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Hazard/Risk Assessment

No Adverse Effects of Stacked *Bacillus thuringiensis* Maize on the Midge *Chironomus riparius*

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Abstract: Material from genetically engineered maize producing insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*) may enter aquatic ecosystems and expose nontarget organisms. We investigated the effects on life table parameters of the midge *Chironomus riparius* (Diptera: Chironomidae) of SmartStax maize leaves, which contain six different Cry proteins targeting Lepidoptera and Coleoptera pests, in two plant backgrounds. For midge development and emergence, 95% confidence intervals for the means of six conventional maize lines (Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258, and EXP 262), were used to capture the natural range of variation. For reproduction, lowest and highest means were used. The natural range of variation allows one to judge whether observed effects between *Bt* maize and the closest non-*Bt* comparator are likely to be of biological relevance. No adverse effects on *C. riparius* were observed with any *Bt* maize line compared with the respective non-*Bt* counterpart. Development time was shorter when females were fed *Bt* maize than when they were fed non-*Bt* maize, but this effect was not considered adverse. Development time, emergence ratio, sex ratio, and larvae/egg rope measured for Bt maize were within the natural range of variation. Fecundity for the Bt lines was equal to or higher than that for the conventional lines. Future risk assessment studies may consider plant background effects and the natural range of variation to judge the relevance of observed differences between particular genetically engineered and non-genetically engineered plants. *Environ Toxicol Chem* 2022;41:1078–1088. © 2022 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

Most insect-resistant transgenic crops that are grown today produce Cry proteins from the bacterium *Bacillus thuringiensis* (*Bt*; International Service for the Acquisition of Agri-biotech Applications, 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 and eventually cause death (Jurat-Fuentes & Crickmore, 2017; Vachon et al., 2012). The advantage of current *Bt* crops over conventional insecticides is their high specificity with minimal effects on nontarget organisms (Romeis et al., 2019). Whereas early *Bt* plants expressed one *cry* gene, many modern plants express multiple stacked genes that target similar or different pests. One commercial product (in the United States) is SmartStax maize, which expresses six insecticidal Cry proteins and two herbicide tolerance genes (Head et al., 2013).

The environmental risk assessment of *Bt* crops has focused on terrestrial nontarget organisms (Romeis et al., 2019), whereas relatively few studies have investigated potential effects on aquatic species in agricultural landscapes. The *Bt* proteins from genetically engineered crops can enter water bodies through pollen deposition, rhizosphere secretion, postharvest crop residues, and other forms of diffusion, so that aquatic organisms are principally exposed (Carstens et al., 2012; X. P. Chen et al., 2013). Concentrations of *Bt* proteins in aquatic ecosystems, however, are generally low compared with concentrations in plants. Nevertheless, some previous studies

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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 & Coats, 2008; Rosi-Marshall et al., 2007), and other aquatic nontarget species (Venter & Bøhn, 2016).

One difficulty of nontarget studies using plant material as test substance is that effects of the *Bt* protein cannot be separated easily from effects of the plant background. Another problem is that dosing is limited to the concentrations in plant tissue. For these reasons, nontarget studies to support environmental risk assessments are conducted with purified test substances provided in artificial diet whenever possible. However, studies with plant material might be warranted if artificial diet studies cannot exclude risks, if no test systems with artificial diet are available, or if *in planta* studies are required by legislation (European Food Safety Authority, 2010; Romeis et al., 2011; Rose, 2007). When studies with plant material are conducted, appropriate control treatments should include similar amounts or concentrations of non-*Bt* plant material or extracts, ideally from the nearest nontransformed line (near-isoline). Plant background effects are more likely if the *Bt* plant is a different variety than the non-*Bt* plant. However, even if *Bt* and non-*Bt* controls are near-isolines, compositional differences may arise from the steps necessary to regenerate and breed 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; Y. Chen et al., 2021a).

In general, performance of nontarget species can differ substantially when the organisms are 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; Y. Chen et al., 2021b). 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 one to judge whether observed differences between a *Bt* plant and its non-*Bt* comparator are of biological relevance (Y. Chen et al., 2021a, 2021b).

Benthic macroinvertebrates have frequently been used to assess aquatic ecosystem integrity (Ferrari & Faburé, 2017). The species richness of Chironomidae is among the highest of aquatic insect families (Ferrington, 2008), and larvae represent an important part of macrozoobenthic communities. Nonbiting midges of the genus *Chironomus* (Diptera: Chironomidae) have often been used for ecotoxicological testing, because several species can be reared relatively easily in the laboratory and their life cycle is completed in a few weeks (Lopes et al., 2005; Péry et al., 2002). As holometabolic insects, *Chironomus* spp. undergo a full metamorphosis with distinct egg, larval, pupal, and adult stages (Bertin et al., 2014). For *Chironomus* spp., several international validated guidelines are available for assessing the toxicity of chemicals in water (Organisation for Economic Co-operation and Development [OECD], 2004a, 2010) and the toxicity of sediments (ASTM International, 2005; OECD, 2004b, 2010; US Environmental Protection Agency [USEPA], 2000). Because of these advantages, *Chironomus* spp. were recommended as aquatic test species for the risk assessment of insecticidal genetically engineered plants (Carstens et al., 2012). We selected the European species *Chironomus riparius* for the present study. During the aquatic larval stage, the species lives in muddy substrate and feeds mainly on fresh sediment-deposited detritus (Armitage et al., 1995). The species is multivoltine and overwinters in the fourth larval stage (Groenendijk et al., 1998). In agroecosystems, larvae can thus be exposed to maize pollen as well as plant debris after harvest.

Maize produces several million wind-distributed pollen grains/plant over a flowering period of approximately 2 weeks (Uribelarrea et al., 2002), and the plants have a high biomass that is often left on the field when only cobs are harvested. Maize may thus contribute to a relatively high input of Cry proteins to streams (Carstens et al., 2012; Griffiths et al., 2009; Rosi-Marshall et al., 2007). Despite a generally fast degradation in the aquatic environment, several studies indicate that *Bt* protein released from remnants of *Bt* maize can be measured in water for several months (Douville et al., 2007, 2009; Tank et al., 2010). 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* spp. (Chambers et al., 2010; Rosi-Marshall et al., 2007; Tank et al., 2010). We used *Bt* maize leaves as test material for the present study, because leaves contain high amounts of *Bt* proteins compared with other maize tissues (Y. Chen et al., 2021a). SmartStax maize was selected because it produces six different Cry proteins. SmartStax leaves thus represent a realistic worst-case exposure scenario of insecticidal transgene products that is currently available in one plant.

To our knowledge, effects of *Bt* crops on *Chironomus* spp. have only been tested with *Chironomus dilutus* in acute toxicity tests lasting 4–10 days (Li et al., 2013; Prihoda & Coats, 2008). 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 their 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* traits. In addition, several conventional, unrelated maize lines were added. This allowed us to build a natural range of variation, which helps to interpret the biological relevance of potential differences between the *Bt* and non-*Bt* lines.

MATERIALS AND METHODS

Maize leaf powder

Eight lines of maize were used for the experiments: 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 the *Bt* genes *cry1A.105*, *cry2Ab2*, *cry1F*, *cry3Bb1*, *cry34Ab1*, and *cry35Ab1*, and the herbicide tolerance genes *pat* and *epsps*, genetic background EXP 258), EXP 262 (breeding line), SmartStax + RR (MON87427 × SmartStax, expressing the same genes as SmartStax plus another copy of the herbicide tolerance gene *epsps* with tissue-specific low-level expression in pollen, genetic background EXP 262). Rheintaler, Tasty Sweet, ES-Eurojet, and Planoxx were cultivated together in a glasshouse in 2018 (Y. Chen et al., 2021b). One year later at the same time of the year, all the *Bt* lines and their non-*Bt* counterparts were grown in the same glasshouse (Y. Chen et al., 2021a).

Leaves were collected from all maize lines and prepared according to Y. Chen et al. (2021b). In short, leaves from 7-week-old plants were lyophilized, ground to fine powder, and sieved through a 100-µm mesh. This particle size is suitable as food for *C. riparius* (Faria et al., 2007). The leaf powders were used to make suspensions with a concentration of 50 mg/ml using nonchlorinated water from the tap. The suspensions were stored in 2-ml aliquots at −20 °C.

C. riparius culture

Chironomus riparius were obtained from Innovative Environmental Services (Witterswil, Switzerland), and a culture in our laboratory was established. Larvae were cultured in two plastic trays (10 L) filled with 300 ml of playground sand (particle size less than 500 µm, sterilized by heating at 200 °C for 2 days) and 5 L of nonchlorinated water in a climate chamber (20 °C, 70% relative humidity, 16:8-h light:dark). The trays were gently aerated with approximately 2 bubbles/s. Larvae were fed daily with 5 ml of a 50-mg/ml suspension of finely ground fish food (TetraMin; Tetrawerke). Emerging adults were retained using a breeding cage covering the culture (Bugdorm; MegaView Science, ca. 45 × 45 × 45 cm). Egg ropes were carefully collected and individually placed in six-well plates (CELLSTAR 6-well multiwell plates; Greiner Bio-One). The wells were filled with 10 ml of water from the culture and covered with lids to prevent evaporation. First instars (2 days after hatching) were used to start the feeding experiments with maize leaves.

Chronic effects of maize leaf powder on C. riparius

Experiments were conducted in a climate chamber (20 °C, 70% relative humidity) under a 16:8-h light:dark cycle (light intensity ~1000 lux; OECD, 2010). Test vessels (720-ml jam glass; Müller + Krempel; 14-cm height, 7.5-cm inner diameter) were filled with 450 ml of nonchlorinated water and 80 ml of playground sand (2 cm deep), according to OECD (2010) test guideline 233. Vessels were covered with metal lids to prevent evaporation and emerged midges from escaping. Each lid had an 8-mm opening through which a glass pipette was fitted. Pipettes were connected with silicone tubing to aeration pumps (APS 300; Tetra). After preparation of the test vessels, the sediment–water systems were left under gentle aeration (pipette tips 2–3 cm above the sediment layer, ~2 bubbles/s) for 7 days. Then 20 first-instar *C. riparius* (2 days after hatching) 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 three vessels/maize line (replicates). Each group of larvae was fed with 200 µl of the respective 50-mg/ml maize leaf suspension/glass/day (0.5 mg/larva/day; OECD, 2010). Leftover food suspensions were stored in the refrigerator (~4 °C) and used in the following days. As a control treatment, larvae in three additional vessels were fed suspensions of TetraMin fish food. Every 2 days, 100 ml of overlying water from the test vessels were renewed. Emerged midges were collected once a day, and the sex was identified (males have plumose antennae and a thinner body posture than females; OECD, 2010). All individuals emerging from the three 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 2 weeks. In the breeding cages the adults could swarm, mate, and oviposit into three plastic dishes (11.5 × 11 × 5 cm) in each cage, each one filled with 250 ml of nonchlorinated water and 50 ml of sand. The overlying water of the dishes was renewed every 2 days. Egg ropes were collected from the dishes daily, placed individually in six-well plates filled with 10 ml of water from the dish, and covered with lids to prevent evaporation. Egg ropes were kept for at least 6 days and the hatched larvae/egg rope were counted (OECD, 2010). The experiment was stopped when the last females in the cages had died.

Development time for each sex (days), emergence ratio, sex ratio of fully emerged and alive adults (proportion of males), fecundity (number of egg ropes/cage divided by number of females in the cage), fertility (number of fertile egg ropes/cage divided by number of females in the cage), and number of hatched larvae/egg rope were recorded (OECD, 2010). The experiment was repeated three times, resulting in a total of nine replicates/treatment for developmental parameters and three replicates for reproduction parameters.

Water quality analyses

Toward the end of each experimental repetition, the quality of overlying water in one test vessel randomly chosen from each treatment was measured to make sure the values were within the recommended range of OECD (2010) test guideline 233. The pH value (FiveEasy pH meter FE20; Mettler-Toledo),

total hardness (MColortest Total Hardness Test; Merck), and dissolved oxygen concentration (DOC; FiveGo F4 portable meter; Mettler-Toledo) were measured.

Quantification of Cry proteins

An additional 19-day test was conducted under the same experimental conditions as the chronic feeding experiments to obtain *Bt*-maize-fed *C. riparius* larvae, sediment samples, and water samples for the quantification of Cry proteins. The experiment included three maize lines (SmartStax, SmartStax + RR, EXP 262), with six replicates (test vessels) each. On day 19, 1-ml samples of overlying water were collected and stored at −80 °C. Each test vessel with sand and larvae was poured into a larger glass dish, and all living larvae were picked up with forceps, washed with tap water, dried on a paper towel, and pooled in 2-ml centrifuge tubes (10–12 larvae/tube for each of the two *Bt* maize lines, 10–20 larvae/tube for EXP 262). Each group of larvae was weighed on an electronic microbalance (MX5; Mettler-Toledo) and stored at −80 °C. Finally, after the overlying water had been gently removed, 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.

Because leftover 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 of maize leaf powder) with three replicates/maize line. Two samples of 40 µl each were frozen at −80 °C immediately (day 0) and after 2, 4, and 6 days in the fridge.

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). In addition to water, sediment, insect, and leaf suspension samples, Cry concentrations in leaf powder were measured. The protocol of Y. Chen et al. (2021a) was followed. The proteins from the larvae, sediment, leaf powder and leaf suspension samples were extracted in 800 µl of extraction buffer (phosphate-buffered saline/Tween [PBST] containing 0.55% Tween-20) with a 3-mm tungsten carbide ball using a Tissue Lyser II (Qiagen) at 30 Hz for 30 s. For the first repetition of the ELISA experiment, the water samples were loaded directly on the ELISA plate. For the second repetition, water samples were lyophilized and resuspended in the same amount of extraction buffer to ensure that the samples were in the appropriate buffer when loaded to the ELISA plate.

After centrifugation (13 000× *g* for 5 min at 4 °C), the supernatants were collected. Samples of leaf powder were diluted with extraction buffer: Cry1A.105 and Cry1F 20×, Cry3Bb1 100×, Cry2Ab2, and Cry34Ab1 200×. Purified Cry1A.105, Cry2Ab2, and Cry3Bb1 of certified quality were supplied by Bayer Crop Science, and Cry1F and Cry34Ab1 by Corteva Agriscience. Appropriate dilutions of each protein served as standards for the ELISA (seven concentrations loaded twice on each plate). In addition, at least four extraction buffer blanks were added. After the appropriate enzyme conjugates and the samples had been loaded onto the precoated ELISA plates, the plates were incubated overnight at 4 °C. Next day, the plates were washed with PBST (0.05% Tween-20), the color substrate was added, and the absorbance (optical density) was measured at 620 nm using a plate reader (infinite 200, Tecan Group).

Standard curves were established based on a single rectangular hyperbola model. The concentrations of each Cry protein were calculated with the corresponding standard curve. The limits of detection (LODs) of the test were calculated according to Y. Chen et al. (2021a) based on buffer-only blanks of multiple ELISA plates of the same batch of kits.

Data analysis

Data were analyzed using R, Ver 4.0.2 (R Foundation for Statistical Computing). All data are presented as mean ± standard error, 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, SmartStax, EXP 262, SmartStax + RR) using two-factorial generalized linear models (GLM), generalized linear mixed effect models (GLMER), or linear mixed effect models (LMER). The conventional lines Rheintaler, Tasty Sweet, ES-Eurojet, and Planoxx were used for the interpretation of potential differences among *Bt* and control lines (see the next paragraph). They were not included in the statistical models, because they did not match the two-factorial design and because the conventional lines were grown in a different year and not side by side with the *Bt*/comparator lines. Development time (days) was analyzed for each sex using nested 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 and the same factors. Because all egg ropes collected in the experiment hatched, fecundity (number of egg ropes/female) and fertility (number of fertile egg ropes/female) were identical, further referred to as fecundity. Fecundity was analyzed with GLM assuming Poisson distribution with plant background (EXP 258, EXP 262) and *Bt* (Bt^+, Bt^-) as factors. The number of hatched larvae/egg rope was analyzed using LMER with plant background (EXP 258, EXP 262) and *Bt* (Bt^+, Bt^-) as fixed factors and cage as nesting 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 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 (95%

CI) of the mean was calculated for each assessed parameter. The natural range of variation was then defined as the range from the lowest to the highest boundary of the 95% CI (Y. Chen et al., 2021b). No 95% CI was calculated for fecundity and larvae/egg rope, because the low number of replicates ($n = 3$) would inflate the intervals. For those parameters, we used the lowest and highest mean as the natural range of variation.

For the design of future experiments, it is informative to calculate the detectable differences for *C. riparius* life table parameters. With a given mean, standard deviation, and sample size in the control treatment, one can estimate how large a treatment effect needs to be for a statistical test to detect it. Detectable differences were calculated for EXP 258 maize, EXP 262 maize, and TetraMin fish food based on two-sample *t*-tests with a significance level of 5% and a power of 80% (pwr package).

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

RESULTS

Overlying water quality

The pH of water collected toward the end of the experiment was between 7.9 and 8.2, the DOC was between 6.2 and 10.5 mg/L, and total hardness was between 120 and 170 mg/L (Supporting Information, Table S1). All values were within the range demanded in OECD (2010) test guideline 233, that is, pH 6–9, DOC greater than 5.46 mg/L, and total hardness less than 400 mg/L.

Performance of C. riparius in the control treatment

When *C. riparius* were fed with TetraMin fish food, the first adults emerged on day 15, and the last on day 31. All introduced larvae emerged as adults (no mortality). The 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/egg rope was 236.6 ± 30.09 (Supporting Information, Table S2). Lowest detectable differences were calculated for development time (7% for males, 11% for females), followed by proportion of males (25%). Highest values were obtained for reproductive parameters (fecundity 47%, larvae/egg rope 68%).

Performance of C. riparius when fed maize leaves

Mean values and 95% CI of the life table parameters of *C. riparius* fed leaves from the eight maize lines are presented in the Supporting Information, Table S2. In the following, analyses for the two *Bt* lines and their corresponding control lines representing two different plant backgrounds are presented. Female development time was significantly affected by the factor *Bt* ($\chi^2 = 4.4$, $p = 0.04$), but not by the factor plant background ($\chi^2 = 2.5$, $p = 0.1$; Figure 1A). The interaction of both factors was not significant ($\chi^2 = 0.2$, $p = 0.7$). When fed the two *Bt* lines, *C. riparius* females emerged earlier compared with the non-*Bt* comparators. The natural range of variation based on 95% CI for female development time was between 29.5 and 40.5 days. The male development time was not affected by the factors *Bt* ($\chi^2 = 3.3$, $p = 0.07$) or plant background ($\chi^2 = 2.1$, $p = 0.1$), and there was no interaction ($\chi^2 = 1.4$, $p = 0.2$; Figure 1B). The natural range of variation for the male development time was between 23.4 and 35.0 days.

Emergence ratio was not affected by *Bt* ($\chi^2 = 2.8$, $p = 0.09$) or plant background ($\chi^2 = 1.9$, $p = 0.2$) in the main analysis, but the interaction of *Bt* and plant background was significant ($\chi^2 = 4.7$, $p = 0.03$; Figure 2A). Subsequent separate analyses for each factor, however, did not show significant differences (all $p \geq 0.09$). The natural range of variation for the emergence ratio was between 0.86 and 1.00. No differences in the sex ratio

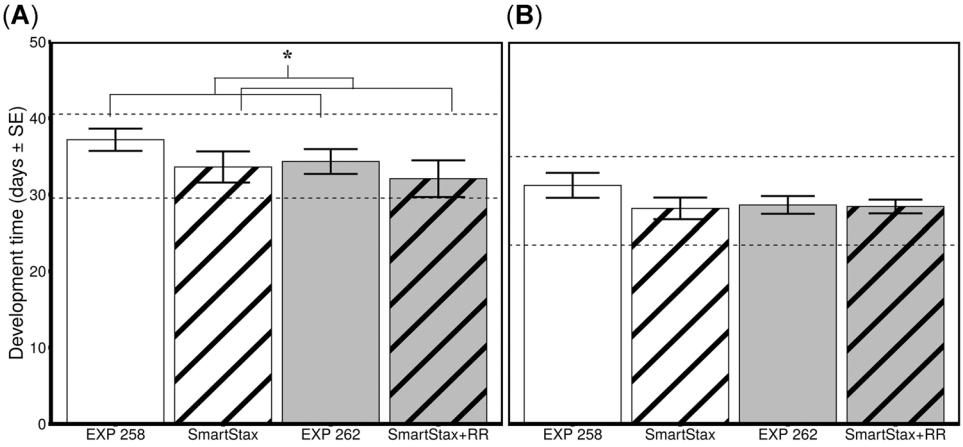

FIGURE 1: Female (**A**) and male (**B**) development time of *Chironomus riparius* fed maize leaves from *Bt* maize (SmartStax, SmartStax + RR) and respective controls (EXP 258, EXP 262). Dashed lines illustrate the natural range of variation from six conventional maize lines (Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258, and EXP 262) represented by the highest and lowest boundary of the 95% confidence interval for each maize line ($n = 9$). The asterisk indicates a significant difference ($p < 0.05$). SmartStax = MON89034 × TC1507 × MON88017 × DAS-59122-7; SmartStax + RR = MON87427 × SmartStax.

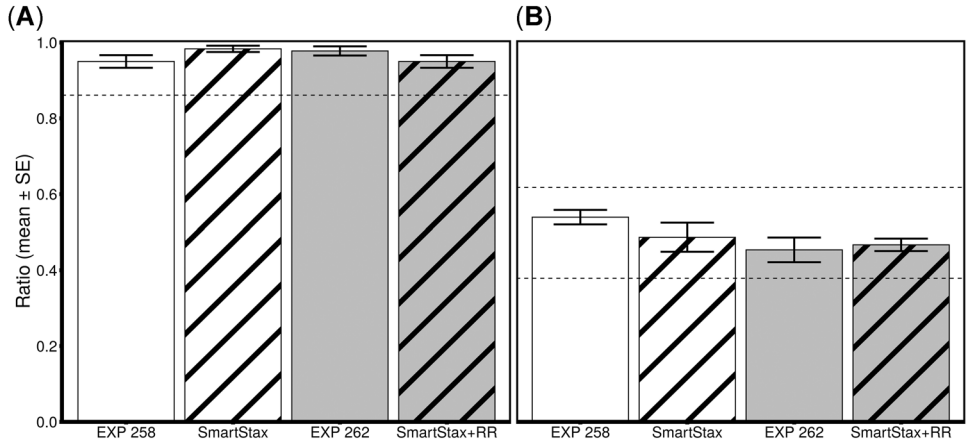

FIGURE 2: Emergence (**A**) and sex ratio (**B**) of *Chironomus riparius* fed maize leaves from *Bt* maize (SmartStax, SmartStax + RR) and respective controls (EXP 258. EXP 262). The sex ratio represents the proportion of males (1 = all males). Dashed lines illustrate the natural range of variation from six conventional maize lines (Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258, EXP 262) represented by the highest and lowest boundary of the 95% confidence interval for each maize line ($n = 9$). SmartStax = MON89034 × TC1507 × MON88017 × DAS-59122-7; SmartStax + RR = MON87427 × SmartStax.

of adults was observed for *Bt* ($\chi^2 = 0.9$, $p = 0.3$) or plant backgrounds ($\chi^2 = 2.4$, $p = 0.1$), and there was no interaction ($\chi^2 = 0.7$, $p = 0.4$; Figure 2B). The natural range of variation for sex ratio ranged from 0.38 to 0.62.

Fecundity was not affected by *Bt* ($\chi^2 = 2.3$, $p = 0.1$) and plant background ($\chi^2 = 0.02$, $p = 0.9$), and there was no interaction ($\chi^2 = 0.04$, $p = 0.8$; Figure 3A). The lowest mean for conventional maize lines was 0.19 and the highest 0.34. The number of hatched larvae/egg rope was also not affected by *Bt* ($\chi^2 = 0.3$, $p = 0.6$), plant background ($\chi^2 = 1.2$, $p = 0.3$), or interaction ($\chi^2 = 2.0$, $p = 0.2$; Figure 3B). The lowest mean for conventional maize was 141.6, and the highest was 225.5.

For development times and emergence and sex ratios, the values obtained for the two *Bt* maize lines were within the natural range of variation calculated from the 95% CI of the six non-*Bt* maize lines (Supporting Information, Table S2). Similarly, larvae/egg rope for the two Bt lines were within the range of the means of conventional maize lines. When fed SmartStax + RR, fecundity was similar to that of Planoxx, which had the highest value of the conventional maize lines (0.34), and when fed SmartStax, fecundity was slightly higher than that of Planoxx (0.36).

Detectable differences were lowest for emergence ratio (8% for EXP 258, 5% for EXP 262), followed by development time (females: 17% for EXP 258, 20% for EXP 262; males: 22% for EXP 258, 17% for EXP 262) and proportion of males (15% for EXP 258, 30% for EXP 262). Reproductive parameters had higher detectable differences (fecundity: 70% for EXP 258, 111% for EXP 262; larvae/egg rope: 43% for EXP 258, 55% for EXP 262).

Cry protein content

The ELISA assay with maize leaves from SmartStax and SmartStax + RR revealed highest concentrations 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 (nonoverlapping 95% CI).

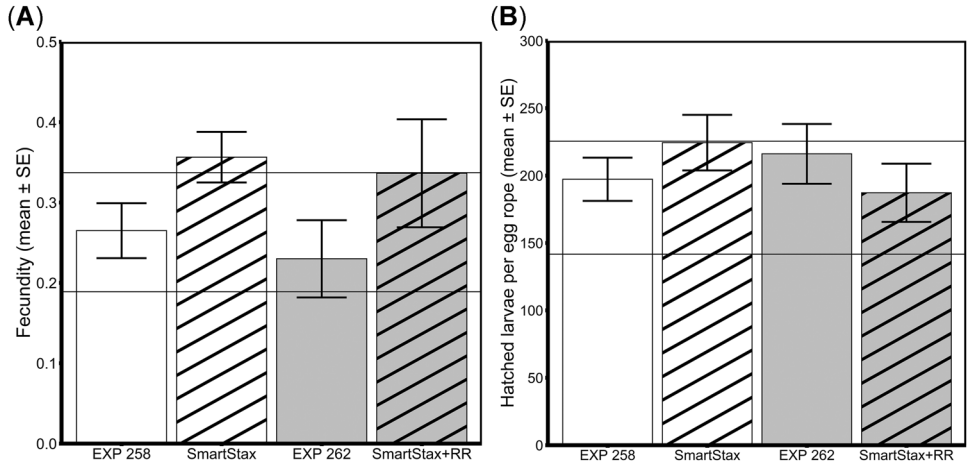

FIGURE 3: Fecundity (number of egg ropes in a cage divided by the number of females in the cage) (**A**) and number of hatched larvae per egg rope (**B**) of *Chironomus riparius* fed maize leaves from *Bt* maize (SmartStax, SmartStax + RR) and respective controls (EXP 258, EXP 262). Thin horizontal lines illustrate the highest and lowest mean from six conventional maize lines (Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258, EXP 262; $n = 3$). SmartStax = MON89034 × TC1507 × MON88017 × DAS-59122-7; SmartStax + RR = MON87427 × SmartStax.

TABLE 1: Cry protein concentrations in maize leaves, sediment, and larvae from two stacked SmartStax hybrids

	Leaves[a]		Sediments[a]		Larvae[b]	
Cry protein	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

Data are presented as median ± 95% confidence interval ($n = 21$ for maize leaves; $n = 12$ for sediments; $n = 24$ for larvae). Values below the limit of detection (LOD) are presented as <LOD.
[a]µg/g dry weight.
[b]µg/g fresh weight.
SmartStax = MON89034 × TC1507 × MON88017 × DAS-59122-7; SmartStax + RR = MON87427 × SmartStax.

The detected Cry proteins in leaf suspensions from the two SmartStax lines showed that the toxin remained relatively stable over 6 days in the refrigerator (Supporting Information, Table S3). The percentages of Cry proteins measured on day 6 compared with day 0 ranged from 60% (Cry2Ab2, SmartStax) to 106% (Cry34Ab1, SmartStax + RR). The Cry2Ab2 and Cry1F tended to degrade more (60%–74%) than Cry1A.105, Cry3Bb1, and Cry34Ab1 (80%–106%; Supporting Information, 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 between the two *Bt* maize lines.

The highest *Bt* protein concentrations in *C. riparius* larvae 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.

The detected Cry proteins in sediments and larvae were low compared with the concentrations in leaves (Supporting Information, Table S4). Approximately 3%–5% of Cry1A.105 in leaves was detected in sediments. For the other Cry proteins, the values were even 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 (based on fresh weight) were lower than in sediments (based on dry weight): 3%–4% for Cry1A.105 and 0.5%–0.8% for Cry2Ab2. Values based on larval dry weight can be estimated to be approximately 10 times higher (Kangur & Tuvikene, 1998).

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

DISCUSSION

The *C. riparius* fed exclusively on maize leaves developed and reproduced. Despite Cry protein exposure, no adverse effects on life table parameters were evident when larvae were fed stacked *Bt* maize leaves compared with non-*Bt* maize leaves.

Experimental conditions

According to OECD (2010) test guideline 233, all measured values for water quality were within the recommended range. In the control treatment with TetraMin, all *C. riparius* larvae that were introduced into the test vessels emerged, and 99% emerged until day 28 (OECD validity criteria: more than 70% emergence until day 28). Furthermore, 93% of the midges emerged between day 12 and day 23 (OECD: more than 85% of emerging adults). The proportion of males was 0.51 (OECD: 0.4–0.6), the number of egg ropes for each

breeding cage was 0.85–1.11 per female added to the breeding cage (OECD: more than 0.6), and all egg ropes were fertile (OECD: more than 0.6). The TetraMin treatment thus demonstrates that the experimental conditions were well suitable and the *C. riparius* larvae used for the experiments were healthy.

When fed only maize leaves, longer development time and reduced fecundity compared with the TetraMin control indicate that green 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% threshold set by OECD (2010). Between 17% and 26% of the adult midges emerged between day 12 and day 23, depending on the maize line. These values were well below the validity criterion of 85%. Similarly, the fecundity (0.19–0.34) remained below the validity criterion of 0.6. A similar result was found for *D. 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; Y. Chen et al., 2021b). Nutritional stress of test animals in feeding studies could lead to confounding effects, which indicates that results of such studies need to be discussed with caution and in the context of appropriate control treatments.

Exposure of *C. riparius* to Cry proteins

The concentrations of Cry proteins in maize leaves were similar to the results of Y. Chen et al. (2021a), except for Cry1A.105 protein, which showed lower values. For the present study, we used the leaves collected by Y. Chen et al. (2021a, 2021b), but we prepared fresh powder from those leaves. The Cry protein concentrations in food suspensions (leaf powder in water) stored in the refrigerator 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 detritus that is deposited on the sediment (Armitage et al., 1995). Compared with 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 with the other Cry proteins (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 the 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 generally low compared with 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 (Meissle & Romeis, 2018; Meissle et al., 2021; Svobodová et al., 2017; Zhang et al., 2017; Zhao et al., 2016). The Cry1A.105 median lethal concentration (LC50) reported for *Ostrinia nubilalis* (Lepidoptera: Crambidae), the main target pest of *Bt* maize, is approximately 0.4 μg/ml artificial diet (USEPA, 2010). Our sediment samples contained approximately 3 μg/g dry weight, which would translate to 0.3 μg/g fresh weight assuming a conversion factor of 10. Although concentrations of other *Bt* proteins in sediment were lower, the total amount of *Bt* proteins in SmartStax sediment are well in the range where effects on the targets would be expected.

To judge the biological relevance of laboratory feeding studies, it is important to relate experimental exposure levels to realistic exposure in the field. Laboratory nontarget 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 confident that our study represents a worst-case Cry protein exposure scenario for *C. riparius* for a number of reasons. As commonly done in laboratory worst-case exposure experiments, leaves were collected from green plants, lyophilized, and processed directly to food suspensions; *C. riparius* was fed exclusively with maize leaves; and new leaves were provided every 24 h to ensure constant exposure. Furthermore, SmartStax maize is currently the plant that produces the most Cry proteins in total while the amounts of individual Cry proteins are comparable to those of nonstacked plants (USEPA, 2009). Finally, SmartStax leaves contained higher Cry protein concentrations than other maize materials, such as pollen or flour (Y. Chen et al., 2021a).

In the field, considerably lower exposure can be expected, because: (1) pollen or late-season maize debris, which would normally enter streams, has lower *Bt* protein concentrations than fresh green leaves (Nguyen & Jehle, 2009; Tank et al., 2010); (2) the stream environment exhibits constant physical abrasion due to water flow as well as diverse invertebrate and microbial activities, which leads to fast degradation (Jensen et al., 2010); and (3) maize debris in streams will likely represent only a small fraction of the diet of *C. riparius*.

Effects of SmartStax maize on *C. riparius*

Female development time in our study was the only parameter for which a significant difference was observed for the two *Bt* maize lines compared with the respective 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. In the literature, reports exist that Cry1 and Cry2 class proteins may show toxicity against Diptera species, such as Cry1Ab, Cry1Ca, and Cry2Ag against *Aedes aegypti* (Culicidae), Cry1Ca against *Anopheles gambiae* and *Culex quinquefasciatus* (Culicidae), Cry1Ac against *Glossina morsitans* (Glossinidae), and Cry1Ba against *Musca domestica* (Muscidae; Van Frankenhuyzen, 2013). For *Chironomus* species, studies with plant material containing Cry proteins exist.

When *C. dilutus* 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 with the 17-ng/ml treatment and a water-only control, whereas growth was unaffected (Prihoda & Coats, 2008). It remains unclear whether 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 extract were included in the study. In acute tests with sediment (10 days) or water (4 days) spiked with cotton seed extract containing Cry1Ac, the LC50 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 with the amounts in the *Bt* treatments was not specified. It can thus not be excluded that the observed effects were caused by the increase in the amount of seed extract and not the Cry proteins per se. In any case, the estimated LC50 concentrations were several orders of magnitude higher than concentrations detected in the field and in aquatic environments. When larvae of the crane fly *Tipula abdominalis* (Diptera: Tipulidae) were fed leaves from non-*Bt* maize, Cry1Ab-containing maize, or stacked Cry1Ab + Cry3Bb1-containing maize of the same plant background (near-isolines) for 30 days, reduced growth was observed in the Cry1Ab treatment compared with the non-*Bt* control, but not in the stacked maize treatment (Jensen et al., 2010). The authors concluded that plant background effects rather than Cry1Ab effects were responsible for the observed differences. Jensen et al. (2010) also fed caddisflies with conditioned *Bt* maize leaf material for 30 days. *Lepidostoma* spp. (Trichoptera: Lepidostomatidae) showed no difference in head capsule growth and dry mass after feeding on the three maize lines. Another species, *Pycnopsyche scabripennis* (Trichoptera: Limnephilidae), even had a higher final dry mass when fed stacked *Bt* maize compared with Cry1Ab-containing maize, or non-*Bt* maize (Jensen et al., 2010). When *Lepidostoma liba* caddisflies were fed conditioned leaf discs of field-collected *Bt* maize (containing Cry1Ab) for 29 days, slower growth was observed compared with non-*Bt* maize (Chambers et al., 2010; Rosi-Marshall et al., 2007). Another caddisfly species, *Helicopsyche borealis* (Trichoptera: Helicopsychidae), was fed algal biofilms and Cry1Ab containing maize pollen for 18 days, and 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 *Bt* and non-*Bt* maize varieties used were either unrelated or not specified, so plant background–related effects are likely.

In summary, previous studies suggesting adverse effects of *Bt* proteins or *Bt* plants on aquatic insects including *Chironomus* species often lack important study design requirements, such as appropriate controls (Romeis et al., 2013). Studies that involved plant material did not always include lines that are closely related to each other (near-isogenic lines; Chambers et al., 2010; Rosi-Marshall et al., 2007). Others did not describe the controls that they used sufficiently, or did not use an appropriate non-*Bt* control treatment at all (Li et al., 2013; Prihoda & Coats, 2008). Most studies that worked with closely related plant material for treatment and control only used one *Bt*/non-*Bt* pair. However, particular studies with plant material bear the risk that differences in plant composition will overlie effects of the introduced *Bt* proteins (as discussed by Jensen et al., 2010). Observed effects in such studies thus cannot be linked directly to the involved Cry proteins. One way to separate plant background effects from *Bt* protein effects is to include the *Bt* trait in multiple backgrounds (Y. Chen et al., 2021a), which we did in the present study. When expression levels are similar among the different backgrounds, *Bt* effects should also be similar. Jensen et al. (2010) addressed this issue by using a single and a stacked *Bt* maize line with the same plant background.

Although low-level effects of some Cry proteins (e.g., Lepidoptera active) on some insect taxa (e.g., Trichoptera) cannot be excluded, the previously reported effects were possibly caused by study design issues and plant background effects. In any case, our study with two SmartStax lines suggests the absence of Cry protein effects on *C. riparius* at realistic worst-case exposure conditions.

Natural range of variation and detectable effect sizes

One 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 nontarget species. A similar approach had been applied in the compositional equivalence studies that support food/feed safety assessment of genetically engineered plants (Anderson et al., 2019, 2020).

In the present study, we included six different non-*Bt* maize lines, that is, Rheintaler, Tasty Sweet, ES-Eurojet, Planoxx, EXP 258, and EXP 262. When sufficient replicates were available ($n = 9$), the natural range of variation was built using 95% CIs around the means of each maize line (Y. Chen et al., 2021b). The range gives an indication of how variable *C. riparius* performance could be when the organisms were fed with different non-genetically engineered maize leaves. In our study, all parameters for *C. riparius* fed with SmartStax and SmartStax + RR were within the natural range of variation. It has to be noted, however, that confidence intervals for parameters with a low sample size ($n = 3$), such as fecundity and larvae/egg rope, would have been unrealistically inflated. This indicates that the 95% CI method may only be informative if a certain number of conventional maize lines are included and the sample size allows a relatively precise estimate of variation for each maize line.

That power to detect effects was comparatively low for the reproductive parameters in our experiment was also evident from the detectable effect sizes calculated for EXP 258, EXP 262, and TetraMin based on *t*-tests. Values for those parameters ranged from 43% to 111%, which indicates that only large effects could be detected statistically with our experimental

setup, in which we pooled adults from three test vessels into one cage (total $n = 3$). More sensitive were the developmental endpoints with $n = 9$, such as development time and emergence ratio, with detectable differences of approximately 20% or lower. This information might be valuable for future experiments; depending on the hypothesis to be tested and the desired statistical power, one might select specific endpoints or increase the sample size accordingly.

CONCLUSIONS

Our one-generation laboratory test with *C. riparius* revealed no adverse effects of stacked *Bt* maize in two plant backgrounds compared with 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 except fecundity in the SmartStax treatment, which was higher than the range. We thus conclude that exposure to stacked *Bt* maize poses no risk for *C. riparius*.

When one is conducting nontarget tests with plant material, using genetically engineered traits in multiple plant backgrounds can help to separate transgene effects from background effects. The biological relevance of observed effects between particular genetically engineered and comparator lines can be evaluated if several non-genetically engineered lines are tested along with the genetically engineered lines (natural range of variation).

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5293.

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Data Availability Statement—All data used for this manuscript are available in the Supporting Information (MS Excel File). For further information, please contact the corresponding author (michael.meissle@agroscope.admin.ch).

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