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Multitrophic Cry-protein flow in a dual-gene Bt-cotton field

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ABSTRACT

The transfer of plant-produced insecticidal Cry-proteins in the arthropod food web can be affected by a number of environmental and ecological factors. Despite this fact, most studies documenting multitrophic Cry-protein acquisition patterns in arthropods are conducted under controlled conditions whereas the number of field studies is limited. Such field studies, however, are valuable to understand multitrophic allocation dynamics of Cry-proteins under ecologically realistic conditions and are therefore important for the interpretation and design of laboratory hazard studies on genetically engineered (GE) crops. We thus sampled arthropods and plant structures in a field planted with GE dual-gene cotton plants producing the Cry-proteins Cry1Ac and Cry2Ab from *Bacillus thuringiensis* Berliner over the growing season. Cry-protein concentrations in field-collected plants, herbivores, and predators were quantified and compared with arthropods subjected to tri-trophic laboratory feeding assays. Both, field studies and laboratory assays showed that Cry-protein concentrations strongly decreased with increasing trophic level to values mostly below the detection limit in predators. Under field conditions, *in-planta* Cry-protein concentrations varied between plant structures and over the season. Concentrations in arthropods were mainly associated with feeding mode, feeding location on the plant, and to a lesser degree seasonality. Compared to plants, arthropods showed lower Cry2Ab:Cry1Ac ratios indicating that Cry2Ab might be less stable than Cry1Ac. Of all predators collected in the field study, we measured highest Cry-protein levels in jumping and crab spiders, predatory flies and some predatory hemipterans. This emphasizes the relevance of these groups for the risk assessment of GE cotton.

1. Introduction

Cotton (*Gossypium* spp.) is cultivated on about 30 million hectares worldwide (USDA, 2017), and harbors a species-rich complex of arthropods (King et al., 1996). About 75% of the globally cultivated cotton is genetically engineered (GE) and produces Cry-proteins from *Bacillus thuringiensis* Berliner ("Bt-cotton"), which protect the plant against certain lepidopteran pests. An increasing number of Bt-cotton plants produce two Cry-proteins, Cry1Ac and Cry2Ab (ISAAA, 2016) with different *in-planta* expression rates (Su et al., 2015) and slightly different target spectra within the Lepidoptera (Sivasupramaniam et al., 2008).

Because Bt-cotton interacts with a multitude of arthropods, a major concern is that the produced insecticidal Cry-proteins might harm non-target species, particularly beneficial arthropods such as natural enemies, pollinators, and decomposers. This potential risk is thus assessed prior to the release of any GE plant. A non-target species will only be at risk if it is exposed to a Cry-protein at hazardous concentrations. Hazard is usually assessed in laboratory assays under worst-case exposure conditions. More realistic studies, such as semi-field or field studies, may then be conducted with Bt-transgenic plant material if the risk of adverse effects cannot be excluded from the worst-case studies (Romeis et al., 2008). Hazard assessments are conducted for selected surrogate species that represent important taxonomic and functional groups for the particular Bt-crop under consideration (Romeis et al., 2013). Detailed information on the movement of Cry-proteins in the arthropod food web would support the selection of relevant surrogate species for laboratory testing.

The multitrophic transfer of Cry-proteins in the field depends on a variety of factors. *In-planta* Cry-protein levels under field conditions differ among plant structures and are affected by seasonal and environmental factors (Greenplate, 1999; Adamczyk and Sumerford, 2001; Kranthi et al., 2005; Knight et al., 2013). Concentrations of Cry-proteins in predators might largely depend on the availability of prey and can thus be influenced by seasonal prey-population dynamics (Kendall et al., 1999). Furthermore, herbivores and predators might contain different Cry-protein concentrations depending on their mode of feeding, their feeding location on the plant, the rate of food uptake

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and excretion, their behavior and possibly also the physiology of the digestive system (Romeis et al., 2009). Finally, studies under controlled conditions found that some Cry-proteins might degrade quicker than others, leading to changing ratios of different Cry-proteins produced by the plant within increasing trophic levels (Tian et al., 2013; Su et al., 2015; Meissle and Romeis, 2017).

Most exposure studies with Bt-cotton have been conducted under controlled laboratory or greenhouse conditions and encompassed few common species (Zhang et al., 2006; Torres and Ruberson, 2008; Meissle and Romeis, 2017). Field studies, on the other hand, which document Cry-protein allocation patterns within realistic cotton food webs are limited (but see Torres et al., 2006), and are even completely lacking for dual-gene cotton. The goals of the current study were therefore to: (1) quantify and compare Cry-protein concentrations in field-grown dual-gene Bt-cotton and arthropods during the season, and (2) compare Cry-protein concentrations in predators collected in the field with those exposed to dual-gene Bt-cotton fed prey in the laboratory.

2. Materials and methods

2.1. Field collection of arthropods and plants

Arthropods and Bt-cotton plants were sampled in a 0.6 ha field planted with Bollgard II Roundup Ready Flex cotton (DP1359B2RF, event MON15985 × MON1445, Monsanto, St. Louis, USA) in Maricopa, Arizona, USA. From 10 randomly selected plants, one sample per plant structure was taken once at pre-flowering, twice at flowering, and once at post-flowering stage (Table S1, Fig. 1). At each sampling event 10 new plants were randomly selected. Pollen was collected from single flowers on 13–15 different plants. Arthropods were randomly collected throughout the field with sweep nets and beat buckets 1–2 times per vegetation stage.

Arthropods and plant samples were transported to the laboratory on ice and stored at −80 °C. Arthropods were sorted by species, or at least family, and frozen until used for Cry-protein measurements (see Table S2 for a list of sampled species). Only adults were kept for Cry-protein analysis except for *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae). For this species no distinction was made between adults an immatures since individuals were hard to handle in frozen condition due to their small size. Furthermore, species for which less than three individuals were collected on a given sampling date were discarded. Species were identified based on Van den Bosch and Hagen (1966) and by using reference collections at the USDA-ARS.

Depending on the size and abundance of the different arthropod species, different numbers of individuals were pooled per sample. Large and less common species, such as the stink bug *Euschistus conspersus* Uhler (Hemiptera: Pentatomidae), were analyzed individually, whereas about 50 individuals of the small and common species *F. occidentalis* were pooled in one sample. For all arthropods and plant structures, the number of samples and the number of pooled individuals per sample used for analyzing Cry-protein concentrations is given in Table S2.

2.2. Laboratory-bioassay

For laboratory bioassays, 9–23 adults of five abundant predatory arthropod species were randomly sampled during the cotton post-flowering stage in an alfalfa field in Maricopa using a sweep net. The field was located about 1.5 km away from the nearest Bt-cotton field. Therefore, dispersal of individuals from Bt-cotton into our alfalfa field was unlikely but could not be excluded completely. The following arthropods were sampled: *Geocoris punctipes* (Say) (Hemiptera: Geocoridae), *Zelus renardii* (Kolenati) (Hemiptera: Reduviidae), *Nabis alternatus* Parshley (Hemiptera: Nabidae), *Collops vittatus* (Say) (Coleoptera: Melyridae), and *Misumenops celer* Hentz (Araneae: Thomisidae). All predators were starved for 1 day. Second instar *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) caterpillars (Frontier Agricultural Sciences, Newmark, USA) were fed for at least 24 h on foliage of Bt-cotton branches collected in the field. Although *S. exigua* is an unlikely prey species to be found in a Bt-cotton ecosystem in Arizona, they depict a good prey-model organism for multitrophic laboratory feeding studies due to their Cry-protein tolerance and relatively high Cry-protein content when fed on Bt-cotton. For the bioassay, predators were kept individually in ventilated Petri dishes (5.5 cm diameter) and were offered 5–7 caterpillars (2nd–3rd instar) between 08:00–09:30 in the morning and again between 16:00–17:30 in the afternoon. At each feeding event, the remaining uneaten caterpillars were removed. Observations confirmed that all predatory species were able to feed on the caterpillars. The assay ended after five feeding events (except for *M. celer,* which were fed only 4 times due to a shortage of suitable caterpillars). All predators were frozen at −80 °C. In addition, two sets of five Bt-cotton fed caterpillars were frozen at each feeding event.

2.3. Quantification of Cry-proteins

The concentrations of Cry1Ac and Cry2Ab in plant and arthropod samples (see Table S2 for details) were measured immunologically using commercial ELISA kits (QualiPlate Combo Kits, Envirologix, Portland, USA). Standard curves for quantitative measurements were created with purity corrected Cry1Ac and Cry2Ab provided by Monsanto.

All samples were lyophilized and dry weights (dw) were determined. Subsequently, Cry1Ac and Cry2Ab concentrations were measured as described in Meissle and Romeis (2017).

The limit of detection (LOD) for each plate was determined based on three times the standard deviation of the optical density of all buffer-only controls from eight ELISA plates multiplied by the slope of the standard curve of each specific plate. The LOD of each sample was calculated based on sample dw, dilution, and amount of added buffer.

2.4. Analysis

Cry-protein concentrations in field-collected cotton plant and arthropod samples were analyzed semi-quantitatively. Medians for Cry-protein concentrations of all replicates of the same plant structure or arthropod species for each plant growth stage were binned to one of the following categories ($\mu g\ g^{-1}$ dw): > 100, 10–100,1–10, 0.1–1, 0.01–0.1, 0.00–0.01, < LOD. Ratios of Cry2Ab to Cry1Ac concentrations were calculated with the medians of data from all plant growth stages pooled.

Cry-protein concentrations of predators collected in Bt-cotton fields were compared to those fed in the lab semi-quantitatively using median concentrations. For field collected arthropods, data pooled for all plant growth stages were used to calculate the medians.

Changes in Cry-protein concentrations in plant tissue were analyzed statistically using R (version 3.2.3, R Core Team, 2017) package agricolae, version 1.2–4 (de Mendiburu, 2016). Kruskal-Wallis tests with Holm-Bonferroni corrections were used to compare concentrations among different plant structures within a given growth stage, and among different growth stages within a given plant tissue.

3. Results

3.1. In-planta Cry-protein concentrations

Cry2Ab concentrations were 20–60 times higher than Cry1Ac (Fig. 1, Table S2). Median concentrations of Cry-proteins in plants ranged from 0.67 in young boll capsules to 9.72 $\mu g\ g^{-1}$ dw in young leaves for Cry1Ac and from 36.6 in mature boll capsules to 176.9 $\mu g\ g^{-1}$ dw in seeds for Cry2Ab (Fig. 1, Table S2). The Cry2Ab:-Cry1Ac ratios in different plant structures varied from 25 in old leaves

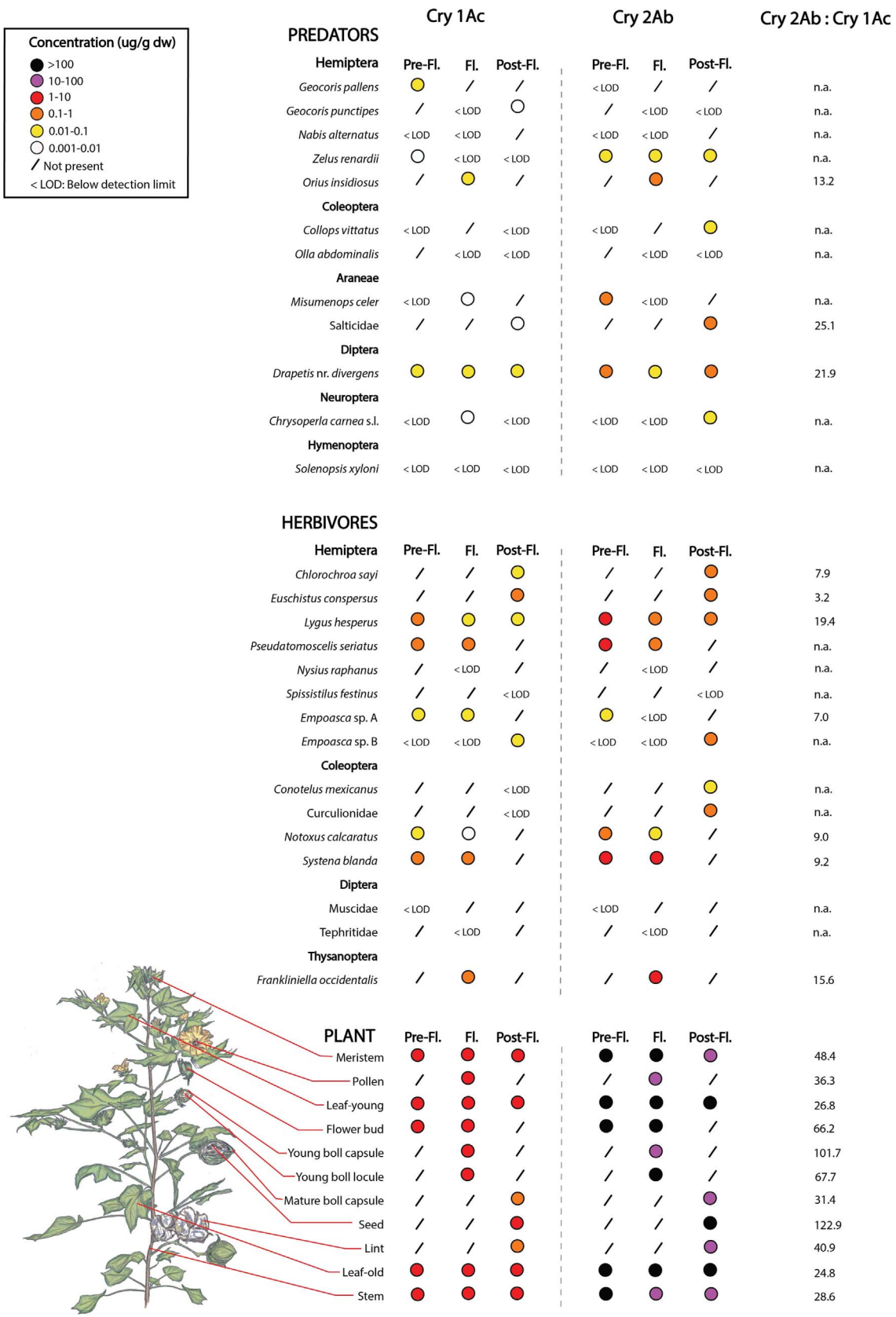

Fig. 1. Median Cry-protein concentrations and ratios of Cry2Ab:Cry1Ac measured in different Bt-cotton (Bollgard II) plant structures and arthropods sampled in a Bt-cotton field, Maricopa, AZ, USA. Concentrations were measured during the pre-flowering (Pre-Fl.), flowering (Fl.) and post-flowering (Post-Fl.) stages in 2015 using ELISA. dw = dry weight.

Table 1
Impact of crop growth stage on Cry-protein concentrations in different Bt-cotton (Bollgard II) plant structures. Medians within the same column and plant structure sharing the same letter are not significantly different ($P > 0.05$; Kruskal-Wallis test with Holm-Bonferroni correction, n = 10). dw = dry weight.

		Cry1Ac	Cry2Ab
Plant structures	Stage	Median (μg g^{-1} dw)	Median (μg g^{-1} dw)
Leaf-young	Pre-flowering	9.72 a	166.69 a
	Flowering	4.72 b	124.96 b
	Post-flowering	3.04 c	115.47 b
		H = 21.997, df = 2, p < 0.001	H = 10.056, df = 2, p = 0.016
Leaf-old	Pre-flowering	4.89 a	102.42 b
	Flowering	7.10 ab	139.79 a
	Post-flowering	3.82 c	161.27 a
		H = 10.113, df = 2, p = 0.0063	H = 8.921, df = 2, p = 0.011
Meristem	Pre-flowering	4.23 a	149.44 a
	Flowering	3.91 a	143.50 a
	Post-flowering	1.26 b	94.47 b
		H = 12.48, df = 2, p = 0.0019	H = 8.255, df = 2, p = 0.016
Stem	Pre-flowering	3.30 a	106.93 a
	Flowering	2.95 a	84.99 ab
	Post-flowering	1.52 b	71.98 b
		H = 18.498, df = 2, p < 0.001	H = 8.123, df = 2, p = 0.017

to 123 in lint (Fig. 1). During pre-flowering and flowering stages, Cry1Ac concentrations were highest in leaves and meristems, whereas reproductive and fruiting structures showed the lowest concentrations. Cry2Ab concentrations showed a similar, albeit less pronounced trend (Table S2). During the post-flowering stage, both Cry-protein concentrations were highest in seeds and leaves and lowest in lint and boll capsules (Table S2). Concentrations tended to decrease in all plant structures over time (Table 1). The only exception were older leaves, which showed higher concentrations during flowering and post-flowering stages than during the pre-flowering stage.

3.2. Multitrophic Cry-protein movement

A total of 15 field-collected herbivore species from four orders and 12 predator species from six orders were analyzed. Cry-protein concentrations were highly variable among species and time of season. Median values in herbivores ranged from below the limit of detection < LOD for both Cry proteins in several species collected at several sampling dates to 0.52 μg g^{-1} dw Cry1Ac and 6.94 μg g^{-1} dw Cry2Ab in *Pseudatomoscelis seriatus* (Reuter) (Hemiptera: Miridae) collected pre-flowering. In predators, Cry protein concentrations ranged from < LOD to 0.035 μg g^{-1} dw Cry1Ac and 0.8 μg g^{-1} dw Cry2Ab in *Drapetis* nr. *divergens* (Diptera: Empididae) collected pre-flowering (Table S2). Herbivores and predators showed generally lower Cry2Ab:Cry1Ac ratios than plants, ranging from 2.4 in *Geocoris pallens* Stäl (Hemiptera: Geocoridae) to 30 in Salticidae (Araneae) (Fig. 1).

Most of the herbivores collected in the field were hemipterans, of which *Lygus hesperus* (Knight) and *P. seriatus* (both Hemiptera: Miridae) together with the thrips *F. occidentalis,* and the flea beetle *Systena blanda* Melsheimer (Coleoptera: Chrysomelidae) contained the highest median concentrations of all herbivores, ranging from 0.022–0.52 μg g^{-1} dw for Cry1Ac and 0.39–6.94 μg g^{-1} dw for Cry2Ab (Fig. 1, Table S2). No Cry-proteins were measured in the false chinch bug *Nysius raphanus* Howard (Hemiptera: Lygaeidae), the treehopper *Spissistilus festinus* (Say) (Hemiptera: Membracidae), and the two dipteran species sampled (all < LOD for Cry1Ac and Cry2Ab). Median values indicated that Cry-protein concentrations decreased over the season in several herbivore species, e.g., *L. hesperus* or *Notoxus calcaratus* Horn (Coleoptera: Anthicidae) (Table S2, Fig. 1).

For predators, most of which were hemipterans, median Cry-protein concentrations were often below the LOD. The predatory fly *D.* nr. *divergens,* the flower bug *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) and Salticidae contained 0.004–0.035 μg g^{-1} dw Cry1Ac and 0.09-0.8 μg g^{-1} dw Cry2Ab (Fig. 1, Table S2). A range of species, i.e. *G. pallens*, *G. punctipes*, *Z. renardii*, *C. vittatus*, *M. celer*, and *Chrysoperla carnea* s.l. (Neuroptera: Chrysopidae), contained no Cry proteins on at least one date that the species were sampled, or contained measurable amounts of one Cry protein, but not of the other. No Cry protein was detected in *N. alternatus*, *Olla abdominalis* (Say) (Coleoptera: Coccinellidae), and *Solenopsis xyloni* McCook (Hymenoptera: Formicidae).

Medians of Cry-protein concentrations measured in laboratory-fed *S. exigua* caterpillars were 1.1 μg g^{-1} dw (Cry1Ac) and 22.3 μg g^{-1} dw (Cry2Ab) (Fig. 2). The median concentrations of both Cry-proteins in *G. punctipes*, *N. alternatus*, and *C. vittatus* from the laboratory-bioassay were below the limit of detection. Laboratory-fed *Z. renardii* contained no measurable Cry1Ac and 0.06 μg Cry2Ab g^{-1} dw. In contrast, *M. celer* contained 0.009 μg Cry1Ac g^{-1} dw and no measurable Cry2Ab (Fig. 2).

4. Discussion

The concentrations of Cry-proteins were highly variable among the different herbivore and predator species. In general, herbivores contained less Cry-protein than plant structures and predators less than herbivores. Most values obtained from predators were below the limit of detection of the ELISA. This is in accordance with findings from other field studies in different Bt-crops including single-gene cotton (Torres et al., 2006), maize (Harwood et al., 2005; Meissle and Romeis, 2009), rice (Li et al., 2017), and soybean (Yu et al., 2014).We furthermore found that herbivores and predators showed lower Cry2Ab:Cry1Ac ratios than plant material. Previous laboratory or greenhouse studies with Bollgard II cotton found similar decreasing ratios with increasing trophic levels (Tian et al., 2013; Su et al., 2015; Meissle and Romeis, 2017) indicating that Cry2Ab is less stable and degrades quicker than Cry1Ac.

A general decline in Cry-protein levels in cotton plants over the season was observed, confirming findings of other studies on Bollgard I cotton producing only Cry1Ac (Greenplate, 1999; Adamczyk and Sumerford, 2001; Kranthi et al., 2005) and Bollgard II cotton (Knight et al., 2013). Similarly, although less pronounced, a seasonal decline in Cry-protein content was also observed for many arthropod species.

Highly varying concentrations of Cry-proteins in herbivores can partly be explained by their feeding mode and feeding location on the plant. Within the Hemiptera, plant bugs, such as *L. hesperus*, *P. seriatus* and *E. conspersus* contained comparatively high Cry-protein concentrations. Many hemipterans feed on enzymatically liquefied plant tissue or on mesophyll cell sap (King et al., 1996), which contains high amounts of Cry-proteins (Dutton et al., 2004). Interestingly, *N. raphanus* contained no measurable Cry-protein, despite the fact that it belongs to the same family as *L. hesperus*. Hopper species in the order of Hemiptera, such as *Empoasca* spp. (Hemiptera: Cicadellidae) or *S. festinus* contained comparatively low concentrations or no measurable Cry-proteins. Many hoppers feed primarily on phloem sap, which contains no or only traces of Cry-proteins, and only occasionally on mesophyll cell sap (Nickel, 2003). With their chewing mouthparts, herbivorous Coleoptera ingest plant tissue and are thus potentially exposed to relatively high concentrations of Cry-proteins. Within the Coleoptera, the leaf beetle *S. blanda* contained most Cry-protein. Lower concentrations were found in *N. calcaratus* and *C. mexicanus*, which preferentially feed on pollen and flowering structures that contain less Cry-protein than leaves (Nishida, 1956). Curculionidae contained medium concentrations of Cry2Ab whereas Cry1Ac levels were below the detection limit. Adult Curculionidae, which were only sampled in the post-flowering season, probably fed on mature boll capsules which contained among

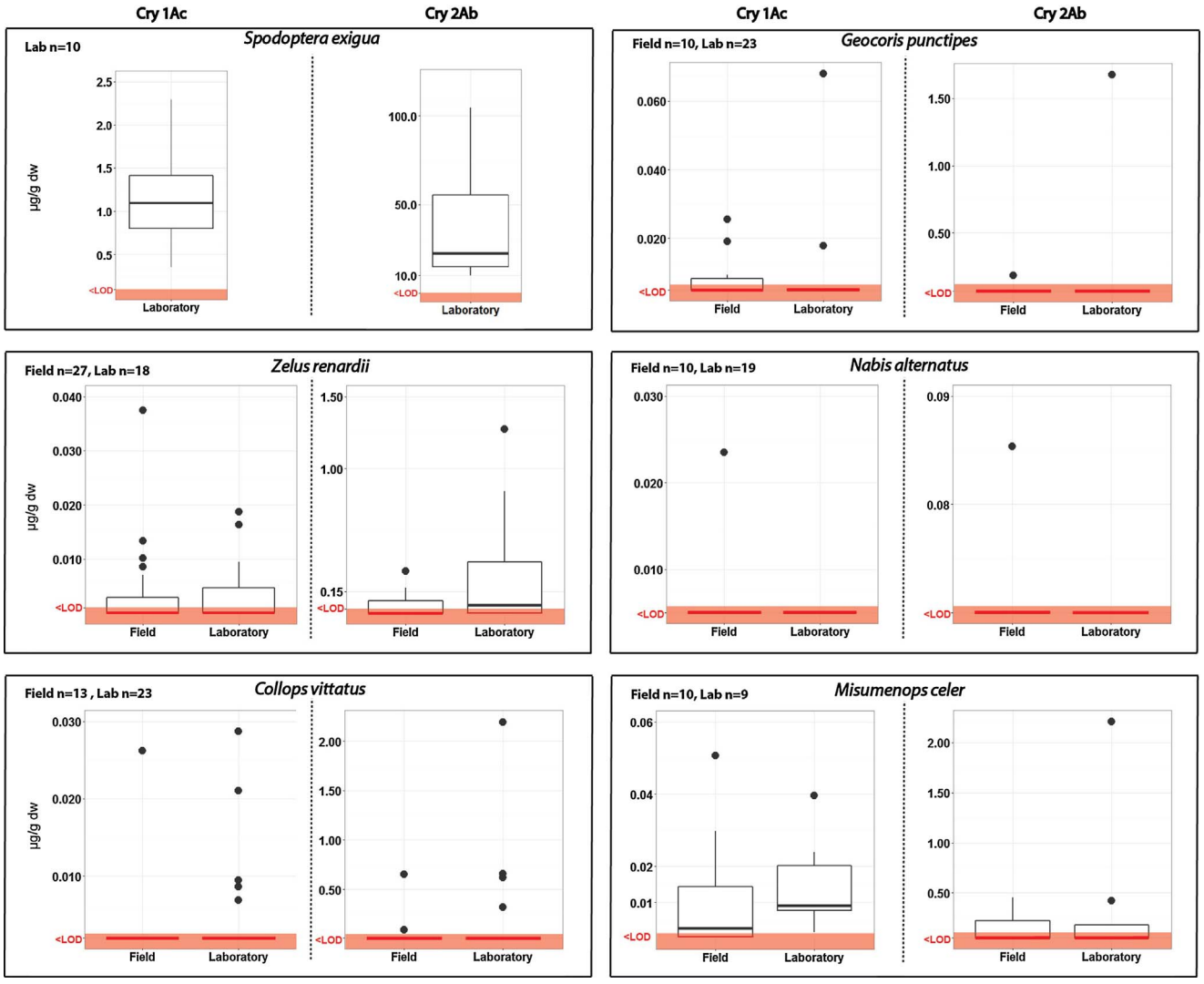

Fig. 2. Laboratory Cry-protein concentrations in caterpillars (*S. exigua*) feeding on Bt-cotton (Bollgard II), and in *G. punctipes, Z. renardii, N. alternatus C. vittatus*, and *M. celer* feeding on the Bt-cotton fed caterpillars in the laboratory. Values from field collections (see Fig. 1 and Table S2) are also included for comparison. Values below the limit of detection (< LOD) are plotted qualitatively in the shaded area at the bottom of each panel. The lines within the boxes indicate the median, the upper and lower boundary of the box the 75th and 25th percentiles, the whiskers extend to the highest and lowest values within 1.5 × inter-quartile range, and circles represent points beyond this range. dw = dry weight.

the lowest Cry1Ac concentrations measured in plant structures and comparatively low Cry 2Ab concentrations (King et al., 1996). The two collected dipteran species contained no measurable Cry-protein because the adults are unlikely to feed directly on cotton tissue. In contrast, Thysanoptera typically suck on the mesophyll tissue of cotton leaves, which explains their relatively high Cry-protein content.

Of all predators, we measured among the highest Cry-protein concentrations in the fly *D.* nr. *divergens,* the flower bug *O. insidiosus,* and the two spider species during at least one sampling period. In addition to prey consumption, facultative feeding on plant structures (including pollen) may contribute to the Cry-protein concentrations in some of those predators (Kiman and Yeargan, 1985; Vogelei and Greissl, 1989; Meehan et al., 2009). Low levels or no measurable Cry-proteins were recorded in the predatory bugs *Geocoris* spp., *N. alternatus*, and *Z. renardii*, the predatory beetles *C. vittatus*, the ladybeetle *O. abdominalis*, and the ant *S. solani*. Apparently, those predators did not consume prey with high Cry-protein concentrations recently during the collection period. The lacewing *C. carnea* s.l. is predaceous in the larval stage, but feeds on pollen and nectar as an adult, which may explain low concentrations of Cry-proteins (Sheldon and MacLeod, 1971). Previous studies confirm our findings on Cry-protein concentrations in predators. Some predatory bug, ladybeetle, lacewing and spider species contained comparatively high concentrations of Cry-proteins, while other species, even in the same families, contained low or no Cry-protein (Harwood et al., 2005; Obrist et al., 2006; Torres et al., 2006; Meissle and Romeis, 2009; Yu et al., 2014; Li et al., 2017). Factors contributing to this variability include the prey spectrum, mode of feeding, developmental stage, time since the last meal, and the physiology of the respective species.

Our results obtained from field collected predators are consistent with the concentrations measured in the laboratory when selected predators were fed with *S. exigua* for 2 days. Cry-protein levels measured in *S. exigua* were higher when compared with Cry-protein levels measured in most of the herbivores from the field. Nevertheless, the Cry-protein levels measured in predators after the comparatively short feeding period in the laboratory were similar, or slightly higher than those in predators from the field. This indicates that tritrophic studies in the laboratory can expose predators to similar or higher Cry-protein concentrations than those occurring in the field, which is important for the interpretation of the relevance and realism of such hazard studies.

Regulatory non-target studies with both Cry1Ac and Cry2Ab have shown that the two proteins have no activity in arthropods outside of the target order of Lepidoptera (ILSI Research Foundation, 2011, 2013). This lack of effect on lethal and sublethal endpoints has been confirmed

in tri-trophic studies using Bollgard II cotton material and non-Bt-sensitive herbivores as toxin carriers for *G. punctipes* (Tian et al., 2014), *O. insidiosus* (Kumar et al., 2014; Tian et al., 2014), *Z. renardii* (Su et al., 2015), *Chrysoperla rufilabris* (Neuroptera: Chrysopidae (Tian et al., 2013), and *Amblyseius andersoni* (Acari: Phytoseiidae) (Guo et al., 2016), and in a bi-trophic test with larvae of *Drosophila melanogaster* (Diptera: Drosophilidae) (Haller et al., submitted). A similar lack of effects has been reported in an artificial diet study at high-dose exposure conditions for *Coleomegilla maculata* (Coleoptera: Coccinellidae (Li et al., 2011). We found that crab and jumping spiders as well as predatory flies showed comparatively high concentrations of Cry-proteins in the field. To our knowledge, no tri-trophic non-target studies with Bollgard II material exist for any of these predators. However, at least for spiders, the broader literature on laboratory feeding studies does not indicate direct negative effects of Cry-protein uptake (Peterson et al., 2011; Svobodová et al., 2017). Meta-analyses of arthropod abundances in Bt-cotton fields with Lepidoptera-active traits (including Bollgard II cotton) revealed a slightly reduced abundance in predators as a group when compared to unsprayed non-Bt-cotton (Naranjo, 2009). In a multi-year study in Arizona (USA), this effect, observed for some spider species, two predatory bugs (*G. punctipes* and *N. alternatus*), one ladybeetle, and the predatory fly *D.* nr. *divergens*, was attributed to a reduction in available prey (Naranjo, 2005). When compared to insecticide-treated non-Bt-cotton, however, predator abundance was significantly increased in the unsprayed Bt-cotton crop (Naranjo, 2005, 2009)

5. Conclusions

Information on Cry-protein movement within a food web, and thus on the exposure rate of non-target arthropods in the field, is not only of value for the interpretation of laboratory hazard studies, but also for the identification of species that might be potentially harmed by future GM-crop events. Our findings indicate that the highest Cry-protein concentrations in herbivores were recorded in certain bug, leaf beetle and thrips species, while other beetle species and plant hoppers contained lower concentrations or no measurable Cry-protein. In the case of predators, certain species of predatory flies, spiders, and flower bugs show particular exposure to the plant-produced Cry-proteins. These findings generally confirm previous results from other Bt-crops including maize (Harwood et al., 2005; Obrist et al., 2006; Meissle and Romeis, 2009), rice (Li et al., 2017) and soybean (Yu et al., 2014). This information supports the non-target risk assessment of future insecticidal GM cotton plants and other crops as it indicates which species and species groups are most likely exposed to the insecticidal trait and are thus most at risk.

Declaration of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.agee.2017.07.009.

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Multitrophic Cry-protein flow in a dual-gene *Bt*-cotton field

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Supplemental material

Table S1
Table S2

Tab. S1: Description of sampled cotton (Bollgard II) plant structures and crop growth stages at the specific sampling periods

Name in study	Description
Plant structures	
Leaf-young	Freshly unfolded leaf of the top plant third
Leaf-old	Fully expanded leaf of the lower plant third
Meristem	Apical meristem (growth point)
Stem	Lignified main stem section at the plant base
Flower bud	Developing flower bud
Young boll capsule	Exocarp of young boll (approx.1.5 cm diameter)
Young boll locule	Endo- and mesocarp of young cotton boll
Pollen	Pollen from 13-15 different flowers
Old boll capsule	Exocarp of fully developed boll
Seed	Seeds of fully developed boll
Lint	Lint of fully developed boll
Crop growth stages	
Pre-flowering Sampled: 26 June 2015	Plants possessed 9-12 branching nodes and started to develop flower buds
Flowering Sampled: 7 July, 3August 2015	Plants possessed 15-17 branching nodes, fully open flowers and first young bolls
Post-flowering Sampled: 25 August 2015	Plants possessed 17-21 branching nodes, only few fully open flowers and fully developed bolls.

Tab. S2: Medians of Cry 1Ac and Cry 2Ab concentrations (µg g^{-1} dry weight) and interquartile ranges (IQR) measured in Bt-cotton (Bollgard II) plant structures and arthropods sampled in the field at three vegetation periods. Plant structure medians within the same column sharing the same letter are not significantly different (P>0.05; Kruskal-Wallis test with Holm-Bonferroni correction).

Concentrations below the limit of detection are indicated in red (median of detection limit shown). n= number of samples. Raw data are provided in File S1

		Pre-Flowering			Flowering			Post-Flowering		
Plant structures	**Ind./ sample**	**n**	**Cry1Ac (IQR)**	**Cry2Ab (IQR)**	**n**	**Cry1Ac (IQR)**	**Cry2Ab (IQR)**	**n**	**Cry1Ac (IQR)**	**Cry2Ab (IQR)**
Leaf-young	1	10	9.72 (7.89;10.8) **a**	167 (153.8;199.2) **a**	10	4.72 (3.89;5.68) **ab**	125.0 (103.8;144.9) **abc**	10	3.04 (1.70;4.68) **ab**	115.5 (83.32;177.3) **abc**
Leaf-old	1	10	4.89 (2.77;6.40) **b**	102.4 (64.26;115.1) **c**	10	7.10 (5.29;8.14) **a**	139.8 (106.7;170) **ab**	10	3.82 (1.82;4.89) **ab**	161.3 (112.5;193.8) **ab**
Meristem	1	10	4.23 (2.72;6.28) **b**	149.4 (98.7;183.1) **ab**	10	4.23 (3.22;4.56) **bc**	143.5 (113.3;152.1) **ab**	10	1.26 (0.98;2.36) **bc**	94.47 (53.61;113.7) **bcd**
Stem	1	10	3.30 (2.87;4.28) **b**	106.9 (81.82;115.2) **bc**	10	2.95 (2.70;3.58) **cd**	84.99 (72.18;118.6) **bcd**	10	1.52 (1.13;1.78) **abc**	71.98 (63.14;82.39) **cd**
Flower bud	1	10	2.11 (1.38;2.61) **c**	141.8 (84.97;160.3) **abc**	10	2.11 (1.39;3.02) **de**	152.5 (106.8;170.9) **a**			
Pollen	13-15				10	1.19 (0.86;1.47) **e**	57.60 (40.86;90.29) **d**			
Young boll locule	1				8	2.45 (1.66;2.95) **de**	165.7 (128.7;196.2) **a**			
Young boll capsule	1				10	0.67 (0.45;1.82) **e**	68.27 (36.69;89.38) **cd**			
Seed	1							10	5.63 (1.37;10.58) **a**	176.9 (96.73;530.7) **a**
Lint	1							10	0.50 (0.25;1.69) **c**	61.05 (31.07;110.6) **cd**
Mature boll capsule	1							10	0.89 (0.52;1.33) **c**	36.60 (26.02;76.93) **d**
			H=30.11, df=4, p<0.001	H=20.52, df=4, p<0.001		H=50.3, df=7, p<0.001	H=35.24, df=7, p<0.001		H=31.23, df=6, p<0.001	H=30.34, df=6, p<0.001
Arthropod herbivores										
Hemiptera										
Chlorochroa sayi	1							5	0.057 (0.035;0.079)	0.45 (0.03;0.05)
Euschistus conspersus	1							8	0.11 (0.087;0.203)	0.36 (0.23;3.67)
Lygus hesperus	3	3	0.226 (-;0.29)	4.43 (2.48;6.52)	7	0.028 (0.012;0.085)	0.53 (0.36;1.09)	3	0.022 (0.011;0.024)	0.39 (0.15;0.5)
Nysius raphanus	6				3	<0.012 (-;-)	<0.220 (-;-)			
Empoasca spp. A	10	4	0.037 (0.035;0.051)	0.052 (-;0.21)	6	0.016 (0.012;0.024)	<0.086 (-;0.1)			
Empoasca spp. B	14/25	7	<0.005 (-;-)	<0.076 (-;-)	6	<0.005 (-;0.002)	<0.095 (-;0.08)	7	0.051 (0.034;0.071)	0.24 (-;0.5)
Pseudatomoscelis seriatus	20	5	0.52 (0.348;0.659)	6.94 (3.49;8.3)	5	0.17 (0.12;0.177)	0.88 (0.43;1.5)			
Spissistilus festinus	3							3	<0.002 (-;-)	<0.026 (-;-)
Coleoptera										
Conotelus mexicanus	15							8	<0.004 (-;0.004)	0.036 (-;0.16)
Curculionidae	20							6	<0.007 (-;0.019)	0.10 (-;0.79)
Notoxus calcaratus	8	10	0.098 (0.078;0.187)	0.80 (0.69;0.98)	3	0.006 (0.005;0.009)	0.039 (-;0.12)			
Systena blanda	8	8	0.47 (0.39;0.53)	3.38 (2.47;4.09)	3	0.38 (0.312;0.383)	4.27 (4.05;4.7)			
Diptera										
Muscidae	7	7	<0.005 (-;-)	<0.088 (-;0.15)						
Tephritidae	6				8	<0.005 (-;-)	<0.090 (-;-)			
Thysanoptera										
Frankliniella occidentalis	ca. 50				5	0.11 (0.06;0.14)	1.63 (0.78;1.72)			

		Pre-Flowering			Flowering			Post-Flowering		
	Ind./ sample	**n**	**Cry1Ac (IQR)**	**Cry2Ab (IQR)**	**n**	**Cry1Ac (IQR)**	**Cry2Ab (IQR)**	**n**	**Cry1Ac (IQR)**	**Cry2Ab (IQR)**
Arthropod predators										
Hemiptera										
Geocoris pallens	6	5	0.032 (0.012;0.035)	<0.066 (-;0.28)	0			0		
Geocoris punctipes	3	0			4	<0.006 (-;-)	<0.083 (-;-)	6	0.005 (-;0.021)	<0.15 (-;-)
Nabis alternatus	4	5	<0.003 (-;0.012)	<0.050 (-;0.04)	8	<0.002 (-;-)	<0.042 (-;-)	0		
Zelus renardii	2	7	0.004 (-;0.01)	0.062 (-;0.16)	10	<0.002 (-;0.007)	0.020 (-;0.073)	10	<0.003 (-;-)	0.084 (-;0.11)
Orius insidiosus	ca.40	0			5	0.011 (0.003;0.022)	0.14 (0.07;0.35)	0		
Coleoptera										
Collops vittatus	2	4	<0.005 (-;0.02)	<0.075 (-;0.49)	0			9	<0.005 (-;-)	0.036 (-;-)
Olla abdominalis	1	0			4	<0.004 (-;-)	<0.067 (-;-)	9	<0.005 (-;-)	<0.092 (-;-)
Araneae										
Misumenops celer	4	4	<0.005 (-;0.038)	0.11 (-;0.36)	6	0.005 (-;0.02)	<0.097 (-;0.28)	0		
Salticidae	4	0			0			5	0.004 (-;0.005)	0.12 (0.04;0.22)
Diptera										
Drapetis nr. *divergens*	20	4	0.035 (0.022;0.075)	0.8 (0.43;1.28)	4	0.017 (-;0.035)	0.089 (-;0.29)	6	0.017 (0.008;0.023)	0.58 (0.27;0.68)
Neuroptera										
Chrysoperla carnea s.l.	3	5	<0.003 (-;0.004)	<0.047 (-;0.09)	8	0.003 (-;0.008)	<0.039 (-;0.008)	3	<0.003 (-;0.008)	0.044 (-;0.06)
Hymenoptera										
Solenopsis xyloni	7	10	<0.009 (-;-)	<0.15 (-;-)	4	<0.100 (-;-)	<0.16 (-;0.18)	7	<0.008 (-;-)	<0.15 (-;0.23)