Effects of mCry51Aa2-producing cotton on the non-target spider mite *Tetranychus urticae* and the predatory bug *Orius majusculus*

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Abstract

Pierce-sucking insects, such as plant bugs or stink bugs, cause damage in cotton fields worldwide. A novel genetically engineered (GE) cotton (*Gossypium hirsutum*) is protected against hemipteran pests and thrips by producing the modified *Bacillus thuringiensis* (*Bt*) mCry51Aa2 protein. Herbivores that consume insect-protected GE crops, and their natural enemies, can be exposed to plant-produced insecticidal proteins. We investigated tritrophic interactions to evaluate the potential impact of the novel *Bt* cotton on a non-target herbivore, the spider mite *Tetranychus urticae*, and on a generalist predator, the pirate bug *Orius majusculus*. Pirate bugs belong to the same insect order as the target pests and might thus be adversely affected by mCry51Aa2. Enzyme-linked immunosorbent assays showed that levels of mCry51Aa2 in *T. urticae* were one order of magnitude, and those in *O. majusculus* were three orders of magnitude lower than in *Bt* cotton leaves. *O. majusculus* nymphs fed with spider mites collected from *Bt* cotton had lower survival, increased developmental time, and reduced fecundity compared to nymphs fed spider mites from non-*Bt* near-isogenic cotton. Because *Bt* cotton did not affect the survival and growth of the spider mites, we conclude that indirect prey-quality mediated effects of the *Bt* cotton on the predatory bugs are unlikely and that *O. majusculus* are directly affected by the *Bt* protein. Follow-up studies are indicated to assess whether the effects observed under worst-case conditions are mitigated under more realistic exposure conditions where alternative prey with low *Bt* protein levels is available.

Keywords Genetically modified crops · mCry51Aa2 · Tritrophic interactions · Environmental risk assessment · *Bt* plants · Natural enemies

Key message

- Genetically engineered cotton producing a modified protein from the bacterium *Bacillus thuringiensis* is protected against plant bugs and thrips.
- In laboratory experiments, *Bt* cotton was offered to beneficial minute pirate bugs in a plant–spider mite–predator food chain.
- Spider mites containing high amounts of *Bt* protein caused increased mortality, delayed development, and reduced fecundity in the pirate bugs, when fed exclusively from nymph to adult.
- Experiments with more realistic feeding scenarios are indicated to assess the ecological relevance of these findings.

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Introduction

Since the commercial introduction of genetically engineered (GE) crops more than 20 years ago, the global cultivated area has increased to 192 million hectares in 2018 (ISAAA 2018). The majority of today's insect-protected GE crops produce Cry proteins from the bacterium *Bacillus thuringiensis* (*Bt*) that specifically target certain lepidopteran or coleopteran pests. Worldwide, 80% of the cultivated cotton, *Gossypium hirsutum* L., carries GE traits, such as insect protection and/or herbicide tolerance (ISAAA 2018).

Cotton with protection against lepidopteran pests is among the most important GE crops and the plant-produced Cry proteins allowed for reduced applications of broad spectrum insecticides against damaging caterpillars (Klümper and Qaim 2014). While *Bt* cotton is highly efficient against the major lepidopteran pests, beneficial arthropods and non-lepidopteran herbivores remain unaffected. When broad spectrum insecticides are replaced by highly specific *Bt* plants, natural enemies become important to keep populations of secondary pests below thresholds for economic injury (Naranjo et al. 2015; Anderson et al. 2019).

In some *Bt* cotton systems, however, plant bugs (Hemiptera, suborder Heteroptera) are serious pests (Lu et al. 2010; Naranjo 2011). GE cotton event MON 88702 has been developed to target certain plant bugs (Gowda et al. 2016). The *Bt* protein Cry51Aa2 showed activity against *Lygus* spp. (Hemiptera: Miridae), but activity in transgenic cotton plants was not sufficient for effective protection. Therefore, MON 88702 containing a modified version of the protein was developed with more than 200-fold increased lethality to *Lygus* spp. compared to the unmodified Cry51Aa2 (Gowda et al. 2016). MON 88702 has been shown to effectively control *Lygus* spp. and also thrips (*Frankliniella* spp.; Thysanoptera: Thripidae) under field conditions (Akbar et al. 2019).

Before GE crops can be cultivated, they have to pass a risk assessment to evaluate potential effects on the ecosystem including biological control provided by natural enemies (Romeis et al. 2008). This is particularly pertinent in the case of cotton producing mCry51Aa2 since some natural enemies are in the same order (Hemiptera) as the target pests. Natural enemies could be adversely affected by ingested *Bt* proteins, for example when consuming prey containing *Bt* protein, or plant material, such as sap or pollen, to supplement their diet. In addition, indirect, prey-quality mediated effects can occur when predators or parasitoids feed on prey or hosts that are affected by the *Bt* proteins (i.e. "sick" prey/hosts) and thus provide a lower nutritional value for their natural enemies (Romeis et al.

2006; Naranjo 2011; Romeis et al. 2019). Early in the risk assessment process, the assessment of adverse effects on natural enemies is typically based on laboratory studies with purified *Bt* protein in artificial diets (first-tier studies) (Romeis et al. 2008). Such studies have the advantage to test high doses of the toxins provided in artificial diet or GE plant tissue under highly controlled laboratory conditions to enhance the ability to detect toxic effects. Higher tier assessments, including more complex laboratory scenarios, greenhouse studies, and field experiments, are then conducted as deemed necessary based on the outcome of the early tier studies or when required specifically by regulatory agencies (Romeis et al. 2008). In those studies, more realistic exposure scenarios can be simulated to evaluate whether previously identified hazards translate to potential risks in the field. Species to focus on the risk assessment should be representatives of valued taxa in the field that provide important ecological functions. In addition, they should be exposed to the produced insecticidal proteins, most likely sensitive, and available and amenable for testing (Romeis et al. 2013).

Laboratory studies reported that the purified *Bt* protein mCry51Aa2 was harmful to some non-target herbivores and natural enemies (Bachman et al. 2017). Three mirid bug species, one thrips species, and two chrysomelid beetle species were adversely affected by mCry51Aa2. In addition to those pest species, the predatory bug *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) also showed susceptibility to the *Bt* protein. In contrast, no activity was seen for two coccinellid beetles, four lepidopteran species, one hymenopteran parasitoid, the honey bee, one collembolan, and one earthworm. The potential hazard for *O. insidiosus* identified by Bachman et al. (2017) indicates further studies with more realistic exposure scenarios including tritrophic experiments.

Laboratory experiments with previously commercialized *Bt* cotton varieties showed that caterpillars (*Spodoptera* spp.) with low susceptibility to the *Bt* proteins produced in *Bt* cotton as well as spider mites feeding on cotton contained relatively high levels of Cry1Ac and Cry2Ab (Eisenring et al. 2017; Meissle and Romeis 2018). Spider mites even contained higher concentrations of Cry1Ac than the leaf tissue in some studies (Torres and Ruberson 2008; Esteves Filho et al. 2010). In contrast, low or non-detectable levels of *Bt* proteins in aphids and other species feeding on phloem sap were reported (Romeis and Meissle 2011; Meissle and Romeis 2018). In general, *Bt* protein concentrations have been found to decrease from one trophic level to the next and most predators, including *Orius* spp., contained relatively low concentrations of *Bt* protein in the field (Meissle and Romeis 2009a; Eisenring et al. 2017; Romeis et al. 2019).

As a follow-up to the artificial diet study (Bachman et al. 2017), we worked in a tritrophic system that represents a more realistic worst-case exposure scenario for *Orius* spp.

in *Bt* cotton with the spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) as prey. As discussed above, spider mites are appropriate for use as prey since they are important pests in many crops including cotton and they contain relatively high concentrations of *Bt* protein when feeding on *Bt* cotton while not being a target of the novel mCry51Aa2-producing cotton. In addition, they can be easily reared under laboratory conditions and natural enemies, such as *Orius* species, readily consume them.

In the present study, we focus on *Orius* predatory bugs since the genus *Orius* is one of the most dominant predator groups in cotton fields (Cisneros and Rosenheim 1998; Ellington et al. 1997; Eisenring et al. 2017). We selected *Orius majusculus* (Reuter) (Hemiptera: Anthocoridae), which is distributed all over Europe. We worked with a European species (instead of *O. insidiosus*, which is a new world species), mainly because of practical aspects, but the lifestyle is very similar to other widespread *Orius* species.

The tritrophic interactions among *Bt* cotton, *T. urticae*, and *O. majusculus* were tested in response to exposure to the mCry51Aa2 protein. The following research questions were addressed: (1) is *T. urticae* affected by mCry51Aa2-producing cotton? Knowledge on the susceptibility of the prey species for the produced *Bt* protein is important to know whether prey-quality mediated effects on the predator are to be expected (Romeis et al. 2019). (2) Is *O. majusculus* affected when feeding exclusively on spider mite prey from mCry51Aa2-producing cotton? (3) To which concentrations of mCry51Aa2 are spider mites and *O. majusculus* exposed? The generated data are relevant for the non-target risk assessment of this novel GE cotton event.

Methods

Cultivation of cotton

GE cotton expressing the mCry51Aa2 protein from *B. thuringiensis* (event MON 88702) and the non-transgenic near isoline (DP393) were provided by Bayer Crop Science (St. Louis, USA). Seeds were planted in 1.3L pots using heat-treated soil (60 °C for > 24 h) to minimize soil-borne pests and diseases and incubated in a growth chamber at 25 °C, 70% relative humidity, and a 16:8 light to dark cycle. After approximately 3 weeks, plants were transplanted into 3L pots using untreated (not heated) soil; 15 g slow-release fertilizer was applied with the soil (Manna 3 M, Wilhelm Haug GmbH, Ammerbuch, Germany). Subsequently, 0.2–0.8 L of liquid NPK fertilizer was applied weekly (0.2%, Manna, Wilhelm Haug GmbH).

Spider mite colony

A starter population of mixed stages of *T. urticae* spider mites on bean leaves was provided by Syngenta Crop Protection Münchwilen AG (Stein, Switzerland). Colonies were established on *Bt* and non-*Bt* cotton and remained on the respective cotton type for the whole experimental period (approximately 1 year) without deliberate mixing among plant types. The colonies were maintained in glasshouse cabins at approximately 25 °C and 16:8 (L:D) photoperiod and were supplied with cotton plants in the flowering and boll-forming stage. *Bt* and non-*Bt* colonies were either kept in separated glasshouse cabins or together in one large cabin, albeit spatially separated. Consequently, exchange between the two colonies was not completely excluded, but minimized. We measured the concentration of mCry51Aa2 in spider mites reared on *Bt* and non-*Bt* plants to evaluate the rate of exchange between colonies (see below).

Predatory bug culture

Orius majusculus was purchased from Andermatt Biocontrol AG (Grossdietwil, Switzerland) and cultured at Agroscope since October 2016. Colonies were maintained in ventilated 1.3L transparent plastic cylinders. Pods of coco bean (*Phaseolus vulgaris* L.) were provided for oviposition, and water was supplied in glass tubes with cotton plugs. Frozen eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae, Biotop, Livron-sur-Drôme, France) were provided on the sticky section of yellow Postit papers as food. The culture was maintained in the glasshouse at approximately 22 °C and 16:8 (L:D) photoperiod.

Spider mite assay 1: Development and fecundity on *Bt* and non-*Bt* cotton

This experiment was conducted to investigate whether life-table parameters of spider mites feeding on leaf discs of *Bt* cotton were affected when compared to non-*Bt* cotton, with a focus on development and reproduction.

Leaf discs (2.5 cm diameter) were cut from the youngest fully developed leaves of 5–6-week-old plants (typically six fully developed leaves). Each disc was cut from a different plant (20 *Bt* and 20 non-*Bt* plants) and placed upside down in a customized transparent plastic dish (5 cm diameter, 1 cm high) covered with a ventilated lid. The plastic dishes were lined with wet cotton pads (Migros Budget, Migros, Switzerland) that provided moisture to the leaf and prevented spider mites from leaving the leaf disc [method adapted from Li and Romeis (2010) and Shu et al. (2018)]. One female spider mite raised on *Bt* or non-*Bt* plants was placed on each leaf disc of the respective plant type by using a fine paint brush. On the next day, females were removed and all eggs

except one were removed or destroyed (day 0). Egg hatching, survival, larval/nymphal stage, and the gender of adults were recorded daily. Cotton pads were rewetted as necessary throughout the experiment. Spider mites were transferred to new dishes for the first time as soon as larvae hatched. Subsequently, leaf discs were changed every 3–4 days. During the immature phase, disc change was postponed to the next day if the spider mite was in the process of moulting (immobile). Data collection ended for males after they reached the adult stage. Newly emerged females were paired with one male either from the experiment or from the respective culture for 2 days. Then, males were removed. Eggs were counted daily and either removed or destroyed except on days when the disc was changed. The old discs with the eggs were incubated to determine egg fertility. After 5 days, the number of unhatched eggs was counted. The experiment ended when the last female died. The whole experiment was conducted three times consecutively.

Spider mite assay 2: Development and adult measures on *Bt* and non-*Bt* cotton

A second series of assays with spider mites was conducted as follows: (1) due to the rather high juvenile mortality observed in the first assay (Table 1), juvenile development was once more investigated with improved methodology that avoided handling of juveniles. (2) The second experiment ended once nymphs became adults, which allowed for data collection on additional measures of adults, such as weight and size. (3) To assess quality of spider mites in long-term cultures (approximately 1 year), we tested whether the origin of the mothers of the juveniles used in the assay (*Bt* or

non-*Bt* culture) influenced their performance on *Bt* or non-*Bt* cotton.

Leaf discs of 6–8-week-old plants were cut from 25 *Bt* and 25 non-*Bt* plants and placed in transparent plastic dishes as described previously. One female spider mite raised on *Bt* or non-*Bt* plants was placed on each leaf disc of the respective plant type. On the next day, females were removed (day 0). After 3 days, two eggs per leaf disc were cut out using fine scissors: one egg was transferred to a *Bt* leaf disc and the other transferred to a non-*Bt* disc. This resulted in 25 replications of each combination of plant-type mother origin and plant-type food (100 eggs altogether). Subsequently, egg hatching, survival, and development were recorded until adults emerged. Leaf discs were not changed during juvenile development to minimize handling effects. Adults were sexed, frozen, and later measured (body length and width) using a binocular microscope fitted with a scaled ocular. The weight of individual females was recorded on a microbalance (Mettler-Toledo MX5, Greifensee, Switzerland). The weight of males was too low to be measured individually with sufficient precision. Therefore, groups of 3–5 males from the same treatment were pooled and weighed as a group. For statistical analysis, the calculated average weight per male was used. This experiment was also conducted three times consecutively.

Predator assay 1: Survival on prey from *Bt* and non-*Bt* cotton

We first established that *O. majusculus* can develop on spider mites as exclusive food. A preliminary assay demonstrated that neonates consumed on average six non-*Bt* spider mites per day, increasing to more than 50 for fifth instars. All

Table 1 Spider mite assay 1: Development and fecundity of spider mites (*Tetranychus urticae*) raised on leaf discs of *Bt* (MON 88702) and near-isogenic non-*Bt* cotton plants (DP393)

	<i>Bt</i>	Non- <i>Bt</i>	Statistic <i>Bt</i> /non- <i>Bt</i>	% det. diff.
Egg hatching first gen. (%)	98.3 (60)	100 (60)	GLMER, $\text{Chi}^2 < 0.001$, $p = 1.0$	–
Egg hatching time (days)	3.9 ± 0.05 (59)	3.9 ± 0.05 (60)	GLMER, $\text{Chi}^2 = 0.009$, $p = 0.9$	5
Juvenile survival (%)	66 (59)	79 (58)	GLMER, $\text{Chi}^2 = 2.53$, $p = 0.1$	20
Juvenile dev. time (days)	5.1 ± 0.11 (39)	5.2 ± 0.13 (46)	GLMER, $\text{Chi}^2 = 0.06$, $p = 0.8$	10
Gender (% females)	67 (39)	52 (46)	GLMER, $\text{Chi}^2 = 1.81$, $p = 0.2$	28
Female longevity (days)	9.7 ± 0.65 (23)	8.9 ± 0.65 (24)	GLMER, $\text{Chi}^2 = 0.57$, $p = 0.4$	30
Total fecundity (# eggs)	53.8 ± 5.98 (24)	48.3 ± 6.15 (24)	LMER, $\text{Chi}^2 = 0.31$, $p = 0.6$	52
Daily fecundity (# eggs)	6.3 ± 0.38 (24)	5.9 ± 0.41 (24)	LMER, $\text{Chi}^2 = 0.52$, $p = 0.5$	28
Egg hatching second gen. (%)	98.7 ± 0.49 (23)	96.4 ± 1.34 (23)	LMER ^a , $\text{Chi}^2 = 0.85$, $p = 0.4$	–

Values are presented as mean $\pm$ SE (*N*). *N* is the number of individuals (replicates). Egg hatching time, juvenile development time, and female longevity were analysed with generalized linear mixed effect models (GLMER) assuming Poisson distribution, juvenile survival and gender with GLMER assuming binomial distribution (logit link function), and fecundity with linear mixed effect models (LMER). Plant type (*Bt*/non-*Bt*) was modelled as fixed factor and experimental repetition (runs 1–3) as random factor. Detectable differences are based on data (*N*, mean and SD) from the non-*Bt* control treatment

^aStatistics performed with arcsin(sqrt) transformed data

stages of spider mites (including eggs) are consumed by the predator (Figure S1, Supplementary Online Material). The preliminary assay confirmed that *O. majusculus* can develop well with exclusive spider mite prey.

The purpose of the first predator assay was to test whether neonates differ in their development to adulthood when exclusively fed with spider mites from *Bt* cotton or non-*Bt* cotton. To obtain neonates, fresh bean pods were placed in the culture vessels for egg laying. After 24 h, the pods with newly laid eggs were incubated in a growth chamber (Panasonic MLR 352 H-PE, Labtech Services, Villmergen, Switzerland) at 25 °C, 70% RH, and 16:8-h photoperiod until neonates hatched. Similar to the spider mite assays, 5 cm plastic dishes with ventilated lids were lined with moist cotton pads. One *Bt* or non-*Bt* cotton leaf disc (diameter 38 mm) was placed in each dish, and one *O. majusculus* neonate (< 24 h after hatching) was introduced. Spider mites from *Bt* or non-*Bt* cotton were provided to the respective leaf discs. To ensure ad libitum feeding, the amount of spider mites was increased during the experiment from approximately 10 for neonates to at least 60 for fifth instar *O. majusculus* (see Fig. S1, Supplemental Online Material). Leaf discs, dishes, and cotton pads were changed every 3–4 days to ensure continuously high exposure of the spider mites to the *Bt* protein.

Survival and developmental stage of *O. majusculus* were recorded daily until the nymphs became adults or died. Moultings were determined by the presence of exuvia. The experiment ended with adult emergence. It was conducted twice with ten replicates and another two times with 20 replicates per plant treatment for a total of 60 replicates per treatment.

Predator assay 2: Fecundity on prey from *Bt* and non-*Bt* cotton

To be able to compare fecundity of *O. majusculus* on *Bt* versus non-*Bt* cotton, a second assay was conducted. Because of the high mortality in the *Bt* treatment of the previous predator assay, *O. majusculus* neonates, which were most susceptible to *Bt* proteins, were raised on *E. kuehniella* eggs for 5 days. Then, each nymph was weighed (Mettler-Toledo MX5) and placed either on a *Bt* or a non-*Bt* cotton leaf disc as described for the previous predator assay. Spider mites of the respective cotton plant type were provided ad libitum, and the leaf discs were changed every 3–4 days throughout the experiment. When nymphs became adults, the sex was determined and the body weight was measured. One female and one male were placed together on a larger leaf disc (diameter 54 mm) in a ventilated plastic dish (diameter 75 mm, height 23 mm) lined with a moist cotton pad. Males were removed once the first eggs were laid. If no eggs were laid after 4 days, the male was replaced. The replacement of males was repeated every 4 days until eggs were laid or the

female died. During the oviposition period, leaf discs were replaced every 2 days and the old discs were kept separately and monitored for egg hatching. Numbers of fresh eggs on the new discs and hatched nymphs on the old discs were recorded daily until all females died. The experiment was conducted twice. The first series started with 15 individuals in each treatment. Because of a lower number of females in the *Bt* treatment, the second series started with 20 individuals on non-*Bt* and 30 individuals on *Bt*. Nymphs that died 1 or 2 days after the start of the experiment were assumed to be damaged by handling and excluded (4 non-*Bt*, 2 *Bt*).

Determination of Cry protein in leaves, spider mites, and predatory bugs

During each experimental repetition of both spider mite assays, samples of leaves (5 mm diameter discs) were taken directly from the plants and frozen for the determination of Cry protein concentrations. In addition, in the second and third repetition of the first spider mite assay, leaf discs were incubated under the conditions of the experiment and samples were taken 4 days later. In the second spider mite assay (all repetitions), additional samples were collected after 7 days of incubation. Those matured leaf samples in comparison with the fresh leaf samples allow an estimation of the stability of the Cry protein during the time period between leaf disc changes.

At the end of each predator assay 1 (repetition 3 and 4) and assay 2 (both repetitions), leaf and spider mite samples were collected from the spider mite culture. At each time period, samples were collected from five groups of *Bt* cotton plants. To get an idea of the vertical distribution of Cry protein, samples were taken from the lower third, the middle third, and the upper third of the plants. Spider mite samples consisted of ca. 5 mg, leaf samples of ca. 10 mg. Sampled plants in the spider mite culture were in the flowering or boll-forming stage, but not necessarily of uniform age across the sampling periods.

To estimate Cry protein concentrations in the predator, neonates of *O. majusculus* were first reared on *E. kuehniella* eggs for 5 days. Subsequently, they were transferred to *Bt* cotton leaf discs and spider mites from the *Bt* culture were provided ad libitum. When the nymphs reached the fifth instar (after approximately 4 days), they were fed one more day with spider mites and then five samples consisting of six nymphs each were collected for Cry protein analysis. This procedure was repeated three times.

We also sampled leaves of non-*Bt* plants throughout the year and spider mites from the non-*Bt* culture as described previously. Cry protein concentrations in all five collected non-*Bt* leaf samples were below the limit of detection (LOD) of 0.0015 µg/g fresh weight (FW). Concentrations in all five spider mite samples were below the LOD when

the *Bt* and non-*Bt* cultures were in separate glasshouse cabins ($<0.0011 \mu\text{g/g}$ FW). When both cultures were in one large glasshouse cabin, Cry protein was detected in the three collected spider mite samples from the non-*Bt* culture, but concentrations were below 1% of those from the *Bt* culture ($0.2\text{--}0.7 \mu\text{g/g}$ FW).

All samples were weighed (FW) and stored at -80°C . For the extraction of Cry protein, $1 \times$ tris–borate buffer with 0.05% Tween20 was added at a ratio of at least 50:1 buffer (μL) to sample fresh weight (mg). After adding one 3 mm tungsten carbide ball, the tissues were macerated in a Qiagen TissueLyser II (Qiagen, Hombrechtikon, Switzerland) fitted with 24-tube adapters for microreaction tubes (Qiagen) at 30 Hz for 2 min. Macerated samples were centrifuged at $13,000 \times g$ for 5 min, and the supernatant was used either undiluted (*O. majusculus* samples and all non-*Bt* samples) or diluted with $1 \times$ PBST + 0.5% BSA depending on the expected concentration in the samples (*Bt* cotton leaves $500\text{--}1000 \times$, spider mites $200 \times$). The mCry51Aa2 protein concentrations were subsequently measured with a sandwich enzyme-linked immunosorbent assay (ELISA). On each ELISA plate, 2×8 purified mCry51Aa2 standards between 0.08 and 10 ng/mL were included, together with six blanks (PBST + 0.5% BSA), two positive controls (*Bt* cotton leaf extract of known concentration), and two negative controls (non-*Bt* cotton leaf extract).

96-well transparent flat bottom plates (nunc, Thermo Scientific, Rochester, NY, USA) were coated with $100 \mu\text{L}$ mouse anti-Cry51Aa antibody in 50 mM carbonate–bicarbonate + 150 mM NaCl buffer ($4 \mu\text{g}$ antibody/ mL buffer). Next day, the plates were washed three times with PBST (wash buffer, also used for subsequent washing steps) and blocked with $200 \mu\text{L}$ PBST + 1% (w/v) bovine serum albumin (BSA) for 1–2 h at 37°C . After $3 \times$ washing, $100 \mu\text{L}$ samples, standards, and blanks were loaded and incubated for 50–70 min at 37°C . The plates were $3 \times$ washed, loaded with $100 \mu\text{L}$ detection antibody in PBST + 0.5% BSA [goat anti-Cry51Aa(IgG)-biotin, $0.1 \mu\text{L}$ antibody/ mL buffer], and incubated for 50–70 min at 37°C . Plates were washed $3 \times$, reconstituted NeutrAvidin (enzyme conjugate, 100 units/mL , diluted $15,000 \times$ with PBST + 0.5% BSA) was loaded, and another incubation step at 37°C for 50–70 min followed. After another $3 \times$ washing, $100 \mu\text{L}$ TMB substrate solution was loaded and the colour reaction was developed at room temperature for 9–11 min. $100 \mu\text{L}$ 6 M phosphoric acid was added to stop the colour reaction, and absorbance was read at 450 nm with an infinite F200 plate reader (Tecan, Männedorf, Switzerland). The concentrations of mCry51Aa2 in each sample were determined with the standard curves using regression analyses based on a hyperbola model. The LOD was calculated based on $3 \times \text{SD}$ of the OD values of the blanks of each plate. At least, five blanks were loaded on each plate.

Data analysis

For both spider mite assays, the following parameters were analysed statistically: egg hatching rate (first generation), days from egg laying to hatching, juvenile survival from egg hatching to adult emergence, days from egg hatching to adult emergence, and gender of adult. For spider mite assay 1, we also analysed days from female emergence to death (female longevity), number of eggs laid from female emergence to death (total fecundity), number of eggs laid per day (total number of recorded eggs divided by female longevity), and egg hatching rate (second generation). To assess the egg hatching rate of the second generation (fertility), the proportion of unhatched eggs compared to the total number of incubated eggs was calculated for each female. For spider mite assay 2, we analysed male and female length, width, and weight. For both predator assays, statistical analyses were conducted for juvenile survival from the start of the experiment to emergence of adults. For predator assay 1, the development time of each nymphal stage was analysed separately, because of the high mortality in the *Bt* treatment. For predator assay 2, days from the start of the experiment to emergence of adults, gender, female weight, female longevity, and the percentage of fecund females, preoviposition time (days from emergence of female to first eggs), total fecundity, daily fecundity, and percentage of hatching eggs were analysed.

All data used for statistical analysis, tables, and figures for this publication can be found in the Supplementary Online Material (Supplementary Data Sheet 1). Data from the spider mite and predator performance assays were analysed with linear mixed effect models (LMER) or generalized linear mixed effect models (GLMER) using R statistical software (R version 3.5.1, The R Foundation for Statistical Computing, Vienna, Austria). For all categorical factors, contrasts were set to orthogonal. Time data (egg hatching time, juvenile development time, female longevity, preoviposition time) were analysed by GLMER with Poisson distribution from the lme4 package. Binomial data (gender, juvenile survival, egg hatching of the first generation, proportion of fecund females) were analysed by GLMER with binomial distribution (logit link function). Weight, length, width, and number of eggs were analysed by LMER. Egg hatching of the second generation was analysed by LMER after arcsin square root transformation. Effects of factors and interactions were determined from an ANOVA table with Type III sum of squares (car package). All GLMER and LMER models included “plant-type food” as a fixed factor and “experimental repetition” as a random factor.

Spider mite assay 2 used a full factorial model with the second fixed factor “plant-type mother origin” and the additional random factor “mother identity”.

In the first predator assay, survival analysis was conducted with tools from the survival package. A survival object was created (Surv function), Kaplan–Meier estimates for *Bt* and non-*Bt* cotton were calculated (survfit), and a log-rank test was applied to test for differences between both food plant types. A survival plot was created with ggsurvplot from the survminer package. The duration of individual developmental stages was analysed with GLMER.

Power analyses were performed to estimate detectable differences. Effect sizes (d) for time and fecundity data were based on two-sided t tests, true sample sizes, means and SDs of the non-*Bt* treatment, a power of 80%, α level of 0.05, and assuming equal sample sizes (package pwr). Using d , hypothetical second means were calculated from the mean of the non-*Bt* group. The detectable differences were then calculated from the percentage difference of the hypothetical means relative to the non-*Bt* means. For ratios (e.g. gender), effect size was similarly calculated based on a test of two proportions (for details of effect size calculations, see Shu et al. 2018).

Results

Spider mite assay 1: Development and fecundity on *Bt* and non-*Bt* cotton

Spider mites fed with *Bt* and non-*Bt* cotton leaves showed similar life-table parameters (Table 1). None of the measured parameters was significantly different between the two cotton treatments ($p \geq 0.1$, Table 1). The estimates of detectable differences based on power analysis (t test) revealed that egg hatching time was the most sensitive parameter, where a 5% difference from the control treatment could be detected (low variation, high N). The least sensitive parameter (highest variation, low N) was total fecundity, with a statistically detectable difference of 52%.

Spider mite assay 2: Development and adult measures on *Bt* and non-*Bt* cotton

The second spider mite assay showed similar results as the first assay (Table 2). Once more, none of the measured parameters was significantly different between the two cotton treatments ($p \geq 0.1$, Table 2).

Plant type, on which the mother of the tested spider mite was reared, was significant for male width ($p = 0.03$). A weak interaction of plant-type mother origin $\times$ plant-type food was present for juvenile survival ($p = 0.02$).

Table 2 Spider mite assay 2: Development and adult measures of spider mites (*Tetranychus urticae*) raised on leaf discs of *Bt* (MON 88702) and near-isogenic non-*Bt* cotton plants (DP393)

	<i>Bt</i>	Non- <i>Bt</i>	Statistic <i>Bt</i> /non- <i>Bt</i>	Sig. factors ^a	% det. diff.
Egg hatching first gen. (%)	98.5 (134)	96.3 (134)	GLMER, $\text{Chi}^2 = 0.005$, $p = 0.9$		6
Egg hatching time (days)	4.1 $\pm$ 0.02 (132)	4.1 $\pm$ 0.02 (129)	GLMER, $\text{Chi}^2 = 0.001$, $p = 1.0$		2
Juvenile survival (%)	91.7 (132)	92.3 (129)	GLMER, $\text{Chi}^2 = 0.71$, $p = 0.4$	$M \times P$	9
Juvenile dev. time (days)	5.0 $\pm$ 0.06 (121)	5.0 $\pm$ 0.07 (119)	GLMER, $\text{Chi}^2 = 0.01$, $p = 0.9$		6
Gender (% females)	48.8 (121)	57.1 (119)	GLMER, $\text{Chi}^2 = 1.91$, $p = 0.2$		18
Male length (μm)	292.1 $\pm$ 1.95 (61)	294.6 $\pm$ 1.70 (49)	LMER, $\text{Chi}^2 = 0.03$, $p = 0.9$		2
Female length (μm)	405.8 $\pm$ 5.01 (54)	403.8 $\pm$ 4.51 (66)	LMER, $\text{Chi}^2 = 0.20$, $p = 0.7$		5
Male width (μm)	166.0 $\pm$ 1.20 (61)	168.3 $\pm$ 1.23 (49)	LMER, $\text{Chi}^2 = 0.002$, $p = 1.0$	M	3
Female width (μm)	249.0 $\pm$ 1.62 (54)	249.9 $\pm$ 1.66 (66)	LMER, $\text{Chi}^2 = 2.73$, $p = 0.1$		3
Male weight (μg) ^b	3.6 $\pm$ 0.18 (17)	3.8 $\pm$ 0.12 (13)	LMER, $\text{Chi}^2 = 1.19$, $p = 0.3$		13
Female weight (μg)	12.5 $\pm$ 0.43 (54)	13.0 $\pm$ 0.44 (65)	LMER, $\text{Chi}^2 = 1.85$, $p = 0.2$		13

Values are presented as mean $\pm$ SE (N). N is the number of individuals (replicates). Egg hatching time and juvenile development time were analysed with generalized linear mixed effects models (GLMER) assuming Poisson distribution, egg hatching rate, juvenile survival and gender with GLMER assuming binomial distribution (logit link function), and length, width, and weight data with linear mixed effects models (LMER). Unless otherwise stated, the models were full factorial with plant-type food (*Bt*/non-*Bt*) and plant-type mother origin as categorical factors and experimental repetition (runs 1–3) and mother ID as random factors. Statistics of the comparison plant-type food are presented. Significance of the factor plant-type mother origin and interactions are also listed. Detectable differences are based on data (N , mean and SD) from the non-*Bt* control treatment

^a M plant-type mother origin, P plant-type food

^bCalculated from groups of pooled males (3–5 individuals per replicate). MotherID not included in the model

Fig. 1 Survival of *Orius majusculus* when fed exclusively spider mites from *Bt* (MON 88702, red) or non-*Bt* cotton (DP393, blue). **a** Kaplan–Meier survival estimates with 95% confidence limits. Nymphs reaching adulthood are censored (marked with X). **b** Number of nymphs becoming adults (number of censored individuals) on each day of the experiment. Data are pooled from four experimental repetitions with a total $N=60$

The most sensitive parameters (based on power analysis) were egg hatching time (2% detectable difference) and the length and width measurements (2–5%). The least sensitive parameter was gender (18%).

Predator assay 1: Survival on prey from *Bt* and non-*Bt* cotton

Survival of *O. majusculus* was reduced when fed prey from *Bt* compared to prey from non-*Bt* cotton (log-rank test, $\text{Chi}^2=44$, $p<0.0001$, Fig. 1a). In the non-*Bt* treatment, nymphs became adults between days 10 and 13 (Fig. 1b). In the *Bt* treatment, only six nymphs moulted into adults (days 13–16) (Fig. 1b). Developmental time in the *Bt* treatment was longer in all nymphal stages, but statistically different only for instars 1 and 3 (Table S1, Supplementary Online Material).

Predator assay 2: Fecundity on prey from *Bt* and non-*Bt* cotton

In contrast to the previous experiment with neonates, the 5-day-old nymphs survived well when fed with spider mites from *Bt* cotton (95%) (Table 3). Females were ca. 15% lighter when fed with prey from *Bt* cotton compared to prey from non-*Bt* cotton ($p=0.0001$), and longevity was reduced by approximately one-third ($p<0.0001$). Total and daily fecundity in the *Bt* treatment was only 20% and 30% of the non-*Bt* treatment, respectively ($p<0.0001$).

Table 3 Predator assay 2: Life table parameters of *Orius majusculus* nymphs fed with spider mites raised on *Bt* (MON88702) and near-isogenic non-*Bt* cotton plants (DP393) exclusively from day 5 onwards

	<i>Bt</i>	Non- <i>Bt</i>	Statistic <i>Bt</i> /non- <i>Bt</i>	% det. diff.
Start weight 5d (μg)	191.4 $\pm$ 3.99 (42)	187.5 $\pm$ 3.45 (31)	LMER, $\text{Chi}^2=0.13$, $p=0.7$	7
Juvenile survival (%) ^a	95.2 (42)	100.0 (31)	GLMER, $\text{Chi}^2<0.001$, $p=1.0$	–
Juvenile dev. time (days)	6.2 $\pm$ 0.14 (39)	6.1 $\pm$ 0.17 (31)	GLMER, $\text{Chi}^2=0.12$, $p=0.7$	11
Gender (% females)	41.0 (39)	64.5 (31)	GLMER, $\text{Chi}^2=3.5$, $p=0.1$	31
Female weight (μg)	461.6 $\pm$ 11.49 (16)	537.3 $\pm$ 14.50 (20)	LMER, $\text{Chi}^2=15.5$, $p=0.0001$	11
Female longevity (days)	15.5 $\pm$ 2.02 (16)	24.6 $\pm$ 2.14 (19)	GLMER, $\text{Chi}^2=21.5$, $p<0.0001$	35
Fecund females (%)	84.6 (13)	77.8 (18)	GLMER, $\text{Chi}^2=0.22$, $p=0.6$	33
Preoviposition time (days)	5.8 $\pm$ 0.70 (11)	5.8 $\pm$ 0.97 (14)	GLMER, $\text{Chi}^2=0.001$, $p=1.0$	69
Total fecundity (# eggs)	14.5 $\pm$ 4.47 (11)	67.6 $\pm$ 8.36 (14)	LMER, $\text{Chi}^2=43.8$, $p<0.0001$	51
Daily fecundity (# eggs)	0.9 $\pm$ 0.25 (11)	2.9 $\pm$ 0.28 (14)	LMER, $\text{Chi}^2=39.8$, $p<0.0001$	40
Egg hatching (%)	85.1 $\pm$ 3.63 (9)	92.4 $\pm$ 0.84 (14)	LMER ^b , $\text{Chi}^2=2.6$, $p=0.1$	–

Values are presented as mean $\pm$ SE (N). N is the number of individuals (replicates). Juvenile development time, female longevity, and preoviposition time were analysed with generalized linear mixed effects models (GLMER) assuming Poisson distribution, juvenile survival, and the proportion of fecund females with GLMER assuming binomial distribution (logit link function), and start weight of 5-day-old nymphs, female weight, total fecundity, daily fecundity, and the proportion of hatching eggs with linear mixed effects models (LMER). Plant-type food (*Bt*/non-*Bt*) was modelled as a fixed factor and experimental repetition (1 or 2) as a random factor. Detectable differences are based on data (N , mean and SD) from the non-*Bt* control treatment

^aJuveniles that died 1 or 2 days after the start of the experiment were excluded from analysis and assumed to have died because of handling

^bStatistics performed with arcsin(sqrt) transformed data

Fig. 2 Mean daily oviposition per female of *Orius majusculus* when fed exclusively spider mites reared on *Bt* (MON 88702) or non-*Bt* cotton (DP393) from 5-day-old nymphs onwards. Numbers in the plot represent *N* of each data point. Grey vertical lines represent SE

Fig. 3 mCry51Aa2 concentrations in cotton leaves, spider mites (*Tetranychus urticae*), and predatory pirate bugs (*Orius majusculus*). Note that the y-axis is log scale. For more details of the individual samples, see Tables S3–S4. Samples were taken in parallel to the feeding assays with pirate bugs. Black dots represent individual values; red vertical lines represent medians

Body weight at the beginning of the experiment, juvenile development time, gender ratio, number of females that produced offspring, preoviposition time, and egg hatching rate were similar between *Bt* and non-*Bt* cotton (Table 3). Oviposition was highest at the beginning of

the reproductive period and decreased continuously until death in both cotton treatments (Fig. 2).

Cry protein in leaves, spider mites, and predatory bugs

The mCry51Aa2 levels in matured leaves (at the time of replacement) collected during the spider mite assays differed from −54% to +12% compared to fresh leaves (Table 2, Supplementary Online Material). The mCry51Aa2 levels in leaves collected during the experiments with pirate bugs generally increased from the bottom third of the plant to the top third of the plant (Table S3, Supplementary Online Material). Measured concentrations in leaves ranged from 93 to 911 with a median of 207 µg/g FW (Fig. 3). The mCry51Aa2 levels in spider mites, which were sampled from the same plants, also increased from bottom to top (Table S3). Spider mites contained approximated one order of magnitude less mCry51Aa2 than leaves, ranging from 9.7 to 128 with a median of 29 µg/g FW (Fig. 3).

Finally, late *O. majusculus* nymphs that were kept on *Bt* cotton for 4 days contained two orders of magnitude lower concentrations than their prey with a median of 0.24 µg/g FW (Fig. 3, Table S4, Supplementary Online Material).

Discussion

Effects of *Bt* cotton on life history traits of herbivores and predators

In our study, *Bt* cotton producing mCry51Aa2 protein did not affect the survival, development, and growth of spider mites. Also, no effects were observed on spider mites after multiple generations feeding on *Bt* cotton, as demonstrated in the second spider mite assay, where eggs from mothers raised on *Bt* and non-*Bt* cotton were tested on both cotton types. Therefore, our findings indicate that *T. urticae* is not susceptible to plant-produced concentrations of mCry51Aa2 and indirect prey-quality mediated effects of the *Bt* cotton on the predatory bugs are unlikely (Romeis et al. 2019). These results are in line with those from previous studies demonstrating that spider mites are not sensitive to other *Bt* proteins, such as Cry1, Cry2, Cry3, or Cry34/35 (Dutton et al. 2002; Guo et al. 2016; Li and Romeis 2010; Lozzia et al. 2000; Shu et al. 2018).

Our study demonstrates that survival and development of first instar *O. majusculus* were negatively affected after feeding on *Bt* cotton-reared spider mites, while 5-day-old *O. majusculus* were not affected. Additionally, *O. majusculus* adults exhibited lower weight, shorter longevity, and decreased fecundity. Bachman et al. (2017) reported that survival of 5-day-old *O. insidiosus* was negatively

affected after feeding on mCry51Aa2 protein incorporated in artificial diet (67% compared to 98% on diet without mCry51Aa2). Interestingly, development time to adulthood was not affected (10.9 ± 0.2 days on mCry51Aa2-containing diet and 10.5 ± 0.1 days on control diet) in that study. Several bi- and tritrophic studies have shown that *Orius* spp. were not affected by *Bt* maize producing Cry1Ab (Al-Deeb et al. 2001; Zwahlen et al. 2000) or Cry1F (Tian et al. 2014), *Bt* cotton producing Cry1Ac (Torres and Ruberson 2008) or Cry1Ac/Cry2Ab (Tian et al. 2014), purified Cry3Bb1 (Duan et al. 2008) or Cry34Ab1 + Cry35Ab1 (De Schrijver et al. 2016) mixed into artificial *Orius*-diet, or purified Cry1Ac, Cry1Ab or Cry2Ab provided in a tritrophic system (Gonzalez-Zamora et al. 2007). In contrast, Lumbierres et al. (2012) reported that fecundity and development time of *O. majusculus* were positively affected after feeding on Cry1Ab-expressing maize tissues (pollen and leaves) or on spider mites reared on *Bt* maize. They hypothesize that characteristics of the plant material other than the Cry1Ab were responsible for those effects.

In the present study, however, we worked with plants producing a modified Cry protein that is intended to reduce damage by heteropteran pests. In contrast to previous studies, susceptibility of predatory bugs can thus be hypothesized based on the target spectrum of mCry51Aa2 (Bachman et al. 2017).

mCry51Aa2 protein concentrations in *Bt* cotton and arthropods

During the course of the spider mite experiments, the mCry51Aa2 protein concentrations, and consequently exposure of spider mites and predators, remained relatively stable. mCry51Aa2 protein levels in cotton leaves generally decreased from young leaves at the top to old leaves at the bottom. In general, protein contents tend to be reduced as plants age (Sachs et al. 1998). Halfhill et al. (2003) demonstrated that the concentrations of Cry1Ac in the leaves of *Bt* oilseed rape (*Brassica napus*) decreased as leaves aged. In addition, field data show that older cotton plants expressing Cry1Ac and Cry2Ab2 contained less *Bt* protein than young plants (e.g. Greenplate 1999; Eisenring et al. 2017; Knight et al. 2013).

Spider mites are among the herbivores that contain the highest amounts of *Bt* protein when feeding on *Bt* crops. Meissle and Romeis (2018) showed that Cry2Ab concentrations in spider mites were approximately half of the concentrations measured in the cotton leaves, and Cry1Ac concentrations even exceeded the concentrations in leaves by a factor of 7. In addition, Torres and Ruberson (2008) have demonstrated that the Cry1Ac concentrations in spider mites exceeded the concentrations in the leaves by about 17 times and Esteves Filho et al. (2010) reported four times

higher concentrations. These laboratory studies are supported by findings that most of Cry1Ab in *Bt* maize is present in mesophyll cells, which is the tissue that is mainly consumed by the spider mites (Dutton et al. 2004). In our study, mCry51Aa2 concentrations in spider mites were approximately one order of magnitude lower than in leaves. This demonstrates that expression patterns, digestion, and excretion dynamics vary among *Bt* proteins and plants. We believe that to date, our ELISA data are the first published with mCry51Aa2 in a herbivore, so comparison with other species is not possible. However, based on the literature available for other *Bt* proteins, we are confident that the concentrations measured in spider mites represent relatively high exposure to generalist predators (Romeis et al. 2019).

We also demonstrated that mCry51Aa2 protein is transmitted along the food chain to the predatory bug. Accordingly, the mCry51Aa2 concentration in *O. majusculus* represented approximately 1% of the concentration in the spider mite prey and thus 0.1% of the concentration in the leaves. Tian et al. (2014) found that adult *O. insidiosus* contained 3–4% of the *Bt* protein measured in *Bt*-resistant lepidopteran prey species (*Trichoplusia ni* and *Spodoptera frugiperda*) from laboratory-grown *Bt* plants (Cry2Ab-producing cotton, Cry1F-producing maize). In addition, Torres and Ruberson (2008) reported 17% of the Cry1Ac content of the western flower thrips on *Bt* cotton in *O. insidiosus*. In *O. insidiosus* collected in a *Bt* cotton field, Eisenring et al. (2017) detected the presence of Cry1Ac and Cry2Ab, while concentrations were approximately three orders of magnitude lower than in plant material. This confirms that *Orius* spp. do ingest *Bt* proteins in the cotton field, and the concentrations were similar to our tritrophic laboratory results.

For *Bt* maize producing Cry1Ab or Cry3Bb1, it was furthermore demonstrated that biological activity of the *Bt* proteins was maintained when *T. urticae* reared on *Bt* maize was fed to sensitive insects (Meissle and Romeis 2009b, Obrist et al. 2006). We thus assume that *O. majusculus* was also exposed to relatively high concentrations of bioactive mCry51Aa2 in the present study.

Conclusions

Our tritrophic bioassays with *Bt* cotton leaves, spider mites, and *O. majusculus* provide an experimental design for an early tier laboratory study that represents tritrophic worst-case exposure conditions through obligate feeding on spider mites. Based on the results of our study, *O. majusculus* can be reared on an exclusive diet of spider mites from neonate to adult, though this is unlikely to happen in a field scenario considering the generalist feeding behaviour of *Orius* spp. The continuous and high exposure to the mCry51Aa2 protein when feeding exclusively on spider mites that ingested

Bt cotton led to lethal and sublethal effects on *O. majusculus* nymphs. While this indicates a potential hazard for pirate bugs, our results do not support a conclusion of risk in the field. Pirate bugs prey on many kinds of small arthropod species, and they can supplement their diet with plant materials, such as pollen (Dicke and Jarvis 1962; Kiman and Yeargan 1985). We selected spider mites as prey to ensure that *O. majusculus* ingest high amounts of *Bt* protein. In contrast, studies with other *Bt* crops have revealed that whiteflies and aphids contained no or at most traces of *Bt* protein (Romeis and Meissle 2011). Thus, pirate bugs in the field are likely to feed on a mix of different quality prey species with high, medium, and low mCry51Aa2 concentrations. The effects observed under worst-case tritrophic conditions might thus be mitigated when alternative, high-quality prey with low mCry51Aa2 levels is available. Follow-up studies including more realistic exposure conditions are currently being conducted to further understand the impact of mCry51Aa2-producing cotton on *O. majusculus*.

Authors' contributions

MM, JR, and YJK conceived and designed the research. YJK and SK conducted experiments. MM and YJK analysed data. YJK and MM wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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