

Appropriate Analytical Methods Are Necessary to Assess Nontarget Effects of Insecticidal Proteins in GM Crops Through Meta-Analysis (Response to Andow et al. 2009)

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As we described in our rebuttal in *Transgenic Research* (Shelton et al. 2009), we think that the meta-analysis approach used by Lövei et al. (2009) suffers from important methodological limitations relative to risk assessment that led them to reach conclusions that are in conflict with those of several recent comprehensive reviews and meta-analyses about the effects of Cry proteins on natural enemies. In particular, we believe that in their analyses they often attributed hazard to a protein rather than, more accurately, to poor prey or host quality. The rebuttal by Andow et al. (2009) does not correct this mistaken comparison or address our other major concerns.

In this response to their letter we clarify misrepresentations of our original statements, refocus the discussion on methodology, and re-emphasize the additional main points of our initial rebuttal that Andow et al. (2009) did not address in their response.

Value of Meta-Analyses

Andow et al. (2009) base much of their rebuttal on the claim that we have “fundamental criticisms of meta-analysis.” This is a red herring. Actually, we think that meta-analyses, when applied correctly, have a critical and appropriate function, especially in the area of environmental risk assessment (Marvier 2008, Duan et al. 2009). In short, we believe meta-analysis is an efficient and robust means of quantitatively summarizing the results of numerous similar studies in such a way that much more statistically powerful inferences can be drawn than is possible from any single study. In fact, one of the authors of this letter (Naranjo) has been involved in three recent meta-analyses focused on both laboratory and field studies of invertebrate nontargets of Bt crops (Wolfenbarger et al. 2008, Duan et al. 2009, Naranjo 2009). These meta-analyses have advanced our collective understanding of the potential risks of Bt crops for nontarget organisms by identifying negative, neutral, and positive effects of the technology in both laboratory and field studies. Thus, the accusation that we recognize no non-neutral effects of genetically modified (GM) crops is false, and we did not make such a claim in Shelton et al. (2009). In addition, several of the authors have worked extensively with proteinase inhibitors (PIs) and lectins and have documented many non-neutral effects of these more broad-spectrum proteins (Burgess et al. 1996; Malone et al. 2000; Bell et al. 2001a, b; Ferry et al. 2003, 2005; Romeis et al. 2003; Hogervorst et al. 2006; Mulligan et al. 2006; Li and Romeis 2009). Our concern was and continues to be focused on the limitations of the meta-analysis performed by Lövei et al. (2009).

Factors Affecting the Quality of Meta-Analyses

The adage that the analysis is only as good as the data included in the analysis applies to meta-analyses as well as it does to any review, synthesis, or original research study (Gurevitch and Hedges 1993). There are two nonmutually exclusive approaches that can be

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used to ensure that a meta-analysis accurately addresses the question at hand: strict criteria to determine which studies should be included in the analysis and, if all studies related to the topic are included, the use of heterogeneity analysis within a meta-analysis framework to identify effect sizes that can be used to indicate whether the responses belong to two or more different populations. Most of the meta-analyses on the effects of *Bt* proteins on nontarget organisms conducted to date have followed this second alternative. Within the context of our debate here, one or both of these approaches are needed to accurately assess and/or delineate the difference between direct *Bt* protein toxicity to natural enemies versus the indirect effects of prey or host quality when they ingest *Bt* proteins and are subsequently exposed to parasitoids or predators. The meta-analytic approach of Lövei et al. (2009) did not use either of these powerful approaches and thus failed to accurately assess effects of *Bt* proteins, and probably non-*Bt* proteins, on natural enemies.

Alternative 1: Study Quality and Tri-Trophic Interactions. As we stated in Shelton et al. (2009), there is a basic factor of "study quality" that should be considered when deciding to include a study in the analysis. If a particular study had a poorly formulated hypothesis, experimental design, or testing method, it cannot lead to reliable results no matter how many times it is replicated. One approach to a more accurate analysis of true effects would be to exclude it from a meta-analysis. This is exemplified with studies that are not able to separate direct and indirect effects of toxins, which is clearly shown by early studies on the larvae of *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae). Hilbeck et al. (1998a, b) published some of the first studies that purported to show harm to natural enemies by a *Bt* protein. They suggested that the reduced fitness of *C. carnea* larvae was associated with CryIAb when they fed on *Bt* maize-reared lepidopteran larvae and that CryIAb was toxic to this chrysopid. Andow and his colleagues have repeatedly used studies by Hilbeck and her colleagues to suggest there is a hazard to this predator by CryIAb (Andow and Hilbeck 2004, Andow and Zwahlen 2006, Andow et al. 2006). In fact, such studies should not be included in a meta-analysis to test for direct toxin effects on nontarget organisms because the "experimental design did not permit a distinction between a direct effect due to the *Bt* protein on the predator versus an indirect effect of consuming a sub-optimal diet consisting of sick or dying prey that had succumbed to the *Bt* protein" (U.S. EPA 2000). In other words, it is important to use only studies that can show a clear cause and effect. Later studies (Romeis et al. 2004, Rodrigo-Simón et al. 2006, Lawo and Romeis 2008) avoided the pitfall of mistakenly attributing hazard to the protein rather than to poor prey quality and, therefore, showed lack of toxicity of CryIA to *C. carnea* when appropriate methods were used, including feeding the toxin directly to the predator and assessing whether the predator had binding sites for the toxin. Moreover, in addition to the flaws eventually shown in

the original studies of Hilbeck and colleagues on the effects of CryIAb on *C. carnea*, subsequent field studies have shown no negative effects of *Bt* crops on this species (Wolfenbarger et al. 2008).

The lack of effect of Cry proteins on predators and parasitoids has also been shown in tri-trophic studies using lepidopteran larvae that are resistant/tolerant to certain CryI proteins (Schuler et al. 2003, 2004; Chen et al. 2008a, b) or other hosts that are simply not susceptible (Dutton et al. 2002; Ferry et al. 2006, 2007; Álvarez-Alfageme et al. 2008), thus removing the effect of poor host quality. Any studies in which parasitized host larvae die (thus killing the internal parasitoid) when feeding on a Cry protein should be seen for what it is—an indirect effect that is common to any pest control action, including removal of the larva by a predatory insect, a bird, or a human hand. We believe it is inappropriate to combine meta-analysis studies that measure indirect and direct effects and that this largely was the reason for the erroneous conclusions by Lövei et al. (2009).

Alternative 2: Heterogeneity of Effects and Tri-Trophic Interactions. If all studies related to the topic are included, another powerful method within the meta-analysis toolbox is the ability to estimate within-group variability or heterogeneity in effect sizes. This approach requires that multiple characteristics of each study be coded in the overall database so that variables leading to heterogeneity can be examined. The example that Andow et al. (2009) presents on eggshells of two bird species being differentially affected by the same compound provides a simple way to show the value of heterogeneity analysis. A meta-analysis begins with the estimation of effect size, which is a metric that places all studies included in the analysis on a common scale that is weighted by study sample size and variance (Hedges and Olkin 1985). Thus, we would begin by estimating the effect size associated with 25% thicker or thinner shell. We might estimate a mean effect size over all the studies, but at the same time would estimate heterogeneity to assess whether all the effect size belongs to the same population. In the example posed, such an analysis would point to significant heterogeneity, which would prompt further analyses of the two (in this case) bird species that were lumped incorrectly into a single meta-analysis. Thus, a meta-analysis does not stop with the estimation of a mean effect size but continues with further exploration of factors affecting responses if heterogeneity is found (see Wolfenbarger et al. 2008 for an example). It was heterogeneity in the response of natural enemies in tri-trophic exposure studies that led to additional analyses by Naranjo (2009) and subsequently the delineation of host/prey quality as a key factor in interpreting the responses observed in these studies. This study, which we described in our rebuttal (Shelton et al. 2009), showed that overall effects on natural enemies were neutral or even positive when high-quality, uncompromised prey/hosts exposed to *Bt* proteins were provided (see Fig. 3 in Naranjo 2009 or Fig. 1 in Shelton et al. 2009). This fact had previously been described by Romeis et al. (2006), who per-

formed a detailed analysis of all published studies at that time looking for evidence of direct and indirect harmful effects of *Bt* Cry proteins on natural enemies.

With the additional analyses provided by Andow et al. (2009; Table 1), they acknowledge bi-trophic and tri-trophic effects but continue to ignore the paramount importance of prey/host quality as it bears on the apparent toxicity of Cry proteins to natural enemies exposed to treated prey/hosts. From the additional analyses presented in Andow et al. (2009), we calculate that >73% of all observations reflect tri-trophic exposure for *Bt* proteins and >82% for non-*Bt* proteins. We can further calculate from Naranjo (2009) that ≈63% of the observations from tri-trophic exposures used prey or hosts that were sublethally compromised by *Bt* proteins. Thus, the vast majority of tri-trophic-based observations in the dataset of Lövei et al. (2009) likely reflect effects of prey/host quality and not intoxication by *Bt* proteins. According to our analysis of their database, nearly one half (46%) of all observations on natural enemies reflect the effect of prey quality rather than direct toxicity of *Bt* proteins. An accurate assessment of toxicity of *Bt* proteins to natural enemies simply cannot be done while ignoring prey/host-mediated effects. Such effects could have been identified easily through heterogeneity analysis. Interestingly, heterogeneity analysis is sensitive to changes in the distribution of effect sizes and could have been more effectively used to detect the types of effects that Andow et al. (2009) argue can only be found using the methods of Lövei et al. (2009).

Lumping Studies That Test Different Toxins. Another major concern is that Lövei et al. (2009) combined proteins with different modes of action. This is not justified from a biological standpoint and goes against the internationally agreed principle of case-by-case risk assessment of GM crops (Romeis et al. 2008). Lövei et al. stated, "All of the PIs were combined and included aprotinin, jackbean lectin (concanavalin A), CpTI, GNA, the barley cystatin (HvCPI), and oryzacystatin I." Andow et al. (2009) defended this strategy, despite the fact that the mode of action and spectrum of activity of these proteins differ substantially (Malone et al. 2008). Their reasoning to do so is that "distinguishing among kinds of proteinase inhibitors (would be) desirable" but "data do not allow at present (such) an analysis." This is not true. A review of the nontarget impacts of all non-*Bt* insecticidal proteins clearly identified the protein and its contribution in each study (Malone et al. 2008). Furthermore, for most of these proteins, there also is extensive literature describing their biochemistry and biological activities. It is true that, compared with *Bt* Cry proteins, fewer studies have been performed for each of these other proteins. This is a consequence of non-*Bt* insecticidal proteins being a large group of very diverse proteins with many different modes of action and the fact that the compounds are not currently expressed in commercialized insecticidal GM crop varieties; it is in no way a justification for lumping them together and performing a meta-analysis as Lövei et al. (2009) have done. Although pooling the

data resulted in a larger data set, the ensuing analysis was not informative and even misleading. Which "PI" had an effect and which didn't? The methods used by Lövei et al. (2009) can not answer this question. Although some lumping of dissimilar studies is inevitable in most meta-analyses, a priori knowledge of the modes of action of the different "PIs" examined by Lövei et al. (2009) would point to separate analyses of each class of compound. At the very least, heterogeneity should have been estimated to assess if responses were derived from two or more populations.

Additional Statistical Aspects of Meta-Analyses

Nonindependence of the Data. As noted by Andow et al. (2009), independence is a central issue in meta-analyses just as it is in any statistical analysis. They point to an example of multiple species in a single field experiment not being independent because of interspecific interactions to illustrate their point. Although we agree that some dependency may reside in this situation, it pales in comparison to using multiple measures of life history and behavioral characteristics on the same cohort of organisms (see Gurevitch and Hedges 1993, pp. 384–385). Their own analyses (Lövei et al. 2009; Table 2) point to these nonindependence issues in developmental and survival rates on individual instars by showing that the majority of these rates are correlated. Even without such an analysis, one could reasonably assume high correlations among multiple measures on the same cohort and would thus want to guard against these interdependencies. As we noted previously (Shelton et al. 2009), most previous meta-analyses of the effects of *Bt* crops have gone to great length to reduce dependency issues for the purpose of increasing the rigor and power of inference. Lövei et al. (2009) have gone in the opposite direction; "more data points provide a more accurate picture of the literature" (Lövei et al. 2009, p. 295, column 2, third paragraph). To defend this further, they (Andow et al. 2009) again point to instar-specific rates of development and survival in lieu of total immature rates as being more meaningful. Although there might be situations in which the duration or survival of individual stadia is of interest, detailed knowledge of ecological interactions in the field would be needed to determine their meaning. We re-emphasize that, in classic demography (Carey 1993), it is the number of organisms that survive to reproduce and the total time it takes to reach maturation that matters in population growth. If they existed, the "complex instar-specific mortality schedules and patterns of development times" offered by Lövei et al. (2009, p. 295, column 2, top) would be accurately reflected in total developmental duration and survival. Furthermore, we argue that total immature development and survival would provide a more robust measure of potential toxin effect because of longer and more complete exposure. Andow et al. (2009) suggested that their use of nonindependent, correlated data may lead to higher type II error rates and thus provide for a more conservative assessment of toxicity. However, neither Andow et al. (2009) nor

Lövei et al. (2009) provided any evidence that this is the case nor do they discuss the potentially more problematic issue of nonindependence in reproductive parameters. By introducing more variability in response through the use of nonindependent data, they may just as easily be reducing statistical power despite the increase in sample size. We agree that further study of nonindependent effects in meta-analysis is warranted, but we disagree that the interim solution should be to ignore it when assessing toxicity. As to the philosophy of how of laboratory studies should be used in risk assessment, a recent meta-analysis (Duan et al. 2009) using independent laboratory survival data showed that laboratory studies either accurately predicted the effects of *Bt* toxins in the field or showed negative effects that were subsequently found to be absent in field-based assessment. Thus, appropriate analyses of laboratory data can serve to both extrapolate effects to the field and determine the need for further evaluation in the field, a key assumption of the tiered approach in regulatory risk assessment (Romeis et al. 2008).

Analytical Philosophy and Approach. Andow et al. (2009) pointed to one of the major strengths of meta-analysis—statistical power. Even a well-crafted study may suffer from small sample size and thus lack the statistical power needed to delineate true experimental differences. The main virtue of meta-analysis is its ability to set aside the limited inferences possible from any single study in favor of a more robust inference based on a larger sample size. This power is further enhanced by combining individual effect sizes into a cumulative or aggregated analysis using time-tested tools and theories such as general linear models (Gurevitch and Hedges 1993, Rosenberg et al. 2000) that allow estimation of weighted means, confidence intervals, and statistical comparisons among subgroup means. This aggregated analysis takes full advantage of sample size for improved inference and also has the property of diluting poor studies that should have been eliminated (see discussion above) and also allows estimation of heterogeneity in population response (also discussed above). The analytical approach of looking only at the distribution of effect sizes derived from individual studies (Lövei et al. 2009) negates the most significant virtues of modern meta-analysis. Their analysis is really only weighted vote-counting.

Another strength of meta-analysis is the use of effect size as a weighted (by variance and sample size) metric of each study that puts all study results on a common scale. Lövei et al. (2009) indicated the use of an effect size estimator similar to Hedges' *g* (p. 293, column 2, bottom) and Andow et al. (2009) now provide the details of their estimator. Hedges' *d*, which provides additional protection against small sample size bias, would have been more appropriate (Hedges and Olkin 1985). In the laboratory database analyzed by Naranjo (2009), which covered many of the same *Bt*-related studies as Lövei et al. (2009), ≈68% of observations had sample sizes ≤15, and 42% had sample sizes ≤5. Another advantage of an aggregated

meta-analysis is the further weighting by the inverse variance of the effect size estimator in a fixed- or mixed-effects model (Rosenberg et al. 2000). The analysis based on weighting of the effect size estimator (which is itself weighted) assures enhanced quality in the final analysis by automatically de-emphasizing individual studies with high variance and/or low sample size. By focusing on only the distribution of individual effect sizes, what Lövei and Arpaia (2005) originally called a "rough bean-counting algorithm" (p. 2, top of column 2), Lövei et al. (2009) have lost the main advantages of meta-analyses (sample size and variance weighting) and in turn the power to arrive at more accurate inferences.

A final constraint of the distribution approach of Lövei et al. (2009) is the limited inference afforded by the analysis. The 4 df *g*-test they perform tests only that the distribution is non-normal. This test allows no inference concerning the magnitude of the five individual classifications (e.g., "negative not significant," "positive not significant") created and thus is of no use for determining whether the magnitude of specific non-neutral effects are larger or smaller than expected.

Ecological Context

Laboratory studies on the effects of insecticidal proteins on beneficial arthropods are mainly conducted to assess the potential impact of transgenic crops expressing those proteins on nontarget organisms in the field. Properly conducted laboratory studies provide a powerful tool to assess direct toxic effects of an insecticidal protein, and the resulting data allow conclusions about whether the abundance and/or ecological function of natural enemies may be altered when such plants are grown in the field (Romeis et al. 2008, Duan et al. 2009). It is therefore unfortunate that Lövei et al. (2009) and Andow et al. (2009) do not put their results in an ecological context, despite the abundance of published information, including meta-analyses of field data (Marvier et al. 2007, Wolfenbarger et al. 2008, Naranjo 2009) that clearly show the environmental benefits of *Bt* crops relative to current management alternatives. They would have most probably come to the same conclusion that *Bt* transgenic plants "are still more environmentally friendly than most if not all chemical insecticides" (quote from Hilbeck et al. 1998b). We share this view and believe that well-designed studies support this opinion not only for predators but also parasitoids. For example, in the case of parasitoids, strains of the herbivore *Plutella xylostella* L. (Lepidoptera: Plutellidae) resistant to a Cry protein or several commonly used insecticides were allowed to become parasitized by *Diadegma insulare* (Cresson) (Hymenoptera: Ichneumonidae), an important endoparasitoid of *P. xylostella* (Chen et al. 2008a). Only the parasitoids that fed on *P. xylostella*, which had consumed the Cry protein, but not other insecticides, suffered no harm, emerged as adults, and killed the host. This was the first study that used such resistant insects to show the lack of hazard of a Cry

protein to a parasitoid compared with traditional insecticides. Laboratory studies with predators have also shown insect-resistant transgenic crops to have significantly lower risk to predators compared with conventional insecticide treatments (Mulligan et al. 2006, 2009). Unfortunately, some of these recent studies were not included in the dataset of Lövei et al. (2009), nor were they acknowledged and discussed by Andow et al. (2009).

Summary

We strongly restate our criticisms of the report by Lövei et al. (2009). (1) They failed to account for the critical importance of well-described prey/host-quality mediated effects in the studies included in their analyses. Studies that failed to delineate toxicity of *Bt* proteins from poor prey quality should have either been eliminated from the analysis or coded so that heterogeneity analysis could have been conducted to reveal true treatment effects. (2) They included multiple nonindependent measures of various life history and behavioral traits in their analyses. (3) They used a distribution approach that negates much of the power of a meta-analysis and the subsequent inferences possible. (4) They lumped together proteins that have entirely different modes of action and host ranges into a single category (i.e., proteinase inhibitors, lectins) (5) They failed to provide any ecological context for their assessments and they disregarded actual field studies that have shown the lack of harm to natural enemies in environments in which *Bt* plants have been grown.

To reiterate, the suggestion by Andow et al. that we have “fundamental criticisms of meta-analysis” is a red herring that diverts attention away from the real debate over the merits of different meta-analytic approaches. Our criticism is directed to the meta-analysis by Lövei et al. (2009) and not to all meta-analyses per se. Additionally, the seven “findings” added to the end of Andow et al. (2009) with the phrase that that “they were not disputed by Shelton et al. (2009)” works counter to a full and objective debate in the scientific literature. Our initial rebuttal (Shelton et al. 2009) was limited by page length, as is this letter. Because we did not address each of these issues does not mean we agree with them or find them without fault.

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