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ORIGINAL ARTICLE

Prey-mediated effects of mCry51Aa2-producing cotton on the predatory nontarget 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

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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) (Luttrell *et al.*, 1994; Anderson *et al.*, 2019). Today's Bt cotton is protected against lepidopteran pests, while nontarget 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 (Musser *et al.*, 2009; Lu *et al.*, 2010; Zeilinger *et al.*, 2011). 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 nontarget 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 5-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 5-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 harbor 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 (Lattin, 1999; Ballal & Yamada, 2016). Plant pollen can also be suitable as a food source for *Orius* 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 (Torres & Ruberson, 2008; Meissle & Romeis, 2018). In contrast, *Aphis gossypii* Glover (Hemiptera: Aphididae) aphids exhibit negligible concentrations when reared on Bt cotton (Zhang *et al.*, 2006; Lawo *et al.*, 2009; Meissle & Romeis, 2018). In addition, eggs of the Mediterranean flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were used as a model for nonmobile 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.

Materials and methods

Cultivation of cotton

Seeds from cotton expressing *mcry51Aa2* (event MON 88702, "Bt cotton") and the nontransgenic 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 24 h) to minimize soil-borne pests and diseases. The heat-treated soil was soaked for at least 15 min with water before 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 3 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% P_2O_5, 6% K_2O, 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 16 h 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 m^2, 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 2 months). In this time period, aphids were transferred twice to new plants (5–6 weeks old). Both colonies were maintained in one climate chamber (6 m^2, 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, 16 h 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 24 h, 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 (< 24 h 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 & González Zamora, 1994) and all 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* and 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 2-day 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 24 h and subsequently fed to neonate *O. majusculus*. Larvae were replaced daily. The amount of prey larvae was increased to eight in the preadult stage of the predator. To test if feeding on high-quality food in the first 2 days of development decreases prey handling problems and increases survival of *O. majusculus*, additional treatments were included where nymphs were fed for 2 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 preadults. 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 (<24 h old) on a non-Bt leaf disc: *E. kuehniella* (10 eggs), spider mites (10 females), lepidopteran larvae (4 larvae fed 24 h on cotton), or cotton aphids (8 adults). After 6, 12, and 24 h, the number of consumed prey items was recorded. Consumed prey was restocked after 6 and 12 h. 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) versus spider mites (17 females), *E. kuehniella* (10 eggs) versus lepidopteran larvae (3 larvae), cotton aphids (7 individuals) versus spider mites (17 females), and cotton aphids (7 individuals) versus 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

(<24 h 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 h, the consumed prey items per prey type and cotton type were recorded. Consumed prey was restocked after 6 and 12 h. The choice tests were replicated 30 times for each cotton type for the prey combination eggs versus spider mites and 20 times for each cotton type for the prey combinations eggs versus lepidopteran larvae, aphids versus spider mites, and aphids versus lepidopteran larvae.

Data analysis

The data sets analyzed 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 analyzed 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 analyzed by LMER and development time by GLMER with Poisson distribution including 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 analyzed 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 nonoverlapping 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 *N*s 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 × C was significant ($\chi^2 = 6.2$, $P = 0.04$), differences between prey types were separately analyzed 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

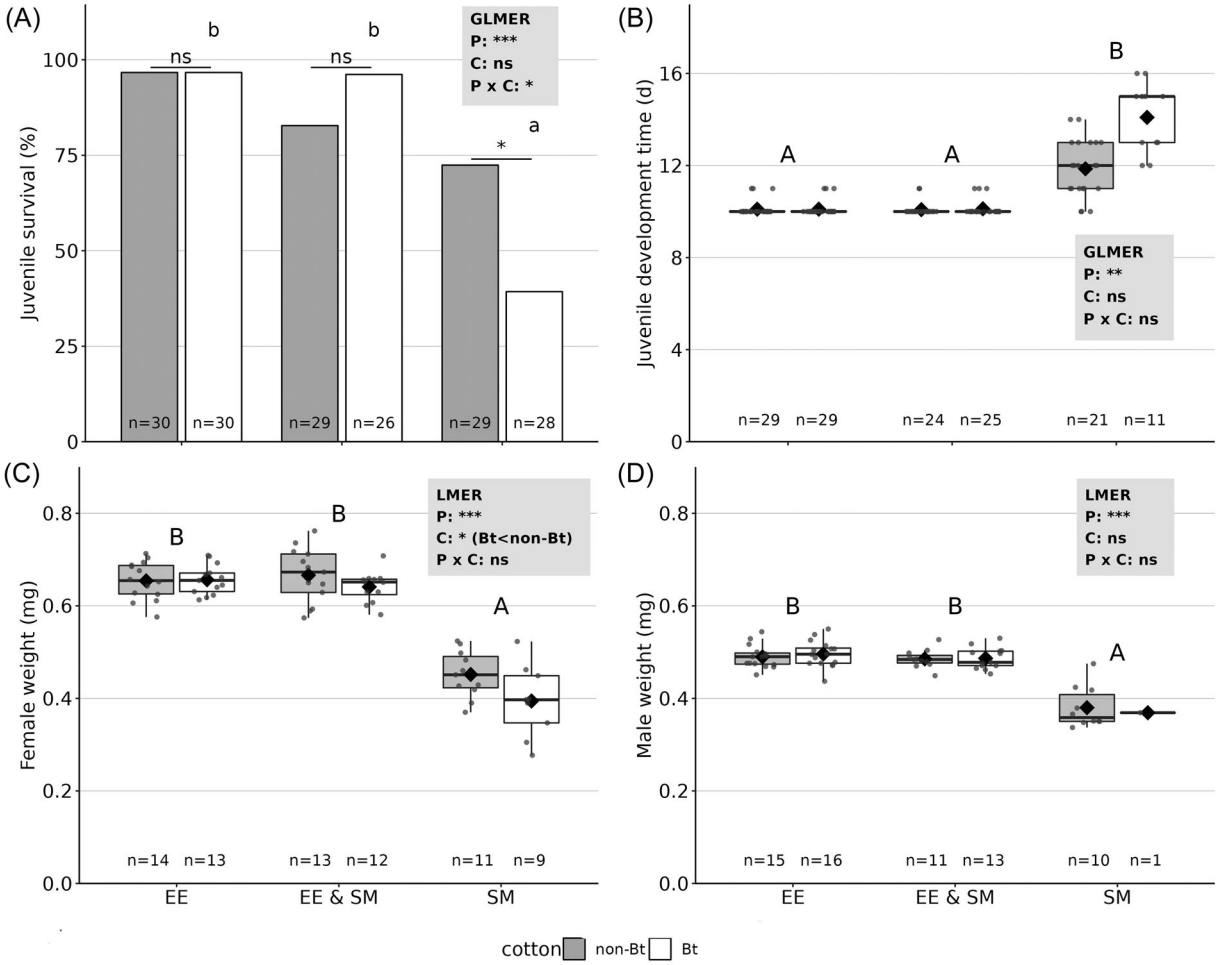

Fig. 1 Experiment 1. Juvenile survival (A), development time (B), female weight (C), and male weight (D) of *Orius majusculus* raised on different prey types: *Ephestia kuehniella* eggs (EE), *Tetranychus urticae* spider mites (SM), or a mixture of both (EE & SM). Spider mites were reared either on mCry51Aa2-producing 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 × 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 gray boxes. Capital letters display sigificant differences among prey types. For survival (panel A), the two cotton types were analyzed separately because of a significant P × C interaction and differences among prey types were observed for Bt cotton (small letters). Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

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 × 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

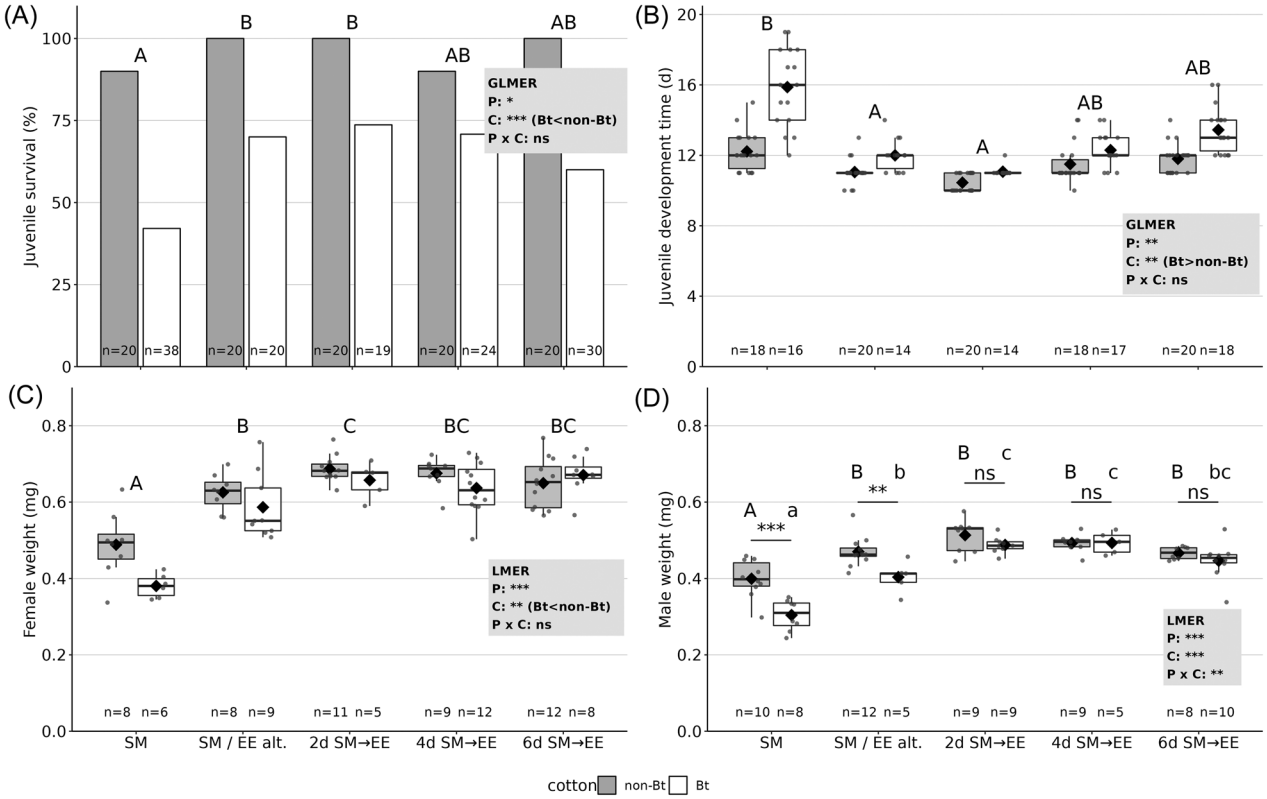

Fig. 2 Experiment 2. Juvenile survival (A), development time (B), female weight (C), and male weight (D) of *Orius majusculus* raised for different time periods on *Ephestia kuehniella* eggs (EE) or *Tetranychus urticae* spider mites (SM). Spider mites were reared either on mCry51Aa2-producing cotton (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 (alt.) every 2 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 × 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 gray boxes. Letters in panels (A)–(C) display sigificant differences among prey types. For male weight (panel D), the two cotton types were analyzed separately because of a significant P × C interaction and differences among prey types were observed for non-Bt (capital letters) and Bt cotton (small letters). Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

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 analyzed 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

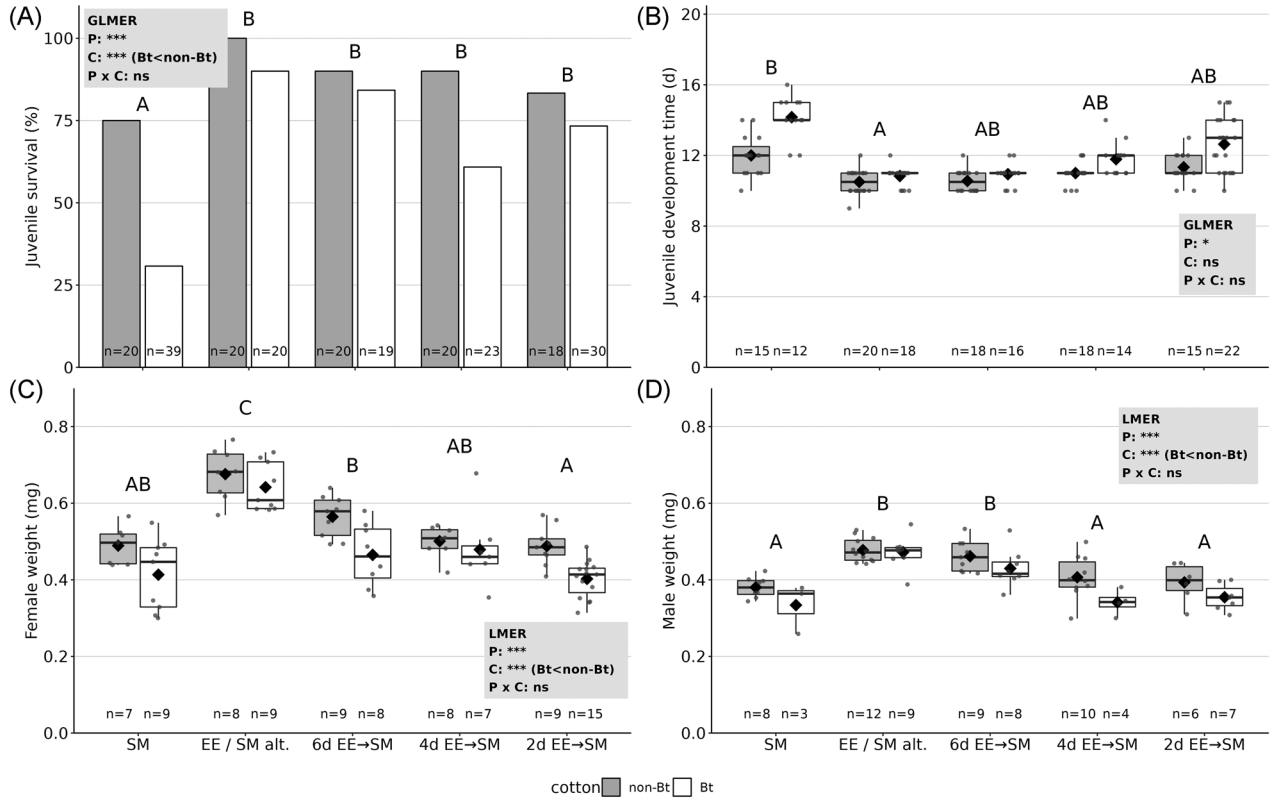

Fig. 3 Experiment 3. Juvenile survival (A), development time (B), female weight (C), and male weight (D) of *Orius majusculus* raised for different time periods on *Ephestia kuehniella* eggs (EE) or *Tetranychus urticae* spider mites (SM). Spider mites were reared either on mCry51Aa2-producing cotton (Bt) or near isogenic cotton (non-Bt). Treatments included feeding SM exclusively, feeding EE for 2, 4, or 6 days followed by SM, and feeding EE or SM alternating (alt.) every 2 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 × 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 gray boxes. Letters display sigificant differences among prey types. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

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

Table 1 Experiment 4.

Life-table parameter	LL		2-day 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 ± 2.6 (16)	11.8 ± 1.0 (9)	10.6 ± 0.9 (18)	10.4 ± 1.1 (17)	ns	ns
Female weight (mg)	0.62 ± 0.04 (9)	0.59 ± 0.04 (5)	0.58 ± 0.07 (7)	0.62 ± 0.09 (7)	ns	ns
Male weight (mg)	0.44 ± 0.06 (7)	0.43 ± 0.03 (4)	0.43 ± 0.05 (11)	0.39 ± 0.06 (9)	ns	ns

Note: 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 (2-day EE→LL). Neonate *S. littoralis* had fed on leaves of Bt or non-Bt cotton for 24 h. Values are means ± 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, ***$P < 0.001$).

Table 2 Experiment 5.

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

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

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. 3D). A feeding period of 2 or 4 days on eggs followed by spider mites or exclusive spider mite feeding 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 afterward 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 ≥80%. There was no difference in survival, sex ratio, development time, and weight of females and males 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 h), 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 h 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

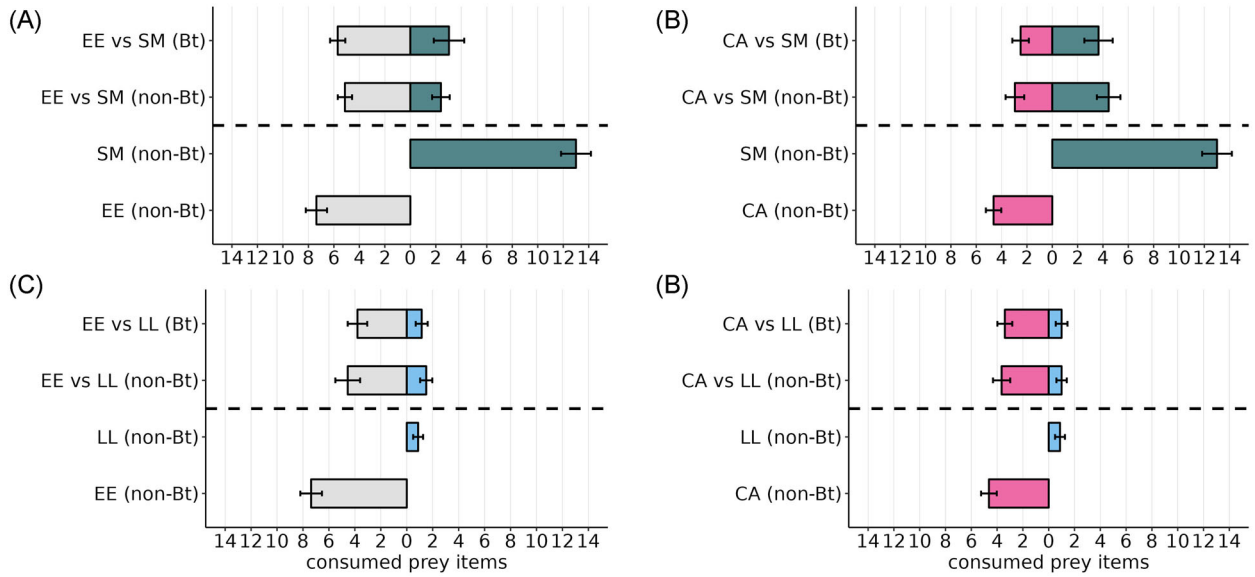

Fig. 4 Prey items consumed by neonate *Orius majusculus* after a feeding period of 24 h in a no choice (bottom part of each panel) and choice (upper part) situation. Values represent means and error bars 95% confidence intervals. Choice experiments were conducted with prey from Bt and non-Bt cotton presented 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)

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 24 h). Similar to the no-choice situation, prey consumption after 6, 12, and 24 h 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* 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 (Obrist *et al.*, 2006; Torres & Ruberson, 2008; Meissle & Romeis, 2018). In the current study, median mCry51Aa2-concentrations in spider mites (30–103 μg/g FW) were on average half of the median concentrations in cotton leaves (109–162 μg/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 μg/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 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 nontarget 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 (Corey *et al.*, 1998; Lattin, 1999; Ballal & Yamada, 2016). 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 2-day 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 2 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.

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; Aragón-Sánchez *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 days) 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 behavior (affecting encounter probability and prey handling time) as well as nutritional value of the prey types contributed to the observed prey consumption behavior. 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 behavior 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 toward 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* toward 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.

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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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.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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 (Chi^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 h 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 6, 12, and 24 h).

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

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

Table S7 Median mCry51Aa2 concentrations (in μg/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 μg/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 Data sets analyzed during the current study.

Open Access

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

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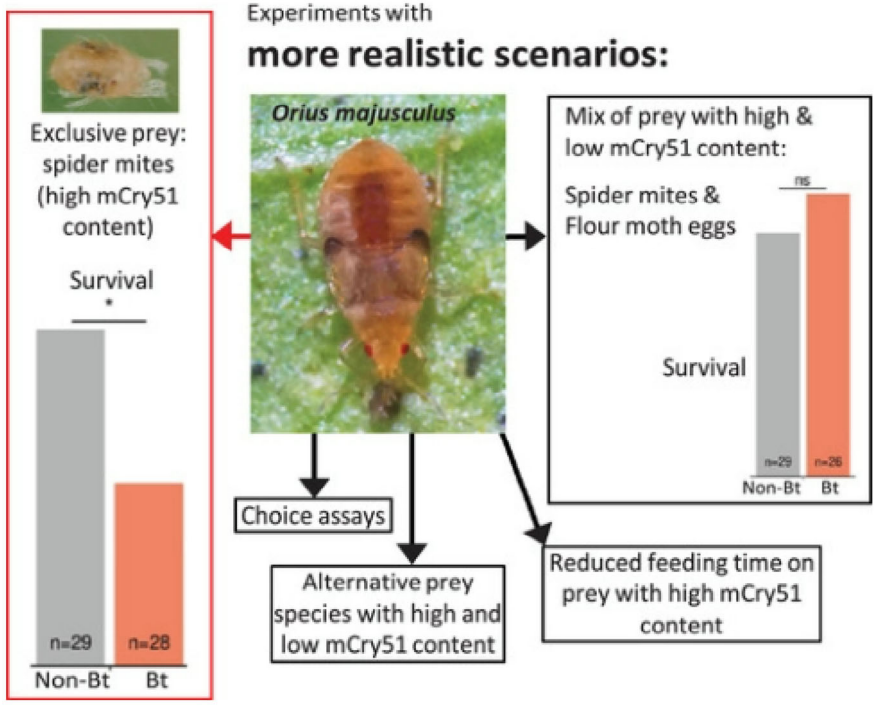

While genetically engineered Bt cotton producing insecticidal mCry51Aa2 is protected against plant bugs, beneficial predatory pirate bugs are also sensitive if exposed continuously to high concentrations. When a mix of prey with high and low toxin content was provided, when alternative prey species with 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.

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Insect Science

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

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ONLINE RESOURCE 1

Similarity of prey reared on MON 88702 and non-Bt cotton

Material and methods

The following experiments were carried out to identify possible differences in prey performance while either feeding on MON 88702 or on non-Bt cotton. Female spider mites (*Tetranychus urticae*) and adult cotton aphids (*Aphis gossypii*) reared on Bt or non-Bt cotton were collected randomly from the culture. After immobilizing the spider mites and cotton aphids temporarily with carbon dioxide, the weight of both prey species in groups of ten individuals was determined with a MX5 microbalance (Mettler Toledo, Greifensee, Switzerland). This procedure was conducted twice with 8 replicates for each plant type and prey species.

To test for differences between cotton leafworm (*Spodoptera littoralis*) larvae reared on Bt or non-Bt cotton, the initial weight of groups of five freshly hatched larvae was determined. The larvae were then placed into 5 cm plastic dishes with ventilated lids containing a moist cotton pad and either a Bt or non-Bt leaf disc (diameter 38 mm, cotton plants 7-9 weeks old). After 24 and 48h, the weight of the five grouped larvae was determined again. The individuals stayed in the experimental setup until they molted into second instar and the development time to L2 also was recorded. This experiment was conducted twice with 10 replicates for each plant type. In three replicates (one non-Bt and two Bt), one larva died between 24h and 48h and those replicates were analysed as a group of four individuals.

The weight of each group of prey species was divided by the number of individuals in the group and analysed with linear mixed-effects models (LMER). Time data (days until L2) of each *S. littoralis* larva were summarized per group and analysed with generalized linear mixed-effects model (GLMER) assuming Poisson distribution. Effects of factors were determined from an ANOVA table with Type III

sum of squares. The models included "cotton type" as fixed factor and "experimental repetition" as random factor.

Results

The weight of female spider mites either reared on Bt or non-Bt cotton did not differ ($p = 0.4$) (Table S1). In contrast, cotton aphids reared on Bt plants showed a higher body weight than those reared on non-Bt plants (41.7 µg and 39.0 µg respectively, $p = 0.002$). Weight of cotton leafworm larvae did not differ between the two cotton treatments at any time point ($p \geq 0.8$). Development time until L2 was similar on both plant types ($p = 0.9$).

The results do not provide evidence for susceptibility of the prey species to mCry51Aa2, because of the similar weight of the prey specimens from Bt and non-Bt cotton. The higher aphid weight on Bt cotton, did not seem to affect *O. majusculus* as there was no difference in predator performance in the Bt and non-Bt treatment (see main manuscript, Experiment 5).

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. Spider mites (*Tetranychus urticae*), cotton aphids (*Aphis gossypii*) and larvae of the African cotton leafworm (*Spodoptera littoralis*) were analysed. Values are presented as means ± SD of individuals and the number of replicates (n) is given. Weight data were analysed with linear mixed-effects models (LMER), time data with generalized linear mixed-effects model (GLMER) assuming Poisson distribution. Cotton type was modelled as fixed factor and experimental repetition as random factor.

	Cotton type				**Statistic Bt *vs.* non-Bt**
	Non-Bt	**n**	**Bt**	**n**	
Spider mites					
Weight (µg)	20.6 ± 1.65	16	21.0 ± 1.58	16	$Chi^2 = 0.6$, $p = 0.4$
Cotton aphids					
Weight (µg)	39.0 ± 1.62	16	41.7 ± 3.03	16	$Chi^2 = 10{,}0$ $p = 0.002$
Cotton leafworm larvae					
Weight 0h (µg)	38.5 ± 1.68	20	38.5 ± 1.84	20	$Chi^2 = 0.005$, $p = 0.9$
Weight 24h (µg)	182.6 ± 21.19	20	180.6 ± 22.91	20	$Chi^2 = 0.09$, $p = 0.8$
Weight 48h (µg)	548.2 ± 68.53	20	543.0 ± 73.84	20	$Chi^2 = 0.05$, $p = 0.8$
Days until L2 (d)	3.1 ± 0.31	20	3.1 ± 0.26	20	$Chi^2 = 0.01$, $p = 0.9$

Insect Science

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

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ONLINE RESOURCE 2

Supplemental Tables and Figures

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

Table S3: Statistical ANOVA outputs (Chi^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.

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

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

Table S2: Set-up of tritrophic feeding experiments with *Orius majusculus*. Given is an overview of the conducted experiments, the diet treatments included in each experiment, the number of replicates set-up per repetition, the number of replicates excluded for statistical comparisons (escaped or damaged nymphs), and the total number of replicates used for analyses. SM = spider mites (*Tetranychus urticae*), EE = *Ephestia kuehniella* eggs, LL = lepidopteran larvae (*Spodoptera littoralis*), CA = cotton aphids (*Aphis gossypii*), Bt = MON 88702 cotton producing mCry51Aa2, non-Bt = non-transformed near isogenic line.

Experiment	**Diet treatment**	**Replicates set up per repetition**	**Replicates excluded per repetition**	**Replicates total**
Experiment 1	EE Bt	10/10/10	0/0/0	30
	EE non-Bt	10/10/10	0/0/0	30
	SM Bt	10/10/10	1/1/0	28
	SM non-Bt	10/10/10	0/1/0	29
	SM Bt & EE	10/10/10	3/0/1	26
	SM non-Bt & EE	10/10/10	0/0/1	29
Experiment 2	EE Bt	5/5	0/0	10
	EE non-Bt	5/5	0/1	9
	SM Bt 2 d→EE	10/10	0/1	19
	SM Bt 4 d→EE	12/12	0/0	24
	SM Bt 6 d→EE	15/15	0/0	30
	SM Bt/EE alternating	10/10	0/0	20
	SM Bt	20/20	2/0	38
	SM non-Bt 2 d→EE	10/10	0/0	20
	SM non-Bt 4 d→EE	10/10	0/0	20
	SM non-Bt 6 d→EE	10/10	0/0	20
	SM non-Bt/EE alternating	10/10	0/0	20
	SM non-Bt	10/10	0/0	20
Experiment 3	EE Bt	5/5	1/0	9
	EE non-Bt	5/5	0/0	10
	EE 6 d→SM Bt	10/10	1/0	19
	EE 4 d→SM Bt	12/12	1/0	23
	EE 2 d→SM Bt	15/15	0/0	30
	SM Bt/EE alternating	10/10	0/0	20
	SM Bt	20/20	0/1	39
	EE 6 d→SM non-Bt	10/10	0/0	20
	EE 4 d→SM non-Bt	10/10	0/0	20
	EE 2 d→SM non-Bt	10/10	0/2	18
	SM non-Bt/EE alternating	10/10	0/0	20
	SM non-Bt	10/10	0/0	20
Experiment 4	EE Bt	5/5	0/0	10
	EE non-Bt	5/5	0/0	10
	LL Bt	15/25	2/2	36
	EE 2 d→LL Bt	10/10	0/0	20
	LL non-Bt	15/15	1/3	26
	EE 2 d→LL non-Bt	10/10	0/0	20
Experiment 5	EE Bt	5/5	0/0	10
	EE non-Bt	5/5	0/0	10
	CA Bt	15/15	0/0	30
	CA non-Bt	15/15	0/1	29

Table S3: Statistical ANOVA outputs (Chi^2 and p-values) of all tested factors and interactions from the 5 tritrophic feeding experiments. Significances are highlighted in bold. EE = *Ephestia kuehniella* eggs, SM = spider mites (*Tetranychus urticae*), LL = lepidopteran larvae (*Spodoptera littoralis*), CA = cotton aphids (*Aphis gossypii*), alt. = both prey types fed alternated.

	Factors and Interactions						
	Prey (P)	**Cotton (C)**	**Sex (S)**	**P×C**	**P×S**	**C×S**	**P×C×S**
Experiment 1 (EE; SM; EE & SM)							
Juvenile survival[a]	**Chi^2=23.7, p<0.0001**	Chi^2=0.02, p=0.9		**Chi^2=6.2, p=0.04**[a]			
Sex ratio	Chi^2=4.3, p=0.1	Chi^2=1.5, p=0.2		Chi^2=3.7, p=0.2			
Development time P×C×S	**Chi^2=10.4, p=0.006**	Chi^2=1.2, p=0.3	Chi^2=0.0001, p=1.0	Chi^2=1.6, p=0.5	Chi^2=0.01, p=1.0	Chi^2=0.05, p=0.8	Chi^2=0.1, p=0.9
Development time P×C	**Chi^2=17.1, p=0.0002**	Chi^2=1.2, p=0.27		Chi^2=2.1, p=0.4			
Weight P×C×S	**Chi^2=210.2, p<0.0001**	Chi^2=2.9, p=0.09	**Chi^2=186.2, p<0.0001**	Chi^2=3.2, p=0.2	**Chi^2=20.7, p<0.0001**	Chi^2=1.7, p=0.2	Chi^2=0.9, p=0.6
Weight females	**Chi^2=310.5, p<0.0001**	**Chi^2=5.4, p=0.02**		Chi^2=3.8, p=0.1			
Weight males	**Chi^2=66.6, p<0.0001**	Chi^2=0.09, p=0.8		Chi^2=0.9, p=0.7			
Experiment 2 (SM; SM / EE alt.; 2d SM→EE; 4d SM→EE; 6d SM→EE)							
Juvenile survival[b]	**Chi^2=9.7, p=0.046**	**Chi^2=43.3, p<0.0001**		Chi^2=5.7, p=0.2			
Sex ratio	Chi^2=2.4, p=0.7	Chi^2=0.06, p=0.8		Chi^2=5.5, p=0.2			
Development time P×C×S	**Chi^2=16.2, p=0.003**	**Chi^2=7.4, p=0.007**	Chi^2=0.09, p=0.8	Chi^2=3.5, p=0.5	Chi^2=0.6, p=1.0	Chi^2=0.1, p=0.8	Chi^2=0.4, p=1.0
Development time P×C	**Chi^2=16.0, p=0.003**	**Chi^2=7.4, p=0.006**		Chi^2=3.1, p=0.5			
Weight P×C×S	**Chi^2=306.9, p<0.0001**	**Chi^2=25.7, p<0.0001**	**Chi^2=398.5, p<0.0001**	**Chi^2=20.0, p=0.0005**	**Chi^2=26.0, p<0.0001**	Chi^2=0.03 p=0.9	Chi^2=3.4, p=0.5
Weight females	**Chi^2=156.2, p<0.0001**	**Chi^2=8.5, p=0.004**		Chi^2=9.3, p=0.06			
Weight males[b]	**Chi^2=177.9, p<0.0001**	**Chi^2=24.7, p<0.0001**		**Chi^2=17.4, p=0.002**[c]			
Experiment 3 (SM; EE / SM alt.; 2d EE→SM; 4d EE→SM; 6d EE→SM)							
Juvenile survival	***Chi^2=23.6, p<0.0001***	***Chi^2=11.1, p=0.0009***		Chi^2=3.6, p=0.5			
Sex ratio	Chi^2=3.5, p=0.5	Chi^2=3.1, p=0.08		Chi^2=1.6, p=0.8			
Development time P×C×S	Chi^2=7.8, p=0.1	Chi^2=2.6, p=0.1	Chi^2=0.1, p=0.7	Chi^2=0.7, p=0.9	Chi^2=0.3, p=1.0	Chi^2=0.003, p=1.0	Chi^2=0.3, p=1.0
Development time P×C	***Chi^2=10.2, p=0.04***	Chi^2=3.2, p=0.07		Chi^2=1.2, p=0.9			
Weight P×C×S	***Chi^2=192.6, p<0.0001***	***Chi^2=30.5, p<0.0001***	***Chi^2=138.1, p<0.0001***	Chi^2=4.2, p=0.4	***Chi^2=24.0, p<0.0001***	Chi^2=1.6 p=0.2	Chi^2=4.4, p=0.4
Weight females	***Chi^2=126.2, p<0.0001***	***Chi^2=20.0, p<0.0001***		Chi^2=5.8, p=0.2			
Weight males	***Chi^2=88.0, p<0.0001***	***Chi^2=12.7, p=0.0004***		Chi^2=4.1, p=0.4			
Experiment 4 (LL; 2d EE→LL)							
Juvenile survival	***Chi^2=16.5, p<0.0001***	Chi^2=3.2, p=0.08		Chi^2=0.9, p=0.3			
Sex ratio	Chi^2=1.2, p=0.3	Chi^2=0.03, p=0.9		Chi^2=0.04, p=0.8			
Development time P×C×S	Chi^2=1.4, p=0.2	Chi^2=0.04, p=0.8	Chi^2=0.04, p=0.8	Chi^2=0.04, p=0.8	Chi^2=0.1, p=0.7	Chi^2=0.0002, p=1.0	Chi^2=0.4, p=0.5
Development time P×C	Chi^2=1.3, p=0.2	Chi^2=0.01, p=0.9		Chi^2=0.001, p=1.0			
Weight P×C×S	Chi^2=0.8, p=0.4	Chi^2=0.7, p=0.4	***Chi^2=126.3, p<0.0001***	Chi^2=0.5, p=0.5	Chi^2=0.4, p=0.6	Chi^2=0.8, p=0.4	Chi^2=2.0, p=0.2

Weight females	Chi^2=0.04, p=0.8	Chi^2=0.004, p=0.9		Chi^2=2.1, p=0.1	
Weight males	Chi^2=1.2, p=0.3	Chi^2=1.3, p=0.2		Chi^2=0.3, p=0.6	
Experiment 5 (CA)					
Juvenile survival		Chi^2=0.07, p=0.8			
Sex ratio		Chi^2=0.3, p=0.6			
Development time C×S		Chi^2=0.03, p=0.9	Chi^2=0.04, p=0.8		Chi^2=0.06, p=0.8
Development time C		Chi^2=0.01, p=0.9			
Weight C×S		Chi^2=3.2, p=0.07	**Chi^2=38.1, p<0.0001**		Chi^2=0.004, p=0.9
Weight females		Chi^2=1.6, p=0.2			
Weight males		Chi^2=3.5, p=0.06			

[a] interaction P×C significant, individual analyses: PREY - non Bt: Chi^2=5.0, p=0.08; **Bt: Chi^2=20.0, p<0.0001**; COTTON - EE: Chi^2=0, p=1; EE & SM: Chi^2=2.1, p=0.1; **SM: Chi^2=6.2, p=0.01**

[b] random factor repetition could not be included in the model because of factor levels

[c] interaction P×C significant, individual analyses: PREY - **non Bt: Chi^2=53.8, p<0.0001; Bt: Chi^2=125.0, p<0.0001**; COTTON - **SM: Chi^2=19.5, p<0.0001; SM / EE (alt.): Chi^2=10.4, p=0.001**; 2d SM→EE: Chi^2=2.8, p=0.1; 4d SM→EE: Chi^2=0.001, p=1.0; 6d SM→EE: Chi^2=1.3, p=0.3

Table S4: Life-table parameters of *Orius majusculus* in Experiments 2-5 that were fed exclusively *Ephestia kuehniella* eggs presented on Bt or non-Bt cotton leaf discs. Values represent means ± SD with the number of replicates (n).

Parameter	Experiment	Non-Bt	n	Bt	n	Total	n
Survival (%)	2	100	9	100	10	100	19
	3	80	10	100	9	89	19
	4	100	10	100	10	100	20
	5	100	10	100	10	100	20
Sex ratio (% females)	2	44	9	50	10	47	19
	3	50	8	22	9	35	17
	4	25	8	40	10	33	18
	5	50	10	60	10	55	20
Development time (d)	2	10.1 ± 0.33	9	10.3 ± 0.48	10	10.2 ± 0.42	19
	3	10.5 ± 0.53	8	10.6 ± 0.53	9	10.5 ± 0.51	17
	4	9.6 ± 0.52	10	9.9 ± 0.57	10	9.8 ± 0.55	20
	5	10.0 ± 0	10	10.2 ± 0.42	10	10.1 ± 0.31	20
Female weight (mg)	2	0.69 ± 0.055	4	0.64 ± 0.033	5	0.66 ± 0.048	9
	3	0.67 ± 0.078	4	0.64 ± 0.050	2	0.66 ± 0.066	6
	4	0.66 ± 0.035	2	0.66 ± 0.094	4	0.66 ± 0.075	6
	5	0.67 ± 0.030	5	0.70 ± 0.064	6	0.68 ± 0.052	11
Male weight (mg)	2	0.52 ± 0.028	5	0.51 ± 0.016	5	0.52 ± 0.022	10
	3	0.50 ± 0.030	4	0.50 ± 0.012	7	0.50 ± 0.019	11
	4	0.52 ± 0.037	6	0.53 ± 0.031	6	0.52 ±0.033	12
	5	0.52 ± 0.038	5	0.55 ± 0.025	4	0.53 ± 0.034	9

Table S5: Life-table parameters of *Orius majusculus* in Experiments 1-5. EE = *Ephestia kuehniella* eggs, SM = spider mites (*Tetranychus urticae*), LL = lepidopteran larvae (*Spodoptera littoralis*), CA = cotton aphids (*Aphis gossypii*), alt. = both prey types fed alternated. Values represent means ± SD with the number of replicates in parenthesis.

Prey	Cotton	Survival (%)	Sex ratio (% female)	Development time (d)	Weight females (mg)	Weight males (mg)
Experiment 1						
EE	Non-Bt	98 (30)	48 (29)	10.10 ± 0.31 (29)	0.65 ± 0.04 (14)	0.49 ± 0.02 (15)
	Bt	97 (30)	45 (29)	10.10 ± 0.31 (29)	0.66 ± 0.03 (13)	0.50 ± 0.03 (16)
SM	Non-Bt	72 (29)	52 (21)	11.86 ± 1.24 (21)	0.45 ± 0.05 (11)	0.38 ± 0.04 (10)
	Bt	39 (28)	90 (10)	14.09 ± 1.51 (11)	0.39 ± 0.08 (9)	0.37 (1)
EE & SM	Non-Bt	83 (29)	54 (24)	10.08 ± 0.28 (24)	0.67 ± 0.06 (13)	0.49 ± 0.02 (11)
	Bt	96 (26)	48 (25)	10.12 ± 0.33 (25)	0.64 ± 0.03 (12)	0.49 ± 0.02 (13)
Experiment 2						
SM	Non-Bt	90 (20)	44 (18)	12.22 ± 1.11 (18)	0.49 ± 0.09 (8)	0.40 ± 0.05 (10)
	Bt	42 (38)	43 (14)	15.88 ± 2.25 (16)	0.38 ± 0.03 (6)	0.30 ± 0.04 (8)
SM / EE alt.	Non-Bt	100 (20)	40 (20)	11.05 ± 0.69 (20)	0.63 ± 0.05 (8)	0.47 ± 0.04 (12)
	Bt	70 (20)	64 (14)	12.00 ± 0.88 (14)	0.59 ± 0.09 (9)	0.40 ± 0.04 (5)
2d SM → EE	Non-Bt	100 (20)	55 (20)	10.45 ± 0.51 (20)	0.69 ± 0.04 (11)	0.51 ± 0.04 (9)
	Bt	74 (19)	36 (14)	11.07 ± 0.27 (14)	0.66 ± 0.05 (5)	0.49 ± 0.02 (9)
4d SM → EE	Non-Bt	90 (20)	50 (18)	11.50 ± 1.10 (18)	0.68 ± 0.04 (9)	0.49 ± 0.02 (9)
	Bt	71 (24)	71 (17)	12.29 ± 0.92 (17)	0.64 ± 0.07 (12)	0.49 ± 0.03 (5)
6d SM → EE	Non-Bt	100 (20)	60 (20)	11.80 ± 0.83 (20)	0.65 ± 0.07 (12)	0.47 ± 0.02 (8)
	Bt	60 (30)	44 (18)	13.44 ± 1.29 (18)	0.67 ± 0.05 (8)	0.45 ± 0.05 (10)
Experiment 3						
SM	Non-Bt	75 (20)	47 (15)	12.00 ± 1.13 (15)	0.49 ± 0.05 (7)	0.38 ± 0.03 (8)
	Bt	31 (39)	75 (12)	14.17 ± 1.19 (12)	0.41 ± 0.09 (9)	0.33 ± 0.07 (3)
EE / SM alt.	Non-Bt	100 (20)	40 (20)	10.50 ± 0.69 (20)	0.68 ± 0.07 (8)	0.48 ± 0.03 (12)
	Bt	90 (20)	50 (18)	10.83 ± 0.51 (18)	0.64 ± 0.06 (9)	0.47 ± 0.4 (9)
2d EE → SM	Non-Bt	83 (18)	60 (15)	11.33 ± 0.82 (15)	0.49 ± 0.05 (9)	0.39 ± 0.05 (6)
	Bt	73 (30)	68 (22)	12.64 ± 1.53 (22)	0.40 ± 0.05 (15)	0.35 ± 0.03 (7)
4d EE → SM	Non-Bt	90 (20)	44 (18)	11.00 ± 0.59 (18)	0.50 ± 0.04 (8)	0.41 ± 0.06 (10)
	Bt	61 (23)	67 (12)	11.79 ± 0.89 (14)	0.48 ± 0.10 (7)	0.34 ± 0.03 (4)
6d EE → SM	Non-Bt	90 (20)	50 (18)	10.56 ± 0.62 (18)	0.56 ± 0.05 (9)	0.46 ± 0.04 (9)
	Bt	84 (19)	50 (16)	10.94 ± 0.57 (16)	0.47 ± 0.08 (8)	0.43 ± 0.05 (8)
Experiment 4						
LL	Non-Bt	62 (26)	56 (16)	11.38 ± 2.63 (16)	0.62 ± 0.04 (9)	0.44 ± 0.06 (7)
	Bt	25 (36)	56 (9)	11.78 ± 0.97 (9)	0.59 ± 0.04 (5)	0.43 ± 0.03 (4)
2d EE → LL	Non-Bt	90 (20)	39 (18)	10.61 ± 0.92 (18)	0.58 ± 0.07 (7)	0.43 ± 0.05 (11)
	Bt	85 (20)	44 (16)	10.41 ± 1.06 (17)	0.62 ± 0.09 (7)	0.39 ± 0.06 (9)
Experiment 5						
CA	Non-Bt	83 (29)	48 (23)	11.42 ± 0.58 (24)	0.45 ± 0.05 (11)	0.36 ± 0.04 (12)
	Bt	80 (30)	57 (23)	11.33 ± 0.56 (24)	0.49 ± 0.07 (13)	0.39 ± 0.02 (10)

Table S6: Consumed prey items by neonate *Orius majusculus* after a feeding period of 24 hours in a no choice and choice situation. Means and 95% confidence intervals are presented. 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 (*Tetranychus urticae*), LL = lepidopteran larvae (*Spodoptera littoralis*), CA = cotton aphids (*Aphis gossypii*).

	Cotton	**n**	**Prey**	**Mean [95% CI]**
No choice	Non-Bt	35	EE	7.4 [6.5; 8.2]
		32	SM	13.0 [11.8; 14.2]
		30	CA	4.6 [4.0; 5.2]
		16	LL	0.9 [0.5; 1.3]
EE *vs.* SM	Non-Bt	30	EE	5.1 [4.6; 5.7]
			SM	2.4 [1.7; 3.1]
	Bt	30	EE	5.7 [5.1; 6.3]
			SM	3.0 [1.8; 4.2]
CA *vs.* SM	Non-Bt	20	CA	3.0 [2.2; 3.7]
			SM	4.5 [3.5; 5.3]
	Bt	20	CA	2.5 [1.8; 3.2]
			SM	3.7 [2.5; 4.8]
EE *vs.* LL	Non-Bt	20	EE	4.6 [3.6; 5.5]
			LL	1.5 [1.0; 2.0]
	Bt	20	EE	3.8 [3.0; 4.6]
			LL	1.2 [0.7; 1.6]
CA *vs.* LL	Non-Bt	20	CA	3.7 [3.0; 4.3]
			LL	1.0 [0.6; 1.4]
	Bt	20	CA	3.4 [2.8; 4.0]
			LL	1.0 [0.5; 1.5]

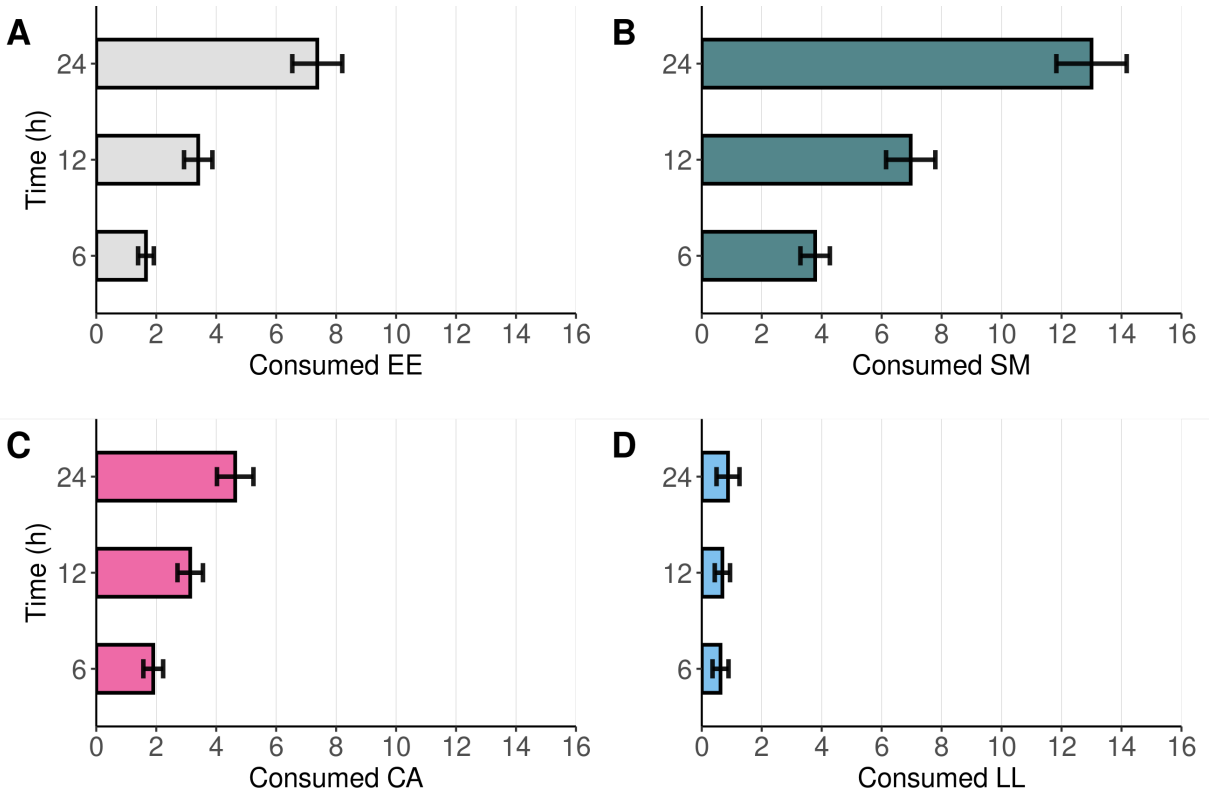

Fig. S1 Prey consumption by neonate *Orius majusculus* in a no choice situation recorded at three different time points (after 6h, 12h, 24h). Mobile prey species were reared on non-Bt cotton. Error bars represent 95 % confidence intervals. EE = *Ephestia kuehniella* eggs, SM = spider mites (*Tetranychus urticae*), LL = lepidopteran larvae (*Spodoptera littoralis*), CA = cotton aphids (*Aphis gossypii*)

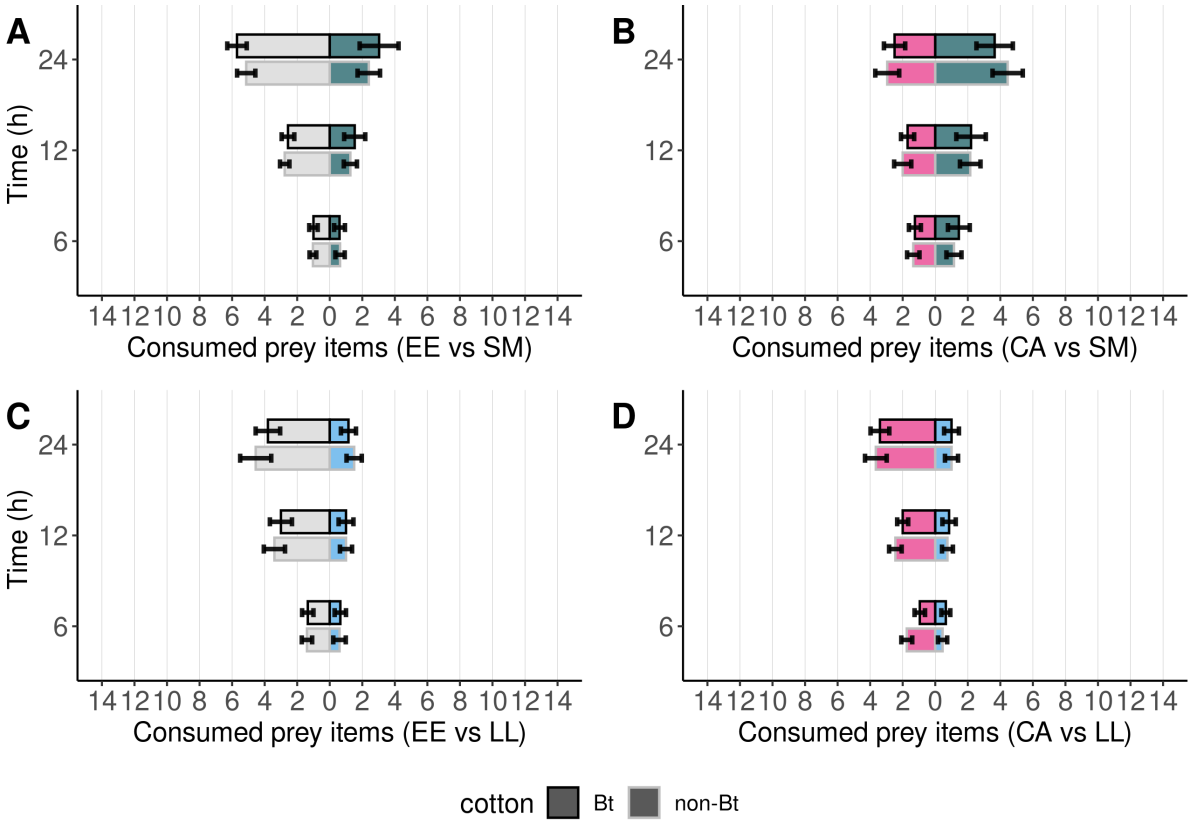

Fig. S2 Consumption rates in a choice situation of neonate *Orius majusculus* nymphs recorded at three different time points (after 6h, 12h, 24h). Two prey species either reared on Bt or non-Bt cotton were offered. Error bars represent 95% confidence intervals. EE = *Ephestia kuehniella* eggs, SM = spider mites (*Tetranychus urticae*), LL = lepidopteran larvae (*Spodoptera littoralis*), CA = cotton aphids (*Aphis gossypii*)

Insect Science

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

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ONLINE RESOURCE 3

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

Materials and methods

At the end of the tritrophic experiments 1-5 (see main manuscript), adult *O. majusculus* were collected for mCry51Aa2 determination (Table S7). In addition, leaf and prey samples were taken throughout the experimental period (see Table S8 for sample sizes). Leaf samples were taken from five Bt and five non-Bt plants of the prey species cultures and frozen at -70°C. Two leaf discs (0.5 cm diameter, ca. 10 mg) collected from one leaf in middle height of the plants represented one sample. In the experiments with lepidopteran larvae, samples were taken from fresh leaves and from leaves incubated on cotton pads for 24h. During experiments involving spider mites, five samples of 2-4 mg spider mites each were collected from Bt and non-Bt plants of the rearing. Five samples of 2-3 mg cotton aphids were collected from Bt and non-Bt plants of the rearing once between the two experimental repetitions. Seventeen and eight samples of caterpillars, each 4-5 mg, were collected after 24h feeding on Bt or non-Bt plants, respectively.

For the determination of mCry51Aa2 concentrations, Enzyme Linked Immunosorbent Assays (ELISA) were conducted as described by Kim et al. (2021). In short, the fresh weight of all samples was measured before storage at -70°C. Proteins were extracted in tris-borate buffer with a bead mill. Bt cotton leaf samples were diluted 1000×, spider mite samples (Bt and non-Bt) and caterpillars (Bt) 200×, and all other samples remained undiluted. ELISA plates were coated with anti-Cry51Aa mouse antibody. The detection antibody was a goat anti-Cry51Aa(IgG)-biotin construct. The colour reaction with TMB

substrate solution was stopped after 10 minutes with 6M phosphoric acid. Absorbance was read at 450 nm with an infinite F200 plate reader (Tecan, Männedorf, Switzerland). The concentration of mCry51Aa2 in each sample was determined by regression analyses based on a hyperbola model with all the R^2 being greater than 0.99. The limit of detection (LOD) was calculated based on 3× SD of the optical density (OD) values of the blanks combined of all plates that were used (at least 5 blanks were loaded per plate, in total 8 plates were prepared and measured). For each sample, a specific LOD was calculated based on the determined LOD value, the regression parameters of the respective standard curves, the dilution, the sample weight and the amount of added buffer. Samples below the LOD were set to 0.

Table S7: Median mCry51Aa2 concentrations (in µg/g FW) of freshly developed ***Orius majusculus*** **adults** that were reared on diet from Bt or non-Bt cotton on the respective leaf discs. Minimum and maximum values are given in parenthesis. n is the number of analysed samples. SM = spider mites (*Tetranychus urticae*), EE = *Ephestia kuehniella* eggs, LL = lepidopteran larvae (*Spodoptera littoralis*), CA = cotton aphids (*Aphis gossypii*), Bt = genetically engineered cotton producing mCry51Aa2 (MON 88702), non-Bt = non-transformed near isogenic line.

		Non-Bt		**Bt**	
Experiment	**Diet treatment**	**mCry51Aa2**	**n**	**mCry51Aa2**	**n**
Experiment 1	EE	0	7	0	8
	EE & SM	0 (0-0.007)	5	0 (0-0.012)	8
	SM	0.009 (0-0.013)	4	0.080 (0-0.114)	4
Experiment 2	SM	0	4	0.208 (0-0.528)	4
	SM/EE alternating	0	4	0.020 (0.007-0.039)	3
	2d SM→EE	0	4	0	3
	4d SM→EE	0	4	0	4
	6d SM→EE	0	4	0 (0-0.041)	4
	EE	0	2	0	2
Experiment 3	SM	0	4	0.064 (0.059-0.101)	3
	EE/SM alternating	0	4	0	4
	EE	0	2	0	2
	6d EE→SM	0	4	0.189 (0.018-0.202)	4
	4d EE→SM	0	4	0.298 (0.237-0.390)	3
	2d EE→SM	0	4	0.051 (0.015-0.184)	5
	EE	0	2	0	2
Experiment 4	LL	0	4	0.037 (0.013-0.797)	3
	2 days EE→LL	0	4	0.279 (0.232-0.326)	4
	EE	0	2	0.008 (0-0.015)	2
Experiment 5	CA	0	5	0	6
	EE	0	2	0	2

Results

Concentrations of mCry51Aa2 in *O. majusculus* adults were highly variable (Table S7). When nymphs fed exclusively spider mites from Bt cotton, median mCry51Aa2 concentrations ranged from 0.06 in Experiment 3 to 0.20 µg/g fresh weight (FW) in Experiment 2. Similar concentrations were measured in Experiment 3, when nymphs were switched from 2, 4 or 6 days *E. kuehniella* feeding to spider mites

until adulthood (0.05-0.30 µg/g). When spider mites and eggs were fed in alternation, nymphs in Experiment 2 contained 0.02 µg/g Cry protein and in Experiment 3 no measurable amounts. Median concentrations were below the limit of detection when spider mites and eggs were provided at the same time (Experiment 1) and when *O. majusculus* were switched from 2, 4, or 6 days spider mite feeding to *E. kuehniella* eggs until adulthood (Experiment 2). When nymphs fed lepidopteran larvae exclusively (0.037 µg/g) or when nymphs fed 2 days *E. kuehniella* eggs and then lepidopteran larvae (0.28 µg/g), Cry protein concentrations were comparable to the concentrations observed after spider mite feeding. No Cry protein was detected in nymphs fed exclusively cotton aphids or exclusively *E. kuehniella* eggs, even when prey was offered on Bt cotton leaf discs (except one sample in the Bt EE treatment in Experiment 4). Median mCry51Aa2-concentrations in *O. majusculus* fed with prey from non-Bt cotton were all zero.

Table S8: Median mCry51Aa2 concentrations (in µg/g FW) of **cotton leaves and prey species** collected during the tritrophic *Orius majusculus* experiments for Bt and non-Bt treatments. Minimum and maximum values are given in parenthesis. n is the number of analysed samples. Bt = genetically engineered cotton producing mCry51Aa2 (MON 88702), non-Bt = non-transformed near isogenic line.

		Non-Bt		**Bt**	
		mCry51Aa2	**n**	**mCry51Aa2**	**n**
Experiment 1	Cotton leaves	0 (0-154)	13	109 (84-248)	15
	Spider mites	11 (5.5-18)	10	30 (5.4-68)	10
Experiment 2	Cotton leaves	0.008 (0-0.066)	10	162 (117-206)	10
	Spider mites	8.7 (6.1-98)	10	74 (19-103)	10
Experiment 3	Cotton leaves	0	5	113 (96-148)	5
	Spider mites	0.17 (0.05-0.45)	5	103 (13-162)	5
Experiment 4	Cotton leaves	0 (0-0.05)	8	93 (76-154)	17
	Cotton leaves 24h			128 (70-182)	17
	Lepidopteran larvae	0 (0-0.01)	8	18 (3.5-27)	17
Experiment 5	Cotton leaves	0	5	123 (99-147)	5
	Cotton aphids	0	5	0 (0-0.004)	5

Median concentrations in leaves from Bt cotton ranged from 93 to 162 µg/g FW (Table S8). Spider mites contained approximately half of the concentration in leaves with medians ranging from 30 in Experiment 1 to 103 µg/g in Experiment 3. Lower Cry protein concentrations were measured in lepidopteran larvae (18 µg/g). Median Cry protein concentrations in cotton aphids reared on Bt cotton were below the limit of detection. Median concentrations in non-Bt cotton leaves were below the LOD although some samples contained measurable concentrations (Table S8). In spider mites reared on non-Bt cotton, Cry protein was detected with median concentrations ranging from 0.17 µg/g in Experiment 3 to 11 µg/g in Experiment 1 (Note that spider mite colonies on Bt and non-Bt cotton were maintained in the same climate chamber, so some exchange between colonies was to be expected). Median concentrations in caterpillars and cotton aphids from non-Bt cotton were below the LOD.

Discussion

ELISA results of newly emerged *O. majusculus* adults revealed medians between 0.04 and 0.3 µg mCry51Aa2/g FW when spider mites from Bt cotton were fed either exclusively or towards the end of the nymphal period, or when lepidopteran larvae served as prey. This represents a dilution factor of 100 to 1000 from prey to predator. Thus, similar to other Cry proteins, mCry51Aa2 gets highly diluted when transferred along the food chain (Kim et al. 2021, Kumar et al. 2014, Meissle and Romeis 2018, Obrist et al. 2006, Svobodová et al. 2017, Tian et al. 2014, Torres and Ruberson 2008). When spider mites were fed early in the nymphal period and *E. kuehniella* eggs later on, no mCry51Aa2 was detected in newly emerged adult *O. majusculus*. This confirms earlier findings that Cry proteins are digested and excreted by arthropods within a few days without evidence for accumulation (Obrist et al. 2006, Romeis et al. 2019). When cotton aphids were served as prey, no Cry protein was measured in *O. majusculus* adults because the aphids contained no Cry protein.

Until now, there are no published ELISA data for *Orius* spp. collected from mCry51Aa2-producing cotton fields. Previous studies with Bollgard II cotton revealed that *O. insidiosus* contained approximately 0.2% of the Cry1Ac and 0.1% of the Cry2Ab concentrations in leaves when sampled at the flowering stage (Eisenring et al. 2017). During anthesis, *Orius* spp. collected in maize producing Cry3Bb1 contained ca. 2% (Meissle and Romeis 2009) and in maize producing Cry1Ab ca. 10% of the concentration in leaves (Obrist et al. 2006).

Tritrophic laboratory experiments with Bollgard II, *Thrips tabaci* Lindemann and *O. insidiosus* showed that the predator acquired approximately 20% of the Cry1Ac and 13% of the Cry2Ab measured in the prey, which equals 4% and 0.3% of the concentrations in leaves, respectively (Kumar et al. 2014). The dilution factors from prey to predator for Cry1Ac (5) and Cry2Ab (8) were thus much lower than for mCry51Aa2 in our study (ca. 300). Cry1Ac concentrations in thrips were reported to be approximately 20 times lower than in spider mites (Torres and Ruberson 2008). When spider mites from Cry1Ab-producing maize were fed to *O. majusculus*, approximately 10% of the Bt protein was found in the bugs (Obrist et al. 2006).

With the high variability in plant expression, feeding mode and physiology of different prey species, and Cry protein stability, it is difficult to conclude from studies with other Cry proteins and prey species to the actual exposure situation in mCry51Aa2 producing cotton. Field investigations are thus warranted for a realistic exposure assessment.

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Insect Science

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

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ONLINE RESOURCE 4

Survival of *O. majusculus* in absence of prey

Material and methods

Orius majusculus has been reported to consume plant material (pollen, leaves) in the absence of prey. We therefore tested survival of *O. majusculus* nymphs when provided Bt or non-Bt leaf discs or when provided no plant material. Those treatments were included in Experiment 1 and Experiment 4, as described in the main manuscript. Experiment 1 included 10 replicates of each treatment (no disc, disc non-Bt, disc Bt) in each of 4 experimental repetitions. Experiment 2 included 5 replicates of the leaf disc treatments (non-Bt, Bt) in each of 2 experimental repetitions (Table S9).

The number of days until death among treatments was analysed with a GLMER with fixed factor treatment and random factor experimental repetition, assuming Poisson distribution.

Results

In the absence of food, *O. majusculus* nymphs died on average after 2.3-2.7 days. There was no difference between the treatments ($p = 0.6$, Table S9). Without leaf disc (plastic dish only contained a moist cotton pad), all nymphs were found dead after 1 to 4 days. With a non-Bt cotton disc, nymphs died after 1 to 7 days and with a Bt cotton disc, nymphs died after 2 to 6 days. Altogether 15 nymphs disappeared after 1 or 2 days. Either they managed to escape the plastic dish or they died and their body was not found.

The results provide no evidence that neonate *O. majusculus* can profit significantly from cotton leaf material compared with a moist cotton pad provided without leaf material. There was also no difference between cotton leaf discs from Bt or non-Bt cotton.

Table S9: Survival of *Orius majusculus* without prey on Bt (MON 88702 producing mCry51Aa2) or non-Bt cotton leaf discs or without leaf discs. Given is the total number of replicates (n), the number of nymphs that were not found after 1 or 2 days (n censored), the number of nymphs found dead (n dead), the mean number of days until death (± SD), and the results of the GLMER (Poisson distribution).

Treatment	n total	n censored	n dead	Days to death	GLMER
No disc	40	6	34	2.32 ± 0.77	$X^2 = 0.87$, $p = 0.6$
Disc non-Bt	50	7	43	2.58 ± 1.16	
Disc Bt	50	2	48	2.65 ± 1.04	