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Putative effects of Cry1Ab to larvae of *Adalia bipunctata* - reply to Hilbeck et al. (2012)

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Abstract

In their recent study, Hilbeck et al. (2012) report that Cry1Ab causes lethal effects on larvae of the ladybird beetle *Adalia bipunctata* when fed directly to the predator. Such toxic effects were not previously observed in a direct feeding study conducted by us (Álvarez-Alfageme et al. 2011). Because Hilbeck et al. (2012) claim that our study design did not allow us to detect any adverse effects we provide arguments for the value and relevance of our study in this commentary. Furthermore we discuss two additional published studies that have not revealed any direct effects of Cry1Ab on larvae of *A. bipunctata* and are not mentioned by Hilbeck et al. (2012). One of the studies was conducted in our laboratory under more realistic exposure conditions (Álvarez-Alfageme et al. 2011). Feeding *A. bipunctata* larvae with spider mites reared on Bt maize did not reveal any adverse effects on lethal and sublethal parameters of the predator. This was despite the fact that the larvae had ingested high amounts of biologically-active Cry1Ab protein. Thus, we do not see verified evidence that *A. bipunctata* larvae are sensitive to Cry1Ab at realistic worst-case exposure concentrations. This, together with the fact that *A. bipunctata* will be little exposed to Cry1Ab under field conditions, allows us to conclude that the risk of Bt maize to this predator is negligible. Support for this comes from the results of many Bt maize field studies that have not revealed evidence for direct Cry1Ab-effects on non-Lepidoptera species.

Keywords: Bt maize, environmental risk assessment, ladybird beetles, MON810, non-target organisms

Background

In 2009, Schmidt et al. [1] reported that larvae of *A. bipunctata* suffered increased mortality during the first larval stage when ingesting the Cry1Ab protein that is expressed in some of today's Bt maize varieties including MON810. This study has been criticized for its design, execution, and data interpretation by several scientists [2,3] and by the Central Commission on Biological Safety that advises the Federal Government of Germany [4,5]. Two subsequent studies in which *A. bipunctata* larvae were directly fed with Cry1Ab conducted by our group [6] and by Porcar et al. [7] could not confirm this toxic effect. Furthermore we did not detect adverse effects of Bt maize expressing Cry1Ab on larvae of *A. bipunctata* in a higher tier, tri-trophic study using Bt maize-fed spider mites as prey [6].

In their paper "A controversy re-visited: Is the coccinellid *Adalia bipunctata* adversely affected by Bt toxins?", Hilbeck et al. [8] confirm the findings from their earlier study [1] but do not convincingly address the critical issues regarding study design and execution. While Hilbeck et al. [8] criticize the design and execution of our direct feeding experiment [6], they do not acknowledge our tri-trophic feeding study and the study by Porcar et al. [7].

In this letter we respond to the main points of criticism by Hilbeck et al. [8] on our direct feeding study and discuss the available data in a wider risk assessment context. The fact that we do not address certain statements and claims made by Hilbeck et al. [8] does not imply that we agree with them.

Discussion

Direct feeding studies assessing the impact of Cry1Ab on larvae of *Adalia bipunctata*

In total, three studies prior to Hilbeck et al. [8] have assessed the effect of Cry1Ab on *A. bipunctata* larvae using different test protocols.

In the study by Schmidt et al. [1], Cry1Ab was dissolved in a buffer solution and deposited on eggs of

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Ephestia kuehniella, which were continuously provided to *A. bipunctata* larvae. The Cry1Ab was applied at three different concentrations (5, 25, and 50 μg/ml). First instar larvae in the Bt treatments showed a significant increase in mortality, but no sublethal effects on development time or weight.

Spraying Bt protein on *E. kuehniella* eggs with the purpose of exposing *A. bipunctata* larvae has raised concern about whether this method was appropriate considering the feeding mode of this species [3,6]. We observed the feeding behavior of young larvae in our study and report that "...visual observations revealed that, when preying on *E. kuehniella* eggs, both first and second instars of *A. bipunctata* sucked out their contents until they were completely depleted. No larva was observed consuming whole eggs or even parts of the egg shell. [...] In no case did the larvae consume the egg shell." [6]. This mode of feeding of young ladybird larvae is well known (e.g., [9]), and has been confirmed by Hilbeck et al. [8].

As the egg shells themselves are not consumed by young *A. bipunctata* larvae, the ingestion of Cry1Ab deposited on *E. kuehniella* eggs is thus nil or very limited. Hilbeck et al. [8] claim that they have confirmed Cry1Ab ingestion by means of Agdia Bt-Cry1Ab/1Ac ImmunoStrips® (Agdia Inc., Elkhart, IN, USA). In their study, larvae were provided with cotton balls moistened with a Cry1Ab sucrose solution for 24 h and then fed Cry1Ab-coated *E. kuehniella* eggs. We do not find the Cry1Ab analysis convincing evidence for the ingestion of toxin because the authors do not indicate that larvae were washed prior to the analysis. It is highly probable that the body surface of the larvae has been contaminated with Cry1Ab from contact with the moistened cotton ball and the Cry1Ab-coated *E. kuehniella* eggs. Even if toxin was ingested, it remains unclear whether the larvae ingested the Cry1Ab by feeding on the Bt-coated *E. kuehniella* eggs or by feeding on the Cry1Ab sucrose solution from the cotton ball, or both. Thus the data presented do not allow one to conclude whether *A. bipunctata* in the original study by Schmidt et al. [1] had ingested the Cry1Ab protein. Moreover, the results from the ImmunoStrips® assay do not provide any information on the amount of Cry1Ab toxin involved, which disallows the comparison with the quantitative ELISA results from our study [6]. As Schmidt et al. [1] did not use a positive control (a substance which is known to be toxic to *A. bipunctata*) it is impossible to affirm that the larvae had ingested Bt toxin at all.

We [6] intentionally used a test protocol that differed from that by Schmidt et al. [1] because: (i) we wanted to use a system that ensures that substantial amounts of Bt protein were ingested, and (ii) the information on the test protocol used by Schmidt et al. [1] was not sufficient to repeat their experiment. While the concentration of the Cry1Ab solutions used to treat the *E. kuehniella* eggs is provided, the amount of toxin solution actually applied to the eggs is not known. Unfortunately, Hilbeck et al. [8] again do not provide this essential piece of information.

In our study, larvae of *A. bipunctata* were provided exclusively with a sucrose solution containing Cry1Ab during the first 24 h in each of their larval instars. The fact that no prey was provided ensured that the larvae consumed the sucrose solution. During the remaining time of the larval stages they were fed exclusively with untreated *E. kuehniella* eggs to continue their development (termed exposure/recovery protocol by Hilbeck et al. [8]). Since *A. bipunctata* has four larval instars, the test insects were exposed four times 24 h each. We recorded lethal (mortality) and sublethal (development time, weight) parameters.

For our experiment, a Cry1Ab concentration of 45 μg/ml was selected. This concentration was 10-fold higher than that measured in Bt maize-fed spider mites, an occasional prey species under field conditions that is known to contain high amounts of toxin. This increased concentration should provide an additional margin of safety for this toxicological assay and also control for the fact that larvae were not continuously exposed [10,11].

Two positive control treatments were included in the bioassay, i.e., the inorganic toxin potassium arsenate and an insecticidal protein (snowdrop lectin, GNA). Ingestion of both toxins revealed adverse effects on the recorded parameters of the *A. bipunctata* larvae. Thus, these positive control treatments confirmed that the test compounds were ingested and that the test system is able to detect treatment effects. The inclusion of positive control (or reference) treatments is an important factor to consider in ecotoxicological studies [10-12].

Porcar et al. [7] provided the Cry1Ab protein at a concentration of 50 μg/ml mixed into an artificial diet. *Adalia bipunctata* larvae were continuously fed the Cry1Ab-containing diet for a total of six days. No effect of Cry1Ab-feeding on larval mortality was detected when compared to the untreated control diet. As in our study, a positive control treatment confirmed the ingestion of the test diet.

None of the three studies quantified the actual dose of Cry1Ab that was ingested by the *A. bipunctata* larvae. Thus a direct comparison of the different studies is difficult. This is particularly the case for the studies by Schmidt et al. [1] and our study [6] because they used very different methods to expose the larvae. Consequently, the comparison of the Cry1Ab concentrations provided to *A. bipunctata* larvae in the two studies [1,6] provided in Figure 1 by Hilbeck et al. [8] has no value. The studies by Porcar et al. [7] and Schmidt et al. [1] are more comparable because they both exposed the larvae

continuously to the Cry1Ab toxin. In contrast to the result of Schmidt et al. [1], however, Porcar et al. [7] did not record a toxic effect of Cry1Ab that was provided at a comparable concentration.

Relevance of the direct feeding study conducted by Alvarez-Alfageme et al. [6]

We are confident that our direct feeding bioassay should have detected adverse effects of Cry1Ab on *A. bipunctata* larvae if present, despite the fact that we did not continuously provide the larvae with Cry1Ab. According to good ecotoxicological practice [10-12], we included two positive control treatments in the bioassay which we knew would cause an effect on the life-table parameters that we estimated. The results from these two positive controls verified that our bioassay set-up was sensitive enough to detect adverse effects.

In respect to the fact that we have only exposed the *A. bipunctata* larvae to Cry1Ab during the first 24 h of each larval stage, Hilbeck et al. [8] state "When this exposure/recovery protocol was applied to a highly sensitive target insect, *Ostrinia nubilalis*, the lethal effect was either significantly reduced or disappeared altogether." We do not see, however, how the experiment conducted with *O. nubilalis* larvae provides evidence that our interval-feeding assay could not reveal adverse effects of Cry1Ab for the following four reasons:

i) While the data presented show that 4-day old *O. nubilalis* larvae can recover from one day feeding on Bt maize, larvae that have fed on Bt toxin sprayed maize plants still showed an increased mortality compared to the control. Thus, Hilbeck et al. [8] did show that it is actually possible to detect a treatment effect after 24 h exposure despite a subsequent period of recovery. The different responses to the two Bt treatments is most likely due to differences in the amount of Cry1Ab that was ingested by the *O. nubilalis* larvae. Unfortunately, Hilbeck et al. [8] have not quantified the amount of Cry1Ab delivered to the *O. nubilalis* larvae through Bt maize or Bt toxin-sprayed maize.

ii) The authors used 4-day old *O. nubilalis* larvae even though it is known that neonates are much more sensitive to Cry1Ab [13], a fact that is also acknowledged by Hilbeck et al. [8]. Because we used neonate *A. bipunctata*, it would have been better to work with neonate *O. nubilalis*. We are convinced that neonates would have suffered significant mortality after 24 h feeding on Bt maize. For example, Huang et al. [13] reported a >95% mortality of neonate *O. nubilalis* after 2 day exposure to Bt maize. We thus wonder why Hilbeck et al. [8] have chosen a less sensitive larval stage of *O. nubilalis* for this experiment.

iii) Hilbeck et al. [8] should have measured a sublethal endpoint such as larval weight when working with 4-day old *O. nubilalis* larvae. It is well established for the impact of Cry1Ab on *O. nubilalis* larva, that growth inhibition data are much more sensitive than mortality data (i.e., EC_{50} values are about one order of magnitude lower than LC_{50} values) [14]. Further, it is well established that older larvae suffer less mortality from Cry1Ab compared to neonates, but show sublethal effects such as reduced growth. For example, Huang et al. [13] reported that third instar *O. nubilalis* did not suffer mortality when feeding on a Cry1Ab-containing diet (0.5 μg/g) for a period of 7 days, while their weight gain was significantly reduced by 93%. For *A. bipunctata*, we recorded sublethal parameters such as development time and larval weight [6]. We thus wonder why Hilbeck et al. [8] have chosen to measure only mortality, which is a relatively insensitive endpoint in this case.

iv) The experiment conducted by Hilbeck et al. [8] does not follow the protocol of our interval-feeding experiment. The *O. nubilalis* larvae were exposed to the Cry1Ab protein for 24 h only and then allowed to recover. In our direct feeding experiment with *A. bipunctata*, the larvae were exposed to the Cry1Ab-containing sucrose solution during the first 24 h of each of their larval instars. The test insects were thus exposed four times 24 h each, which contrasts with the one-time 24-h exposure in the Hilbeck et al. [8] study.

Higher tier study to assess the risk of Bt maize expressing Cry1Ab to *Adalia bipunctata*

As a common practice in ecotoxicology and in the nontarget assessment of GM plants [10,15], we designed an experiment in which we exposed *A. bipunctata* larvae to more realistic concentrations of Bt maize-expressed Cry1Ab using a prey herbivore (the spider mite *Tetranychus urticae*) as a toxin carrier. This was done to test whether the putative hazard reported by Schmidt et al. [1] can be observed under a more realistic route of exposure. Schmidt et al. [1] themselves suggested such studies: "Initial experiments, like the ones conducted in this study, provide important data on toxicological responses of nontarget organisms to Bt toxins. Nonetheless, they cannot simply be extrapolated to describe the potential ecological impacts of Cry proteins in the field, but have to be complemented with experiments under ecologically more realistic conditions." We are thus surprised that the results from our tri-trophic feeding study are not mentioned by Hilbeck et al. [8].

We fed larvae of *A. bipunctata* continuously with Bt maize-reared spider mites. This herbivore is an acceptable food source for young ladybird larvae. The experiment was thus restricted to the first two larval stages. We regard this as sufficient given the fact that Schmidt

et al. [1] have only observed toxic effects of Cry1Ab on the first instar. We recorded lethal (mortality) as well as sublethal (development time, weight) parameters. None of these parameters were affected in *A. bipunctata* larvae fed with Bt maize-reared prey compared to larvae that had received prey reared on control maize.

Spider mites served as an ideal toxin carrier because they are prey of ladybird larvae and are known to contain very high amounts of Cry protein when compared to other herbivores [6,16–18]. Furthermore, we had shown in a previous sensitive insect bioassay that the Cry1Ab ingested by spider mites is biologically active [19], and the spider mites themselves are not affected when feeding on Cry1Ab-expressing Bt maize [16]. By using a quantitative ELISA, we demonstrated that ladybird larvae contained 0.7 and 0.5 μg Cry1Ab/g fresh weight in the first and second instar, respectively, when fed continuously with Bt maize-reared spider mites. In parallel we also tested spider mites that had fed on another Bt maize line expressing the Coleoptera-active Cry3Bb1 toxin [6]. There we were able to compare the ELISA values to those from ladybird beetle larvae that were collected in field with the same Bt maize [18]. This comparison revealed that the Cry protein concentration detected in larvae from our laboratory bioassay was between 160- and 330-fold higher than that measured in field-collected larvae [6]. This confirms that our tri-trophic feeding assay provides realistic worst-case exposure conditions and adds certainty to the conclusion that *A. bipunctata* is unlikely to be affected by Bt maize expressing Cry1Ab under field conditions.

Conclusions

We reject the statement by Hilbeck et al. [8] that we were not able to detect adverse effects of Cry1Ab on *A. bipunctata* larvae in our direct feeding bioassay due to the fact that we only exposed the larvae to the toxin at certain intervals and not in a continuous way as done by Schmidt et al. [1]. The statement made by Hilbeck et al. [8] is based on a bioassay with *O. nubilalis* that is not convincing because it included (i) 4 day-old and thus less sensitive *O. nubilalis* larvae; (ii) only one dose of 24 h feeding compared to 4 doses of 24 h feeding in our study; (iii) only mortality as an endpoint, which is known to be less sensitive than sublethal parameters such as growth inhibition used in our study. Even under those conditions, Hilbeck et al. [8] detected a significant difference between the Bt sprayed leaves and the control leaves, which demonstrates that the assay is suitable to detect effects if present. In addition, Hilbeck et al. [8] ignore two important studies in which *A. bipunctata* larvae were continuously exposed to Cry1Ab without revealing any direct lethal or sublethal effects [6,7].

Furthermore, we showed in our tri-trophic feeding study that Bt maize expressing Cry1Ab does not cause adverse effects to *A. bipunctata* larvae under realistic worst case exposure conditions. This together with the fact that many ladybird species, including *A. bipunctata*, mainly feed on aphids that are known to contain, at best, trace amounts of Cry protein when feeding on Bt maize [20] leads to the conclusion that Cry1Ab-expressing Bt maize poses a negligible risk to this predator. This conclusion is supported by a large body of evidence from field studies in different parts of the world showing that Cry1Ab-expressing Bt maize does not cause harm to ladybird beetles or any other non-Lepidoptera species under field conditions [21-23].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JR, FAA, and FB jointly wrote the commentary. All authors read and approved the final manuscript.

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