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Limitations in dose–response and surrogate species methodologies for risk assessment of Cry toxins on arthropod natural enemies

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Abstract Dose–response assays and surrogate species are standard methods for risk analysis for environmental chemicals. These assume that individuals within a species have unimodal responses and that a surrogate species can predict responses of other related taxa. We exposed immature individuals of closely related aphidophagous coccinellid predators, *Cycloneda sanguinea* and *Harmonia axyridis*, to Cry1Ac and Cry1F toxins through uniform and constant artificial tritrophic exposure through *Myzus persicae* aphids. Both toxins were detected in coccinellid pupae, with individual and interspecific variation. Uptake was significantly higher in *H. axyridis* than in *C. sanguinea*, both in the proportion of individuals and the concentrations per individual. We also observed bimodal uptake of the Cry toxins by *H. axyridis*, which indicated that some individuals had low bioaccumulation and some had high bioaccumulation. This suggests that standard dose–response assays need to be interpreted with caution and future assays should examine the modality of the responses. In addition, the similarity in the biological effects of the Cry toxins in the two predators was due to different biological exposure mechanisms. The majority of *H. axyridis* were exposed both internally and in the gut, while *C. sanguinea* was exposed primarily in the gut. Thus, despite their close phylogenetic relatedness, these species would not be good surrogates for each other and the surrogate species methodology should be tested more rigorously.

Keywords Coccinellid · GM crops · Predator · Surrogacy

Introduction

Many studies have been conducted to evaluate the direct or indirect effects of Cry toxins expressed by genetically modified (GM) plants on non-target beneficial insects, such as natural enemies, due to their important ecosystem service of pest regulation. The primary method to evaluate non-target effects has been based on quantifying a "dose–response relationship" through bi- and tritrophic exposure (Groot and Dicke 2002) on a small number of surrogate species, and extrapolating the results to broader taxa of interest (Garcia-Alonso et al. 2006; Suter 2007; Romeis et al. 2008). This relies on two key assumptions: individual responses to uniform and constant exposure are unimodal, and the surrogate accurately predicts responses of other taxa. The unimodal response presumption has not been evaluated for non-target effects of GM plants, and extrapolation based on surrogate species has been shown to be inaccurate (Suter 2007; Banks et al. 2014) and imprecise (Elmegaard and Akkerhuis 2000).

In the last 15 years, laboratory studies have demonstrated that some natural enemy species can uptake and even bioaccumulate Cry toxins delivered from their prey (Couty et al. 2001; Zhang et al. 2006; Gao et al. 2010;

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Paula and Andow 2015). Uptake is the absorption of a chemical from the food (USEPA 2013), i.e. the continued presence of a toxin after exposure has stopped and gut contents eliminated. However, there are no studies comparing the variability in the uptake of Cry toxins by a natural enemy species and by a closely related one, and its possible ecological significance. Of course, all published studies in this area report variation in presence of Cry toxins among individuals as error, and they assume that the variation is unimodal and without biological meaning. If the variation in uptake of Cry toxins by a natural enemy is instead multimodal, then accurate interpretation of dose–response experiments requires examination of the modality of the response. Moreover, if there is a large difference in uptake of Cry toxins between closely related natural enemy species, then the species will not be good surrogates for each other, because they have different exposure and effects mechanisms (Lewis 1995; Godoy et al. 2015).

To test the robustness of the dose–response and surrogate species methodologies for risk assessment of Cry toxins on insect pest natural enemies, we exposed two closely related coccinellid aphidophagous predator species using an artificial tritrophic system with uniform and constant concentration of Cry toxins. We investigated how much variability in uptake of the Cry toxins occurs among individuals from the same species and across closely related species, and compared the population responses of related species to evaluate how well one can act as a surrogate for the other.

Methods

Insect rearing

The prey aphid, *Myzus persicae* (Hemiptera: Aphididae), was reared on collard plants in a greenhouse at 13 h photophase at 25 ± 4 °C at 60 ± 10 % RH. The aphidophagous predators *Cycloneda sanguinea* and *Harmonia axyridis* (Coleoptera: Coccinellidae) are common in Brazil and were chosen because they are closely related in the same tribe, Coccinellini (Giorgi et al. 2008). They were reared in plastic cages, 10 × 15 cm, containing a daily supply of water in wet cotton balls and leaves containing aphids collected from the field. Their egg masses were transferred to separate cages and inspected daily to collect the neonate larvae (less than 24 h old) for use in the bioassays.

Bioassay preparation

The trypsinized and purified Cry1Ac and Cry1F toxins (both ca 65 kDa) were purchased from Dr. M. Pusztai-Carey (Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio) and their biological activities were confirmed in tests against caterpillars of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) as described in Nakasu et al. (2013). The Cry toxins were solubilized in a 50 mM Na_2CO_3 solution containing 100 mM NaCl, pH 10 with 10 mM dithiothreitol added before use. This solution was added to a liquid holidic diet for aphids (Dadd and Mitter 1996), which was sterilized by filtration at 0.22 µm in a laminar flow hood. This diet (150 µl) was placed in a sterilized sachet of two pieces of stretched Parafilm M attached to one end of a 25 mm acrylic tubular cage (Douglas and van Emden 2007). Between 10 and 50 aphids were transferred to each cage, according to the predator instar, to feed on the diet of each treatment (see below) for at least 24 h before exposure to the predators in the bioassays (Wright et al. 1985). Cages with aphids were provided daily to the predators. All bioassays were conducted inside a controlled environment chamber (25 ± 2 °C and 13 h photophase).

Exposure of larval predators

Unfed neonate predator larvae were individually transferred to cages containing *M. persicae* aphids feeding on diet of one of the treatments: 1. Control (no Cry toxin added); 2. Cry1Ac 20 µg/ml (C20); 3. Cry1F 20 µg/ml (F20); 4. Cry1Ac 20 + Cry1F 20 µg/ml (C20:F20); 5. Cry1Ac 20 + Cry1F 70 µg/ml (C20:F70); and 6. Cry1Ac 70 + Cry1F 20 µg/ml (C70:F20). These Cry1Ac and Cry1F concentrations were similar to that in leaves of WideStrike® cotton (Siebert et al. 2009), and were chosen to investigate the possibility of synergisms between the toxins. Water was supplied daily in each cage on wet filter paper (1 cm^2). The cages were inspected daily until the pupal stage to evaluate survival and developmental stage. Pupae (within 24 h of pupation) were weighed and stored at −20 °C for ELISA.

Cry1Ac and Cry1F detection

The Cry toxins were detected and quantified using enzyme-linked immunosorbent assay (ELISA). We measured the concentrations in undiluted diet to confirm toxin concentrations (see Supplementary Material, SM-1). To determine if Cry1Ac and Cry1F decayed in the diets during the experiment, toxin was quantified in paired diets 24 and 72 h after diet addition to the cages with three technical replicates for each diet cage. In addition, for each diet at least six replicates of 100 *M. persicae* were fed for 24 h, collected and weighed for Cry quantification. All predators from the two bioassays described above were analyzed individually. All insect samples were macerated using a

glass pestle and homogenized in PBST in a volume (in µl) corresponding to 70× the fresh weight (FW) (in mg) to normalize the amount of total protein across samples. The samples were centrifuged at 15,500×*g* for 15 min and the supernatant was used for the analysis. Each sample was applied (100 µl/well) in duplicate or triplicate technical replicates on a double sandwich ELISA PathoScreen plate (Agdia, USA) for Cry1Ac and Cry1F detection and quantification according to manufacturer's instructions. Cry1Ac and Cry1F from the same source as used in the bioassays at 0, 0.0625, 0.125, 0.25, 0.5, and 1.0 ng/well were used as calibration standards on each plate. All standards were replicated in duplicate on each plate and a linear calibration curve was estimated for each plate separately. For each toxin, we averaged the slopes of the calibration curves across plates to estimate toxin concentrations from observed absorbances. The absorbance was measured at 630 nm with a microtiter plate reader (TP Reader NM Thermo Plate®, USA).

The limit of detections (LODs) for Cry1Ac and Cry1F detection in the predator samples were calculated using the standard deviation and slope method. The Cry1Ac LOD was 0.0016 ng/mg FW based on 19 predator samples tested in duplicate, and the Cry1F LOD was 0.0004 ng/mg FW based on 29 predator samples tested in duplicate. Based on the dilutions and the technical specifications of the reader, the linear part of the standard curve indicated accurate estimations for the predator samples of Cry1Ac up to 5 ng/mg FW and for Cry1F up to 20 ng/mg FW.

Statistical analysis

Each ELISA plate was set up to contain multiple blanks, standards and controls that matched the aphid and predator samples of each Cry treatment. ELISA absorbances for each well with a sample (diets, aphids, and predator pupae) were normalized for each plate by subtracting the mean of the blanks. Then the corresponding normalized, averaged controls on each plate were subtracted from each of these normalized samples. Technical replicates were averaged to estimate the absorbance for each Cry treatment sample. These absorbances were transformed to concentrations of Cry toxin (ng of Cry/mg FW) using the average slope of the standard curve. The mean concentration of Cry toxin was calculated for each species by averaging values for all individuals in a treatment. Total sample sizes for each of the statistical analyses are provided in the Supplementary Material.

To determine the proportion of positive samples, standard errors and degrees of freedom for each individual sample were estimated from the standard deviations of the blanks, controls and technical replicates using the Welch–Satterthwaite formula, which allows that the respective variances for the blanks, controls and technical replicates are not equal. Positive presence of Cry toxin was determined using Welch's *t* test (for unequal sample size and unequal variance). The proportion of positive samples and larval survival were analyzed using logistic regression (Proc Genmod, SAS 9.4). Standard deviations for the proportion of positive samples were estimated using the Wilson score interval.

Differences in the proportion of positive samples among the species were compared using the *z*-approximation (SAS 9.4), and differences among Cry treatments were compared using a priori linear contrasts for unequal sample sizes. For Cry1Ac the contrasts were: 1. C70:F20 versus the others (to test if a higher concentration of Cry1Ac had a higher detection rate); 2. C20 versus the mean of C20:F20 and C20:F70 (to test if the presence of Cry1F influenced the detection of Cry1Ac); and 3. C20:F20 versus C20:F70 (to test if the concentration of Cry1F influenced detection of Cry1Ac). Analogous contrasts were examined for Cry1F detections. Survival rate of predators for the entire larval period were analyzed using Proc Genmod.

Cry concentrations were analyzed using ANOVA with the same a priori linear contrasts (Proc GLM, SAS 9.4) as described above. Differences among species were analyzed by Tukey's HSD. Development time (from time of hatch to time of pupation) and pupal weight of the predators was also analyzed using Proc GLM. All sufficient statistics for all of the logistic regressions and ANOVAs are provided in the Supplementary Material.

The correlation between Cry1Ac and Cry1F concentrations in individual predators was examined using the corrected absorbances for each Cry treatment sample. All individuals from the C20:F70 diets were excluded and only individuals with positive detection of either Cry1Ac or Cry1F were included because including the individuals from the C20:F70 diets or those negative for both toxins inflated the correlations. Pearson's correlation coefficient was calculated and the Fisher transformation was used to test statistical significance.

In addition, the distributions of the estimated Cry concentrations for each predator species from the treatments with 20 µg toxin/ml in the aphid diet were tested for bimodality by fitting one or two gamma distributions to the data and testing the improvement with the small sample Akaike information criterion (ΔAIC_c) using Mathematica 8. This bimodality test was used to determine if the individuals from a predator species came from a homogeneous population with an unimodal distribution or from a heterogeneous population with a bimodal distribution.

Results

Differential uptake of the Cry toxins

The diet concentrations of Cry toxins did not change between 24 and 72 h (Cry1Ac: $t_6 = -1.80$, $P = 0.115$; Cry1F: $t_4 = 0.65$, $P = 0.543$, see SM-1). Cry toxins were detected in the prey and predators in all Cry treatments, although not in all samples (Fig. 1, SM-2). Cry1Ac and Cry1F were detected more frequently in *H. axyridis* pupae (Cry1Ac, $P = 1.30 \times 10^{-5}$; Cry1F, $P = 0.0002$) than in *C. sanguinea* pupae. About 74 and 70 % of *H. axyridis* pupae had Cry1Ac and Cry1F respectively, while only 9 and 14 % of *C. sanguinea* did. The detection of the toxins in the predator pupae is a proof of uptake (Paula and Andow 2015), as prior to pupation, insects like coccinellids empty their guts, and during pupation they shed both the external cuticle and the cuticular lining of the gut (Chapman 1998). Thus, most *H. axyridis* were exposed internally to Cry toxins, while most *C. sanguinea* were not.

Although the exposure to the toxins via the aphid food was the same, *H. axyridis* pupae had higher concentrations of Cry1Ac and Cry1F than *C. sanguinea* pupae (Cry1Ac, $P < 0.00005$; Cry1F, $P = 0.0001$), and also higher than the prey *M. persicae* (Cry1Ac, $P = 0.0119$; Cry1F, $P = 0.0062$) (Fig. 2, SM-3). This indicates bioaccumulation of the toxins in *H. axyridis* (Bryan 1979; Paula and Andow 2015). The Cry1Ac and Cry1F concentrations were not different in *C. sanguinea* pupae and the prey *M. persicae* (Cry1Ac, $P = 0.2246$; Cry1F, $P = 0.6082$), indicating no bioaccumulation in *C. sanguinea*. The predator pupae and prey had their highest concentrations of Cry1F when exposed to the C20:F70 treatment (Fig. 2b, $P = 2.07 \times 10^{-9}$). While *H. axyridis* had a much higher Cry1F concentration in C20:F70 treatment compared to the F20 treatments, *C. sanguinea* did not (Fig. 2b).

Bimodality in Cry toxin uptake

Among the *H. axyridis* pupae that were exposed to both toxins during the full larval period, those that had a higher concentration of Cry1F also had a higher concentration of Cry1Ac (Fig. 3a, SM-4, $P = 0.0007$). While the *H. axyridis* were derived from a seemingly homogeneous source population, individuals showed substantial differences in the uptake of Cry toxins (bimodally distributed). Therefore, two kinds of *H. axyridis* individuals were observed: ones that had high concentrations of both toxins and ones that had no or low toxin (Fig. 4, SM-5, bimodal distributions, Cry1Ac, $P = 0.0116$; Cry1F, $P = 0.0009$). Although a similar correlation in the uptake of the two toxins was also

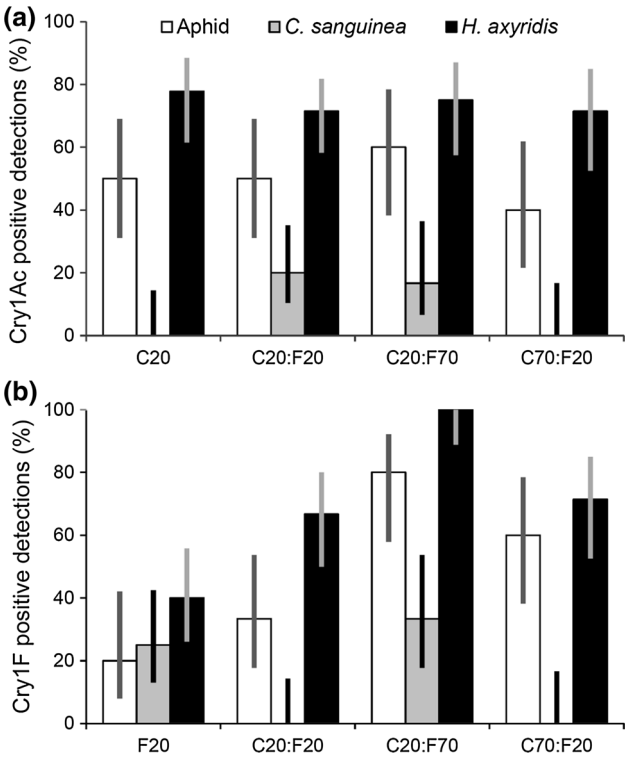

Fig. 1 Percent of positive Cry detections by ELISA in the aphid prey and predator pupae (mean ± SE): **a** Cry1Ac in different concentrations, with and without Cry1F; **b** Cry1F in different concentrations, with and without Cry1Ac

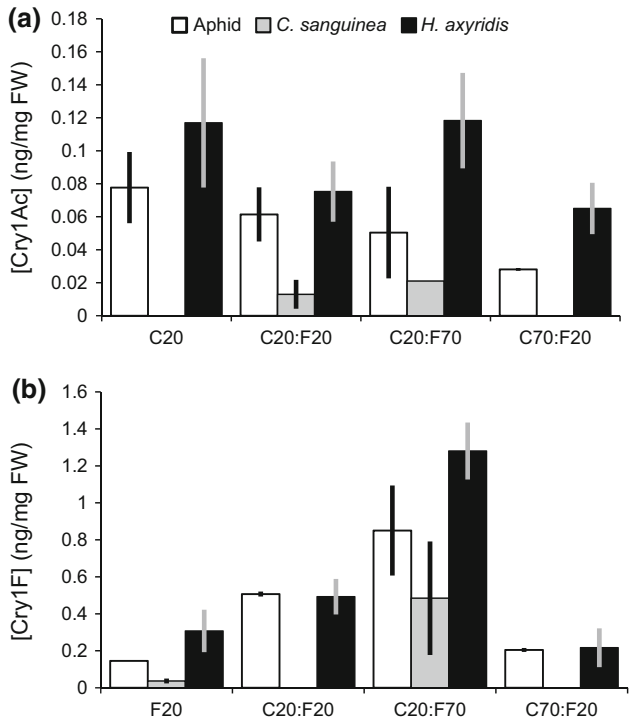

Fig. 2 Concentration (mean ± SE) in the aphid prey and predator pupae of **a** Cry1Ac in different concentrations, with and without Cry1F; **b** Cry1F in different concentrations, with and without Cry1Ac

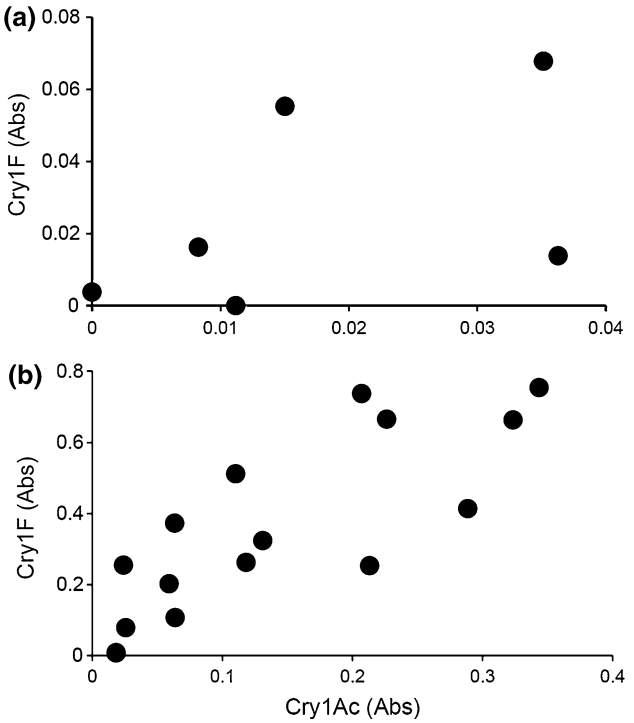

Fig. 3 Simultaneous detection of Cry1Ac and Cry1F in the predators **a** *C. sanguinea* and **b** *H. axyridis*. Each *dot* is an individual predator pupa. Only individuals with positive detection of either Cry1Ac or Cry1F are displayed

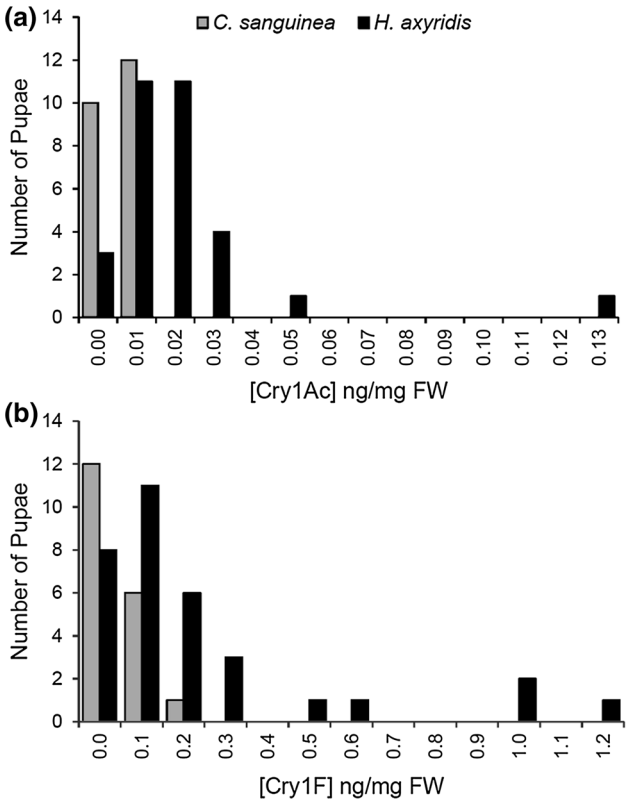

Fig. 4 Histograms of concentrations of **a** Cry1Ac in individuals of two species of predators, *C. sanguinea* and *H. axyridis*, and **b** Cry1F in individuals of the same species

seen for *C. sanguinea*, it was not significant probably due to the small sample size (Fig. 3b, $P = 0.493$). In contrast to *H. axyridis*, only one kind of *C. sanguinea* was observed (Fig. 4, unimodal distributions, Cry1Ac, $P = 0.1935$; Cry1F, $P = 0.8976$).

Similar ecological effects in related predators

Cry1Ac and Cry1F, separately or combined, did not have any detectable ecological effects on either predator (Fig. 5, SM-6). Development time ($P = 0.088$), pupal fresh weight ($P = 0.222$), and larval survival rate ($P = 0.553$) did not differ among the treatments for the predator species.

Discussion

Despite using a constant exposure system, we demonstrated individual variation in uptake of Cry toxins by the predaceous coccinellids in all of the diet treatments, and also interspecific variation between the related predator species. The observed variation might have several causes, including variation in delivery of the Cry toxins from the prey, and a differential ability within and across species to uptake the Cry proteins, although the former is unlikely to be the main cause. While some of the aphid samples did not have detectable Cry toxin, new aphids were provided almost every day. So predators were exposed via aphids during the bioassay at an estimated average daily exposure of 0.028 ± 0.010 ng Cry1Ac/mg aphid FW, and 0.111 ± 0.059 and 0.680 ± 0.170 ng Cry1F/mg aphid FW for the F20 and F70 diets respectively (Fig. 3). This resulted in a total cumulative exposure during the entire larval stage (Table 1) at least three orders of magnitude higher than the LOD. In addition, the standard error for the estimated total cumulative exposure (Table 1) was small enough that all individuals were likely to be exposed to close to this estimated level.

Thus, the main cause of the observed variation in uptake of Cry toxins within and between predator species is a differential ability to uptake the Cry toxins. With regard to the within species variation, the bimodal distributions for *H. axyridis* for both Cry1Ac and Cry1F showed that there were two groups of individuals, one with low uptake and bioaccumulation and one with high uptake and bioaccumulation. Those with high uptake simultaneously had high uptake of both Cry toxins. The bimodal uptake of Cry toxins by *H. axyridis* suggests that published literature about standard dose–response assays should be interpreted

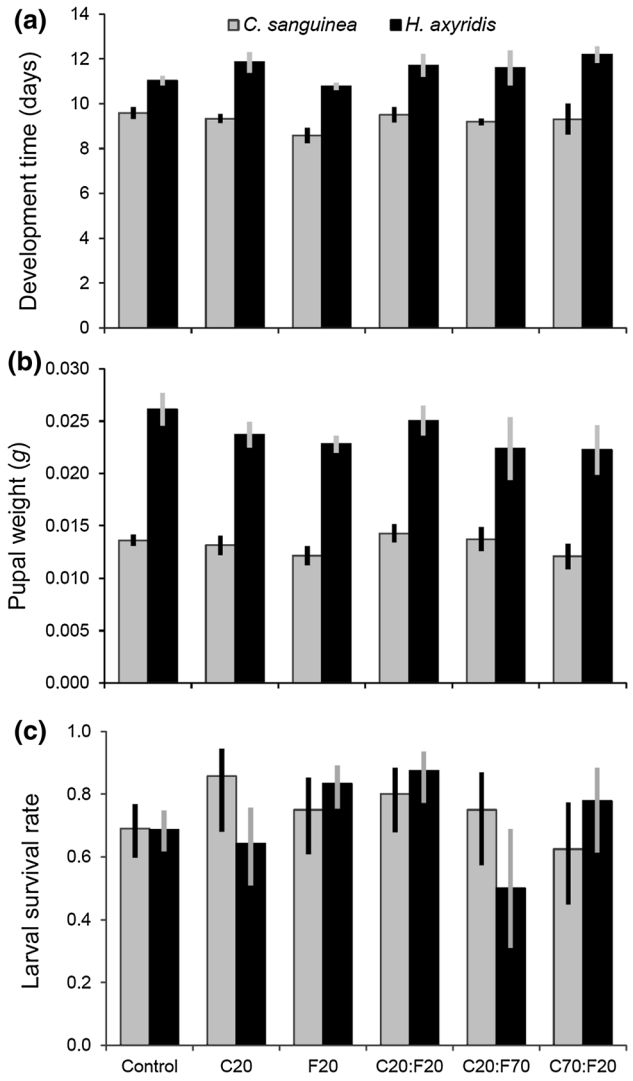

Fig. 5 Ecological performance of coccinellids exposed during the larval stage to aphids feeding on a liquid diet containing different concentrations of Cry1Ac and Cry1F, separately and combined: **a** development time (mean ± SE); **b** pupal weight (mean ± SE); **c** larval survival rate (mean ± SD)

with caution. At the same time, future dose–response assays should be designed to test the modality of the biological response parameter. This will prevent faulty interpretations derived from bimodal biological responses among individuals from the same population, which could influence the conclusions about potential ecological effects. This variation might be a potential cause for why different research groups have reported contrasting findings from laboratory concentration–response experiments on the same species of natural enemy, even though these have been previously considered to be due to contamination, methodological differences, or methodological error (Romeis and Meissle 2011; Hilbeck et al. 2012; Romeis et al. 2012, 2014).

Regarding the interspecific differences between the predator species in uptake and bioaccumulation of Cry toxins, our study showed that the surrogate species methodology for studying potential ecological effects of Cry toxins on insect pest natural enemies should be used with caution. Despite being close relatives in the tribe Coccinellini, uptake was significantly higher in *H. axyridis*, both in the proportion of individuals and the concentrations per individual. The majority of *H. axyridis* took up both Cry toxins, and when they did, they bioaccumulated from their aphid food, especially on the F70 diet. In contrast, few *C. sanguinea* larvae took up either Cry toxin, and when uptake did occur, the concentration was low. This indicates that the lack of ecological effects in the two species stemmed from different mechanisms. The majority of *H. axyridis* were exposed both internally and in the gut, indicating that the absence of detectable effects is related to a lack of significant impacts on internal processes and in the gut. For *C. sanguinea*, the majority were exposed only in the gut, so absence of detectable effects may be related primarily to processes (or their absence) in the gut.

Superficially, the two species might have appeared to be reliable surrogates for each other because both had similar ecological effects (both lacked detectable effects), but in reality, the absences of detectable effects stemmed from very different biological mechanisms. A *sine qua non* condition for the use of surrogate species is that the species should respond to the toxin with similar physiological processes (Lewis 1995; Godoy et al. 2015), so the two species are not reliable surrogates for each other, and phylogenetic relatedness does not guarantee surrogacy for risk assessments of Cry toxins on non-target natural enemies. This surrogacy assumption needs to be examined more thoroughly for Cry toxins before non-target risk

Table 1 Estimated average dose of Cry toxin (ng) exposure to larval predators during their entire larval stage (±SE) via aphids

Predator	Cry1Ac (ng)	Cry1F (F20, ng)	Cry1F (F70, ng)
Cycloneda sanguinea	1.57 ± 0.19	6.13 ± 1.07	37.65 ± 3.09
Harmonia axyridis	2.29 ± 0.24	8.91 ± 1.39	54.76 ± 4.03

Calculated from the average Cry toxin concentration in aphids (ng/mg FW) and the average total number of aphids provided to the predators during larval development (mg FW)

assessment methodologies based on it are widely adopted (e.g., Garcia-Alonso et al. 2006; Romeis et al. 2008).

On the other hand, it is impractical and unnecessary to test every non-target species from the local biodiversity. Instead, methods that do not rely on surrogacy should be used, such as an ecologically based method (Andow et al. 2013). This approach identifies the local biological diversity, classifies it into ecological functional groups, and selects key ecological functions for risk assessment. Non-target species likely to be highly exposed to the environmental stressor and to contribute significantly to the ecological function are selected. For example, *C. sanguinea* is a common arthropod predator in cotton in Brazil (Faria et al. 2006) and provides important biological control of cotton aphids. As it is likely to be exposed to *Bt* cotton through bi- or tritrophic exposure, it could be used to evaluate the potential effects of *Bt* cotton on biological control agents in Brazil.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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