For: Environmental Entomology

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Letter to the Editor

Appropriate Analytical Methods are Necessary to Assess Non-target 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 mis-representations of our original statements, re-focus 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 (see 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

66 single study. In fact, one of the authors of this letter (Naranjo) has been involved in three recent
67 meta-analyses focused on both laboratory and field studies of invertebrate non-targets of Bt
68 crops (Wolfenbarger et al. 2008, Duan et al. 2009, Naranjo 2009). These meta-analyses have
69 advanced our collective understanding of the potential risks of Bt crops for non-target organisms
70 by identifying negative, neutral and positive effects of the technology in both laboratory and
71 field studies. Thus, the accusation that we recognize no non-neutral effects of GM crops is false
72 and we did not make such a claim in Shelton et al. (2009). In addition, several of the authors
73 have worked extensively with proteinase inhibitors (PI's) and lectins and have documented many
74 non-neutral effects of these more broad-spectrum proteins (e.g., Burgess et al. 1996, Malone et
75 al. 2000, Bell et al. 2001a, b, Ferry et al. 2003, 2005, Romeis et al. 2003, Hogervorst et al. 2006,
76 Mulligan et al. 2006, Li and Romeis 2009). Our concern was and continues to be focused on the
77 limitations of the meta-analysis performed by Lövei et al. (2009).

78 **Factors Affecting the Quality of Meta-Analyses**

79 The adage that the analysis is only as good as the data included in the analysis applies to
80 meta-analyses as well as it does to any review, synthesis or original research investigation
81 (Gurevitch and Hedges 1993). There are two non-mutually exclusive approaches that can be
82 used to ensure that a meta-analysis accurately addresses the question at hand: strict criteria to
83 determine which studies should be included in the analysis and, if all studies related to the topic
84 are included, the use of heterogeneity analysis within a meta-analysis framework to identify
85 effect sizes that can be used to indicate whether the responses belong to two or more different
86 populations. Most of the meta-analyses on the effects of Bt proteins on non-target organisms
87 conducted to date have followed this second alternative. Within the context of our debate here,
88 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 utilize 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 illustrated by early studies on the larvae of *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae). Hilbeck and colleagues published one 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 CrylAb when they fed on Bt maize-reared lepidopteran larvae and that CrylAb was toxic to this chrysopid (Hilbeck et al. 1998a, b). Andow and his colleagues have repeatedly used studies by Hilbeck and her colleagues to suggest there is a hazard to this predator by CrylAb (e.g., Andow and Hilbeck 2004, Andow and Zwahlen 2005, Andow et al. 2006). In fact, such studies should not be included in a meta-analysis to test for direct toxin effects on non-target 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" (US EPA 2000). In other words, it is important to

use only studies that can demonstrate 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 CrylA 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 Cry1Ab 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 illustrated in tri-trophic studies using lepidopteran larvae that are resistant/tolerant to certain Cry1 proteins (e.g., Schuler et al. 2003, 2004; Chen et al. 2008a, b), or other hosts that are simply not susceptible (e.g., 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 for 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, then 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)

135 presents on eggshells of two bird species being differentially affected by the same compound
136 provides a simple way to illustrate the value of heterogeneity analysis. A meta-analysis begins
137 with the estimation of effect size, which is a metric that places all studies included in the analysis
138 on a common scale that is weighted by study sample size and variance (Hedges and Olkin 1985).
139 Thus, we would begin by estimating the effect size associated with 25% thicker or thinner shell.
140 We might then estimate a mean effect size over all the studies, but at the same time would
141 estimate heterogeneity to assess whether all the effect size belongs to the same population. In
142 the example posed, such an analysis would point to significant heterogeneity, which would
143 prompt further analyses of the two (in this case) bird species that were lumped incorrectly into a
144 single meta-analysis. Thus, a meta-analysis does not stop with the estimation of a mean effect
145 size but continues with further exploration of factors affecting responses if heterogeneity is
146 found (see Wolfenbarger et al. 2008 for an example). It was heterogeneity in the response of
147 natural enemies in tri-trophic exposure studies that led to additional analyses by Naranjo (2009)
148 and subsequently the delineation of host/prey quality as a key factor in interpreting the responses
149 observed in these studies. This study, which we described in our rebuttal (Shelton et al. 2009),
150 showed that overall effects on natural enemies were neutral or even positive when high quality,
151 uncompromised prey/hosts exposed to Bt proteins were provided (see Fig. 3 in Naranjo 2009 or
152 Fig. 1 in Shelton et al. 2009). This fact had previously been described by Romeis et al. (2006)
153 who performed a detailed analysis of all published studies at that time looking for evidence of
154 direct and indirect harmful effects of Bt Cry proteins on natural enemies.
155 With the additional analyses provided by Andow et al. (2009, Table 1) they acknowledge
156 bi-trophic and tri-trophic effects but continue to ignore the paramount importance of prey/host
157 quality as it bears on the *apparent* toxicity of Cry proteins to natural enemies exposed to treated

158 prey/hosts. From the additional analyses presented in Andow et al. (2009) we calculate that over
159 73% of all observations reflect tri-trophic exposure for Bt proteins and over 82% for non-Bt
160 proteins. We can further calculate from Naranjo (2009) that about 63% of the observations from
161 tri-trophic exposures used prey or hosts that were sub-lethally compromised by Bt proteins.
162 Thus, the vast majority of tri-trophic based observations in the Lövei et al. (2009) dataset likely
163 reflect effects of prey/host quality and not intoxication by Bt proteins. According to our analysis
164 of their database, nearly half (46%) of all observations on natural enemies reflect the effect of
165 prey quality rather than direct toxicity of Bt proteins. An accurate assessment of toxicity of Bt
166 proteins to natural enemies simply cannot be done while ignoring prey/host mediated effects.
167 Such effects could have been identified easily through heterogeneity analysis. Interestingly,
168 heterogeneity analysis is sensitive to changes in the distribution of effect sizes and could have
169 been more effectively used to detect the types of effects that Andow et al. (2009) argue can only
170 be found using the methods of Lövei et al. (2009).

171 **Lumping Studies that Test Different Toxins.** Another major concern is that Lövei et
172 al. (2009) combined proteins with different modes of action. This is not justified from a
173 biological standpoint and goes against the internationally agreed principle of case-by-case risk
174 assessment of GM crops (Romeis et al. 2008). Lövei et al. stated, “All of the PIs were combined
175 and included aprotinin, jackbean lectin (concanavalin A), CpTI, GNA, the barley cystatin
176 (HvCPI), and oryzacystatin I”. Andow et al. (2009) defended this strategy, despite the fact that
177 the mode of action and spectrum of activity of these proteins differ substantially (Malone et al.
178 2008). Their reasoning to do so is that “distinguishing among kinds of proteinase inhibitors
179 (would be) desirable” but “data do not allow at present (such) an analysis”. This is not true. A
180 review of the non-target 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. While 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't answer this question. While 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

Non-Independence 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 due to interspecific interactions to illustrate their point. While 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 non-independence 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 required to determine their meaning. We re-emphasize that in classic demography (e.g., 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 due to longer and more complete exposure. Andow et al. (2009) suggest that their use of non-independent, 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) provide any evidence that this is the case nor do they discuss the potentially more problematic issue of non-independence in reproductive parameters. By introducing more variability in response through the use of non-independent data they may just as easily be reducing statistical power despite the increase in sample size. We agree that further

investigation of non-independent 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 demonstrated 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) point 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

249 significant virtues of modern meta-analysis. Their analysis is really only weighted vote-
250 counting.

251 Another strength of meta-analysis is the use of effect size as a weighted (by variance and
252 sample size) metric of each study that puts all study results on a common scale. Lövei et al.
253 (2009) indicate the use of an effect size estimator similar to Hedges' *g* (p. 293, column 2, bottom)
254 and Andow et al. (this issue) now provide the details of their estimator. Hedges' *d*, which
255 provides additional protection against small sample size bias, would have been more appropriate
256 (Hedges and Olkin 1985). In the laboratory database analyzed by Naranjo (2009), which
257 covered many of the same Bt related studies as Lövei et al. (2009), about 68% of observations
258 had sample sizes ≤ 15, and 42% had sample sizes ≤ 5. Another advantage of an aggregated
259 meta-analysis is the further weighting by the inverse variance of the effect size estimator in a
260 fixed- or mixed- effects model (Rosenberg et al. 2000). The analysis based on weighting of the
261 effect size estimator (which is itself weighted) assures enhanced quality in the final analysis by
262 automatically de-emphasizing individual studies with high variance and/or low sample size. By
263 focusing on only the distribution of individual effect sizes, what Lövei and Arpaia (2005)
264 originally called a "rough bean-counting algorithm" (p. 2, top of column 2), Lövei et al. (2009)
265 have lost the main advantages of meta-analyses (sample size and variance weighting) and in turn
266 the power to arrive at more accurate inferences.

267 A final constraint of the distribution approach of Lövei et al. (2009) is the limited
268 inference afforded by the analysis. The 4 d.f. g-test they perform tests only that the distribution
269 is non-normal. This test allows no inference concerning the magnitude of the five individual
270 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 non-target 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 then 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 to a parasitoid, compared to traditional

294 insecticides. Laboratory studies with predators have also shown insect resistant transgenic crops
295 to have significantly lower risk to predators compared to conventional insecticide treatments
296 (e.g., Mulligan et al. 2006, 2009). Unfortunately, some of these recent studies were not included
297 in the Lövei et al. (2009) dataset nor were they acknowledged and discussed by Andow et al.
298 (2009).

299 **Summary**

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

312 To reiterate, the suggestion by Andow et al. that we have "fundamental criticisms of
313 meta-analysis" is a red herring that diverts attention away from the real debate over the merits of
314 different meta-analytic approaches. Our criticism is directed to the meta-analysis by Lövei et al.
315 (2009), not to all meta-analyses *per se*. Additionally, the seven "findings" added to the end of
316 Andow et al. (2009) with the phrase that that "they were not disputed by Shelton et al. (2009)"

317 works counter to a full and objective debate in the scientific literature. Our initial rebuttal
318 (Shelton et al. 2009) was limited by page length, as is this letter. Because we did not address
319 each of these issues does not mean we agree with them or find them without fault.

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