Appendix A: *D. magna* life table parameters and statistical analysis

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

Table A.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) (LMER)	Time: $\chi^2 = 292.8$, $p < 0.0001$ 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) (LMER)	Time: $\chi^2 = 216.4$, $p < 0.0001$ 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 (#) (GLMER)	Plant: $\chi^2 = 0.01$, $p = 0.9$ *Bt*: $\chi^2 = 0.001$, $p = 0.9$ P x B: $\chi^2 = 0.08$, $p = 0.8$		
First Offspring Time (d) (GLMER)	Plant: $\chi^2 = 0.01$, $p = 0.9$ *Bt*: $\chi^2 = 0.005$, $p = 0.9$ P x B: $\chi^2 = 0.007$, $p = 0.9$		
Individuals in first clutch (#) (GLMER)	Plant: $\chi^2 = 0.2$, $p = 0.6$ *Bt*: $\chi^2 = 0.5$, $p = 0.5$ P x B: $\chi^2 = 0.06$, $p = 0.8$		
Total clutches (#) (GLMER)	Plant: $\chi^2 = 4.5$, $p = 0.03$ *Bt*: $\chi^2 = 6.2$, $p = 0.01$ P x B: $\chi^2 = 8.1$, $p = 0.004$	Bt^-: $\chi^2 = 4.3$, $p = 0.04$ Bt^+: $\chi^2 = 3.7$, $p = 0.06$	EXP 258: $\chi^2 = 6.2$, $p = 0.01$ EXP 262: $\chi^2 = 2.4$, $p = 0.1$
Total offspring (#) (LMER)	Plant: $\chi^2 = 0.6$, $p = 0.4$ *Bt*: $\chi^2 = 3.8$, $p = 0.051$ P x B: $\chi^2 = 5.6$, $p = 0.02$	Bt^-: $\chi^2 = 0.6$, $p = 0.4$ Bt^+: $\chi^2 = 6.0$, $p = 0.01$	EXP 258: $\chi^2 = 3.2$, $p = 0.08$ EXP 262: $\chi^2 = 2.4$, $p = 0.1$
Offspring per clutch (#) (LMER)	Plant: $\chi^2 = 4.6$, $p = 0.03$ *Bt*: $\chi^2 = 0.1$, $p = 0.7$ P x B: $\chi^2 = 0.2$, $p = 0.6$		

Table A.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) (LMER)	Time: $\chi^2 = 315.0$, $p < 0.0001$ 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) (LMER)	Time: $\chi^2 = 240.7$, $p < 0.0001$ 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 (#) (GLMER)	Plant: $\chi^2 = 0.06$, $p = 0.8$ *Bt*: $\chi^2 = 0.01$, $p = 0.9$ P x B: $\chi^2 = 0.081$ $p = 0.9$		
First Offspring Time (d) (GLMER)	Plant: $\chi^2 = 0.07$, $p = 0.8$ *Bt*: $\chi^2 = 0.4$, $p = 0.6$ P x B: $\chi^2 = 0.9$, $p = 0.4$		
Individuals in first clutch (#) (GLMER)	Plant: $\chi^2 = 3.5$, $p = 0.06$ *Bt*: $\chi^2 = 0.02$, $p = 0.9$ P x B: $\chi^2 = 0.002$, $p = 0.9$		
Total clutches (#) (GLMER)	Plant: $\chi^2 = 2.7$, $p = 0.1$ *Bt*: $\chi^2 = 9.5$, $p = 0.002$ P x B: $\chi^2 = 10.2$, $p = 0.001$	Bt^-: $\chi^2 = 2.6$, $p = 0.1$ Bt^+: $\chi^2 = 8.2$, $p = 0.004$	EXP 258: $\chi^2 = 9.4$, $p = 0.002$ EXP 262: $\chi^2 = 2.4$, $p = 0.1$
Total offspring (#) (LMER)	Plant: $\chi^2 = 6.0$, $p = 0.01$ *Bt*: $\chi^2 = 2.7$, $p = 0.1$ P x B: $\chi^2 = 3.4$, $p = 0.06$		
Offspring per clutch (#) (LMER)	Plant: $\chi^2 = 3.2$, $p = 0.07$ *Bt*: $\chi^2 = 1.9$, $p = 0.2$ P x B: $\chi^2 = 1.7$, $p = 0.2$		

Appendix B: Cry protein quantification: methods, results, and discussion

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). 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 B.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).

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 B.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.

Throughout the 48 h exposure period, the concentrations of most *Bt* proteins decreased (Table B.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 (Fig. B.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).

Table B.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)

Table B.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%

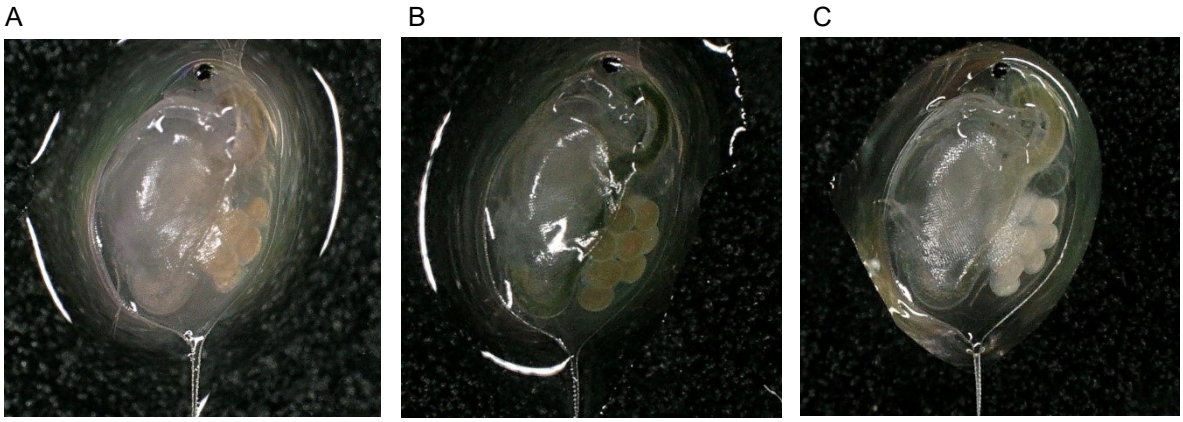

Fig. B.1. Photographs of *D. magna* after feeding on SmartStax maize A) flour, B) leaves, or C) pollen. Note the different color of the gut for the different maize materials.

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CHAPTER IV

No adverse effects of stacked *Bt* maize on the midge *Chironomus riparius*

Abstract: Material from genetically engineered (GE) maize producing insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*) may enter aquatic ecosystems and expose non-target organisms. In this study, we investigated effects of SmartStax maize leaves that contain six different Cry proteins targeting Lepidoptera and Coleoptera pests, in two plant backgrounds, on life table parameters of the midge *Chironomus riparius* (Diptera: Chironomidae). Using 95% confidence intervals for the means of the six conventional maize lines (Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258, EXP 262) for all measured parameters of *C. riparius* performance in our study, we captured the natural range of variation (NV), which allows to judge if observed effects between GE and non-GE maize are likely to be of biological relevance. No adverse effects on *C. riparius* were observed with both *Bt* maize lines compared to the respective non-*Bt* counterparts. Female development time was shorter when fed *Bt* maize than when fed non-*Bt* maize, but this effect was not considered adverse. All the parameters measured for *Bt* maize were within the natural range of variation. Future risk assessment studies may consider plant background effects and the natural range of variation to judge the relevance of observed differences between particular *Bt* and non-*Bt* plants.

Based on: Yi Chen, Jörg Romeis, Michael Meissle. No adverse effects of stacked *Bt* maize on the midge *Chironomus riparius*. In preparation.

1. Introduction

Most insect-resistant transgenic crops that are grown today produce Cry proteins from the bacterium *Bacillus thuringiensis* (*Bt*) (ISAAA, 2019). In sensitive insects of the target orders Lepidoptera or Coleoptera, the Cry proteins bind to specific receptors in the midgut, lead to membrane perforation, eventually causing death (Vachon *et al*., 2012; Jurat-Fuentes and Crickmore, 2017). The advantage of *Bt* crops over conventional insecticides is their high specificity with minimal effects on non-target organisms (Romeis *et al*., 2019). While early *Bt* plants expressed one *cry* gene, many modern plants express multiple stacked genes that provide similar or different traits. One commercial product (in the USA) is SmartStax maize expressing 6 insecticidal Cry proteins and 2 herbicide tolerance genes (Head *et al.*, 2013).

The environmental risk assessment of *Bt* crops has focused on terrestrial non-target organisms (Romeis *et al*., 2019), whereas relatively few studies investigated potential effects on aquatic species in agricultural landscapes. *Bt* proteins from genetically engineered (GE) crops can enter water bodies through pollen deposition, rhizosphere secretion, post-harvest crop residues and other forms of diffusion, so that aquatic organisms are principally exposed (Carstens *et al.*, 2012; Chen *et al.*, 2013). Previous studies that tested plant material or extracts from *Bt* crops have indicated adverse effects on aquatic insects, such as caddisflies (Trichoptera) and midges (Diptera) (Chambers *et al.*, 2010; Jensen *et al.*, 2010; Li *et al*., 2013; Prihoda and Coats, 2008; Rosi-Marshall *et al*., 2007).

One difficulty of non-target studies using plant material as test substance, however, is that *Bt* protein effects cannot be separated easily from effects of the plant background. Appropriate control treatments should include similar amounts or concentrations of non-*Bt* plant material or extracts, ideally from the nearest non-transformed line (near isoline). Plant background effects are more likely if the *Bt* plant is a different variety than the non-*Bt* plant. But even if *Bt* and non-*Bt* controls are near-isolines, compositional differences may arise from the breeding steps necessary to regenerate the plant after transformation or from the transformation process itself. Ways to separate plant background effects from *Bt* effects include: 1) use of the same *Bt* trait in different plant backgrounds; 2) use of different transformation events with the same *Bt* protein (e.g., MON810 and Bt11, both expressing the *cry1Ab* gene); or 3) use of different plant tissues with different concentrations of the *Bt* protein (e.g., leaves and pollen) (Chen *et al*., submitted).

In general, performance of non-target species can differ substantially when fed different conventional varieties. For example, maize materials from different conventional

lines had different impacts on growth and reproduction of the water flea *Daphnia magna* (Cladocera: Pulicidae) (Chen *et al*., 2021). Differences among conventional lines, however, are generally not considered a risk for the environment. Therefore, the natural range of variation among conventional lines might allow to judge if differences between a *Bt* plant and its non-*Bt* comparator are of biological relevance (Chen *et al*., 2021).

Benthic macroinvertebrates have been used frequently to assess aquatic ecosystem integrity (Ferrari and Faburé, 2017). Species richness of Chironomidae is among the highest of aquatic insect families (Ferrington, 2008) and larvae represent an important part of macrozoobenthic communities. Non-biting midges of the genus *Chironomus* (Diptera: Chironomidae) have been used frequently for ecotoxicological testing, because several species can be reared relatively easily in the laboratory and their life-cycle is completed in a few weeks (Péry *et al*., 2002; Lopes *et al*., 2005). As holometabolic insects, *Chironomus* spp. undergo a full metamorphosis with distinct eggs, larval, pupal and adult stages (Bertin *et al*., 2014). For *Chironomus* species, several international validated guidelines are available for assessing the toxicity of chemicals in water (OECD, 2004b; OECD, 2010) and the toxicity of sediments (OECD, 2004a; OECD, 2010; EPA, 2000; ASTM, 2005). Because of these advantages, *Chironomus* spp. were recommended as aquatic test species for the risk assessment of insecticidal GE plants (Carstens *et al.,* 2012). We selected the European species *C. riparius* for the current study. During the aquatic larval stage, the species lives in muddy substrate and feeds mainly on fresh sediment-deposited detritus (Armitage *et al*., 1995).

Maize has a high biomass and produces a lot of wind-distributed pollen. It may thus contribute to a relatively high input of Cry-proteins to streams (Rosi-Marshall *et al.*, 2007; Carstens *et al*., 2012; Griffiths *et al*., 2009). Several studies indicate that *Bt* protein released from remnants of *Bt* maize can measured in water for several months (Tank *et al*., 2010; Douville *et al.,* 2007, 2009). In addition, *Bt* protein remaining in plant detritus may expose invertebrates feeding on larger particles (e.g., shredders) and ultimately those feeding on smaller particles (e.g., filter feeders, collector-gatherers), including *Chironomus* species (Rosi-Marshall *et al*., 2007; Chambers *et al*., 2010; Tank *et al*., 2010). We used *Bt* maize leaves for the current study, because leaves contain high amounts of *Bt* proteins. SmartStax maize was selected because it produces six different Cry proteins. SmartStax leaves thus represent a worst-case exposure of insecticidal transgene products that is currently available in one plant.

To our knowledge, effects of *Bt* crops on *Chironomus* species have only been tested with *C. dilutus* in acute toxicity tests lasting 4 - 10 days (Prihoda and Coats, 2008; Li *et al*., 2013). However, exposure of aquatic organisms to *Bt* proteins via food may last for

several weeks, albeit at relatively low concentrations. We thus conducted a one generation laboratory feeding study with *C. riparius* providing SmartStax leaves as exclusive food. To separate potential *Bt* effects from plant background effects, we used two plant backgrounds with the same set of Cry proteins (SmartStax) and the respective non-*Bt* controls. Differences in *C. riparius* response to the two *Bt* lines would indicate that effects may derive from the plant background rather than from the *Bt* trait. In addition, several conventional, unrelated lines were added. This allows to build a natural range of variation, which helps to interpret the biological relevance of potential effects between the *Bt* and non-*Bt* lines.

2. Materials and methods

2.1 Maize leaf powder

Eight lines of maize were used for the experiment: Rheintaler (Swiss landrace and population maize), Tasty Sweet (sweet maize), ES-Eurojet (early maturing durum maize), Planoxx (late maturing dent maize), EXP 258 (breeding line), SmartStax (event MON89034×TC1507×MON88017×DAS-59122-7, expressing cry1A.105, cry2Ab2, cry1F, cry3Bb1, cry34Ab1 and cry35Ab1, genetic background EXP 258), EXP 262 (breeding line), SmartStax+RR (MON87427×Smartstax, expressing the same insecticidal proteins as SmartStax plus the herbicide tolerance gene *epsps*, genetic background EXP 262). Rheintaler, Tasty Sweet, ES-Eurojet, and Planoxx were cultivated together in a glasshouse in 2018. The *Bt* lines and their non-*Bt* counterparts were grown in the same glasshouse, but one year later. For details on plant cultivation see Chen *et al*. (2021).

Leaves were collected from all maize lines and prepared according to Chen *et al*. (2021). In short, leaves from seven week old plants were lyophilized, ground to fine powder, and sieved through a 100 µm mesh. This particle size is suitable for *C. riparius* (Faria *et al*., 2007). The leaf powders were used to make suspensions with a concentration of 50 mg/mL using non-chlorinated water from the tap. The suspensions were stored in 2 mL aliquots at −20 °C.

2.2 Chironomus riparius culture

C. riparius were obtained from Innovative Environmental Services (IES) Ltd (Witterswil, Switzerland). Larvae used for the experiment originated from the culture maintained in our laboratory. *C. riparius* were cultured in two plastic trays (10 L) filled with 300 mL playground sand (particle size < 500 µm, sterilized by heating at 200 °C for 2 days) and 5 L non-chlorinated water in a climate chamber (20 °C, 70% RH, 16 h light / 8 h

dark). The trays were gently aerated with approximately two bubbles per second. Larvae were fed daily with 5 mL of a 50 mg/mL suspension of finely ground fish food (TetraMin, Tetrawerke, Melle, Germany). Emerging adults were retained using a breeding cage covering the culture (bugdorm, MegaView Science, Taichung, Taiwan, ca. 45 x 45 x 45 cm). Egg ropes were carefully collected from the culture and individually placed in 6 well plates (CELLSTAR 6 well multiwell plates, Greiner Bio-One, St. Gallen, Switzerland). The wells were filled with 10 mL water from the culture and covered with lids to prevent evaporation. First-instars (two days after hatching) were used to start the experiment.

2.3 Chronic effects of maize leaf powder on C. riparius

Each test vessel (720 mL jam glass, Müller+Krempel, Bülach, Switzerland; 14 cm height, 7.5 cm inner diameter) was filled with 450 mL non-chlorinated water and 80 mL playground sand (2 cm deep, according to guideline OECD233) (OECD, 2010). Vessels were covered with metal lids to prevent emerged midges from escaping. Each lid had a hole (0.8 cm diameter) through which a glass pipette was fitted. The pipettes were connected with silicone tubing to aeration pumps (APS 300, Tetra GmbH, Germany). After preparation of the test vessels, the sediment-water systems were left under gentle aeration (pipette tips 2 - 3 cm above the sediment layer, two bubbles per second) for 7 days. 20 first-instar *C. riparius* (two days after hatching) collected from the culture were introduced to each test vessel. During addition of the larvae to the test vessels and the following 24 h, aeration was stopped to allow the larvae to settle within the sediment (OECD, 2010). The experiment was set up with all eight maize lines (treatments) and 3 vessels per maize line (replicates). Each group of larvae was fed with 200 µL of the respective 50 mg/mL suspension per glass per day (0.5 mg per larvae per day; OECD, 2010). Left-over food suspensions were stored in the fridge (approximately 4°C) and used in the following days. As a control treatment, larvae in 3 additional vessels were fed suspension of TetraMin fish food. 100 mL overlying water from the test vessels were renewed every two days. The emerged midges were collected once per day and the sex was identified (males have plumose antennae and a thinner body posture than females; OECD, 2010). All individuals emerging from the 3 replicates of the same treatment were transferred into one breeding cage (bugdorm). The test vessels for larvae were observed for emerging adults until no more adults emerged over a period of two weeks. In the breeding cages the adults could swarm, mate and oviposit into 3 plastic dishes (11.5 x 11 x 5 cm) per cage, each filled with 250 mL non-chlorinated water and 50 mL sand. The overlying water of the dishes was renewed every two days. Egg ropes were collected from the dishes daily, placed individually in 6 well plates filled with 10 mL water from the dish,

and covered with lids to prevent evaporation. Egg ropes were kept for at least 6 days and the hatched larvae per egg rope were counted (OECD, 2010). The experiment stopped when the last female in the cages died. The experiment was repeated three times resulting in a total of 9 replicates per treatment. Experiments were conducted in a climate chamber (20 °C, 70% RH) under a 16 h light / 8 h dark cycle (intensity ca. 1000 lux) (OECD, 2010).

Development time for each gender (days), emergence ratio, sex ratio of fully emerged and alive adults (proportion of males), fecundity (number of egg ropes per cage divided by number of females in the cage), fertility (number of fertile egg ropes per cage divided by number of females in the cage), and the number of hatched larvae per egg rope were calculated (OECD, 2010).

2.4 Water quality analyses

For each experimental repetition, the quality of overlying water in one test vessel randomly chosen from each treatment was measured towards the end of the experiment to make sure the values were within the recommended range of guideline OECD233 (OECD, 2010). The pH value (FiveEasy pH meter FE20, Mettler-Toledo AG, Greifensee, Switzerland), total hardness (MColortest Total Hardness Test, Merck KGaA, Darmstadt, Germany), and dissolved oxygen concentration (DOC) (FiveGo F4 portable meter, Mettler-Toledo AG) were measured.

2.5 Quantification of Cry proteins

A 19-day-test was conducted with the same experimental conditions as the chronic experiments to obtain *Bt* maize fed *C. riparius* larvae, sediment samples, and water samples for the quantification of Cry proteins. The experiment included 3 maize lines (SmartStax, SmartStax+RR, EXP 262), with 6 replicates (test vessels) each. On day 19, 1 mL samples of overlying water were collected and stored at −80 °C. The glasses with sand and larvae were poured into a larger glass dish and all living larvae of each test vessel were picked with forceps, washed with tap water, dried on a paper towel, and pooled in 2 mL centrifuge tubes (10 - 12 larvae for *Bt* maize, 10 - 20 larvae for EXP 262 maize per tube). Each group of larvae was weighed on an electronic microbalance (MX5, Mettler-Toledo AG) and stored at −80 °C. Finally, after gently removing the overlying water, the detritus on the surface of the sand (referred to as sediment) was collected, lyophilized, weighed, and stored at −80 °C. This experiment was conducted twice with similar experimental conditions.

Because left-over food suspensions were stored in the fridge and used in the

following days, an additional experiment was set up to evaluate the degradation of Cry proteins in the fridge over 6 days. For this, food suspensions of SmartStax and SmartStax+RR were prepared as for the feeding experiments (2 mL aliquots with 50 mg/mL maize leaf powder, 3 replicates per maize line). Two samples of 40 µL each were taken immediately (day 0) and after 2, 4, and 6 days, and frozen at −80°C.

Concentrations of Cry proteins were determined with enzyme linked immunosorbent assays (ELISA), using commercial detection kits (PathoScreen Cry1Ab/Ac for Cry1A.105; Cry1F, Cry2A, Cry3Bb1, and Cry34Ab1, Agdia Inc., Elkhart, USA). In addition to water, sediment, and insect samples, also Cry concentrations in leaf powder and leaf suspension were measured. The protocol by Chen *et al*. (submitted) was followed. The proteins from the larvae, sediment, leaf powder and leaf suspension samples were extracted in 800 µL extraction buffer (PBST + 0.55% Tween-20) and a 3 mm tungsten carbide ball with a Tissue Lyser II (Qiagen, Hombrechtikon, Switzerland) at 30 Hz for 30 s. In the first repetition, the water samples were loaded directly on the ELISA plate. In the second repetition, water samples were lyophilized and resuspended in the same amount of extraction buffer to ensure that the samples are in the appropriate buffer when loaded to the ELISA plate.

After centrifugation (13.000 × g for 5 min at 4 ºC), the supernatants were taken. The samples of leaf powder needed to be diluted with extraction buffer: Cry1A.105 and Cry1F 20 x, Cry3Bb1 100 ×, Cry2Ab2 and Cry34Ab1 200 x. Purified Cry1A.105, Cry2Ab2, and Cry3Bb1 of certified quality were supplied by Bayer Crop Science (St Louis, USA), and Cry1F and Cry34Ab1 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 extraction buffer blanks were loaded per plate. After adding the samples and the appropriate enzyme conjugates to the precoated ELISA plates, the plates were incubated over night at 4 °C. Next day, the plates were washed with PBST, the colour substrate was added, and the absorbance (optical density) was measured at 620 nm using a plate reader (infinite 200, Tecan Group Ltd., Männedorf, Switzerland).

Standard curves were established based on a single rectangular hyperbola model. The concentrations of each Cry protein were calculated on the basis of the corresponding standard curve. The limits of detection (LOD) of the test, were calculated according to Chen *et al*. (submitted) based on buffer-only blanks of multiple ELISA plates of the same batch of ELISA kits.

2.6 Data analysis

Data were analyzed using R, version 4.0.2 (The R Foundation for Statistical Computing, Vienna, Austria). All data are presented as mean ± standard error (SE), unless otherwise indicated. Data from the control treatment (*C. riparius* fed exclusively with TetraMin fish food) were not included in the analyses.

Data were compared among the *Bt* maize lines and their respective controls (EXP 258 *vs*. SmartStax; EXP 262 *vs*. SmartStax+RR). Development time for each gender (days) was analyzed using nested generalized linear mixed effects models (GLMER) assuming Poisson distribution with plant background (EXP 258, EXP 262) and *Bt* (Bt^+, Bt^-) as fixed factors, each glass vessel as nesting factor, and experimental repetition as random factor (lme4 package). Emergence ratio and sex ratio of adults were analyzed by nested GLMER with binomial distribution with the same factors. Because all egg ropes collected in the experiment hatched, fecundity (number of egg ropes per female) and fertility (number of fertile egg ropes per female) were identical, further referred to as fecundity. Fecundity was analyzed with a generalized linear model (GLM) assuming Poisson distribution with plant background (EXP 258, EXP 262) and *Bt* (Bt^+, Bt^-) as factors. The number of hatched larvae per egg rope was analyzed using a linear mixed effects model (LMER) with plant background (EXP 258, EXP 262) and *Bt* (Bt^+, Bt^-) as fixed factors and experimental repetition as random factor. In all models, factor contrasts were set to orthogonal. Differences were considered significant at $p \leq 0.05$. When interactions between the factors plant background and *Bt* were significant in the overall analyses, separate analyses for both factors were conducted.

To assess whether the obtained means of the various parameters of SmartStax hybrids fell within the natural range of variation, a reference range of variation (NV) was calculated from the six conventional lines tested in parallel to the two *Bt* lines (i.e., Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258, EXP 262). For each of those maize lines, the 95% confidence interval (95CI) was calculated for each assessed parameter. The NV was then defined as the range from the lowest to the highest boundary of the 95CI (Chen *et al*. 2021).

For ELISA data, we worked with median concentrations and 95CI. Differences were considered significant for non-overlapping 95CI.

3. Results

3.1 Overlying water quality

All pH values of water collected towards the end of the experiment were between 7.9 and 8.2; DOC values were between 6.2 mg/L and 10.5 mg/L; the hardness values were between 120 mg/L and 170 mg/L (Table S1). All values were within the range demanded in OECD233 (OECD, 2010), i.e. pH 6 - 9, DOC > 5.46 mg/L and total hardness < 400 mg/L.

3.2 Performance of C. riparius in the control treatment

When *C. riparius* was fed with Tetra-Min fish food, the first adults emerged on day 15, and the last on day 31. All introduced larvae emerged as adults. The sex ratio (proportion of males) was 0.51 ± 0.03. The mean development time was 21.1 ± 0.37 days for females and 19.5 ± 0.49 days for males. Fecundity was 0.95 ± 0.08, and the mean number of hatched larvae per egg rope was 236.6 ± 30.09 (Table S2).

3.3 Performance of C. riparius when fed maize leaves

Mean values and 95CI of the life table parameters of *C. riparius* fed leaves from the eight maize lines are presented in the supplemental online material (Table S2).

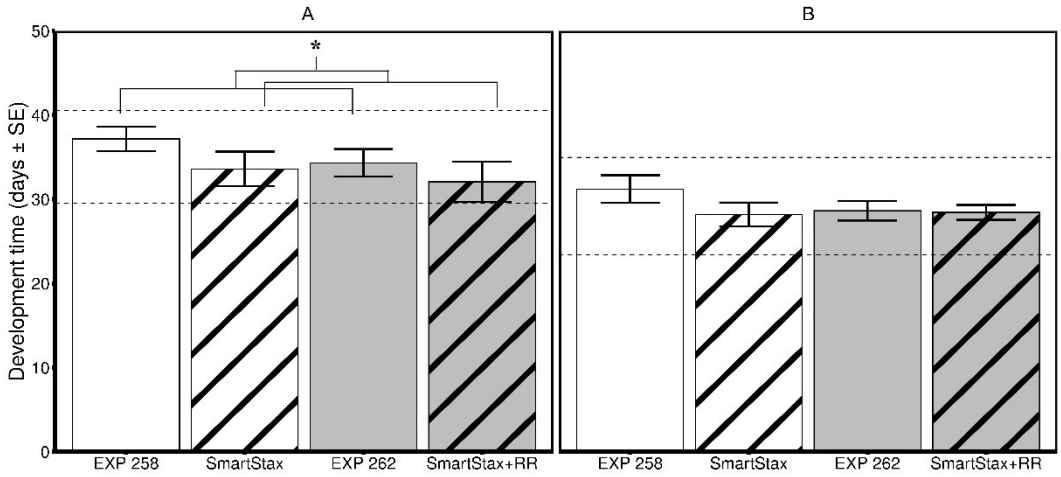

Fig. 1. Female (A) and male (B) development time of *Chironomus riparius* fed maize leaves from *Bt* maize (SmartStax, SmartStax+RR) and respective controls (EXP 258, EXP 262). Dashed lines illustrate the natural range of variation from six conventional maize lines (Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258, EXP 262) (n = 9). Asterisks indicate significant differences (p < 0.05).

In the following, analyses for the two *Bt* lines and their corresponding control lines representing two different plant backgrounds are presented. The female development time was significantly affected by the factor *Bt* (χ^2 = 4.4, p = 0.04), but not by the factor plant background (χ^2 = 2.5, p = 0.1). The interaction of both factors was not significant (χ^2 = 0.2, p = 0.7). *C. riparius* females emerged earlier on the two *Bt* lines when compared to the non-*Bt* comparators (Fig. 1A). The NV for female development time was between 29.5 and 40.5 days. The male development time was not affected by the factors *Bt* (χ^2 = 3.3, p = 0.07) or plant background (χ^2 = 2.1, p = 0.1) and there was no interaction (χ^2 = 1.4, p = 0.2) (Fig. 1B). The NV for the male development time was between 23.4 and 35.0 days.

The emergence ratio was not affected by *Bt* (χ^2 = 2.8, p = 0.09) or plant background (χ^2 = 1.9, p = 0.2) in the main analysis, but the interaction of *Bt* and plant background was significant (χ^2 = 4.7, p = 0.03) (Fig. 2A). Subsequent separate analysis for each factor, however, did not show significant differences (all $p \geq 0.09$). The NV for the emergence ratio was between 0.86 and 1.00. No differences in the sex ratio of adults was observed for *Bt* (χ^2 = 0.9, p = 0.3) or plant backgrounds (χ^2 = 2.4, p = 0.1) and there was no interaction (χ^2 = 0.7, p = 0.4) (Fig. 2B). The NV for sex ratio ranged from 0.38 to 0.62.

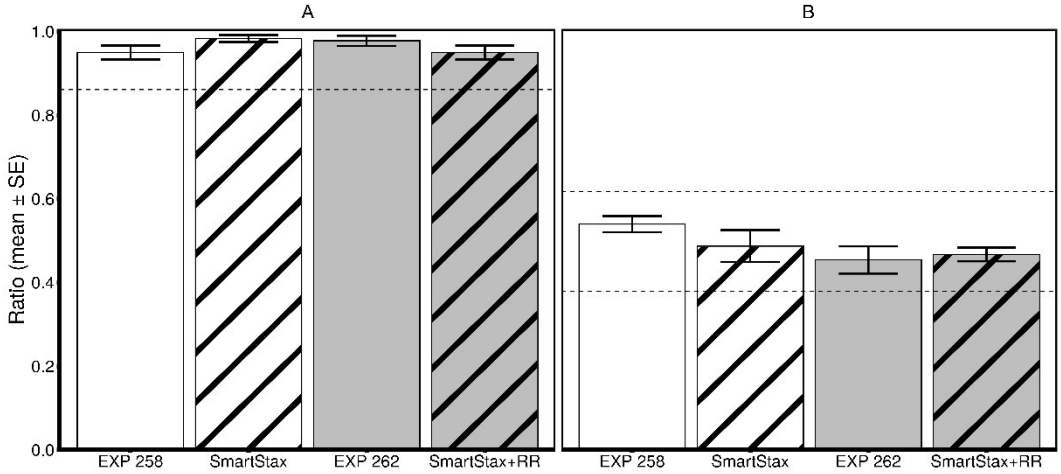

Fig. 2. Emergence (A) and sex (1 = all males) (B) ratio of *Chironomus riparius* fed maize leaves from *Bt* maize (SmartStax, SmartStax+RR) and respective controls (EXP 258. EXP 262). Dashed lines illustrate the natural range of variation from six conventional maize lines (Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258, EXP 262) (n = 9).

The fecundity was not affected by *Bt* (χ^2 = 2.3, p = 0.1) and plant background (χ^2 = 0.02, p = 0.9) and there was no interaction (χ^2 = 0.04, p = 0.8) (Fig. 3A) with a NV between 0.01 and 0.67. The number of hatched larvae per egg rope was also not affected by *Bt* (χ^2 = 0.3, p = 0.6), plant background (χ^2 = 1.2, p = 0.3), or interaction (χ^2 = 2.0, p = 0.2) (Fig. 3B). The NV for the number of hatched larvae was between 100.3 and 347.2.

For all parameters, the values obtained for the two *Bt* maize lines were within the NV calculated from the 95CI of the six non-*Bt* maize lines (Table S2).

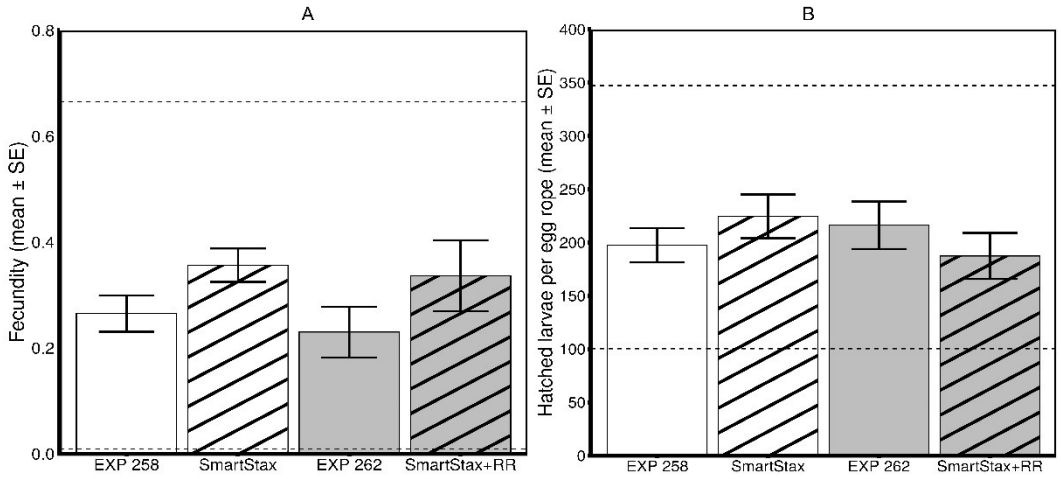

Fig. 3. Fecundity (number of egg ropes in a cage divided by the number of females in the cage) (A) and number of hatched larvae per egg rope (B) of *Chironomus riparius* fed maize leaves from *Bt* maize (SmartStax, SmartStax+RR) and respective controls (EXP 258, EXP 262). Dashed lines illustrate the natural range of variation from six conventional maize lines (Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258, EXP 262) (n = 3).

3.4 Cry protein content

The ELISA assay with maize leaves from SmartStax and SmartStax+RR revealed that the highest concentrations were measured for Cry3Bb1 and Cry34Ab1, and the lowest for Cry1F. SmartStax+RR leaves contained significantly more Cry1A.105 and Cry1F protein than SmartStax leaves (Table 1). No differences among the two *Bt* maize lines were evident for the other Cry proteins (non-overlapping 95CI).

The detected Cry proteins in leaf suspensions from two SmartStax lines of different time points showed that the toxin in the suspensions remained relatively stable over six days (Table S3). The percentages of Cry proteins measured on day 6 compared to day 0 ranged from 60% (Cry2Ab2, SmartStax) to 106% (Cry34Ab1, SmartStax+RR). Cry2Ab2 and Cry1F tended to degrade more (60 - 74%) than Cry1A.105, Cry3Bb1 and Cry34Ab1 (80 - 106%) (Table S3).

The concentrations of Cry proteins in overlying water from SmartStax and SmartStax+RR were all below the LOD of the ELISA assay. The LODs for each Cry

protein were: 0.8 ng/mL for Cry1A.105; 0.1 ng/mL for Cry1F; 0.02 ng/mL for Cry2Ab2; 0.1 ng/mL for Cry3Bb1; and 0.04 ng/mL for Cry34Ab1.

The concentration of Cry proteins in sediments from the SmartStax and SmartStax+RR treatments were highest for Cry1A.105, and lowest for Cry34Ab1 (Table 1). There were no significant differences among the two *Bt* maize lines.

The highest concentrations in *C. riparius* larvae fed *Bt* maize leaves were measured for Cry1A.105, followed by Cry2Ab2. There were no significant differences for the median concentrations of Cry1A.105 or Cry2Ab2 in larvae between SmartStax and SmartStax+RR. Concentrations for Cry1F, Cry3Bb1, and Cry34Ab1 were below the LOD of the ELISA assay (Table 1): 0.002 µg/g; 0.001 - 0.002 µg/g; 0.0006 µg/g, respectively.

Table 1. Cry protein concentrations in maize leaves, sediment and larvae from two stacked SmartStax hybrids. Data are presented as median ± 95CI (n = 21 for maize leaves; n = 12 for sediments; n = 24 for larvae). Values below the limit of detection (LOD) are presented as < LOD.

	Leaves[a]		Sediments[a]		Larvae[b]	
	SmartStax	**SmartStax+RR**	**SmartStax**	**SmartStax+RR**	**SmartStax**	**SmartStax+RR**
Cry1A.105	49.4 (43.0; 57.2)	85.9 (79.0; 89.5)	2.6 (1.4; 5.8)	2.8 (1.1; 5.2)	0.07 (0.06; 0.1)	0.1 (0.1; 0.2)
Cry1F	16.8 (15.7; 20.2)	29.9 (27.6; 33.0)	0.01 (0.007; 0.03)	0.02 (0.01; 0.08)	< 0.002	< 0.002
Cry2Ab2	71.5 (68.3; 83.3)	66.4 (62.2; 72.9)	0.1 (0.02; 0.3)	0.05 (0.006; 0.1)	0.0005 (0.0003; 0.0008)	0.0004 (0.0002; 0.001)
Cry3Bb1	94.7 (82.8; 113.9)	121.8 (106.7; 133.5)	0.04 (0.02; 0.1)	0.06 (0.03; 0.1)	< 0.002	< 0.001
Cry34Ab1	107.4 (98.0; 110.6)	108.2 (102.6; 113.4)	0.003 (0.001; 0.006)	0.002 (0.0005; 0.008)	< 0.0006	< 0.0006
Total	339.8	412.2	2.8	2.9	0.07	0.1

[a] µg/g dry weight
[b] µg/g fresh weight

The detected Cry proteins in sediments and larvae are low compared to the concentrations in leaves (Table S4). About 3 - 5% of Cry1A.105 in leaves were detected in sediments. For the other Cry proteins, the values were lower: Cry1F 0.06 - 0.07%, Cry2Ab2 0.08 - 0.1%, Cry3Bb1 0.04 - 0.05%, and Cry34Ab1 0.002 - 0.003%. Furthermore, concentrations in larvae were lower than in sediments: 3 - 4% for Cry1A.105 and 0.5 - 0.8% for Cry2Ab2 in fresh larvae compared to dry sediment samples (values for dry larvae are approximately 10 times higher, Kangur and Tuvikene, 1998).

No Cry proteins were detected in EXP 262 leaves, overlaying water, sediments, or *C. riparius* larvae fed with EXP 262 leaves.

4. Discussion

C. riparius fed exclusively on maize leaves can develop and reproduce. Despite Cry protein exposure, no adverse effects on life table parameters were evident when fed stacked *Bt* maize leaves compared to non-*Bt* maize leaves.

4.1 Experimental conditions

According to the guideline OECD233 (OECD, 2010), all measured values for water quality were within the recommend range.

In the control treatment with TetraMin, almost all *C. riparius* larvae (99%) that were introduced into the test vessels emerged until day 28 (OECD validity criteria: >70% emergence until day 28). Furthermore, 93% of the midges emerged between day 12 and day 23 (OECD: > 85% of emerging adults). The proportion of males was 0.51 (OECD: 0.4 - 0.6), the number of egg ropes for each breeding cages was 0.85 - 1.11 per female added to the breeding cage (OECD: > 0.6), and all egg ropes were fertile (OECD: > 0.6). The TetraMin treatment thus demonstrates that the experimental conditions were well suitable and the *C. riparius* larvae used for the experiment were healthy.

C. riparius can survive, grow and reproduce when fed only maize leaves. However, longer development time and reduced fecundity compared to the TetraMin control indicates that maize leaves are a suboptimal food for *C. riparius* causing nutritional stress. At day 28, the mean emergence ratio in the maize leaf treatments was 33 - 49% and thus below the 70% set by OECD. Between 17 and 26% of the adult midges emerged between day 12 and day 23 depending on the maize line. These values, however, were well below the validity criterion of 85% according to OECD. Similarly, the fecundity was relatively low (0.19 - 0.34) and remained below the validity criterion of 0.6. A similar result was found for *Daphnia magna* which had a smaller body size, a lag for reproduction, a reduced fecundity and a reduced intrinsic rate of increase, compared with the optimal food treatment (green algae) (Chen *et al*., 2021). Nutritional stress of test animals in feeding studies could lead to confounding effects, which warrants that results of such studies need to be treated with care.

4.2 Exposure of C. riparius to Cry proteins

The concentration of Cry proteins in maize leaves were similar to the results of Chen *et al*. (submitted), except for Cry1A.105 protein which showed lower values. For the current study, we used the leaf material collected by Chen *et al.* (2021; submitted) and made fresh leaf powder. Cry protein concentrations in food suspensions (leaf powder in water) stored in the fridge over 6 days remained relatively stable (60 - 100% of the Cry protein on day 0). Larvae of *C. riparius* build tubes in the sediment and feed on fresh detritus that is deposited on the sediment (Armitage *et al*., 1995). Compared to fresh leaf powder, sediment collected from the sand surface in our experiment contained only 0.7 - 0.8% of the total Cry protein. Interestingly, Cry1A.105 concentrations in sediment were much higher compared to the other Cry proteins (3.3 - 5% of the concentrations in leaf

powder). Lowest values were observed for Cry34Ab1 (0.002 - 0.003%). Concentrations of Cry1F, Cry2Ab, and Cry3Bb1 were in between (0.04 - 0.14%). This demonstrates different degradation dynamics of the different Cry proteins in the experimental water system, with lowest degradation of Cry1A.105 and highest of Cry34Ab1.

Similarly, Cry1A.105 showed highest concentrations in *C. riparius* larvae, followed by Cry2Ab2. Concentrations of the other Cry proteins were below the LOD. Our ELISA measurements thus demonstrate that *C. riparius* larvae ingested Cry proteins, but exposure was very low compared to the leaf material that was introduced to the test vessels. High dilution factors and fast degradation is typical for aquatic environments (Carstens *et al.*, 2012). It is further known that the concentrations of Cry proteins in arthropods are lower than in their food because of digestion and excretion (Svobodová *et al.*, 2017; Meissle *et al.*, 2021; Meissle and Romeis, 2018; Zhang *et al.*, 2017; Zhao *et al.*, 2016).

To judge the biological relevance of laboratory feeding studies, it is important to relate experimental exposure levels to realistic exposure in the field. Laboratory non-target risk assessment studies usually aim at creating worst-case exposure conditions to add a margin of safety to the assessment. Although the measured Cry protein contents in sediments and larvae were several orders of magnitude lower than in lyophilized maize leaves, we are still confident that our study represents a worst case Cry protein exposure scenario for *C. riparius*, because: 1) SmartStax maize is currently the plant with the most Cry proteins available; 2) maize leaves contained the highest Cry protein concentrations among maize materials (Chen *et al.*, submitted); 3) maize leaves were collected from green plants, lyophilized and processed directly to food suspensions, while in agricultural fields, maize debris, which would normally enter streams, would be degraded to some extent (Tank *et al.*, 2010); 4) the stream environment exhibits constant physical abrasion due to water flow as well as diverse invertebrate and microbial activities, which may lead to faster degradation than in our experimental study (Jensen *et al.*, 2010); 5) *C. riparius* was fed exclusively with maize leaves in this study, while in streams maize debris will likely represent only a small fraction of their diet; and 6) new maize leaves were provided every 24 h to ensure constant exposure to fresh material.

4.3 Effects of SmartStax maize on C. riparius

Female development time in our study was the only parameter where a significant difference was observed for the two *Bt* maize lines compared to the non-*Bt* comparators. Female *C. riparius* fed with SmartStax or SmartStax+RR maize leaves needed less time to become adult, so the effect was not adverse. One indication for the hypothesis that

SmartStax maize might affect *C. riparius* comes from the literature, where reports exist that Cry1 and Cry2 class proteins may show toxicity against Diptera species, such as *Aedes aegypti* (Cry1Ab, Cry1Ca, Cry2Ag), *Glossina morsitans* (Cry1Ac), *Musca domestica* (Cry1Ba), *Anopheles gambiae* (Cry1Ca), or *Culex quinquefasciatus* (Cry1Ca) (van Frankenhuyzen, 2013).

In addition, studies with *Bt* maize-derived test material have reported putative effects on aquatic insects. When *Lepidostoma liba* (Trichoptera: Lepidostomatidae) caddisflies fed conditioned leaf discs of field-collected *Bt* maize (containing Cry1Ab) for 29 days, slower growth was observed compared to non-*Bt* maize (Chambers *et al*., 2010; Rosi-Marshall *et al.*, 2007). When another caddisfly species, *Helicopsyche borealis* (Trichoptera: Helicopsychidae), was fed algal biofilms and Cry1Ab containing maize pollen for 18 days, no effects on mortality were observed at the mean daily aerial input rates that were measured by the authors in the field. Increased mortality, however, was observed at pollen concentrations two to three times higher than maximum aerial input rates (Rosi-Marshall *et al.*, 2007). In both studies, the used *Bt* and non-*Bt* maize varieties were either unrelated or not specified. Jensen *et al.* (2010) also fed caddisflies with conditioned *Bt* maize material for 30 days. *Lepidostoma* spp. showed no difference in head capsule growth and dry mass after feeding on non-*Bt* maize, Cry1Ab containing maize, or stacked Cry1Ab + Cry3Bb1 containing maize of the same plant background (near-isolines). Another species, *Pycnopsyche scabripennis* (Trichoptera: Limnephilidae), even had a higher final dry mass when fed stacked *Bt* maize compared to Cry1Ab containing maize, or non-*Bt* maize (Jensen *et al.*, 2010). When larvae of the crane fly *Tipula abdominalis* (Diptera: Tipulidae) were fed the same three maize lines for 30 days, reduced growth was observed in the Cry1Ab treatment compared to the non-*Bt* control, but not in the stacked maize treatment (Jensen *et al*., 2010). In another study, the benthic detritus feeding midge *Chironomus dilutes* (Diptera: Chironomidae) was exposed to Cry3Bb1 containing maize root extracts mixed with fish food flakes at nominal concentrations of 17, 30, and 48 ng/mL for 10 days. Survival was lower in the 30 and 48 ng/mL treatments compared to the 17 ng/mL treatment and a water-only control, while growth was unaffected (Prihoda and Coats, 2008). It remains unclear if the observed effect was caused by the Cry3Bb1 protein or by other compounds present in the root extract, because no treatments with non-*Bt* root extracts were included in the study. In acute tests with sediment (10 days) or water (4 days) spiked with cotton seed extract containing Cry1Ac, the median lethal concentration (LC_{50}) for *C. dilutus* was 155 ng/g dry weight and 201 ng/mL, respectively (Li *et al*., 2013). Although one control treatment with sediment or water spiked with non-*Bt* cotton seed extract was included, the amount of seed extract in the control compared to the amounts

in the *Bt* treatments was not specified. It can thus not be excluded that effects observed where caused by the increase in the amount of seed extract and not by the Cry proteins *per se*. In any case, the estimated LC_{50} concentrations were several orders of magnitude higher than concentrations detected in the field and in aquatic environments.

In general, previous studies with aquatic organisms often lack important study design requirements of non-target toxicity studies, such as well characterized test substances, confirmed exposure, or appropriate controls (Romeis *et al*., 2013). In particular studies with plant material bear the risk that differences in plant composition overlay effects of the introduced *Bt* proteins. One way to separate plant background effects from *Bt* protein effects is to include the *Bt* trait in multiple backgrounds. When expression levels are similar among the different backgrounds, also *Bt* effects should be similar. In our study, however, no adverse effects were observed with any of the two *Bt* lines.

4.4 Natural range of variation

Another way of judging the biological relevance of observed effects among two particular maize lines is to look at the variation among a range of different maize lines that had been bred conventionally and are therefore not seen as posing a potential risk to non-target species. A similar approach had been applied in the compositional equivalence studies that support food/feed safety assessment of GE plants (Anderson *et al.*, 2019, 2020).

In the current study, we included six different non-*Bt* maize lines, Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258 and EXP 262. The natural range of variation (NV) was built using 95% confidence intervals (Chen *et al*. 2021). The range gives an indication how variable *C. riparius* performance could be when fed with non-GE maize leaves. In our study, all parameters for *C. riparius* fed with SmartStax and SmartStax+RR were within the range. It has to be noted, however, that the calculated confidence intervals were very broad for parameters with a low sample size of N=3 (fecundity and larvae per egg rope), which indicates that this method may only be informative if a certain number of conventional maize lines is included and the sample size allows a relatively precise estimate of variation for each maize line.

5. Conclusions

Our one-generation laboratory test with *C. riparius* revealed no adverse effects of stacked *Bt* maize in two plant backgrounds compared to non-*Bt* maize on development time, emergence ratio, sex ratio, and fecundity. Furthermore, all parameters measured for *Bt* maize lines were within the estimated natural range of variation. We thus conclude that exposure to *Bt* maize debris poses no risk for *C. riparius*.

Acknowledgments

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Table S1. Water quality parameters during the feeding experiment with *Chironomus riparius*: pH value; dissolved oxygen concentration (DOC); total hardness. Treatments included a control (TetraMin fish food) and eight maize lines. Data are presented as mean ± SE (n = 3).

Variety	pH value	DOC (mg/L)	Total hardness (mg/L)
Rheintaler	8.1 ± 0.06	8.1 ± 0.08	160 ± 10.0
Tasty Sweet	8.1 ± 0.02	8.1 ± 0.72	147 ± 12.0
ES-Eurojet	8.0 ± 0.03	8.7 ± 0.52	152 ± 1.7
Planoxx	8.1 ± 0.04	7.7 ± 0.78	140 ± 10.0
EXP 258	8.1 ± 0.02	7.0 ± 0.38	153 ± 7.3
EXP 262	8.1 ± 0.07	8.3 ± 0.60	137 ± 8.8
SmartStax	8.1 ± 0.05	7.2 ± 0.81	162 ± 1.7
SmartStax+RR	8.1 ± 0.02	7.8 ± 0.71	153 ± 3.3
TetraMin	8.1 ± 0.03	8.4 ± 1.08	158 ± 4.4

Table S2. Life table parameters of *Chironomus riparius* fed leaves from eight maize lines or TetraMin fish food. *Bt* maize lines are indicated in italics. Data are presented as means ± SE with the 95CI in parenthesis. The lowest and highest boundary values of the non-*Bt* maize lines (bold) represent the natural range of variation.

Parameters	Maize lines	
Female development time (d)	Rheintaler	34.63 ± 1.63 (30.86; 38.40)
(n=9)	Tasty Sweet	31.80 ± 0.98 (**29.54**; 34.05)
	ES-Eurojet	33.47 ± 1.50 (30.01; 36.93)
	Planoxx	32.97 ± 1.37 (29.80; 36.13)
	EXP 258	37.18 ± 1.46 (33.82; **40.53**)
	EXP 262	34.33 ± 1.63 (30.58; 38.07)
	SmartStax	*33.61 ± 2.04 (28.90; 38.31)*
	SmartStax+RR	*32.08 ± 2.40 (26.54; 37.61)*
	TetraMin	21.10 ± 0.37 (20.24; 21.96)
Male development time (d)	Rheintaler	29.65 ± 1.53 (26.12; 33.18)
(n=9)	Tasty Sweet	26.22 ± 0.99 (23.94; 28.50)
	ES-Eurojet	26.75 ± 1.45 (**23.41**; 30.09)
	Planoxx	26.40 ± 0.96 (24.18; 28.63)
	EXP 258	31.20 ± 1.63 (27.44; **34.97**)
	EXP 262	28.64 ± 1.16 (25.96; 31.32)
	SmartStax	*28.19 ± 1.42 (24.92; 31.45)*
	SmartStax+RR	*28.44 ± 0.90 (26.36; 30.51)*
	TetraMin	19.48 ± 0.49 (18.36; 20.60)
Emergence ratio	Rheintaler	0.97 ± 0.012 (0.94; 0.99)
(n=9)	Tasty Sweet	0.97 ± 0.015 (0.94; 1.00)
	ES-Eurojet	0.92 ± 0.026 (**0.86**; 0.98)
	Planoxx	0.93 ± 0.015 (0.89; 0.96)
	EXP 258	0.95 ± 0.017 (0.91; 0.99)
	EXP 262	0.98 ± 0.012 (0.95; **1.00**)
	SmartStax	*0.98 ± 0.0083 (0.96; 1.00)*
	SmartStax+RR	*0.95 ± 0.017 (0.91; 0.99)*
	TetraMin	1.00 ± 0.00 (1.00; 1.00)
Sex ratio	Rheintaler	0.51 ± 0.047 (0.40; **0.62**)
(n=9)	Tasty Sweet	0.50 ± 0.027 (0.44; 0.56)
	ES-Eurojet	0.51 ± 0.025 (0.46; 0.57)
	Planoxx	0.52 ± 0.026 (0.46; 0.58)
	EXP 258	0.54 ± 0.019 (0.50; 0.58)
	EXP 262	0.45 ± 0.032 (**0.38**; 0.53)
	SmartStax	*0.49 ± 0.038 (0.40; 0.57)*
	SmartStax+RR	*0.47 ± 0.016 (0.43; 0.50)*
	TetraMin	0.51 ± 0.030 (0.44; 0.58)
Fecundity	Rheintaler	0.22 ± 0.018 (0.14; 0.30)
(n=3)	Tasty Sweet	0.28 ± 0.052 (0.053; 0.50)
	ES-Eurojet	0.19 ± 0.033 (0.048; 0.33)
	Planoxx	0.34 ± 0.076 (**0.0089**; **0.67**)
	EXP 258	0.26 ± 0.034 (0.12; 0.41)
	EXP 262	0.23 ± 0.048 (0.023; 0.44)
	SmartStax	*0.36 ± 0.032 (0.22; 0.49)*
	SmartStax+RR	*0.34 ± 0.067 (0.047; 0.63)*
	TetraMin	0.95 ± 0.083 (0.60; 1.31)
Larvae per egg rope	Rheintaler	141.64 ± 9.62 (**100.25**; 183.04)
(n=3)	Tasty Sweet	204.25 ± 13.28 (147.10; 261.40)

ES-Eurojet	225.51 ± 28.28 (103.84; **347.18**)
Planoxx	172.76 ± 4.71 (152.51; 193.01)
EXP 258	197.28 ± 16.06 (128.18; 266.37)
EXP 262	216.10 ± 22.21 (120.55; 311.66)
SmartStax	*224.46 ± 20.60 (135.84; 313.09)*
SmartStax+RR	*187.24 ± 21.60 (94.29; 280.19)*
TetraMin	236.59 ± 30.09 (107.13; 366.05)

Table S3. Cry protein concentrations (ng/mL) of leaf suspension from two SmartStax maize lines (SmartStax, SmartStax+RR) at different time points. Samples were taken at 0 d, 2 d, 4 d and 6 d. Data are presented as median ± 95CI for each time point (n=6 for each time point).

Cry protein	Time (d)	SmartStax	SmartStax+RR
Cry1A.105	0	1.44 (0.79; 2.58)	3.68 (3.26; 4.21)
	2	1.24 (0.69; 2.39)	3.56 (3.11; 3.99)
	4	1.25 (0.63; 2.69)	3.38 (3.13; 3.56)
	6	1.15 (0.77; 1.96)	3.70 (3.08; 3.90)
% day6 / day0		80%	100%
Cry1F	0	0.61 (0.39; 0.93)	1.32 (1.17; 1.48)
	2	0.50 (0.32; 0.82)	1.14 (1.04; 1.29)
	4	0.45 (0.25; 0.87)	1.06 (0.99; 1.11)
	6	0.37 (0.24; 0.64)	0.97 (0.91; 1.05)
% day6 / day0		61%	73%
Cry2Ab2	0	4.02 (2.20; 5.37)	4.21 (3.59; 4.97)
	2	2.86 (1.70; 4.51)	3.61 (3.06; 4.89)
	4	2.75 (1.80; 3.94)	3.25 (2.37; 3.98)
	6	2.43 (1.53; 3.24)	3.10 (2.49; 3.88)
% day6 / day0		60%	74%
Cry3Bb1	0	4.40 (2.72; 6.87)	7.36 (6.86; 8.01)
	2	4.24 (2.53; 6.89)	6.64 (6.23; 7.28)
	4	3.94 (2.72; 6.39)	6.38 (6.03; 6.87)
	6	4.32 (2.70; 6.03)	6.84 (6.32; 7.32)
% day6 / day0		98%	93%
Cry34Ab1	0	5.30 (3.69; 7.12)	5.86 (5.01; 7.60)
	2	5.23 (4.14; 6.26)	6.13 (5.62; 7.41)
	4	5.50 (3.58; 9.32)	6.83 (4.97; 10.00)
	6	5.01 (3.91; 5.52)	6.23 (5.84; 6.71)
% day6 / day0		95%	106%

Table S4. Percentage (%) of the detected concentrations of Cry proteins in sediments compared to leaves (A) and in larvae compared to sediments (B). Data are from Table 1 in the main manuscript.

Cry protein	A		B[a]	
	SmartStax	SmartStax+RR	SmartStax	SmartStax+RR
Cry1A.105	5%	3%	3%	4%
Cry1F	0.06%	0.07%		
Cry2Ab2	0.1%	0.08%	0.5%	0.8%
Cry3Bb1	0.04%	0.05%		
Cry34Ab1	0.003%	0.002%		

[a] Cry proteins in larvae were measured based on fresh weight, while sediment and leaf samples were measured based on dry weight. Assuming a dry matter content of 10% in larvae, the percentages based on dry matter of larvae can be estimated to be 10 times higher.

CHAPTER V

General conclusions and discussion

No risk of stacked Cry proteins produced in SmartStax maize to two aquatic arthropods, *D. magna* and *C. riparius*

Our feeding bioassays of *D. magna* (Chapter II) with three maize materials (flour, leaves, pollen) from five different maize lines showed that maize materials are suboptimal foods for *D. magna* causing nutritional stress and plant background effects affect the performance of *D. magna*. When fed only with maize materials, *D. magna* can survive, grow and reproduce, but showed a lower fitness in the life table parameters compared with the algae treatment (optimal food). Different maize lines and different maize materials have different effects on the performance of *D. magna*. The observed significant differences in *D. magna* life table parameters were more and the variability was higher in flour treatments than in the pollen and leaves treatments. This is likely because maize pollen and leaves were harvested from plants grown at the same time in the same glasshouse. On the contrary, maize flour was made directely from the original grains, which were produced in different fields and years around the world under different environmental conditions.

Since plant background effects appear to affect *D. magna*, we included the SmartStax traits in two plant backgrounds: EXP 258 (plant background for SmartStax) and EXP 262 (plant background for SmartStax+RR) when assessing the *Bt* toxin effects in Chapter III. 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 again affected by unidentified factors in the maize plant background. By including an in-study range of variation (IRV) of three non-transformed maize lines and an external range of variation (ERV) calculated from the data of five conventional maize lines (Chapter II) differences between *Bt* and non-*Bt* comparators could be interpreted. 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. Similar with the results in Chapter II, 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) in flour treatments rather than in leaf or pollen treatments. Leaf treatments in our study represent a worst-case exposure situation for *D. magna* to Cry proteins and the ELISA measurements revealed that concentrations of all Cry proteins were 8- to 10- times higher in leaf powder than in flour. So the effects observed in the flour treatments were likely not caused by the Cry proteins in the *Bt* maize materials.

The one-generation test using *C. riparius* (Chapter IV) revealed that SmartStax maize poses a negligible risk on this insect. Similar to the results in Chapter II, maize leaves were not optimal food for *C. riparius* causing nutritional stress compared with the TetraMin fish food treatment. The ELISA results demonstrated that *C. riparius* was exposed to Cry proteins for the larval stage, and ingested Cry proteins contained in leaves. All measured lifetable parameters of *C. riparius* in SmartStax maize lines (SmartStax, SmartStax+RR) were within the in-study natural range of variation. The only significant difference was observed for the female development time when fed with the two *Bt* maize lines and their respective non-*Bt* comparators. Female *C. riparius* fed with SmartStax or SmartStax+RR maize leaves needed significant shorter development time than those fed with the two non-*Bt* comparator maize lines, which was not an adverse effect. So *Bt* proteins have no effect on the *C. riparius* even when provided in combinations of six toxins.

Implications for risk assessment

With the rapid development of gene technology, GE crops have been grown on steadily increasing areas worldwide (ISAAA, 2019). Among GE crops, *Bt* crops produce insecticidal Cry or VIP proteins that can not only control Lepidoptera or Coleoptera pests, but may also pose risks to the environment. The potential impact on aquatic ecosystems must be taken into account due to the fact that *Bt* proteins from *Bt* crops can be transferred to streams draining agricultural fields through secretions of the roots, the dispersion of pollen and the spread of crop residues after harvest (Carstens *et al.*, 2012; Chen *et al.*, 2013; Venter and Bøhn, 2016).

For regulatory purposes, the potential environmental risks are commonly assessed by exposing selected species to high doses of purified insecticidal proteins via artificial diet. When the purified protein studies cannot exclude the risks, or there are no suitable test systems with artificial diet, or the legislation specifically required, it is necessary to conduct studies with plant materials (Rose, 2007; EFSA, 2010; Romeis *et al*., 2011). Studies to assess the impact of *Bt* maize on aquatic organisms usually compared tissue from one *Bt* maize line to an conventional counterpart. The adverse effects that were

reported in some studies may be because of plant background differences but not the *Bt* protein itself (Romeis *et al.*, 2013). The studies in this thesis proved that the plant background effects exist and influenced the results in the non-target arthropods studies. Even if the relevant conventional counterpart closest to a given GE plant is selected as the comparator, the differeces in plant composition may still be significant due to the transformation process, the breeding steps and the production of the new GE trait. The effects reported in previous studies were likely caused by differences in the nutrional composition of the *Bt* and comparator non-*Bt* maize lines.

This thesis demonstrate how Cry protein effects can be separated from plant background effects in non-target studies of *Bt* plant material as the test substance. This can be done by including GE crops with several plant backgrounds in studies. If the effects between the GE and non-GE are inconsistent, it is likely due to different plant backgrounds. Alternatively, multiple materials from the same plants can be included in the study. *Bt* effects should correspond to the different Cry protein contents in different materials and be consistent.

Furthermore, this thesis also showed how effects that are detected can be judged for their biological relevance. By emphasizing the importance of study design to address plant background effects in non-target arthropods studies to to minimize the probability of erroneous results. In particular, considering the natural range of variation among conventional plant lines is of importance to interpret the obtained data and statistical differences of a particular GE / control pairing and to define whether they might be of biological relevance. It is feasible by obtaining data from historical references and/or from multiple unrelated conventional varieties in the experiments. A similar approach is followed for the food/feed risk assessment of GM plants (Anderson *et al.*, 2019, 2020).

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Declaration of consent

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Name/First Name: Chen Yi

Registration Number: 17-128-869

Study program: Interuniversity Doctoral Program in Organismal Biology

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Supervisor: Jörg Romeis

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