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Fate of multiple Bt proteins from stacked Bt maize in the predatory lady beetle *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae)☆

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ABSTRACT

Insecticidal Cry proteins from *Bacillus thuringiensis* (Bt) can be transferred from genetically engineered crops to herbivores to natural enemies. For the lady beetle *Harmonia axyridis*, we investigated potential uptake of Cry proteins from the gut to the body and intergenerational transfer. Third and fourth instar *H. axyridis* fed with pollen or spider mites from SmartStax maize contained substantial amounts of Cry1A.105, Cry1F, Cry2Ab2, Cry3Bb1, and Cry34Ab1. Cry protein concentrations in lady beetle larvae were typically one order of magnitude lower than in the food. When *H. axyridis* larvae were fed Bt maize pollen, median amounts of Cry protein in the non-feeding pupae were below the limit of detection except for small amounts of Cry34Ab1. No Cry protein was detected in pupae when spider mites were used as food. Cry protein concentrations decreased quickly after *H. axyridis* larvae were transferred from pollen or spider mites to Bt-free food. Aphids contained very low or no detectable Cry protein, and no Cry protein was found in *H. axyridis* larvae fed with aphids, and in pupae. When *H. axyridis* adults were fed with Bt maize pollen (mixed with *Ephestia kuehniella* eggs), the median concentrations of Cry proteins in lady beetle eggs were below the limit of detection except for Cry34Ab1 in eggs laid later in adult life. No Bt protein was detected in eggs laid by *H. axyridis* females fed with aphids from Bt maize. Our results confirm previous observations that Cry proteins are degraded and excreted quickly in the arthropod food web without evidence for bioaccumulation. Despite the fact that small amounts of Cry proteins were detected in some samples of the non-feeding pupal stage of *H. axyridis* as well as in eggs, we conclude that this route of exposure is unlikely to be significant for predators or parasitoids in a Bt maize field.

1. Introduction

Most genetically engineered (GE) plants with insecticidal properties that are cultivated today produce Cry proteins from the bacterium *Bacillus thuringiensis* Berliner (Bt). When ingested by a sensitive target insect, Cry proteins are proteolytically activated, bind to gut receptors, and lead to a formation of pores in the gut. This causes a disturbance of the ion balance and ultimately death of the insect (Bravo et al., 2013; Jurat-Fuentes and Crickmore, 2017). Cry proteins are only activated under certain physiological conditions in the gut (e.g. pH) and toxicity requires receptors specific for the particular Cry protein. This specificity is one of the most relevant attributes of insecticidal technologies based on Bt proteins (Jurat-Fuentes and Crickmore, 2017). Maize, cotton, soybean, eggplant, cowpea, and sugar cane were transformed with Cry1 and Cry2 class proteins targeting lepidopteran pests and maize was transformed with Cry3 class proteins targeting corn rootworm beetles (*Diabrotica* spp.) (ISAAA, 2018).

Before commercialization of a novel GE plant, an ecological risk assessment is conducted, which includes the assessment of potential risks for non-target species, in particular natural enemies of pest herbivores (Romeis et al., 2008). In the field, a natural enemy is potentially at risk if it is susceptible to the plant-produced insecticidal protein (toxicity) at concentrations that it ingests when feeding directly on plant material or on prey that has consumed the insecticidal protein (exposure). Knowledge on the mode of action, the specificity, spatiotemporal expression patterns in the plant, and the environmental fate guides the non-target risk assessment for novel insecticidal proteins (Jurat-Fuentes and Crickmore, 2017; Romeis et al., 2008).

This study focuses on the exposure of natural enemies and the fate of Cry proteins after ingestion, and not on the sensitivity of

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natural enemies to Cry proteins. Spider mites are one example of herbivores that contain relatively high concentrations of Cry proteins when feeding on Bt plants (Romeis et al., 2019), because they suck on mesophyll cells, which contain a lot of Cry protein (Dutton et al., 2004). In contrast, aphids contain only trace amounts of Cry proteins, because they feed on phloem sap that does not transport those proteins (Romeis and Meissle, 2011). Predators generally contain lower Cry protein concentrations than their food sources, which might consist of prey or of plant material, such as pollen (Romeis et al., 2019). When herbivores and predators were switched from diet containing Cry proteins to a non-Bt diet, no Cry protein was detected within a few days (Kim et al., 2012; Meissle and Romeis, 2012; Obrist et al., 2006; Romeis et al., 2019). In addition, Cry protein concentrations in herbivores and predators after long term feeding exposure were similar or even lower than after short-term feeding exposure (Meissle and Romeis, 2012). These laboratory findings are supported by field data showing lower Cry protein concentrations in higher trophic levels (Romeis et al., 2019). Dilution of Cry proteins along the food chain can be explained by limited prey consumption, digestion in the gut, and excretion (Romeis et al., 2019).

In most previous studies, Cry proteins were measured in the whole arthropod body including the gut where the Cry proteins are concentrated. Little is known, however, on the potential uptake of the Cry proteins from the arthropod gut into other body tissues (Kim et al., 2012; Zhao et al., 2016). Recently, Paula and Andow (2016) detected Cry1Ac and Cry1F proteins in the non-feeding pupal stage of the lady beetle *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), when larvae were fed with aphids from artificial diet containing purified Cry protein. Because lady beetles empty and molt their gut in the process of pupation, the authors concluded that the Cry proteins were sequestered in other tissues. Since the concentrations in lady beetle pupae were even higher than those in aphids, accumulation in the predator body was hypothesized (Paula and Andow, 2016). This contradicts previous findings with other insects that showed rapid degradation rather than bioaccumulation (Meissle and Romeis, 2012; Romeis et al., 2019). Another surprising result reported by Paula et al. (2015) was the intergenerational transfer of Cry proteins from *H. axyridis* females to their eggs and offspring. However, intergenerational transfer of Bt proteins has previously also been suggested for *Propylea japonica* (Thunberg) (Coleoptera: Coccinellidae) (Zhang et al., 2006), *Nilaparvata lugens* (Stal) (Hemiptera: Delphacidae) (Gao et al., 2010), and *Chlosyne lacinia* (Geyer) (Lepidoptera: Nymphalidae) (Paula et al., 2014).

The conflicting results on accumulation of Cry proteins in *H. axyridis* reported by Paula and Andow (2016) and the limited knowledge on uptake of Cry proteins into the body of arthropods and their intergenerational transfer triggered the current study. We worked with a prey-predator system using SmartStax Bt maize that produces Cry1A.105, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1, and Cry35Ab1 (Svobodová et al., 2017). Pollen from Bt maize, spider mites reared on Bt maize, and aphids reared on Bt maize served as different, realistic food sources for the predator *H. axyridis*. First, we tested the hypothesis that uptake of Cry proteins occurs in *H. axyridis* by addressing the following questions:

i) How much of the different Cry proteins produced in SmartStax maize do 3rd and 4th instars of *H. axyridis* contain after continuous feeding on aphids, spider mites, or pollen from Bt maize?
ii) How much of the Cry proteins are detectable in the non-feeding pupal stage?
iii) How do Cry protein concentrations change when 3rd or 4th instars are transferred from Bt maize diet to diet free of Cry proteins?

Second, we tested if *H. axyridis* females can pass Cry proteins on to their offspring by asking:

i) How much of the different Cry proteins do adults of *H. axyridis* contain when exposed to aphids or pollen from SmartStax maize during their developmental and adult stages?
ii) Are the different Cry proteins transferred to the eggs of *H. axyridis*?
iii) Do *H. axyridis* eggs that do not contain Bt protein get contaminated when placed in a cage with *H. axyridis* adults and food from Bt maize?

2. Methods

2.1. Plants and insects

SmartStax maize (event MON89034 × TC1507 × MON88017 × DAS-59122-7, Bayer Crop Science, St Louis, USA, referred to as "Bt maize") and the genetically closest conventional hybrid EXP258 (Bayer, "non-Bt maize") were used in this study. Maize pollen was collected, dried and frozen by Svobodová et al. (2017) and reused for the present study.

Colonies of *Rhopalosiphum padi* Linnaeus (Hemiptera: Aphididae) aphids and *Tetranychus urticae* C.L. Koch (Acari: Tetranychidae) spider mites were started with individuals supplied by Syngenta Crop Protection Münchwilen AG (Stein, Switzerland). The herbivores were reared on Bt maize plants in separate climate chambers at 25 °C, 70% RH, and a 16:8 light:dark cycle according to Shu et al. (2018). Plants were infested with the herbivores when 4–5 weeks old and replaced before anthesis. Aphids were harvested from the maize plants using a paint brush and spider mites were collected by beating leaves over a tray. Eggs of the lady beetle *H. axyridis* were derived from our own continuous culture, which was started with adults collected near Zurich, Switzerland, in 2013. After hatching, neonate lady beetle larvae were allowed to remain on their egg batches to feed on the egg shells. They were used for the experiments after approximately one day when they started to move around freely looking for food. As an optimal, Cry protein-free food source, we used sterilized eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) that were purchased from Biotop (Livron-sur-Drôme, France).

2.2. Experiment 1: fate of Cry proteins in H. axyridis larvae and pupae

Approximately one day old *H. axyridis* neonates were distributed individually in plastic dishes (5 cm diameter, 1 cm high) with ventilated lids. A wet piece of a cotton pad (ca. 1 cm^2) was provided as a source of water. The larvae were subjected randomly to one of the treatments summarized in Table 1.

Experiments with the different food types were conducted consecutively, starting with 3 experimental repetitions using spider mites as food (48–60 neonates each), followed by 2 repetitions using aphids (99 neonates each), and finally 2 repetitions using pollen (90 neonates each). Each experiment with one food type contained all treatments (rows in Table 1). An exception were the experiments with spider mites, where treatment SSS (only spider mites until pupation) was not possible due to high mortality in the 4th instar.

Table 1
Food treatments applied in Experiment 1 to larvae of *Harmonia axyridis*. Pollen, spider mites (*Tetranychus urticae*) and aphids (*Rhopalosiphum padi*) were collected from SmartStax Bt maize. Eggs of *Ephestia kuehniella* contained no Bt protein.

Treatment	Larval stage Instar 1 + 2	Instar 3	Instar 4	Stage collected
PEE/SEE/AEE	Pollen/spider mites/aphids	*Ephestia* eggs	*Ephestia* eggs	Pupa
PE/SE/AE	Pollen/spider mites/aphids	*Ephestia* eggs	–	L4
P/S/A	Pollen/spider mites/aphids	–	–	L3
PPE/SSE/AAE	Pollen/spider mites/aphids	Pollen/spider mites/aphids	*Ephestia* eggs	Pupa
PP/SS/AA	Pollen/spider mites/aphids	Pollen/spider mites/aphids	–	L4
PPP/AAA*	Pollen/aphids	Pollen/aphids	Pollen/aphids	Pupa

*Spider mites were not included here since rearing *H. axyridis* to the pupal stage on spider mites only was not possible because of high mortality.

The experiments were conducted in climate cabinets (MLR-352H-PE, Panasonic Biomedical, Etten-Leur, The Netherlands) at 25 °C, 75% RH, and 16:8 h light:dark. To minimize contamination, larvae that were in a stage where they were fed with *E. kuehniella* eggs or in the pupal stage were kept in a different cabinet than those fed with food from Bt maize.

On a daily basis, new food was provided and the cotton pads were wetted or exchanged. Dead lady beetle larvae were removed from the experiment. The plastic dishes were changed every second day. According to the schedule of the different treatments, a change of food type (from aphids, pollen, or spider mites to *E. kuehniella* eggs) was done on the second day of the 3rd or 4th instar. Larvae scheduled to be collected for Bt protein measurements were weighed and frozen at −80 °C on the second day of the 3rd or 4th instar. Pupae were collected on the second day after pupation. To avoid contamination of the samples with prey remnants sticking to the numerous spines of the larval skin that remained attached to the pupa or remnants of the molted gut, we dissected the anal part off the pupa using two pairs of forceps. The remaining pupal body was washed for at least 10 s with deionized water, dried on tissue paper, checked for contaminants under a binocular microscope, weighed, and frozen at −80 °C. The anal plate with the molted larval skin was also frozen and analyzed. While larvae and pupae were analyzed individually, each analyzed sample of anal plates consisted of three to five pooled plates.

Fresh food samples and food samples exposed for 24 h in the climate cabinet of the lady beetles were collected during each experiment, weighed and frozen at −80 °C for later Cry protein measurements.

2.3. Experiment 2: transfer of Cry proteins from females to eggs

Neonates of *H. axyridis* were reared to pupae as described previously and extended to the adult stage. Food was provided *ad libitum* and consisted either of *R. padi* aphids from Bt maize, or Bt maize pollen mixed with *E. kuehniella* eggs at a ratio of 1:2 (volume:volume) (termed "pollenmix"). The addition of *E. kuehniella* eggs was necessary because preliminary experiments revealed that it was not possible to obtain healthy and fertile *H. axyridis* adults when providing larvae and adults with maize pollen as a sole food source. New food, wet cotton pads and plastic dishes were provided three times per week. After pupation, the plastic dishes were cleaned and the cotton pads were wetted every second day. Shortly before expected emergence, new food was added to ensure that emerging adults were able to feed. After emergence, one female and one male were placed together in a plastic cylinder (4.5 cm diameter, 6 cm high). Gender was distinguished after McCornack et al. (2007). The plastic cylinders contained the respective food (aphids from Bt maize or Bt maize pollenmix), a wet cotton pad, a piece of fine gauze (ca. 1.5 × 13 cm) for egg laying and a piece of coarse gauze (ca. 3 × 5.5 cm) to provide more structure inside the cylinders. The cylinders were closed with a piece of gauze and a lid with a large hole for ventilation. In the aphid treatment, 35 pairs were set up and in the pollen treatment, 39 pairs. Adults were transferred to clean plastic cylinders three times per week. Egg batches laid on the gauze were collected every day, cut out using scissors, and washed gently with deionized water for ca. 5 s using a spray bottle to ensure that the eggs were not washed off or destroyed. After washing, the eggs were dried on tissue paper, checked for contamination with food items under a binocular microscope, and frozen at −80 °C. In the aphid treatment, eggs of each *H. axyridis* pair were collected until the female laid the 6th batch. Females in the pollenmix treatment produced egg batches more frequently, so collection was extended to 10 more egg batches in this treatment. After collecting the last egg batch (6th batch in the aphid treatment and 16th batch in the pollenmix treatment), females and males were weighed and frozen at −80 °C.

For Cry protein measurements, the frozen egg batches were detached carefully from the gauze pieces with a small spatula. In the aphid treatment, all 6 egg batches of each lady beetle pair were pooled to one sample in a new 2 mL tube. In the pollen treatment, the first 6 egg batches and the remaining 10 batches were pooled. After pooling, the eggs were once more checked under the binocular for contamination, weighed, and stored at −80 °C. The frozen adults were washed gently with deionized water for ca. 5 s with a spray bottle, checked also for contamination with aphids or pollen under the binocular, and stored in a new 2 mL tube at −80 °C.

To investigate the role of contamination for Cry protein measurements in eggs despite washing and checking under the binocular, we collected freshly laid egg batches on gauze pieces from the *H. axyridis* culture, which is maintained with *E. kuehniella* eggs without contact to Bt proteins. These Bt-free eggs were encircled with a pen on the gauze. The gauze pieces with the marked egg batches were placed in plastic cylinders with *H. axyridis* couples as described previously. Aphids (ca. 100 per day) or pollenmix (ca. 30 mg per day) served as food. A wet cotton pad and another piece of gauze was also added. After 2 days, the marked eggs were recollected and processed as described previously. To obtain samples with similar weights for ELISA, 3 to 7 egg batches depending on the size were combined. This resulted in 12 samples for the aphid treatment and 19 samples for the pollen treatment.

2.4. Cry protein measurements

To ensure that positive ELISA readings indicate the presence of Bt protein and not unspecific binding of the antibodies to any *H. axyridis* protein, we collected different stages of *H. axyridis* that never came in contact with Bt protein. Neonates from the culture were set up individually as described previously and fed *ad libitum* with *E. kuehniella* eggs until they were sampled. Fourth instars were sampled one day after the molt (10 individuals), pupae were sampled one day after pupation (9 individuals), males and females were sampled one day after emergence (10 individuals each), and egg batches were sampled as described in experiment 2 (17 pooled

samples).

For protein extraction, 800 μl of phosphate-buffered saline with 0.55% TWEEN-20 (PBST) and one 3 mm tungsten carbide ball was added to each sample in 2 mL tubes. Tissues were macerated in a bead mill (TissueLyser II, Qiagen, Hombrechtikon, Switzerland) for 2–3 min at 25–30 Hz. After centrifugation at 13′000×*g* for 5 min at 4 °C, the supernatants were collected. According to expected concentrations in lady beetle larvae (Svobodová et al., 2017), supernatants were used directly or diluted 5, 25, or 100 times with PBST. Samples from pupae were not diluted. Samples were kept on ice throughout processing. Cry proteins were quantified with antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA). Individual kits were used for the detection of Cry1A.105 (Cry1Ab/1Ac kit), Cry1F, Cry2Ab2, Cry3Bb1, and Cry34Ab1 (Agdia, Elkhart, IN, USA). Purified Cry proteins of certified quality, provided by Bayer or Corteva Agriscience (Indianapolis, IN, USA), were used to construct standard curves with 5–7 concentrations. Antibody-coated plates were loaded with enzyme conjugate, (diluted) sample extracts and Cry protein standards according to the manufacture's protocol. The plates were incubated overnight at 4 °C and washed seven times with PBST (0.05% TWEEN-20). The color reagent was added according to protocol and optical densities were measured after 20 min with a plate reader (Spectrafluor-Plus, Tecan, Switzerland).

2.5. Data analysis

The concentrations of Cry proteins were determined with standard curves using regression analyses based on a 2-parameter hyperbola model (SigmaPlot 13, Systat Software Inc., San Jose, USA). R^2 of fitted curves was >0.98 (except one case where the lowest R^2 was 0.91 because of a shift between the standards on the left and the right side of the plate). The limit of detection (LOD) for the clear separation of positive readings from the variation of buffer-only blanks was calculated based on 3 standard deviations of the blank OD values from 4 to 6 ELISA plates. Based on these values, the LOD was calculated for each sample using the regression parameters of the respective standard curves, the dilution, the weight and the amount of added buffer. Samples below the LOD were set to 0. Individual measurements and LODs are provided in the supplementary online material (**Data file**). Statistical analyses were conducted with R (version 3.6.3, The R Foundation for Statistical Computing, Vienna, Austria). Each ELISA sample served as a replicate in the analyses. The measured Cry proteins in lady beetle larvae and pupae are presented as median total amounts in ng per individual (Figure 1, S1, Table S1). We wanted to follow the fate of the Bt proteins in each larva and thus amounts per individual are more illustrative than concentrations, which are influenced by body weight. For better comparability with published values, however, we also provide median concentrations in μg/g fresh weight (FW) in the online supplementary material (Table S2). Median concentrations were presented for measurements in food, females and eggs. Because of many zero values (i.e., measurements below the LOD), we worked with medians and 95% confidence intervals (CI), which were calculated for each treatment and Cry protein using the groupwiseMedian function of the rcompanion package. Values of treatments were considered significantly different when the CI were not overlapping. Cry protein concentrations in fresh and 24 h incubated samples were compared with nonparametric pairwise Mann-Whitney U-tests using the wilcox.test function. Spearman's rank correlation analysis was conducted for Cry protein concentrations in female *H. axyridis* fed pollenmix and concentrations in their late eggs (ggpubr package). We correlated the late eggs with the females because they were laid closer to the time point when the females were frozen.

3. Results

3.1. Experiment 1: fate of Cry proteins in H. axyridis larvae and pupae

When *H. axyridis* larvae were fed with Bt maize pollen from neonate to 3rd instar (P-L3), the highest amount of Cry protein was measured for Cry34Ab1 (median of 52 ng per L3 larva), followed by Cry1F (14 ng), Cry3Bb1 (7.0 ng), Cry1A.105 (3.6 ng), and Cry2Ab2 (0.2 ng) (Fig. 1A, Table S1). In 4th instars (PP-L4), values for Cry1A.105, Cry1F, Cry2Ab2, and Cry3Bb1 were not significantly higher compared to 3rd instars (overlapping CI), despite the fact that the larval weight doubled (Table S1). In contrast, 3 × more Cry34Ab1 was measured in L4 compared to L3 (non-overlapping CI). When *H. axyridis* larvae were fed pollen from neonate to pupation (PPP), the medians of the Cry proteins in the pupal body were below the LOD except for Cry34Ab1. However, 13 and 11 of 28 samples for Cry1F and Cry3Bb1, respectively, were above the LOD. In contrast, all samples of the anal part (molted skin and anal plate), that was dissected from the pupal body, contained Cry1F, Cry2Ab2, Cry3Bb1, and Cry34Ab1, while no sample contained measurable amounts of Cry1A.105. When larvae were switched from pollen to *E. kuehniella* eggs on the second day of the 4th instar (PPE), Cry protein medians in pupae were below the LOD except for Cry34Ab1. Amounts in anal parts were approximately 20 × lower than in anal parts when larvae fed pollen continuously. When larvae were switched from Bt maize pollen to *E. kuehniella* eggs on the second day of the 3rd instar (PE-L4), the 4th instars contained 6 × less Cry34Ab1 than the L3, 13 × less Cry3Bb1, 47 × less Cry1F, and median amounts of Cry1A.105 and Cry2Ab2 were below the LOD. When those L4 larvae were reared on *E. kuehniella* eggs further to pupation (PEE), Cry protein medians were below the LOD in the pupal bodies. In the anal parts, no Cry proteins were measured except for Cry34Ab1.

The second food source was *T. urticae* spider mites from Bt maize. Third instar *H. axyridis* that were fed exclusively with spider mites (S-L3) contained 20 ng Cry3Bb1, 14 ng Cry34Ab1, 6.8 ng Cry1A.105, 2.9 ng Cry1F, and 0.2 ng Cry2Ab2 (Fig. 1B, Table S1). The 4th instars (SS-L4) had more than doubled their larval weight compared to the L3 **(Table S1)**. Similarly, the amount of Cry protein has increased by a factor of 1.6–3.8 (significant for Cry1A.105, Cry1F, and Cry34Ab1). Because of high mortality, pupae could not be obtained when lady beetle larvae were continuously fed spider mites. When larvae were switched from spider mites to *E. kuehniella* eggs on the second day of the 4th instar (SSE), no Cry protein was measured in the pupae in any sample except for Cry34Ab1 (7 of 15 samples positive). In addition, the anal parts contained Cry3Bb1 and Cry34Ab1, while the other Cry proteins were not detected. When larvae were switched to *E. kuehniella* on the second day of L3 (SE-L4), the 4th instars contained significantly less of the Cry proteins compared to the L3: 4 × less Cry34Ab1, 17 × less Cry3Bb1, 29 × less Cry1F, and median amounts of Cry1A.105 and Cry2Ab2 were below the LOD. No Cry protein was detected in pupal bodies and anal parts, when L4 were further reared on *E. kuehniella* eggs (SEE).

With the third food source, *R. padi* aphids, all median Cry protein amounts in the 3rd and 4th instar as well as in pupal bodies and anal parts were below the LOD (Figure S1, Table S1).

Cry protein concentrations were measured in fresh prey items (shortly after collection from the plants) and after 24 h exposure under experimental conditions (25 °C, 16 h light). After 24 h, spider mites contained 4 × less Cry1A.105, 5 × less Cry1F, 8 × less Cry3Bb1, 14 × less Cry34Ab1, and 20 × less Cry2Ab2 compared to mites directly collected from Bt maize (Table 2). This difference was significant for all Cry proteins (Mann-Whitney *U* test, $p < 0.0001$).

In aphids, median concentrations of Cry1A.105, Cry2Ab2, and Cry34Ab1 were below the LOD in both freshly collected aphids and in aphids after 24 h incubation. Concentrations of Cry1F and Cry3Bb1 were above the LOD in freshly collected aphids, but below the LOD after incubation ($p \leq 0.0007$ for both Cry proteins) (Table 2). Bt maize pollen contained similar concentrations of Cry proteins after 24 h compared to pollen that was not incubated ($p \geq 0.09$ for all Cry proteins) (Table 2).

3.2. Experiment 2: transfer of Cry proteins from females to eggs

When lady beetles were fed pollenmix as a food source for their whole life (1:2 ratio of Bt maize pollen to *E. kuehniella* eggs), the first 6 egg batches were produced within 11–22 days after emergence and the next 10 egg batches within further 6–18 days. The adults were frozen 19–35 days after emergence, once the 16th egg batch was produced. As expected, Cry protein concentrations in the pollenmix were approximately one third of the concentrations in pure Bt maize pollen (Fig. 2, Table 2, Table S3). Median concentrations of Cry1A.105 and Cry2Ab2 in male lady beetles were below the LOD of 0.035 and 0.001 μg/g FW, respectively. The concentrations of Cry1F, Cry3Bb1, and Cry34Ab1 were between 14 × and 50 × less than the concentrations in the pollenmix. In females, median concentrations were above the LOD for all Cry proteins, but between 10 × (Cry3Bb1) and 33 × (Cry1F & Cry2Ab2) less than in the pollenmix. Females contained more Cry1A.105, Cry2Ab2, and Cry3Bb1 than males (non-overlapping CI) (Fig. 2, Table S3). In the first 6 egg batches, median concentrations of all Cry proteins were below the LOD. Some individual values, however, were above the LOD, in particular for Cry34Ab1 (14 of 35 values). In egg batches 7 to 16 (late eggs), the median Cry protein concentrations were also below the LOD with the exception of Cry34Ab1, where 26 values above the LOD resulted in a median of 0.0015 μg/g FW. There was no correlation between any of the Cry proteins in females and in their late eggs (Spearman's rank correlation, $p \geq 0.2$) (Table S4). Median concentrations in control eggs from lady beetles reared on *E. kuehniella* eggs that were exposed to pollenmix and lady beetle couples for 2 days were below the LOD for all Cry proteins (Fig. 2). Confidence intervals for Cry34Ab1 of early and late eggs overlapped with those of potentially "contaminated" control eggs. All other confidence intervals of egg samples were 0.

In the experiment with aphids from SmartStax maize as food, the first 6 egg batches were collected between 18 and 43 days. Median concentrations above the LOD were detected in aphids for Cry1F (0.01 μg/g FW), Cry2Ab2 (0.004 μg/g), Cry3Bb1 (0.006 μg/g), and Cry34Ab1 (0.003 μg/g), while the median for Cry1A.105 was below the LOD (<0.06 μg/g) (Figure S2, Table S3). When fed with aphids, median concentrations of all Cry proteins in *H. axyridis* males, females, and eggs were below the LOD except Cry1F in females with a median of 0.0014 μg/g FW. Eggs from lady beetles fed exclusively with *E. kuehniella* eggs (control eggs) did not contain Cry proteins despite exposure to aphids and *H. axyridis* adults for 2 days.

4. Discussion

4.1. Cry protein uptake by H. axyridis

Larvae of *H. axyridis* were exposed to Cry proteins throughout the experiment when fed pollen and spider mites from SmartStax maize. While Cry protein concentrations in pollen were very stable within the 24 h exposure period under experimental conditions, spider mites digested and excreted the Cry proteins, leading to 4 × (Cry1A.105) to 20 × (Cry2Ab2) lower concentrations after 24 h. Overall exposure in the spider mite assay was thus somewhat lower than in the field where spider mites are consumed directly from the plant. When feeding on aphids, predator larvae were only exposed to traces or undetectable amounts of Cry protein. That aphids contain no or very small amounts of Bt protein when feeding on Bt crops was known from several previous studies (Romeis and Meissle, 2011; Romeis et al., 2019). Nevertheless, we decided to include aphids in the present study because they are an important prey for *H. axyridis* and because Paula and Andow (2016) reported Bt protein accumulation in a laboratory system with aphids as prey that had consumed Cry protein-containing artificial diet.

When 3rd and 4th instars of *H. axyridis* fed on Bt maize pollen or on spider mites from Bt maize, they contained 4 × (Cry3Bb1 and Cry1A.105 in L3 and Cry34Ab1 in L4 fed pollen) to 300 × (Cry2Ab2 in L3 fed spider mites) lower concentrations than their (fresh) food. That significantly lower amounts of Cry protein were detected in *H. axyridis* than in their food was reported previously (Chang et al., 2017; Dutra et al., 2012; Meissle and Romeis, 2018; Svobodová et al., 2017). *Harmonia axyridis* larvae fed with aphids contained no measurable Cry protein, which was expected because of the low or undetectable amount of Cry protein in aphids.

Fourth instar *H. axyridis* approximately doubled their body weight compared to third instars and also the amount of Bt protein measured per larva generally increased from L3 to L4. However, this increase was more pronounced when spider mites were provided as food (significant for Cry1A.105, Cry1F, and Cry34Ab1) compared to pollen (significant only for Cry34Ab1). The reasons why some Cry proteins increased more than others and the difference among food sources remains unclear, but changes in digestion physiology between L3 and L4 and different consumption patterns between pollen and spider mites could be an explanation.

When third instar *H. axyridis* were switched from Bt maize pollen or spider mites to high quality non-Bt diet (*E. kuehniella* eggs), the amount of Bt protein measured in the fourth instars decreased by a factor of 3 or more and medians of Cry1A.105 and Cry2Ab2 were below the LOD. Since the development of third instar *H. axyridis* takes approximately 3 days (Svobodová et al., 2017), this confirms that Bt proteins are excreted and digested rapidly. Rapid excretion and/or digestion of Cry protein was also shown for other insects, such as lacewing larvae (Obrist et al., 2005; Romeis et al., 2004), predatory bugs (Obrist et al., 2006; Torres and Ruberson, 2008; Torres et al., 2006), caterpillars (Wei et al., 2008; Zhao et al., 2016), mealworm larvae (Kim et al., 2012), and spiders (Meissle and Romeis, 2012).

Pollen and particularly spider mites were suboptimal food sources for *H. axyridis*. This is evident from the lower weights of larvae and pupae fed continuously with spider mites or pollen, compared to the higher weights after third or fourth instars were switched to *E. kuehniella* eggs. In contrast, aphids were a better food source resulting in similar weights of *H. axyridis* pupae in all treatments, confirming the findings by Svobodová et al. (2017). That maize pollen is a suitable, yet suboptimal food source has earlier been demonstrated for another lady beetle species, *Coleomegilla maculata* De Geer (Lundgren and Wiedenmann, 2004).

When *H. axyridis* larvae were continuously fed with Bt maize pollen, median amounts of Cry protein in the pupae (without anal parts, i.e., anal plate and molted larval skin) were below the limit of detection, except for Cry34Ab1. In addition, also 13 and 11 of 28 samples were positive for Cry1F and Cry3Bb1, respectively. Pupae contained no measurable Cry protein when L3 or L4 fed with pollen or spider mites were switched to non-Bt diet. Once more, an exception was Cry34Ab1, which was measured in pupae when L4 were switched from Bt pollen to *E. kuehniella* eggs. When the larvae molt into the non-feeding pupal stage, they also empty their guts, where the food and thus most of the Bt proteins are located. Our results confirm that almost none of the Bt protein measured in

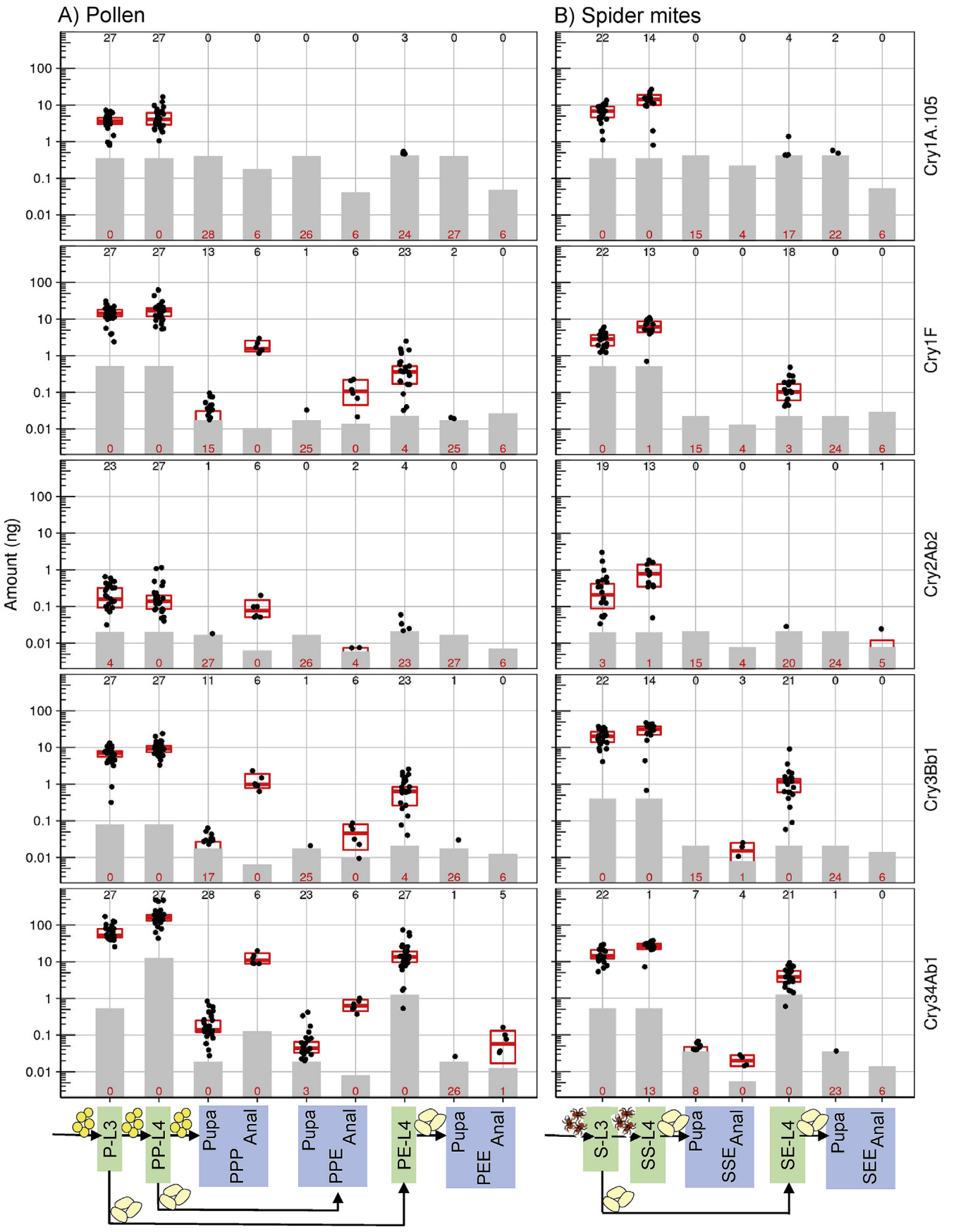

Fig. 1. Amount (ng per individual) of different Cry proteins in individual larvae and pupae of *Harmonia axyridis* feeding on A) pollen (P), B) *Tetranychus urticae* spider mites (S) from SmartStax Bt maize. Sterilized eggs of *Ephestia kuehniella* (E) were used as a non-Bt food. x-axis: L3 and L4 indicate the larval stage of the lady beetle, arrows the feeding sequence.

Table 2
Concentration (μg/g fresh weight) of different Cry proteins in SmartStax Bt maize pollen, spider mites (*Tetranychus urticae*) reared on Bt maize, and aphids (*Rhopalosiphum padi*) reared on Bt maize. "Fresh" indicates that herbivores were collected from the plants, weighed, and immediately frozen for ELISA analysis. "24 h" means that herbivores were collected from the plants, incubated for 24 h at 25 °C without food and then weighed and frozen. Pollen was collected, dried at ambient conditions, and frozen. "Fresh" means that pollen was taken from the freezer, separated into several technical samples, weighed and frozen again for analysis. "24 h" means that the technical samples were incubated for 24 h at 25 °C before being frozen for analysis. Values are medians with 95% confidence intervals. Fresh samples were compared to 24 h samples with Mann-Whitney U-tests.

	Fresh	24 h	*U* Test
Pollen (N = 12)			
Cry1A.105	2.7 (1.7; 3.4)	2.2 (1.9; 2.9)	W = 61, p = 0.6
Cry1F	15.2 (10.3; 18.8)	17.4 (14.5; 19.5)	W = 84, p = 0.5
Cry2Ab2	1.0 (0.8; 1.2)	1.0 (0.8−1.2)	W = 81, p = 0.6
Cry3Bb1	5.5 (4.0; 6.5)	5.6 (5.3; 7.4)	W = 75, p = 0.6
Cry34Ab1	57.5 (49.0; 60.5)	61.1 (60.4; 61.9)	W = 94, p = 0.09
Spider mites (N = 20)			
Cry1A.105	35.4 (28.6; 37.8)	8.4 (8.0; 8.8)	W = 0; p < 0.0001
Cry1F	10.5 (9.2; 10.8)	2.2 (1.9; 2.6)	W = 0, p < 0.0001
Cry2Ab2	16.7 (9.8; 18.1)	0.8 (0.6; 1.0)	W = 0, p < 0.0001
Cry3Bb1	44.0 (32.6; 49.3)	5.9 (5.6; 8.2)	W = 3, p < 0.0001
Cry34Ab1	46.1 (37.4; 48.6)	3.2 (2.9; 3.7)	W = 0, p < 0.0001
Aphids (N = 16)			
Cry1A.105	<0.01	<0.01	NA
Cry1F	0.003 (0.002; 0.005)	<0.0006 (0; 0.0006)	W = 11, p < 0.0001
Cry2Ab2	<0.0006	<0.0006	NA
Cry3Bb1	0.001 (0; 0.002)	<0.0007	W = 56, p = 0.0007
Cry34Ab1	<0.0007	<0.004	NA

larvae is detectable anymore in pupae and we can thus rule out accumulation of Bt proteins in *H. axyridis*. Nevertheless, our results also suggest that small amounts of some Bt proteins (in particular Cry34Ab1) might have been taken up by the larval body and persist to the pupal stage. In contrast, relatively high amounts of Bt protein were detected in the anal parts that were dissected from the pupal body. *H. axyridis* prepupae attach themselves to the plants with a gluey substance, where pollen grains and potentially also feces get stuck easily. In addition, the old larval skin with their spines can trap pollen and prey remnants. Pollen grains were clearly visible with a binocular microscope on the anal part of the pupae after washing (Figure S3). Dissecting the pupal body from the anal plate and the molted skin was thus necessary to minimize contamination by pollen grains.

Paula and Andow (2016) detected Cry1Ac and Cry1F in *H. axyridis* pupae when larvae were fed aphids from artificial diet containing purified Cry protein at concentrations similar to leaves of WideStrike Bt cotton. Concentrations detected in the pupae were 2−4 × higher than in the aphid prey and more pupae samples were positive than aphid samples. The reasons for the obvious differences between our results and those by Paula and Andow (2016) are unclear. However, a major shortcoming of the study by Paula and Andow (2016) is the artificial diet system that they used to expose the aphid prey to Cry protein. While this system should provide constant and high exposure to the Cry proteins, they were only detected in 20−50% of the analyzed aphid samples, despite the fact that each sample consisted of 100 aphids. It is also unclear if lady beetle larvae might have fed directly on the artificial diet or on potentially Cry protein-rich honeydew excreted by the aphids. The actual exposure of *H. axyridis* in the study by Paula and Andow (2016) thus remains unclear.

Another study has assessed Cry protein concentrations in *H. axyridis* larvae and pupae. Chang et al. (2017) fed *H. axyridis* larvae with *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) caterpillars reared on *cry1Ab/cry2Aj* expressing maize kernels and lady beetle larvae contained 5 × less Cry protein than the prey, which is in line with our findings. Interestingly, the authors indicate that also pupae and freshly emerged adult lady beetles contained Bt protein (2.5 × less than in larvae). However, it was not mentioned whether the molted skin and anal plate was dissected from the pupal body. Eggs contained no measurable Cry protein. Statistical comparisons revealed that there was no difference between Cry1Ab in pupae, adults (containing Cry protein) and eggs (containing no Cry protein). When Zhang et al. (2014) exposed larvae of the lady beetle *P. japonica* to purified Cry1Ab, Cry1Ac and Cry1F added to a diet based on rapeseed pollen, no Cry protein was detected in the pupae. Potential Cry protein uptake was also investigated in other arthropods. Zhao et al. (2016) did not detect Cry1Ac in pupae and adults of Cry1Ac-resistant *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), but in the hemolymph and fat body, where it was metabolized within 48 h. Finally, Kim et al. (2012) did not find Cry1A in the hemolymph of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) after feeding on Bt maize grain powder.

4.2. Transfer of Cry proteins to the eggs of H. axyridis

Cry protein concentrations in *H. axyridis* males and females were at least one order of magnitude lower than in their food. Because a mix of pollen with *E. kuehniella* eggs was provided as food, exposure was approximately one third compared to pure pollen. In aphids used for this experiment with adults, slightly higher concentrations of Cry protein were detected compared to the experiment with larvae (Experiment 1) with median concentrations that were low, but above the LOD for all Cry proteins except Cry1A.105. Nevertheless, when fed aphids from Bt maize, median concentrations in male and female *H. axyridis* were below the LOD for all Cry proteins except Cry1F in females. Males generally contained less Cry protein than females which is likely due to the fact that females require

Pupal bodies (Pupa) were analyzed separately from the anal plate and the remnants of the molted skin (Anal). Note log scale of the y-axis. Grey bars represent the median of the limit of detection (LOD) of the ELISA for each sample. Black dots represent individual Cry protein concentrations above the LOD of the respective sample. Values below the LOD were set to 0. Red lines indicate the median, red boxes the 95% confidence intervals (CI) around medians. Medians and CI of 0 (>50% of samples below LOD) are not displayed. Black numbers at the top of the panels denote samples above the LOD, red numbers at the bottom samples below the LOD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

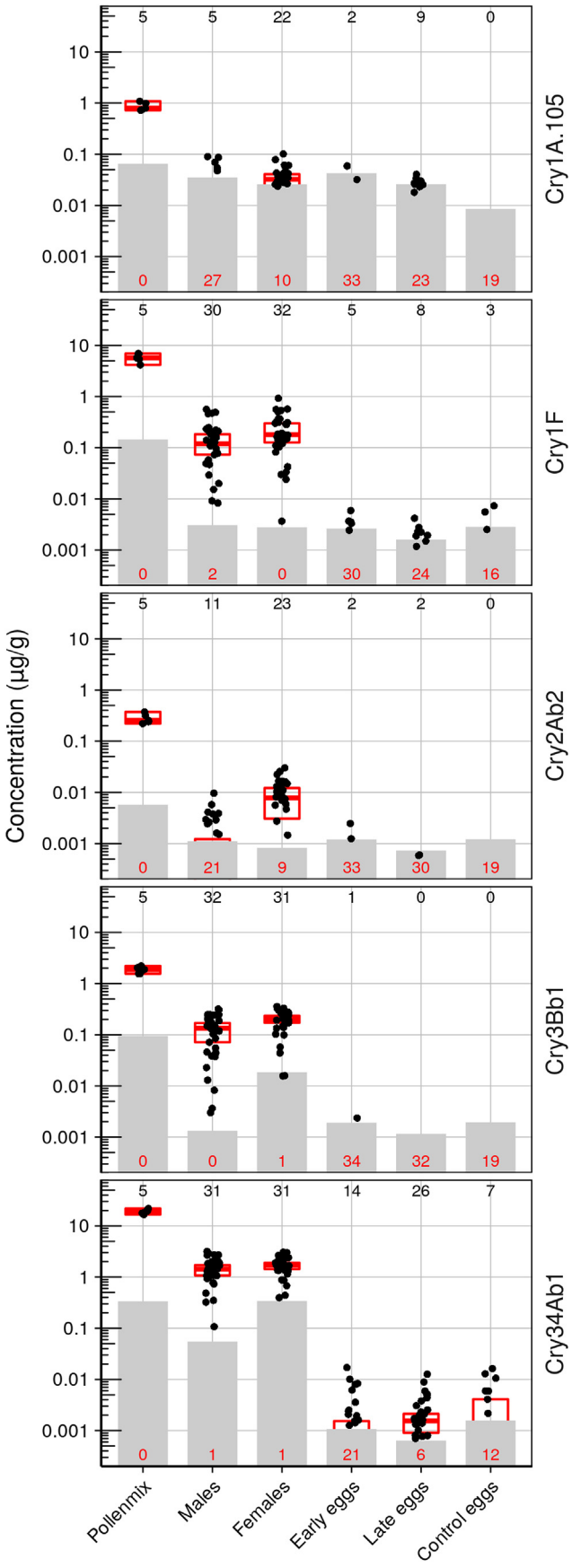

Fig. 2. Concentration (µg/g fresh weight) of different Cry proteins in pollenmix (SmartStax maize pollen mixed with *E. kuehniella* eggs at a ratio of 1:2, v:v), individual males and females of *Harmonia axyridis* fed with pollenmix for their whole life, early eggs (first 6 egg batches), late eggs (egg batch 7–16), and control eggs (Cry-protein free eggs incubated for 2 days with *H. axyridis* pairs and pollenmix). Note log scale of the y-axis. Grey bars represent the median of the limit of detection (LOD) of the ELISA for each sample. Black dots represent individual Cry protein concentrations above the LOD of the respective sample. Values below the LOD were set to 0. Red lines indicate the median, red boxes the 95% confidence intervals (CI) around medians. Medians and CI of 0 are not displayed. Black numbers at the top of the panels denote samples above the LOD, red numbers at the bottom samples below the LOD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

more food for egg production. This has, for example, also been reported for adult *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) (Li et al., 2010). In the experiment with aphids, the time range needed for the production of 6 egg batches (18–43 days) was delayed and broader than in the experiment with pollenmix (11–22 days), indicating lower food quality of aphids for female *H. axyridis* compared with the pollenmix.

When *H. axyridis* larvae and adults were fed with pollenmix, the median concentrations of Cry proteins in their eggs were below the LOD, except for Cry34Ab1 in egg batches 7–16 (late eggs). Compared to the concentrations in the pollenmix, however, the concentrations of Cry34Ab1 in the late eggs were 13′000 × (4 orders of magnitude) lower and compared to the concentrations in the females 1′100 × (3 orders of magnitude) lower. In addition, 9 samples were also positive for Cry1A.105, 8 for Cry1F and 2 for Cry2Ab2. When females were fed with aphids from Bt maize, only 6 out of 135 egg samples were positive (while none of the control egg samples were positive). Nevertheless, the confidence intervals for all Cry proteins in eggs from lady beetles fed with pollenmix or aphids either overlapped with those of the control eggs, or were zero. Therefore, we cannot conclude with certainty that Cry protein was transferred from females to eggs. Paula et al. (2015) reported that Cry1F concentrations in *H. axyridis* females were positively correlated with the amount of Cry1F detected in eggs (and subsequently neonates). In our experiments, no correlation between the concentration of any of the five Cry proteins in *H. axyridis* females and the concentration in the respective (late) egg batches was present.

Intergenerational transfer of Bt proteins has previously been suggested for *P. japonica* lady beetles feeding on aphids from Bt cotton (Zhang et al., 2006), *N. lugens* leafhoppers feeding on Bt rice (Gao et al., 2010), and *C. lacinia* butterflies feeding on Bt protein enriched honey solution (Paula et al., 2014). In contrast, no Cry1Ab was detected in eggs of *H. axyridis* when *S. exigua* from Bt maize was provided as prey (Chang et al., 2017).

Contamination and false positive readings are an issue with the highly sensitive ELISA tests, in particular when the measured concentrations are close to the LOD. We cannot exclude that contamination in the rearing containers (e.g., grooming of eggs by females, pollen, feces, or honey dew sticking to eggs or pupal bodies), during the different processing steps (dissecting, washing, weighing, transfer in new tubes, etc.), and on the ELISA plates may also have contributed to some positive measurements, despite the fact that special care was taken to avoid contamination. Furthermore, Cry protein detection by ELISA does not necessarily indicate that the proteins are present in a bioactive structure. The detection antibodies may also bind to degraded and non-functional Cry protein fragments.

While the focus of our study was on exposure of *H. axyridis* to Cry proteins and the fate of the proteins, Svobodová et al. (2017) also addressed toxicity using the same bi- and tri-trophic system as in the present study. No effects on survival, development and weight of *H. axyridis* and other predators (i.e., lacewings and spiders) were reported when fed aphids, spider mites or pollen from

SmartStax maize. Similarly, Ali et al. (2016) reported no deleterious effects when *H. axyridis* larvae were exposed to Cry1Ac, Cry2Ab, Cry1F, Cry1Ca, or Vip3A in an artificial diet and Chang et al. (2017) found no effects of Cry1Ab/Cry2Aj producing maize on life table parameters of *H. axyridis* when exposed *via* caterpillar (*S. exigua*) prey or *via* pollen. Wang et al. (2018) reported that no specific receptors for Cry1Ac and Cry2Aa could be found in *H. axyridis* larvae and adults, indicating that toxicity of those Bt proteins is unlikely. Despite their finding of relatively high concentrations of Cry1F in *H. axyridis* females and eggs in the artificial diet system, Paula et al. (2015) reported no sublethal effects on pre-oviposition period, number of eggs produced, age-specific fecundity, egg development time, fertility rate, and hatching rate. Thus transovarial transfer of Cry proteins, if occurring, did not appear to impact the performance of the predator.

5. Conclusions

Our results confirm previous observations that Cry proteins are degraded and excreted quickly in the arthropod food web without evidence for bioaccumulation. Nevertheless, the presence of some Cry proteins (in particular Cry34Ab1) in *H. axyridis* pupae indicates uptake of small amounts from the gut to other tissues of the lady beetle. Sequestration of the proteins to reproductive tissues, and finally intergenerational transfer to eggs can neither be confirmed nor excluded based on the results of our study. In any case, in a Bt maize field, Cry protein is omnipresent in plant tissue, herbivores and predators and we conclude that potential trace concentrations in lady beetle pupae and eggs, several orders of magnitude lower than in plant tissue, are unlikely to represent a significant route of exposure for other predators or parasitoids.

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CRediT authorship contribution statement

Michael Meissle: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Stefanie Kloos:** Investigation, Methodology, Writing - review & editing. **Jörg Romeis:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing.

Declaration of competing interest

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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Appendix B. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2020.115421.

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Supplementary online material

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Fate of multiple Bt proteins from stacked Bt maize in the predatory lady beetle *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae)

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Table S1: Amount (ng per individual) of different Cry proteins in *Harmonia axyridis* larvae and pupae after feeding on SmartStax maize and/or *Ephestia kuehniella* eggs

Table S2: Concentrations (µg/g fresh weight) of different Cry proteins in *Harmonia axyridis* larvae and pupae after feeding on SmartStax maize and/or *Ephestia kuehniella* eggs

Table S3: Concentrations (µg/g fresh weight) of different Cry proteins in pollenmix (SmartStax maize pollen mixed with *Ephestia kuehniella* eggs at a ratio of 1:2, v:v) or aphids reared on SmartStax maize, individual males and individual females of *Harmonia axyridis* fed with pollenmix or aphids for their whole life, early eggs (first 6 egg batches), late eggs (7th to 16th egg batch), and control eggs (Cry-protein free eggs incubated for 2 days with *H. axyridis* pairs fed pollenmix or aphids)

Table S4: Correlations between Cry protein concentrations in female *Harmonia axyridis* after feeding pollenmix (SmartStax maize pollen mixed with *Ephestia kuehniella* eggs at a ratio of 1:2, v:v) and their late eggs (7th to 16th egg batch)

Figure S1: Amount (ng per individual) of different Cry proteins in individual larvae and pupae of *Harmonia axyridis* feeding on *Rhopalosiphum padi* aphids from SmartStax Bt maize

Figure S2: Concentration (µg/g fresh weight) of different Cry proteins in *Rhopalosiphum padi* aphids from SmartStax maize, individual males and females of *Harmonia axyridis* fed with aphids from SmartStax maize for their whole life, eggs (first 6 egg batches), and control eggs (Cry-protein free eggs incubated for 2 days with *H. axyridis* pairs fed aphids from SmartStax maize).

Figure S3: Photograph of *Harmonia axyridis* anal plate of the pupa, detached from the substrate.

Table S1: Amount (ng per individual) of different Cry proteins in *Harmonia axyridis* larvae and pupae after feeding on SmartStax maize and/or *Ephestia kuehniella* eggs. Given is the median (bold) with 95% confidence intervals. Instead of zero medians, the LOD is given with a < symbol (in italics). N is the number of samples, which equals individuals except for anal part (molted skin and anal plate) samples, were 4-6 individuals were pooled. Weight (in mg) per individual is also provided.

Treatment[1]	N	Weight	**Cry1A.105**	**Cry1F**	**Cry2Ab2**	**Cry3Bb1**	**Cry34Ab1**
Pollen							
P-L3	27	5.9	**3.6** (3.0; 4.5)	**14** (12; 18)	**0.16** (0.093; 0.32)	**7.0** (5.6; 8.0)	**52** (46; 78)
PP-L4	27	12	**4.1** (2.9; 6.1)	**17** (12; 20)	**0.14** (0.086; 0.20)	**9.1** (7.5; 11)	**158** (131; 189)
PPP-Pupa	28	22	*<0.41* (0; 0)	*<0.017* (0; 0.031)	*<0.017* (0; 0)	*<0.017* (0; 0.027)	**0.14** (0.12; 0.25)
PPP-Anal part	6	1.5	*<0.18* (0; 0)	**1.6** (1.3; 2.6)	**0.077** (0.051; 0.15)	**0.98** (0.78; 1.9)	**11** (8.9; 17)
PPE-Pupa	26	31	*<0.41* (0; 0)	*<0.017* (0; 0)	*<0.017* (0; 0)	*<0.017* (0; 0)	**0.043** (0.033; 0.065)
PPE-Anal part	6	1.1	*<0.042* (0; 0)	**0.11** (0.045; 0.22)	*<0.0060* (0; 0.0075)	**0.045** (0.016; 0.080)	**0.63** (0.45; 0.92)
PE-L4	27	28	*<0.43* (0; 0)	**0.36** (0.17; 0.52)	*<0.021* (0; 0)	**0.64** (0.26; 0.84)	**13** (9.7; 19)
PEE-Pupa	27	35	*<0.41* (0; 0)	*<0.017* (0; 0)	*<0.017* (0; 0)	*<0.017* (0; 0)	*<0.019* (0; 0)
PEE-Anal part	6	1.0	*<0.049* (0; 0)	*<0.027* (0; 0)	*<0.0072* (0; 0)	*<0.013* (0; 0)	**0.057** (0.017; 0.13)
Spider mites							
S-L3	22	4.2	**6.8** (4.6; 9.1)	**2.9** (1.9; 3.7)	**0.21** (0.089; 0.42)	**20** (14; 27)	**14** (12; 21)
S-S-L4	14	10	**14** (10; 19)	**6.2** (4.4; 8.8)	**0.79** (0.35; 1.4)	**31** (22; 38)	**26** (22; 31)
S-S-E-Pupa	15	29	*<0.43* (0; 0)	*<0.023* (0; 0)	*<0.021* (0; 0)	*<0.021* (0; 0)	*<0.035* (0; 0.047)
S-S-E-Anal part	4	0.77	*<0.22* (0; 0)	*<0.013* (0; 0)	*<0.0079* (0; 0)	**0.015** (0; 0.025)	**0.020** (0.014; 0.028)
S-E-L4	21	27	*<0.43* (0; 0)	**0.10** (0.061; 0.17)	*<0.021* (0; 0)	**1.2** (0.60; 1.4)	**3.9** (2.8; 5.6)
S-E-E-Pupa	24	33	*<0.43* (0; 0)	*<0.023* (0; 0)	*<0.021* (0; 0)	*<0.021* (0; 0)	*<0.035* (0; 0)
S-E-E- Anal part	6	0.76	*<0.054* (0; 0)	*<0.030* (0; 0)	*<0.0080* (0; 0.012)	*<0.014* (0; 0)	*<0.014* (0; 0)
Aphids							
A-L3	31	11	*<0.51* (0; 0)	*<0.025* (0; 0)	*<0.022* (0; 0)	*<0.018* (0; 0)	*<0.017* (0; 0)
A-A-L4	30	25	*<0.51* (0; 0)	*<0.025* (0; 0)	*<0.022* (0; 0)	*<0.018* (0; 0)	*<0.017* (0; 0)
A-A-A-Pupa	30	37	*<0.46* (0; 0)	*<0.021* (0; 0)	*<0.023* (0; 0)	*<0.027* (0; 0)	*<0.036* (0; 0)
A-A-A-Anal part	6	1.5	*<0.18* (0; 0)	*<0.011* (0; 0)	*<0.0063* (0; 0)	*<0.0064* (0; 0)	*<0.0037* (0; 0.0029)
A-A-E-Pupa	29	37	*<0.53* (0; 0)	*<0.020* (0; 0)	*<0.022* (0; 0)	*<0.022* (0; 0)	*<0.025* (0; 0)
A-A-E-Anal part	7	1.4	*<0.54* (0; 0)	*<0.030* (0; 0)	*<0.0080* (0; 0)	*<0.014* (0; 0)	*<0.014* (0; 0)
A-E-L4	31	35	*<0.46* (0; 0)	*<0.021* (0; 0)	*<0.023* (0; 0)	*<0.027* (0; 0)	*<0.036* (0; 0)
A-E-E-Pupa	31	35	*<0.53* (0; 0)	*<0.020* (0; 0)	*<0.022* (0; 0)	*<0.022* (0; 0)	*<0.025* (0; 0)
A-E-E- Anal part	7	1.1	*<0.054* (0; 0)	*<0.030* (0; 0)	*<0.0080* (0; 0)	*<0.014* (0; 0)	*<0.014* (0; 0)
E. kuehniella							
E-E-L4	10	30	*<0.91* (0; 0)	*<0.054* (0; 0)	*<0.032* (0; 0)	*<0.032* (0; 0)	*<0.019* (0; 0)
E-E-E-Pupa	9	34	*<0.90* (0; 0)	*<0.053* (0; 0)	*<0.032* (0; 0)	*<0.032* (0; 0)	*<0.018* (0; 0)

[1] The different letters stand for the food provided in the larval stages (neonate to L2 – L3 – L4). P = pollen, E = *Ephestia kuehniella* eggs, S = spider mites, A = aphids. Either L3, L4, the pupal body (Pupa) or the anal part (abdomen and molted skin) were frozen for ELISA.

Table S2: Concentrations (µg/g fresh weight) of different Cry proteins in *Harmonia axyridis* larvae and pupae after feeding on SmartStax maize and/or *Ephestia kuehniella* eggs. Given is the median (bold) with 95% confidence intervals. Instead of zero medians, the LOD is given with a < symbol (in italics). N is the number of samples, which equals individuals except for anal part (molted skin and anal plate) samples, were 4-6 individuals were pooled.

Treatment[1]	N	**Cry1A.105**	**Cry1F**	**Cry2Ab2**	**Cry3Bb1**	**Cry34Ab1**
Pollen						
P-L3	27	**0.65** (0.60; 0.75)	**2.4 (**2.2; 3.0)	**0.026** (0.015; 0.059)	**1.3** (1.1; 1.4)	**10** (8.7; 12.5)
P-P-L4	27	**0.36** (0.24; 0.53)	**1.3** (1.0; 1.8)	**0.012** (0.0093; 0.017)	**0.80** (0.69; 0.88)	**13** (12; 15)
P-P-P-Pupa	28	*<0.021* (0; 0)	*<0.00085* (0; 0.0015)	*<0.00089* (0; 0)	*<0.00092* (0; 0.0012)	**0.0062** (0.0056; 0.012)
P-P-P-Anal part	6	*<0.13* (0; 0)	**1.2** (1.1; 1.3)	**0.055** (0.041; 0.080)	**0.77** (0.64; 0.95)	**8.7** (7.0; 9.2)
P-P-E-Pupa	26	*<0.013* (0; 0)	*<0.00057* (0; 0)	*<0.00055* (0; 0)	*<0.00057* (0; 0)	**0.0014** (0.0012; 0.0020)
P-P-E- Anal part	6	*<0.038* (0; 0)	**0.13** (0.042; 0.24)	*<0.0055* (0; 0.0063)	**0.052** (0.015; 0.086)	**0.63** (0.40; 1.1)
P-E-L4	27	*<0.015* (0; 0)	**0.011** (0.0057; 0.018)	*<0.00073* (0; 0)	**0.022** (0.0066; 0.035)	**0.43** (0.29; 0.69)
P-E-E-Pupa	27	*<0.012* (0; 0)	*<0.00049* (0; 0)	*<0.00048* (0; 0)	*<0.00050* (0; 0)	*<0.00054* (0; 0)
P-E-E- Anal part	6	*<0.051* (0; 0)	*<0.028* (0; 0)	*<0.0076* (0; 0)	*<0.013* (0; 0)	**0.066** (0.015; 0.14)
Spider mites						
S-L3	22	**1.55** (1.2; 2.0)	**0.69** (0.51; 0.81)	**0.057** (0.021; 0.093)	**4.4** (3.6; 5.9)	**3.6** (2.9; 4.3)
S-S-L4	14	**1.30** (0.89; 1.7)	**0.55** (0.40; 0.82)	**0.071** (0.031; 0.12)	**2.7** (2.0; 3.9)	**2.3** (2.0; 3.0)
S-S-E-Pupa	15	*<0.015* (0; 0)	*<0.00079* (0; 0)	*<0.00073* (0; 0)	*<0.00072* (0; 0)	*<0.0012* (0; 0.0015)
S-S-E- Anal part	4	*<0.32* (0; 0)	*<0.019* (0; 0)	*<0.011* (0; 0)	**0.022** (0; 0.033)	**0.028** (0.017; 0.034)
S-E-L4	21	*<0.015* (0; 0)	**0.0043** (0.0023; 0.0055)	*<0.00074* (0; 0)	**0.043** (0.026; 0.049)	**0.16** (0.097; 0.19)
S-E-E-Pupa	24	*<0.013* (0; 0)	*<0.00068* (0; 0)	*<0.00063* (0; 0)	*<0.00063* (0; 0)	*<0.0011* (0; 0)
S-E-E- Anal part	6	*<0.068* (0; 0)	*<0.038* (0; 0)	*<0.0095* (0; 0.043)	*<0.018* (0; 0)	*<0.016* (0; 0)
Aphids						
A-L3	31	*<0.046* (0; 0)	*<0.0022* (0; 0)	*<0.0020* (0; 0)	*<0.0015* (0; 0)	*<0.0015* (0; 0)
A-A-L4	30	*<0.022* (0; 0)	*<0.0011* (0; 0)	*<0.00098* (0; 0)	*<0.00076* (0; 0)	*<0.00073* (0; 0)
A-A-A-Pupa	30	*<0.013* (0; 0)	*<0.00059* (0; 0)	*<0.00064* (0; 0)	*<0.00074* (0; 0)	*<0.0010* (0; 0)
A-A-A- Anal part	6	*<0.12* (0; 0)	*<0.0072* (0; 0)	*<0.0043* (0; 0)	*<0.0043* (0; 0)	*<0.0025* (0; 0.0019)
A-A-E-Pupa	29	*<0.014* (0; 0)	*<0.00053* (0; 0)	*<0.00058* (0; 0)	*<0.00060* (0; 0)	*<0.00068* (0; 0)
A-A-E- Anal part	7	*<0.040* (0; 0)	*<0.022* (0; 0)	*<0.0059* (0; 0)	*<0.010* (0; 0)	*<0.010* (0; 0)
A-E-L4	31	*<0.013* (0; 0)	*<0.00062* (0; 0)	*<0.00066* (0; 0)	*<0.00074* (0; 0)	*<0.0010* (0; 0)
A-E-E-Pupa	31	*<0.015* (0; 0)	*<0.00057* (0; 0)	*<0.00062* (0; 0)	*<0.00064* (0; 0)	*<0.00072* (0; 0)
A-E-E- Anal part	7	*<0.044* (0; 0)	*<0.024* (0; 0)	*<0.0065* (0; 0)	*<0.011* (0; 0)	*<0.011* (0; 0)
E. kuehniella						
E-E-L4	10	*<0.030* (0; 0)	*<0.0018* (0; 0)	*<0.0011* (0; 0)	*<0.0011* (0; 0)	*<0.00061* (0; 0)
E-E-E-Pupa	9	*<0.026* (0; 0)	*<0.0016* (0; 0)	*<0.00093* (0; 0)	*<0.00094* (0; 0)	*<0.00054* (0; 0)

[1] The different letters stand for the food provided in the larval stages (neonate to L2 – L3 – L4). P = pollen, E = *E. kuehniella* eggs, S = spider mites, A = aphids. Either L3, L4, the pupal body (Pupa) or the anal part (abdomen and molted skin) were frozen for ELISA.

Table S3: Concentrations (µg/g fresh weight) of different Cry proteins in pollenmix (SmartStax maize pollen mixed with *Ephestia kuehniella* eggs at a ratio of 1:2, v:v) or aphids reared on SmartStax maize, individual males and females of *Harmonia axyridis* fed with pollenmix or aphids for their whole life, early eggs (first 6 egg batches), late eggs (7th to 16th egg batch), and control eggs (Cry-protein free eggs incubated for 2 days with *H. axyridis* pairs fed pollenmix or aphids). Values below the limit of detection (LOD) were set to 0. Given is the median (bold) with 95% confidence intervals. Instead of zero medians, the LOD is given with a < symbol (in italics). N is the number of samples.

Treatment	N	**Cry1A.105**	**Cry1F**	**Cry2Ab2**	**Cry3Bb1**	**Cry34Ab1**
Pollenmix as food						
Pollenmix	5	**0.79** (0.73; 1.1)	**5.7** (4.2; 6.9)	**0.25** (0.22; 0.37)	**1.9** (1.6; 2.2)	**19** (17; 22)
Males	32	*<0.035* (0; 0)	**0.12** (0.073; 0.18)	*<0.0011* (0; 0.0012)	**0.13** (0.071; 0.17)	**1.4** (1.1; 1.7)
Females	32	**0.032** (0.024; 0.041)	**0.18** (0.13; 0.30)	**0.0078** (0.0031; 0.012)	**0.20** (0.17; 0.24)	**1.7** (1.4; 1.9)
Early eggs	35	*<0.043* (0; 0)	*<0.0026* (0;0)	*<0.0012* (0; 0)	*<0.0019* (0; 0)	<0.0011 (0; 0.0015)
Late eggs	32	*<0.026* (0; 0)	*<0.0016* (0; 0)	*<0.00074* (0; 0)	*<0.0012* (0; 0)	**0.0015** (0.00091; 0.0021)
Control eggs	19	*<0.0085* (0; 0)	*<0.0028* (0; 0)	*<0.0012* (0; 0)	*<0.0019* (0; 0)	<0.0016 (0; 0.0041)
Aphids as food						
Aphids	5	*<0.063* (0; 0)	**0.011** (0.0096; 0.013)	**0.0036** (0.0022; 0.013)	**0.0058** (0.0037; 0.022)	**0.0026** (0.0011; 0.0057)
Males	22	*<0.038* (0; 0)	*<0.0021* (0; 0)	*<0.0011* (0; 0)	*<0.0013* (0; 0)	*<0.0011* (0; 0)
Females	24	*<0.028* (0; 0)	**0.0014** (0; 0.0018)	*<0.00081* (0; 0)	*<0.00097* (0; 0)	*<0.00081* (0; 0.0011)
Early eggs	27	*<0.039* (0; 0)	*<0.0025* (0; 0)	*<0.0011* (0; 0)	*<0.0017* (0; 0)	*<0.0011* (0; 0)
Control eggs	12	*<0.048* (0; 0)	*<0.0042* (0; 0)	*<0.0015* (0; 0)	*<0.0017* (0; 0)	*<0.0015* (0; 0)

Table S4: Correlations between Cry protein concentrations in female *Harmonia axyridis* after feeding pollenmix (SmartStax maize pollen mixed with *Ephestia kuehniella* eggs at a ratio of 1:2, v:v) and their late eggs (7^{th} to 16^{th} egg batch). Spearman's rank correlation was used. No calculation was possible for Cry3Bb1 because all values in late eggs were zero (NA).

Toxin	S	rho	P
Cry1A.105	3958	0.20	0.28
Cry1F	6096	-0.23	0.22
Cry2Ab2	4109	0.17	0.36
Cry3Bb1	NA	NA	NA
Cry34Ab1	5950	-0.20	0.28

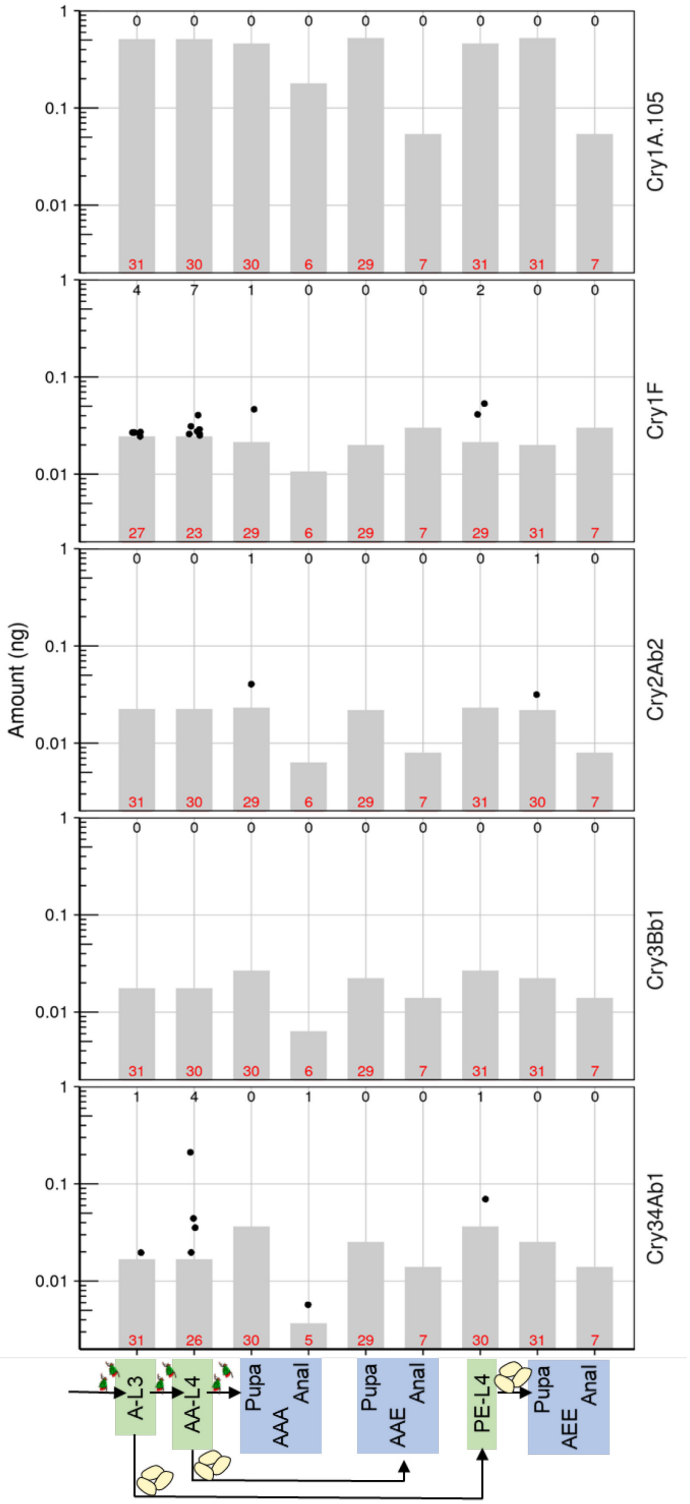

Figure S1: Amount (ng per individual) of different Cry proteins in larvae and pupae of *Harmonia axyridis* feeding on *Rhopalosiphum padi* aphids (A) from SmartStax Bt maize. Sterilized eggs of *Ephestia kuehniella* (E) were used as a non-Bt food source. x-axis: L3 and L4 indicate the larval stage of the lady beetle. Pupal bodies (Pupa) were analyzed separately from the anal plate and the remains of the molted skin (Anal). Note log scale of the y-axis. Grey bars represent the median of the limit of detection (LOD) for each sample. Black dots represent individual Cry protein concentrations above the LOD of the respective sample. Values below the LOD were set to 0. Black numbers at the top of the panels denote samples above the LOD, red numbers at the bottom samples below the LOD. Median concentrations and 95% confidence intervals (CI) around medians were always below the LOD and are thus not shown.

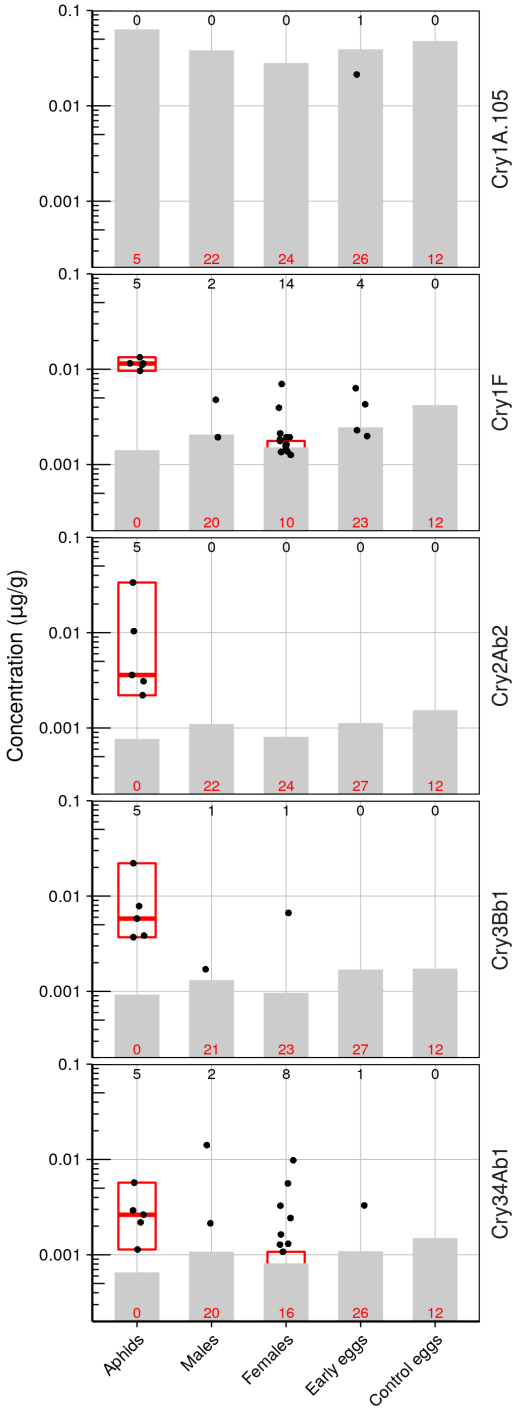

Figure S2: Concentration (µg/g fresh weight) of different Cry proteins in *Rhopalosiphum padi* aphids from SmartStax maize, individual males and females of *Harmonia axyridis* fed with aphids from SmartStax maize for their whole life, eggs (first 6 egg batches), and control eggs (Cry-protein free eggs incubated for 2 days with *H. axyridis* pairs fed aphids from SmartStax maize). Note log scale of the y-axis. Grey bars represent the median of the limit of detection (LOD) for each sample. Black dots represent individual Cry protein concentrations above the LOD of the respective sample. Values below the LOD were set to 0. Red lines indicate the median, red boxes the 95% confidence intervals (CI) around medians. Medians and CI of 0 are not displayed. Black numbers at the top of the panels denote samples above the LOD, red numbers at the bottom samples below the LOD.

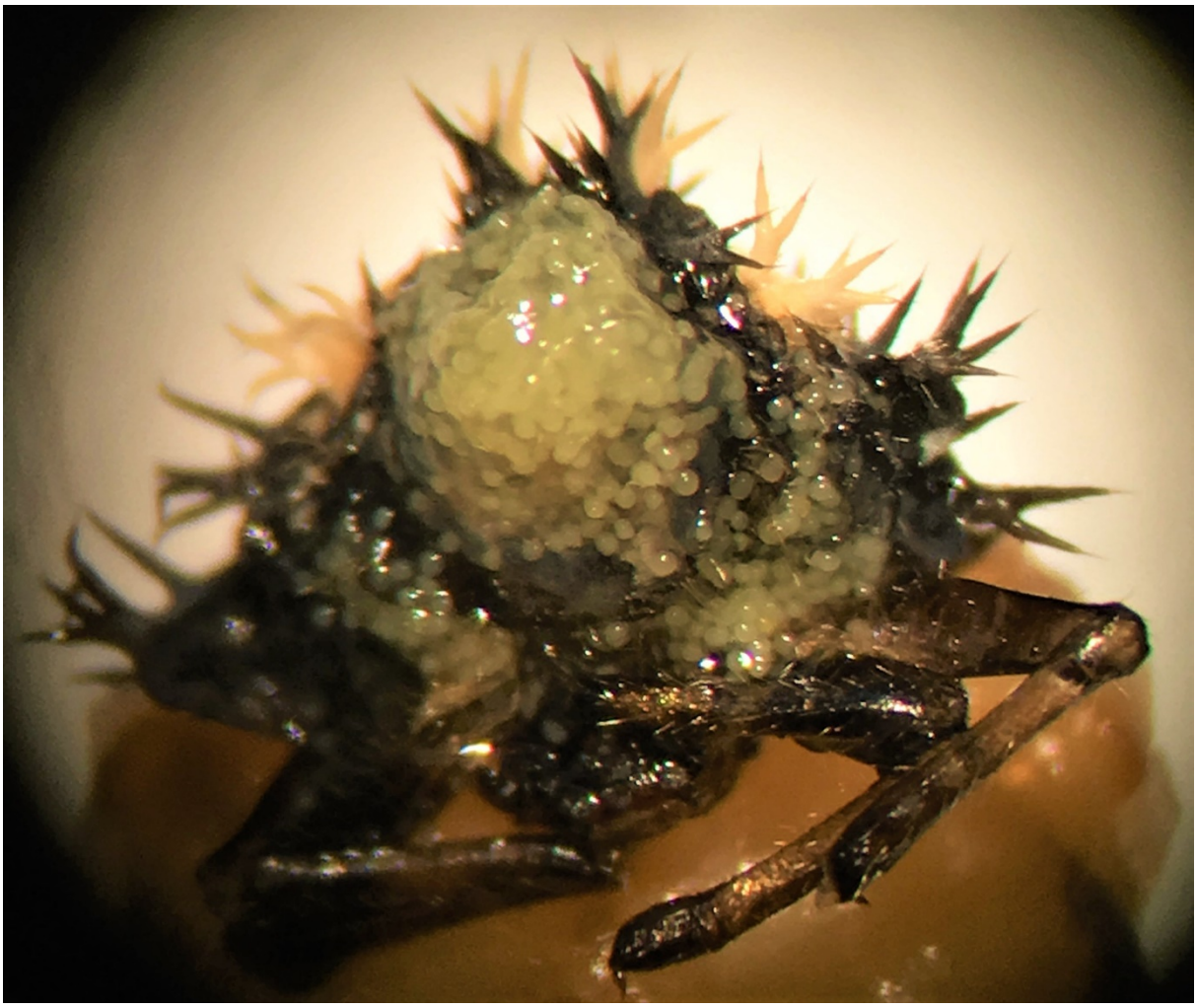

Figure S3: Photograph of *Harmonia axyridis* anal plate of the pupa, detached from the substrate. Pollen grains were provided *ad libitum* as food to larvae. After washing the detached pupae, pollen grains were still trapped to the gluey substance that the prepupae use to attach themselves to the substrate.