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ORIGINAL ARTICLE

Prey-mediated effects of mCry51Aa2-producing cotton on the predatory non-target bug *Orius majusculus* (Reuter)

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Abstract

Genetically engineered (GE) cotton, MON 88702, is protected against certain sucking pests, such as plant bugs and thrips, by producing mCry51Aa2, a modified protein from *Bacillus thuringiensis* (Bt). Predatory pirate bugs (*Orius* spp.), natural enemies contributing to biological pest control, are also sensitive to the insecticidal protein when exposed continuously to high concentrations.

We evaluated effects of MON 88702 on *Orius majusculus* when fed prey types with different mCry51Aa2 concentrations. When neonates were provided exclusively *Tetranychus urticae* spider mites reared on MON 88702 (high mCry51Aa2 content), adverse effects on predator survival and development were confirmed, compared with specimens fed prey from near-isogenic non-Bt cotton. When fed a mixture of *T. urticae* and *Ephestia kuehniella* eggs (mCry51Aa2-free), predator life table parameters were similar to the treatment where eggs were fed exclusively. When mCry51Aa2-containing spider mites were provided for a limited time at the beginning or the end of juvenile development, effects were less pronounced. While pirate bug nymphs showed similar consumption rates for prey from Bt and non-Bt cotton, choice experiments revealed a preference for *E. kuehniella* eggs over spider mites. Lepidopteran larvae (*Spodoptera littoralis*, high mCry51Aa2 content) or cotton aphids (*Aphis gossypii*, mCry51Aa2-free) reared on MON 88702 as alternative prey did not result in adverse effects on *O. majusculus*.

Our study suggests limited risk of mCry51Aa2-producing cotton for *O. majusculus*, because its sensitivity for the Bt protein is relatively low and its natural food consists of diverse prey species with varying concentrations of Bt protein.

Key words ecosystem services; environmental risk assessment; enzyme-linked immunosorbent assay (ELISA); genetically modified crops; Heteroptera: Anthocoridae; tritrophic interactions

Introduction

Genetically engineered (GE) crops have become the fastest adopted crop technology in the history of modern agriculture (Khush, 2012; ISAAA 2019). Most commercialized GE crops provide insect resistance, herbicide tolerance or a combination of both. Insect resistant plants produce crystalline (Cry) proteins or vegetative insecticidal proteins (VIP) from the bacterium *Bacillus thuringiensis* (Bt). Ingested Bt proteins kill susceptible insects by binding to and disrupting the midgut membranes (Bravo *et al.*, 2013; Jurat-Fuentes & Crickmore, 2017). Bt crops can help to reduce the need for insecticides that often have adverse effects on beneficial species, such as decomposers, natural enemies, or pollinators (Naranjo, 2009; Romeis *et al.*, 2019). However, potential risks for beneficial species also need to be evaluated for Bt crops (Romeis *et al.*, 2019). This is one important part of the environmental risk assessment that precedes regulatory approval for field release of any GE crop (Romeis *et al.*, 2008).

Bt cotton is one of the most important biotech crops worldwide, carrying one or multiple genes for insect resistance (ISAAA, 2019). Cotton is attacked by a complex of insects, including aphids, whiteflies, plant bugs, thrips, spider mites, and weevils. The main pests of cotton worldwide, however, are diverse sets of caterpillars (lepidopteran larvae), such as *Heliothis virescens* (Fabricius), *Helicoverpa* spp., (both Noctuidae) and *Pectinophora gossypiella* (Saunders) (Gelechiidae) (Anderson *et al.*, 2019; Luttrell *et al.*, 1994). Today's Bt cotton is protected against lepidopteran pests, while non-target species, such as natural enemies, generally remain unaffected (Naranjo, 2009). Functional biological control by the natural enemy complex is crucial for keeping populations of secondary pests below thresholds for economic injury and integrated pest management principles need to be applied to foster natural pest control (Anderson *et al.*, 2019; Naranjo *et al.*, 2020). In some cotton growing areas, however, plant and stink bugs benefit from reduced applications of broad-spectrum insecticides and reduced competition with caterpillars. Outbreaks of these pests may threaten the economic and ecological benefits of Bt cotton (Lu *et al.*, 2010; Musser *et al.*, 2009; Zeilinger *et al.*, 2011).

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Gowda *et al.* (2016) presented a novel GE cotton, MON 88702 (referred to as “Bt cotton”), for controlling hemipteran pests by producing the modified Bt protein mCry51Aa2. Those cotton plants effectively controlled plant bugs (*Lygus* spp., Miridae) and thrips (*Frankliniella* spp.; Thysanoptera: Thripidae) under field conditions (Akbar *et al.*, 2019). First-tier studies, in which a range of non-target species was exposed to high concentrations of mCry51Aa2, demonstrated selective and limited activity within the insect orders Hemiptera, Thysanoptera, and Coleoptera (Bachman *et al.*, 2017). Most of the tested arthropods, including other herbivores (four lepidopterans), pollinators (honey bee), decomposers (collembolan and earthworm), and natural enemies (two coccinellid beetles and one hymenopteran parasitoid), were not affected by mCry51Aa2. An adverse effect, however, was found for the pirate bug *Orius insidiosus* (Say) (Hemiptera: Anthocoridae). This is not surprising given the taxonomic relatedness with the targeted plant bugs (Hemiptera, suborder Heteroptera). When five-day-old juvenile *O. insidiosus* were fed with purified mCry51Aa2 mixed into artificial diet (200 or 400 µg/g diet), survival to adulthood was 67% for both concentrations and significantly lower than for control individuals (98%). Development time was not affected. Kim *et al.* (2021) used a more realistic, tritrophic system and the European species *Orius majusculus* (Reuter). When five-day-old nymphs were fed exclusively with spider mites, *Tetranychus urticae* (Koch) (Acari: Tetranychidae), from MON 88702, no effects on survival and development were observed, but the weight of emerging females was reduced and fecundity was only half compared with a diet consisting of spider mites from near-isogenic non-Bt cotton. Furthermore, survival of freshly hatched pirate bug nymphs to adulthood was less than 10% and development was prolonged by 3 days when fed exclusively spider mites reared on Bt cotton compared with non-Bt cotton (Kim *et al.*, 2021). Pirate bugs of the genus *Orius* are among the most dominant predators in cotton worldwide (Lattin, 1999). Although American and Eurasian cotton fields harbour different species, their biology is similar. For the present study, we selected the European species *O. majusculus*, a polyphagous predator feeding on thrips, aphids, springtails, spider mites, eggs and other soft-bodied arthropods (Ballal & Yamada, 2016; Lattin, 1999). Plant pollen can also be suitable as a food source for *Orius*

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spp. (Lattin, 1999). While Kim *et al.* (2021) worked with spider mites only to simulate worst-case exposure to cotton-produced mCry51Aa2, the goal of the present study was to create additional, more realistic exposure scenarios with different prey types to further characterize the risk. *Tetranychus urticae* spider mites and larvae of the African cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) contain relatively high concentrations of Bt protein when fed Bt cotton (Meissle & Romeis, 2018; Torres & Ruberson, 2008). In contrast, *Aphis gossypii* Glover (Hemiptera: Aphididae) aphids exhibit negligible concentrations when reared on Bt cotton (Lawo *et al.*, 2009; Meissle & Romeis, 2018; Zhang *et al.*, 2006). In addition, eggs of the Mediterranean flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were used as a model for non-mobile and Bt protein-free food sources. All four prey species can be easily reared under laboratory conditions, are readily consumed by *Orius* species, and are not targets of MON 88702.

The following research questions were addressed: (1) How do different prey species reared on MON 88702, fed exclusively or in combinations, affect the survival and the development of *O. majusculus*? (2) What are the preferences of *O. majusculus* nymphs in a choice situation with different combinations of the four prey species and is their choice affected by MON 88702? The generated data add to the understanding of the ecological relevance of differences between *O. majusculus* performance that were observed when spider mites from MON 88702 and non-Bt cotton were fed as exclusive prey.

Material and methods

Cultivation of cotton

Seeds from cotton expressing *mcry51Aa2* (event MON 88702, “Bt cotton”) and the non-transgenic near isoline (DP393, “non-Bt cotton”) were provided by Bayer Crop Science (St. Louis, USA). Cotton was planted individually in 1.3-L pots using heat-treated soil (60°C for at least 24h) to minimize soil-borne pests and diseases. The heat-treated soil was soaked for at least 15 minutes with water before

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planting. The plants were grown in a climate chamber at 25°C, 70% relative humidity (RH), and a 16 : 8 light to dark cycle. Water was provided daily. After approximately three weeks, plants were transferred to 3-L pots using untreated soil mixed with 15 g slow release fertilizer (Manna 3M, Wilhelm Haug GmbH, Ammerbuch, Germany). Liquid fertilizer (10% N, 6% P₂O₅, 6% K₂O, Manna, Wilhelm Haug GmbH) was applied weekly at 0.2%.

Rearing of O. majusculus

Orius majusculus was purchased from Andermatt Biocontrol (Grossdietwil, Switzerland) and cultured at Agroscope since October 2016. Colonies were maintained in ventilated 1.3-L transparent plastic cylinders. Sterilized eggs of *E. kuehniella*, purchased from Biotop (Livron-sur-Drôme, France), served as food. Frozen eggs were fixed on the sticky section of yellow Post-it paper and new food was provided every 2–3 days. Pods of “coco bean” (*Phaseolus vulgaris* L.) were provided for oviposition and water was supplied through a cotton plug spanning from an opening in the bottom of the cylinders to a glass container filled with water. The culture was maintained in the glasshouse at approximately 22°C with additional light to ensure at least 16h light per day throughout the year.

Rearing of prey species

All mobile prey species described below were provided by Syngenta Crop Protection Münchwilen AG (Stein, Switzerland). *Tetranychus urticae* (spider mite) colonies were established on Bt and non-Bt cotton and remained on the respective cotton type for approximately two years without deliberate mixing among plant types. Because both colonies were kept spatially apart in one large climate chamber (12 m², 25°C, 70% RH, 16 h light), exchange between colonies was not completely excluded. New plants in the flowering and boll-forming stage (>11 weeks old) were supplied every 4–5 weeks.

Aphis gossypii (cotton aphid) colonies were established on Bt and non-Bt cotton and remained on the respective cotton type for the experimental period (approximately two months). In this time

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period, aphids were transferred twice to new plants (5–6 weeks old). Both colonies were maintained in one climate chamber (6 m², 23°C, 70% RH, 16 h light), where Bt and non-Bt colonies were spatially separated.

Spodoptera littoralis (cotton leafworm) eggs were shipped as needed for the experiments. Eggs were incubated in a climate chamber (25°C, 70% RH, 16h light) for 3 days in 8.5 cm diameter plastic dishes with ventilated lids. Neonates were transferred into new plastic dishes, lined with a wet cotton pad and a leaf disc from Bt or non-Bt cotton (7–9 weeks old). After the lepidopteran larvae fed on cotton for 24 h, they were used as prey for *O. majusculus* nymphs.

Although all three mobile prey species are not targeted by mCry51Aa2, we confirmed that specimens from both cotton types had a similar weight, which we used as an indicator for the absence of adverse effects of MON 88702 on these species. We conclude that indirect, prey quality-mediated effects on *O. majusculus* are thus unlikely (for details see Online Resource 1).

Tritrophic feeding experiments

All experiments were carried out in a controlled climate cabinet (Panasonic MLR 352 H-PE, Labtech Services, Villmergen, Switzerland) at 25°C, 70% RH and 16 h light. To obtain *O. majusculus* neonates, fresh bean pods were placed in the plastic cylinders with adults for egg laying. After 24h, the pods were removed and incubated until neonates hatched. A moist cosmetic cotton pad (Migros Budget, Switzerland) and a 4 cm diameter leaf disc from either Bt or non-Bt cotton (7–9 weeks old) were placed in 5 cm diameter plastic dishes with ventilated lids. One neonate (< 24h after hatching) was introduced per dish. According to the particular experimental setup (described below), different prey species were added daily to ensure *ad libitum* food supply (Online Resource 2, Table S2). Prey items from the Bt cotton treatment were presented on Bt cotton leaf discs and prey from non-Bt cotton on non-Bt cotton discs. Leaf discs, dishes, and cotton pads were changed every 3–4 days. Survival was recorded daily until nymphs became adults or died. The development time to adulthood and the sex of the adults was determined (Ferragut & Gonz ales Zamora, 1994) and all

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adults were frozen at -70°C for mCry51Aa2 determination by Enzyme Linked Immunosorbent Assay (ELISA) (for details see Online Resource 3). During each experiment, samples of prey species and leaf material also were collected for ELISA (Online Resource 3). Each experiment was repeated 2–3 times with 10–20 neonates per treatment and repetition (Table S2). Because high mortality was observed in certain treatments in the first repetition (in particular in the Bt spider mite treatment), the number of replicates for those treatments was increased for the second repetition. Nymphs that died because of handling and nymphs that escaped the plastic dish were excluded from further analysis (Table S2).

Spider mites as prey

In **Experiment 1**, we tested if nymphs feeding on a mixed diet including prey with high mCry51Aa2 concentrations (spider mites reared on Bt cotton) and prey without Bt protein (*E. kuehniella* eggs) can mitigate adverse effects observed when feeding exclusively on spider mites from Bt cotton. Nymphs of *O. majusculus* were fed exclusively either *E. kuehniella* eggs provided on Bt or non-Bt leaf discs or spider mites reared and provided on Bt or non-Bt cotton. Two additional treatments were represented by mixed feeding with *E. kuehniella* & spider mites from Bt or non-Bt cotton (approximately same numbers of both prey items).

In a second series of experiments, we tested whether the persistence of adverse effects when feeding on spider mites from Bt cotton was limited in time. Neonates were fed either 2, 4, or 6 days with spider mites from Bt or non-Bt cotton and then switched to *E. kuehniella* eggs until adulthood (**Experiment 2**). Similarly, neonates were fed 2, 4, or 6 days with *E. kuehniella* eggs and then switched to spider mites from Bt or non-Bt cotton until adulthood (**Experiment 3**). Both experiments included a treatment where nymphs were fed exclusively spider mites (Bt or non-Bt) over the whole developmental time period and a treatment where spider mites (Bt or non-Bt) and *E. kuehniella* eggs were fed in alternation for 2d periods, starting either with spider mites (Experiment 2) or with *E.*

kuehniella (Experiment 3). As a control treatment, exclusive feeding on *E. kuehniella* eggs was included in both experiments.

Lepidopteran larvae or cotton aphids as prey

Lepidopteran larvae represent a second prey species with a relatively high expected mCry51Aa2 content. We tested for Bt effects when *O. majusculus* nymphs were fed exclusively lepidopteran larvae (**Experiment 4**). Because the larvae are known to defend themselves when attacked by predators, only young larvae were used as prey. Five neonate *S. littoralis* were reared on Bt or non-Bt cotton for 24h and subsequently fed to neonate *O. majusculus*. Larvae were replaced daily. The amount of prey larvae was increased to eight in the pre-adult stage of the predator. To test if feeding on high-quality food in the first two days of development decreases prey handling problems and increases survival of *O. majusculus*, additional treatments were included where nymphs were fed for two days with *E. kuehniella* eggs and then switched to lepidopteran larvae (Bt or non-Bt) until adulthood.

As alternative prey with little to no mCry51Aa2, cotton aphids from Bt or non-Bt plants were fed to *O. majusculus* nymphs (**Experiment 5**). The number of aphids presented to the nymphs ranged from approximately 10 for neonates to at least 40 for pre-adults. In both experiments, a control treatment, where nymphs were fed *E. kuehniella* eggs from neonate to adult, was included.

Consumption rates and choice experiments

To test for prey preferences of *O. majusculus* nymphs, consumption rates of the different prey items under no-choice conditions were determined and choice assays were performed. These experiments took place in the same experimental arenas and under the same climatic conditions as described for the previous experiments. For consumption rates, one of the four prey types reared on non-Bt cotton was offered to *O. majusculus* neonates (< 24h old) on a non-Bt leaf disc: *E. kuehniella* (10 eggs), spider mites (10 females), lepidopteran larvae (4 larvae fed 24h on cotton), or cotton

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aphids (8 adults). After 6, 12 and 24 hours, the number of consumed prey items was recorded. Consumed prey was restocked after 6 and 12 hours. The experiment was conducted at least twice for every prey type (total number of replicates: eggs $N = 35$, spider mites $N = 32$, lepidopteran larvae $N = 16$, aphids $N = 30$). In addition, 10 controls (prey without predator) were set up for each prey type to test for natural mortality and disappearance.

The consumption rates were used to decide on the number of prey items to be provided in the choice experiments. To test for the preferences of neonate *O. majusculus*, four combinations of prey types were tested: *E. kuehniella* (10 eggs) vs. spider mites (17 females), *E. kuehniella* (10 eggs) vs. lepidopteran larvae (3 larvae), cotton aphids (7 individuals) vs. spider mites (17 females), and cotton aphids (7 individuals) vs. lepidopteran larvae (3 larvae). Each pairing consisted of a prey type without Bt protein (cotton aphids, *E. kuehniella* eggs) against a prey type with high Bt protein content (spider mites or lepidopteran larvae) when reared on Bt cotton. One *O. majusculus* neonate (< 24h old) was placed on either a Bt or non-Bt leaf disc containing two prey types reared on the respective cotton type. After 6, 12, and 24 hours, the consumed prey items per prey type and cotton type were recorded. Consumed prey was restocked after 6 and 12 hours. The choice tests were replicated 30 times for each cotton type for the prey combination eggs vs. spider mites and 20 times for each cotton type for the prey combinations eggs vs. lepidopteran larvae, aphids vs. spider mites, and aphids vs. lepidopteran larvae.

Data analysis

The datasets analysed in the current study are available in the Supporting Information to this article (Online Resource 5). Life table data of *O. majusculus* from the tritrophic feeding experiments were analysed with linear mixed-effects models (LMER) or generalized linear mixed-effects models (GLMER) using R statistical software (R version 3.6.3, The R Foundation for Statistical Computing, Vienna, Austria), package lme4. For all categorical factors, contrasts were set to orthogonal. Weight data were analysed by LMER and development time by GLMER with Poisson distribution including

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the fixed factors prey type (P), cotton type (C) and sex (S). Because weight differed between sexes and interactions of sex with other factors were significant, separate LMERS were performed for each sex (Online Resource 2, Table S3). In contrast, for development time, GLMERs were simplified by removing the factor sex and its interactions because they were never significant. Sex and survival were analysed by GLMER with binomial distribution (logit-link function) and the fixed factors prey type (P) and cotton type (C). “Experimental repetition” was included as a random factor in all models. Effects of factors and interactions were determined from ANOVA tables with Type III sum of squares (car package) with $\alpha = 0.05$. Significant differences between individual prey treatments were determined with Tukey’s tests (emmeans package). For differences between survival probabilities, Games Howell test (rstatix package) was used because of a lack of variance in some treatments (when survival was 100%). If significant interactions between prey type and cotton type were observed, additional analyses were performed where prey type was compared for each cotton type and cotton type was compared for each prey type separately.

For consumption rates in no choice and choice experiments, differences between treatments were assessed by non-overlapping 95% confidence intervals.

For experiments 2–5, the treatments where *E. kuehniella* eggs were provided as exclusive food for *O. majusculus* were excluded from statistical analysis and presentation in the graphs and tables, because this treatment served as a check if experimental conditions were suitable (e.g., fitness of test specimens) and comparable among experiments. In all experiments, in the treatment with *E. kuehniella* eggs as exclusive food source, survival was 89%–100%, average development time 9.7–10.5 days, female weight 0.66–0.68 mg, and male weight 0.50–0.53 mg (Online Resource 2, Table S4).

Results

Tritrophic feeding experiments

Mean values, SDs and Ns of *O. majusculus* life table parameters recorded in the tritrophic experiments are presented in Online Resource 2, Table S5. Detailed information on the statistical tests can be found in Table S3.

Experiment 1

When *O. majusculus* nymphs were fed either spider mites (SM), *E. kuehniella* eggs (EE), or a mixture (EE & SM), prey type (P) had a significant effect on survival rates ($\chi^2 = 23.7$, $P < 0.0001$), while cotton type (C) was not significant ($P = 0.9$) (Fig. 1A). Because the interaction $P \times C$ was significant ($\chi^2 = 6.2$, $P = 0.04$), differences between prey types were separately analysed for Bt and non-Bt cotton. For Bt cotton, survival of nymphs fed eggs or a mixture of eggs and spider mites was higher than survival of nymphs fed spider mites ($P < 0.0001$). For non-Bt cotton, no significant difference among prey types was observed ($P = 0.08$). When survival on Bt and non-Bt cotton was compared for each prey type separately, lower survival was observed for nymphs fed Bt spider mites compared with those fed non-Bt spider mites ($P = 0.01$). The sex ratio of the emerging *O. majusculus* adults was not affected by prey or cotton type ($P \geq 0.1$).

The different prey types significantly influenced development time ($\chi^2 = 10.4$, $P = 0.006$), while no effect was evident for cotton type or the interaction ($P \geq 0.3$) (Fig. 1B). Nymphs fed spider mites needed significantly longer to adult emergence than nymphs fed eggs or a mixture of eggs and spider mites ($P \leq 0.0009$).

Female weight was affected by prey type (P , $\chi^2 = 310.5$, $P < 0.0001$) and cotton type (C, $\chi^2 = 5.4$, $P = 0.02$), while the interaction was not significant (Fig. 1C). Female weight was lower on spider mite diet compared with diet consisting of eggs or a mixture of eggs and spider mites ($P < 0.0001$). Females fed prey from Bt cotton were lighter than those fed prey from non-Bt cotton. The weight of

males was similarly affected by prey type ($\chi^2 = 66.6$, $P < 0.0001$), but not by cotton type ($P = 0.8$) (Fig. 1D). Males fed spider mites were lighter than those fed eggs or a mixture of eggs and spider mites ($P < 0.0001$). It has to be noted, however, that the analysis of male weight has to be treated with care because only one replicate was available for the Bt spider mite treatment.

Experiment 2

When *O. majusculus* nymphs were fed either spider mites (SM) exclusively, spider mites for 2, 4, or 6 days followed by *E. kuehniella* eggs (EE), or spider mites and *E. kuehniella* eggs in alternation (SM / EE alt.), prey type (P , $\chi^2 = 9.7$, $P = 0.046$) and cotton type (C , $\chi^2 = 43.3$, $P < 0.0001$) affected survival (Fig. 2A). The interaction $P \times C$ was not significant ($P = 0.2$). Survival of nymphs fed spider mites exclusively was lower than survival of nymphs fed spider mites and eggs in alternation and lower than survival of nymphs fed spider mites for 2 days followed by eggs. Nymphs in the Bt cotton treatments showed lower survival than those in the non-Bt cotton treatment. The sex ratio of the emerging *O. majusculus* adults was not affected by prey or cotton type ($P \geq 0.7$).

The different prey types ($\chi^2 = 16.2$, $P = 0.003$) and cotton types ($\chi^2 = 7.7$, $P = 0.006$) influenced development time without significant interaction ($P = 0.5$) (Fig. 2B). Nymphs fed spider mites exclusively needed significantly longer to adult emergence than nymphs fed spider mites and eggs in alternation and longer than nymphs fed spider mites for 2 days followed by eggs. Nymphs in the Bt cotton treatments needed longer to reach adulthood than those in the non-Bt cotton treatment.

Female weight was affected by prey type ($\chi^2 = 156.2$, $P < 0.0001$) and cotton type ($\chi^2 = 8.5$, $P = 0.004$), while the interaction was not significant ($P = 0.06$) (Fig. 2C). Female weight on exclusive spider mite diet was lower than in any other treatment and diet consisting of spider mites and eggs in alternation resulted in lower weight than spider mite diet for 2 days followed by *E. kuehniella* eggs. Females fed prey from Bt cotton were lighter than those fed prey from non-Bt cotton.

The weight of males was similarly affected by prey type ($\chi^2 = 177.9$, $P < 0.0001$) and cotton type ($\chi^2 = 24.7$, $P < 0.0001$) (Fig. 2D). Because the interaction also was significant ($\chi^2 = 17.4$, $P = 0.002$),

differences between prey types were separately analysed for Bt and non-Bt cotton. In the Bt cotton treatments, males fed spider mites exclusively were lighter than those in any other treatment and males fed alternately with spider mites and eggs were lighter than those fed spider mites for 2 days or 4 days followed by eggs. In the non-Bt cotton treatments, males fed spider mites exclusively were lighter than those in any other treatment. Males fed spider mites from Bt cotton exclusively or in alternation with *E. kuehniella* eggs were lighter than those fed prey from non-Bt cotton.

Experiment 3

When *O. majusculus* nymphs were fed either spider mites exclusively (SM); *E. kuehniella* eggs (EE) 2, 4, or 6 days followed by spider mites; or *E. kuehniella* eggs and spider mites in alternation (EE / SM alt.), prey type (P , $\chi^2 = 23.6$, $P < 0.0001$) and cotton type (C , $\chi^2 = 11.1$, $P = 0.0009$) affected survival rates (Fig. 3A). The interaction was not significant ($P = 0.5$). Survival of nymphs fed spider mites exclusively was lower than survival of nymphs in any other treatment. Nymphs in the Bt cotton treatments showed lower survival than those in the non-Bt cotton treatments. The sex ratio of the emerging *O. majusculus* adults was not affected by prey or cotton type ($P \geq 0.08$).

For development time, prey type was significant ($\chi^2 = 10.2$, $P = 0.04$), while cotton type was above the significance threshold ($\chi^2 = 3.1$, $P = 0.08$) and there was no significant interaction ($P = 0.9$) (Fig. 3B). Nymphs fed spider mites exclusively needed longer to adulthood than nymphs fed eggs and spider mites in alternation.

Female weight was affected by prey type ($\chi^2 = 126.2$, $P < 0.0001$) and cotton type ($\chi^2 = 20.0$, $P < 0.0001$), while the interaction was not significant ($P = 0.2$) (Fig. 3C). When fed 2 days *E. kuehniella* eggs followed by spider mites, female weight was lower than when fed 6 days eggs followed by spider mites. Furthermore, females in the treatment where eggs and spider mites were fed in alternation were heavier than those in any other treatment. Females fed prey from Bt cotton were lighter than those fed prey from non-Bt cotton. Also male weight was affected by prey type ($\chi^2 = 88.0$, $P < 0.0001$) and cotton type ($\chi^2 = 12.7$, $P = 0.0004$), without significant interaction ($P = 0.4$) (Fig.

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3D). A feeding period of 2 or 4 days on eggs followed by spider mites resulted in lower male weight than a feeding period of 6 days on eggs followed by spider mites or a feeding treatment of eggs and spider mites in alternation. Weight of males in the Bt cotton treatments was lower than in the non-Bt cotton treatments.

Experiment 4

Survival rates of *O. majusculus* nymphs were lower when feeding exclusively on lepidopteran larvae (LL) compared with nymphs feeding for 2 days on *E. kuehniella* eggs (EE) and afterwards on lepidopteran larvae ($\chi^2 = 16.5$, $P < 0.0001$, Table 1). Cotton type, however, had no significant effect on survival ($P = 0.08$) and there was no significant interaction between both factors ($P = 0.3$). Sex ratio, development time, and weight of females and males were not affected by prey type or cotton type.

Experiment 5

When *O. majusculus* nymphs were fed cotton aphids for their entire juvenile period, survival was $\geq 80\%$. There was no difference in survival, sex ratio, development time, female weight and male weight between nymphs reared on aphids from Bt cotton and nymphs reared on aphids from non-Bt cotton (Table 2).

Consumption rates and choice experiments

In a no-choice situation, highest consumption rates by *O. majusculus* neonates were observed for spider mites (13.0 individuals in 24 hours), followed by *E. kuehniella* (7.4 eggs), cotton aphids (4.6 individuals) and lepidopteran larvae (0.9 larvae) (Fig. 4, bottom part of each panel; Online Resource 2, Table S6). Prey consumption after 6, 12 and 24 hours increased in an approximately linear way (Online Resource 2, Fig. S1).

For the choice experiments (Fig. 4, upper part of each panel), the pairings always represented prey with a low Cry protein content or no Cry protein (CA, cotton aphids or EE, *E. kuehniella* eggs) against prey with a high toxin content (SM, spider mites or LL, lepidopteran larvae) when reared on Bt cotton. The choice of *O. majusculus*, however, was not affected by cotton type as evident from overlapping 95% confidence intervals in the Bt and non-Bt cotton treatments for all pairings and prey types.

A clear preference for *E. kuehniella* eggs over spider mites was observed. While 25% less eggs were consumed in the choice situation compared to the no choice situation, 80% less spider mites were consumed (Fig. 4A, Table S6). The preference of *O. majusculus* was less clear when cotton aphids and spider mites were presented together (Fig. 4B, Table S6). Consumption of cotton aphids was reduced by approximately 50%, while consumption of spider mites was reduced by approximately 65% in the choice situation compared with the no-choice situation. Lepidopteran larvae were consumed in similar amounts in choice and no-choice experiments (Fig. 4C, D, Table S6). When larvae were paired with *E. kuehniella* eggs, the consumed number of eggs was approximately one third lower compared with the no choice situation (Fig. 4C, Table S6) and when larvae were paired with cotton aphids, a similar number of aphids were consumed in both no choice and choice experiments (Fig. 4D, Table S6). It must be considered, however, that the amount of lepidopteran larvae preyed upon was generally low (approximately 1 per 24h). Similar to the no-choice situation, prey consumption after 6, 12 and 24 hours in the choice experiments increased in a linear way (Online Resource 2, Fig. S2).

Discussion

The tritrophic feeding experiments confirmed the susceptibility of *O. majusculus* to mCry51Aa2 in scenarios with continuously high exposure, represented by spider mite prey collected from Bt cotton. When feeding time on spider mites was reduced or when alternative prey with lower concentrations were provided, the observed effects decreased or disappeared. When given a choice, *O. majusculus*

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nymphs tended to prefer less mobile prey that allowed better performance (“high quality prey”), but did not discriminate between prey containing mCry51Aa2 and prey without.

*Exclusive feeding on spider mites from Bt cotton affects *O. majusculus**

When *O. majusculus* neonates were fed exclusively with spider mites from Bt cotton in the different tritrophic experiments, survival to adulthood was reduced by approximately half, development time prolonged by 2–4 days, and adult weight reduced by approximately 20% compared with spider mite prey from non-Bt cotton. When Kim *et al.* (2021) fed neonate *O. majusculus* exclusively with spider mites from Bt cotton, a higher effect on survival was reported (90%) and a similar effect on development time (3 days). In an additional experiment, Kim *et al.* (2021) started to feed *O. majusculus* nymphs with spider mites when they were already 5 days old. Survival and development time were similar, however, female weight was reduced by 15% and total and daily fecundity on mites from Bt cotton were approximately half compared with fecundity on mites from non-Bt cotton.

Spider mites feed on mesophyll cells (Bensoussan *et al.*, 2016), where Cry proteins are concentrated (Dutton *et al.*, 2004). They are thus among the herbivores that contain the highest concentrations of Bt protein when feeding on Bt crops (Meissle & Romeis, 2018; Obrist *et al.*, 2006; Torres & Ruberson, 2008). In the current study, median mCry51Aa2-concentrations in spider mites (30–103 $\mu\text{g/g}$ FW) were on average half of the median concentrations in cotton leaves (109–162 $\mu\text{g/g}$ FW) (Online Resource 3). Even higher exposure levels can be achieved when purified Cry protein is incorporated into artificial diet and fed to predators. In such assays, Bachman *et al.* (2017) reported 31% lower survival of 5-day-old *O. insidiosus* nymphs, when fed artificial diet containing 200 μg mCry51Aa2 per g compared with diet containing only buffer solution. The same survival was reported for 400 $\mu\text{g/g}$ and both concentrations of purified Cry protein in the diet had no significant effect on development time of *O. insidiosus*. The results of our tritrophic feeding studies and the results by Kim *et al.* (2021) and Bachman *et al.* (2017) demonstrate that *Orius* spp. are susceptible to mCry51Aa2 in such

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exposure scenarios. This is not surprising, given the fact that the target pests of MON 88702 are taxonomically related, also belonging to the suborder Heteroptera. However, susceptibility of the target species *Lygus hesperus* (Knight) was approximately two orders of magnitude higher with a LC_{50} of 3 μg mCry51Aa2 per ml artificial diet (Bachman *et al.*, 2017). We can rule out the possibility that effects observed on *O. majusculus* in the tritrophic experiments with spider mites as prey were indirect and prey-quality mediated (Romeis *et al.*, 2019), because *T. urticae* showed no susceptibility to plant-produced concentrations of mCry51Aa2 (Kim *et al.*, 2021) and the weight of specimens collected from Bt and non-Bt cotton was similar (Online Resource 1).

In contrast to mCry51Aa2, studies with Cry proteins with activity against target species from the orders of Lepidoptera and Coleoptera, such as Cry1Ac, Cry1Ab, Cry1F, Cry2Ab, Cry3A, and Cry3Bb1, did not report adverse effects on *Orius* spp. when purified protein was provided via artificial diet or pollen (Gonzalez-Zamora *et al.*, 2007; Duan *et al.*, 2008), when Bt plant pollen and/or foliage was provided (Armer *et al.*, 2000; Al-Deeb *et al.*, 2001; Pons *et al.*, 2004), and when prey from Bt plants was provided in tritrophic feeding assays (Zwahlen *et al.*, 2000; Gonzalez-Zamora *et al.*, 2007; Kumar *et al.*, 2014; Tian *et al.*, 2014). One study even reported positive effects when *O. majusculus* fed leaf tissue or spider mites from Cry1Ab-producing maize, which was discussed as plant variety-related effects (Lumbierres *et al.*, 2012).

Mixed diet and limited feeding time on spider mites reduces Bt effects

Early tier non-target risk assessment studies aim at high exposure levels to identify hazards. In the field, however, *Orius* species, being generalist predators, consume thrips, aphids, springtails, spider mites, eggs, other soft-bodied arthropods, or even pollen (Ballal & Yamada, 2016; Corey *et al.*, 1998; Lattin, 1999). We have tested if *O. majusculus* nymphs can also feed directly on cotton leaf discs, but we did not find significantly prolonged survival compared with a no-food treatment (Online Resource 4). Because different prey items exhibit very different Bt protein concentrations, overall

realistic exposure will most likely be considerably lower than in a tritrophic situation of feeding on spider mites as exclusive prey (Raybould *et al.*, 2007; Romeis & Meissle, 2011).

Compared with spider mites, feeding on *E. kuehniella* eggs resulted in higher survival to adulthood, reduced development time, and higher body weight of emerged *O. majusculus* adults, confirming superior suitability as prey (Sobhy *et al.*, 2010). In our experiments, *E. kuehniella* eggs represented a model for Bt protein-free and highly nutritious prey. When spider mites from Bt or non-Bt cotton were provided simultaneously with *E. kuehniella* eggs, no adverse effects on life history traits of *O. majusculus* nymphs were detected in the Bt treatment (Experiment 1, Fig. 1). We observed that both Bt and non-Bt spider mites were consumed when presented simultaneously with eggs, but the choice assays demonstrated that the predator preferred eggs.

We also investigated how feeding on spider mites for limited time periods affects *O. majusculus* nymphs (Experiments 2, 3, Figs. 2, 3). When spider mites were provided for 2, 4, or 6 days followed by *E. kuehniella* eggs, when eggs were provided for 2, 4, or 6 days followed by spider mites, or when spider mites and eggs were fed alternately in 2d intervals, *O. majusculus* nymphs performed better compared with exclusive spider mite feeding (analyses of prey type). In addition, in both experiments, performance was better when spider mites were provided that had fed on non-Bt cotton compared to those fed on Bt cotton. However, a significant interaction of prey type and cotton type could only be established for male weight in Experiment 2, which was lower in the Bt cotton treatment compared to non-Bt cotton when spider mites were fed exclusively or in alternation with *E. kuehniella* eggs, but not when spider mites were fed for 2, 4, or 6 days followed by eggs. Nevertheless, longer feeding periods of Bt spider mites tended to result in stronger effects on development time and adult weight, indicating that feeding on high quality prey cannot fully compensate adverse effects of multiple days of exclusive feeding on Bt spider mites. In addition, many *O. majusculus* nymphs died within the first two days when fed spider mites from Bt cotton, demonstrating that they are most susceptible to mCry51Aa2 early in their development. The study by Kim *et al.* (2021) supports this finding.

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No effects of Bt cotton when lepidopteran larvae and cotton aphids served as prey

Lepidopteran larvae were used as another model prey type with comparatively high concentrations of Cry protein (18 μg mCry51Aa2/ g after feeding for 24 h on Bt cotton, Online Resource 3). In a commercial product, however, mCry51Aa2 will most likely be stacked with Lepidoptera-active Cry proteins, thus lepidopteran larvae will generally be scarce in the field (except for species with low susceptibility to those Lepidoptera-active Cry proteins). When neonate *O. majusculus* fed exclusively on lepidopteran larvae, survival in the non-Bt treatment was moderate (62%, Table 1). This confirms that neonates of this predator struggle with the comparatively large and defensive caterpillars (Symondson *et al.*, 2002; Aragon-Sanchez *et al.*, 2018). When *O. majusculus* nymphs were fed *E. kuehniella* eggs for 2 d before being switched to Lepidoptera larvae as prey, survival was significantly improved. This indicates that older nymphs were better capable of handling caterpillars. We did not observe a significant effect of cotton type on any life table parameter, despite the relatively high mCry51Aa2 concentration in lepidopteran larvae (but note that predator survival was reduced to 23% when lepidopteran larvae fed with Bt cotton served as exclusive food). Because lepidopteran larvae showed similar weight gain and development time to L2 when feeding on Bt and non-Bt cotton, we conclude that *S. littoralis* is not sensitive to mCry51Aa2 (Online Resource 2).

Cotton aphids represent a mobile and abundant prey type that is almost free of Cry protein when feeding on phloem of Bt cotton, because Cry proteins are not transported in significant amounts in the phloem (Online Resource 3; Torres *et al.*, 2006; Meissle & Romeis, 2009; Romeis & Meissle, 2011). For *O. majusculus* nymphs, the suitability of cotton aphids as prey seemed to be inferior to *E. kuehniella* eggs indicated by slightly lower survival (80%), longer development time (> 11 d) and lower adult weight (females 0.5 mg, males 0.4 mg), which also has been shown previously for *O. insidiosus* (Mendes *et al.*, 2002). No lethal or sublethal effects, however, were observed when

nymphs consumed aphids reared on Bt cotton compared with non-Bt cotton (Table 2). This confirms studies with other Cry proteins (Romeis & Meissle, 2011).

O. majusculus prefers less mobile, high quality prey, but does not avoid prey with high Bt protein content

Knowledge on the prey spectrum of a predator in the field can help to refine the realistic exposure in environmental risk assessments. When given the choice between two prey types, *O. majusculus* nymphs tended to prefer more nutritious (allowing better performance) and less mobile (less defensive) prey, i.e. *E. kuehniella* eggs and cotton aphids, over spider mites and Lepidoptera larvae. Consequently, the nymphs selected for prey items with low mCry51Aa2 concentrations, which would reduce exposure if our results were transferrable to a field situation. With the simple experimental setup that we used, we cannot assess how the number of available prey items, prey size, mobility, and defensive behaviour (affecting encounter probability and prey handling time) as well as nutritional value of the prey types contributed to the observed prey consumption behaviour. However, all prey types were consumed and the nymphs did not show different feeding patterns when prey was reared on Bt or non-Bt cotton (Fig. 4).

Various studies examined the prey preferences of *Orius* species. Thrips were clearly preferred over spider mites, aphids, or whiteflies, independent of the ratio of the prey species (Arnó *et al.*, 2008; Butler & O'Neil, 2008; Xu & Enkegaard, 2009). In addition, Cry1Ac concentrations in thrips were reported to be approximately 20 times lower than in spider mites (Torres & Ruberson, 2008), which indicates reduced exposure when thrips are present compared with spider mites. Our study with mixed feeding scenarios and with *S. littoralis* larvae as prey showed no adverse effects on *O. majusculus* nymphs when prey was provided that contained less Bt protein than spider mites, indicating relatively low sensitivity to mCry51Aa2. Because thrips are generally more abundant in cotton, are preferred over spider mites, and contain less Bt protein than spider mites, one might speculate that adverse effects on *Orius* spp. are unlikely. However, predator-prey dynamics in the

field are complex. In addition to defensive behaviour and nutritional quality of the prey, predation rates and selection behaviour of *Orius* spp. are influenced by life stage of the predator and prey density (Butler & O'Neil, 2008; Xu & Enkegaard, 2009) as well as patch productivity (Venzon *et al.*, 2002). In addition, MON 88702 showed protection against thrips (Akbar *et al.*, 2018), while thrips, i.e. *Frankliniella occidentalis* (Pergande), were shown to prey on spider mite eggs (Trichilo & Leigh, 1986). This may also influence prey population dynamics in Bt compared with non-Bt cotton fields.

Conclusions

Our results, together with the findings by Kim *et al.* (2021) and Bachman *et al.* (2017), demonstrate that continuous dietary exposure to high concentrations of mCry51Aa2 affect different life history traits of pirate bugs. In our study, no active avoidance of Bt protein-containing prey was observed. However, limited feeding time on Bt protein-containing prey reduced the magnitude of effects and no effects were evident when prey items with high Bt protein concentrations were offered simultaneously with mCry51Aa2-free prey. As a generalist predator, *O. majusculus* is likely to consume a mix of prey items in the field, which will lead to highly variable exposure over time. Prey preference towards less mobile and less defensive prey that allows better performance, such as aphids or eggs, may reduce overall exposure to plant-produced Cry proteins. Because susceptibility of *O. majusculus* towards mCry51Aa2 was only evident under worst-case exposure conditions, effects under realistic (field) conditions, when prey items with high and low Bt protein concentrations are available, are less likely.

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Disclosure

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

Author contribution statement

Anja Boss: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Jörg Romeis:** Conceptualization, Methodology, Writing – Review & Editing, Supervision, Project administration. **Michael Meissle:** Conceptualization, Methodology, Validation, Formal analysis, Writing – Original Draft, Writing – Review & Editing, Supervision.

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Supporting Information

The following Online Resources are available:

Online Resource 1 Similarity of prey reared on MON 88702 and non-Bt cotton.

Table S1 Comparison of body weight and development time of arthropod prey species either reared on Bt (producing mCry51Aa2) or near isogenic non-Bt cotton.

Online Resource 2 Supplemental Tables and Figures.

Table S2 Set-up of tritrophic feeding experiments with *Orius majusculus*.

Table S3 Statistical ANOVA outputs (χ^2 and P -values) of all tested factors and interactions from the 5 tritrophic feeding experiments.

Table S4 Life-table parameters of *Orius majusculus* in Experiments 2–5 that fed exclusively *Ephestia kuehniella* eggs presented on Bt or non-Bt cotton leaf discs.

Table S5 Life-table parameters of *Orius majusculus* in Experiments 1–5.

Table S6 Consumed prey items by neonate *Orius majusculus* after a feeding period of 24 hours in a no choice and choice situation.

Fig. S1 Prey consumption by neonate *Orius majusculus* in a no choice situation recorded at three different time points (after 6h, 12h, 24h).

Fig. S2 Consumption rates in a choice situation of neonate *Orius majusculus* nymphs recorded at three different time points (after 6 h, 12 h, 24 h).

Online Resource 3 Determination of mCry51Aa2 in *O. majusculus*, prey species and cotton leaves (ELISA).

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Table S7 Median mCry51Aa2 concentrations (in $\mu\text{g/g}$ FW) of freshly developed *Orius majusculus* adults that were reared on diet from Bt or non-Bt cotton on the respective leaf discs.

Table S8 Median mCry51Aa2 concentrations (in $\mu\text{g/g}$ FW) of **cotton leaves and prey species** collected during the tritrophic *Orius majusculus* experiments for Bt and non-Bt treatments.

Online Resource 4 Survival of *O. majusculus* in absence of prey.

Table S9 Survival of *Orius majusculus* without prey on Bt (MON 88702 producing mCry51Aa2) or non-Bt cotton leaf discs or without leaf discs.

Online Resource 5 datasets analysed during the current study.

TABLES

Table 1 Experiment 4. Life-table parameters of *Orius majusculus* neonates fed either exclusively lepidopteran larvae (*Spodoptera littoralis*, LL) or *Ephestia kuehniella* eggs for 2 days and then lepidopteran larvae until adulthood (2d EE→LL). Neonate *S. littoralis* had fed on leaves of Bt or non-Bt cotton for 24h. Values are means \pm SD with the number of replicates in parentheses. GLMER and LMER models included prey type (P) and cotton type (C) as fixed factors (ns = not significant, *** $P < 0.001$).

	LL		2d EE→LL		C	P
	non-Bt	Bt	non-Bt	Bt		
Juvenile survival (%)	62 (26)	25 (36)	90 (20)	85 (20)	ns	***
Sex ratio (% females)	56 (16)	56 (9)	39 (18)	44 (16)	ns	ns
Development time (d)	11.4 \pm 2.6 (16)	11.8 \pm 1.0 (9)	10.6 \pm 0.9 (18)	10.4 \pm 1.1 (17)	ns	ns
Female weight (mg)	0.62 \pm 0.04 (9)	0.59 \pm 0.04 (5)	0.58 \pm 0.07 (7)	0.62 \pm 0.09 (7)	ns	ns
Male weight (mg)	0.44 \pm 0.06 (7)	0.43 \pm 0.03 (4)	0.43 \pm 0.05 (11)	0.39 \pm 0.06 (9)	ns	ns

Table 2 Experiment 5. Life-table parameters of *Orius majusculus* neonates fed exclusively cotton aphids (*Aphis gossypii*) reared on Bt or non-Bt cotton. Values are means \pm SD with the number of replicates in parentheses. GLMER and LMER models included cotton type (C) as fixed factor (ns = not significant).

	non-Bt	Bt	C
Juvenile survival (%)	82.8 (29)	80 (30)	ns
Sex ratio (% females)	47.8 (23)	56.5 (23)	ns
Development time (d)	11.4 \pm 0.58 (24)	11.3 \pm 0.56 (24)	ns
Female weight (mg)	0.45 \pm 0.05 (11)	0.49 \pm 0.07 (13)	ns
Male weight (mg)	0.36 \pm 0.04 (12)	0.39 \pm 0.02 (10)	ns

FIGURE CAPTIONS

Fig. 1 Experiment 1. Juvenile survival (A), development time (B), female weight (C) and male weight (D) of *Orius majusculus* raised on different prey types: *Ephestia kuehniella* eggs (EE), *Tetranychus urticae* spider mites (SM), or a mixture of both (EE & SM). Spider mites were reared either on mCry51Aa2-producing cotton (Bt) or near isogenic cotton (non-Bt). All prey types were presented on the respective Bt or non-Bt cotton leaf discs. Dots represent individual values, black rhombuses means, black horizontal lines medians, hinges 25th and 75th percentiles, and whiskers the smallest or largest values no further than $1.5 \times$ IQR from the hinges. The number of replicates (n) is given. Results of GLMER and LMER with fixed factors prey (P) and cotton type (C) are presented in grey boxes. Capital letters display significant differences among prey types. For survival (panel A), the two cotton types were analysed separately because of a significant $P \times C$ interaction and differences among prey types were observed for Bt cotton (small letters). Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, * $P < 0.001$)**

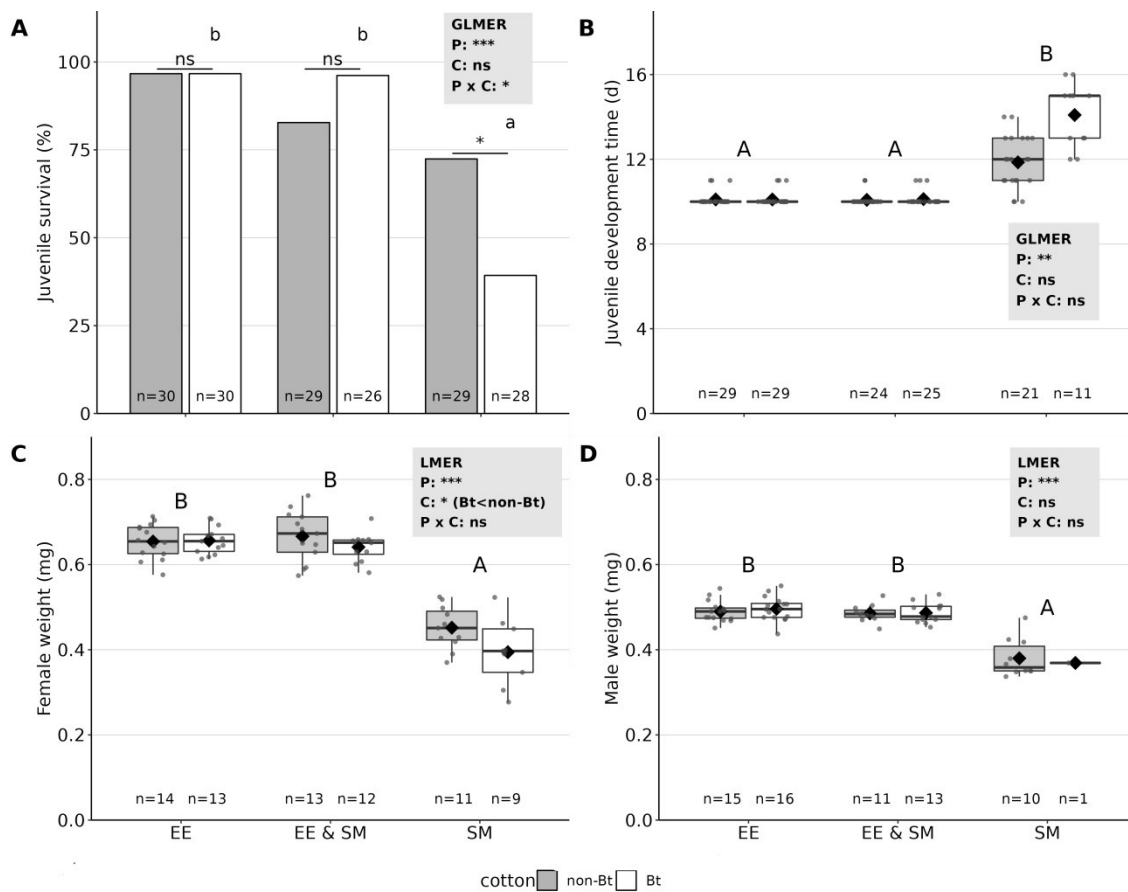


Fig. 2 Experiment 2. Juvenile survival (A), development time (B), female weight (C) and male weight (D) of *Orius majusculus* raised for different time periods on *Ephestia kuehniella* eggs (EE) or *Tetranychus urticae* spider mites (SM). Spider mites were reared either on mCry51Aa2-producing cotton (Bt) or near isogenic cotton (non-Bt). Treatments included feeding SM exclusively, feeding SM for 2, 4 or 6 days followed by EE, and feeding SM or EE alternating every two days. All prey types were presented on the respective Bt or non-Bt cotton leaf discs. Dots represent individual values, black rhombuses means, black horizontal lines medians, hinges 25th and 75th percentiles, and whiskers the smallest or largest values no further than $1.5 \times$ IQR from the hinges. The number of replicates (n) is given. Results of GLMER and LMER with fixed factors prey (P) and cotton type (C) are presented in grey boxes. Letters in panels (A) – (C) display significant differences among prey types. For male weight (panel D), the two cotton types were analysed separately because of a significant $P \times C$ interaction and differences among prey types were observed for non-Bt (capital letters) and Bt cotton (small letters). Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, * $P < 0.001$)**

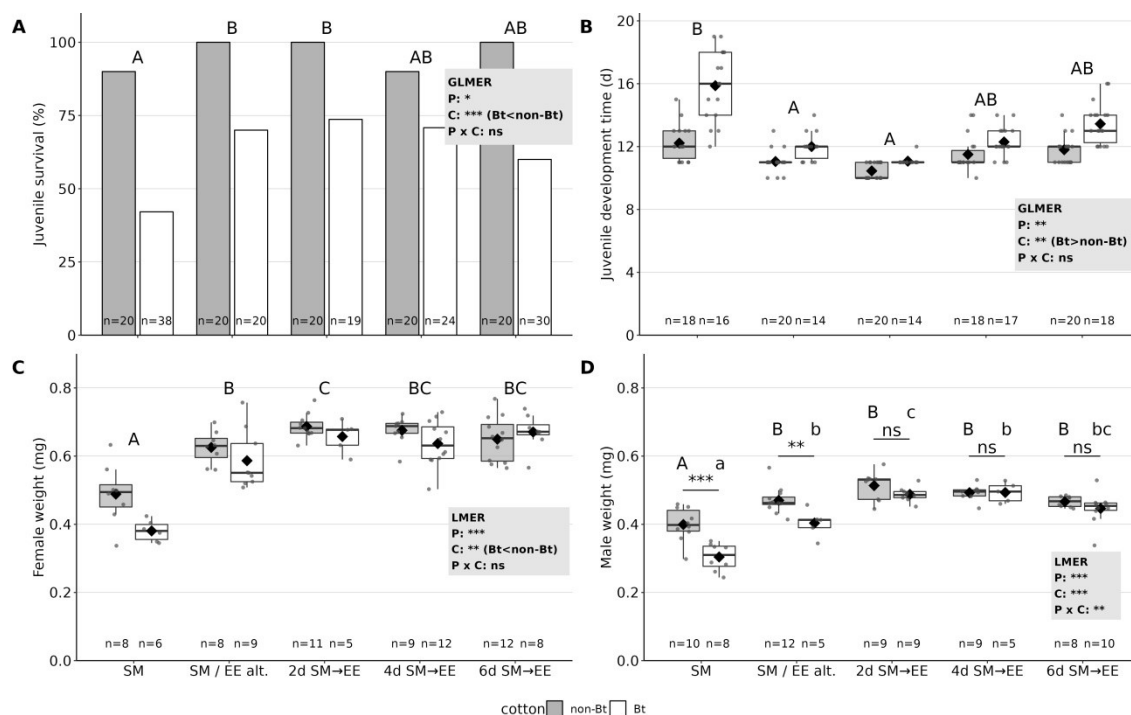


Fig. 3 Experiment 3. Juvenile survival (A), development time (B), female weight (C) and male weight (D) of *Orius majusculus* raised for different time periods on *Ephesthia kuehniella* eggs (EE) or *Tetranychus urticae* spider mites (SM). Spider mites were reared either on mCry51Aa2-producing cotton (Bt) or near isogenic cotton (non-Bt). Treatments included feeding SM exclusively, feeding EE for 2, 4 or 6 days followed by SM, and feeding EE or SM alternating (alt.) every two days. All prey types were presented on the respective Bt or non-Bt cotton leaf discs. Dots represent individual values, black rhombuses means, black horizontal lines medians, hinges 25th and 75th percentiles, and whiskers the smallest or largest values no further than $1.5 \times$ IQR from the hinges. The number of replicates (n) is given. Results of GLMER and LMER with fixed factors prey (P) and cotton type (C) are presented in grey boxes. Letters display significant differences among prey types. Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, * $P < 0.001$)**

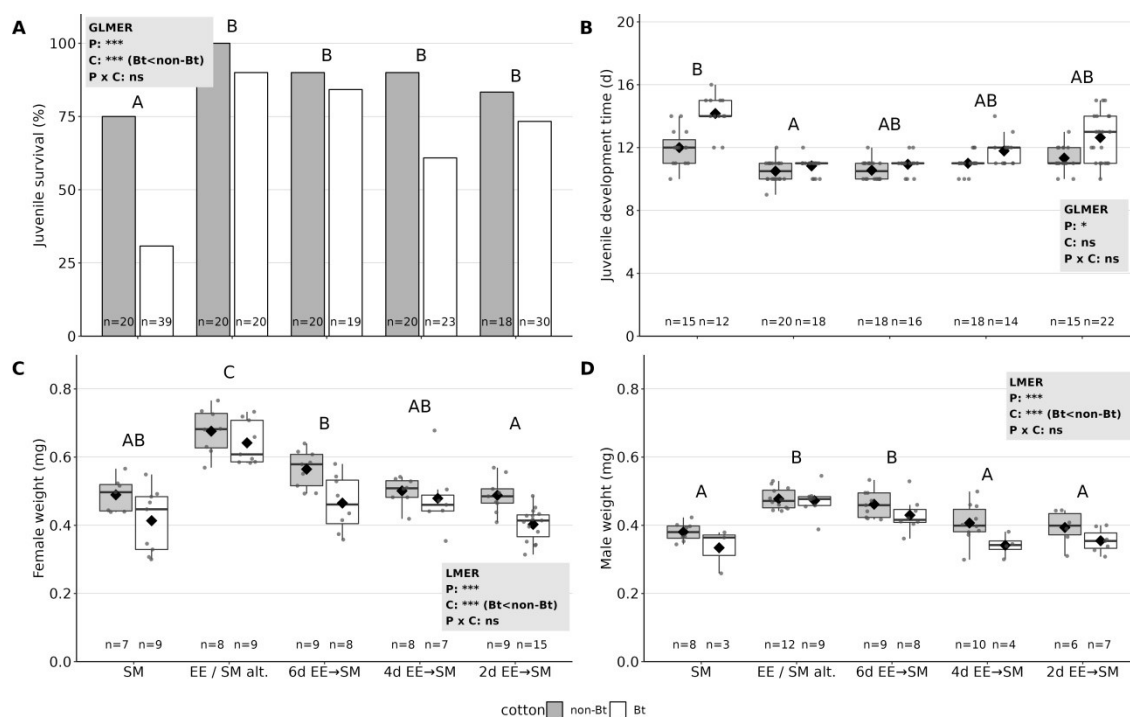
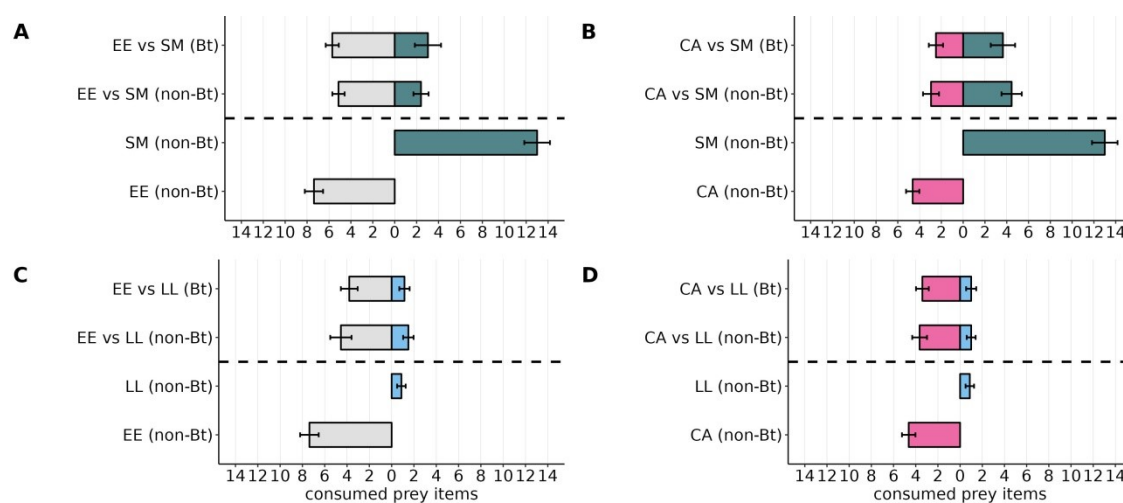


Fig. 4 Prey items consumed by neonate *Orius majusculus* after a feeding period of 24 hours in a no choice (bottom part of each panel) and choice (upper part) situation. Values represent means and error bars 95% confidence intervals. Choice experiments were conducted with prey from Bt and non-Bt cotton that were presented on leaf discs of the respective cotton type. EE = *E. kuehniella* eggs, SM = spider mites, CA = cotton aphids, LL = lepidopteran larvae. Note that for no-choice experiments, each bar is duplicated in two panels (e.g., SM non-Bt in Panel A and B)



Graph Abstract

While genetically engineered Bt cotton producing insecticidal mCry51Aa2 is protected against plant bugs, beneficial predatory pirate bugs are also sensitive if exposed continuously to high concentrations. When a mix of prey with high and low toxin content was provided, when alternative prey species with lower concentrations were provided, or when feeding time on prey with high concentrations was reduced, effects decreased or disappeared. In the field with a diverse prey spectrum, the risk of Bt cotton for *Orius* spp. is likely low.

