August 19, 2004

MEMORANDUM

SUBJECT: Transmittal of Minutes of the FIFRA Scientific Advisory Panel Meeting Held June 8-10, 2004: Product Characterization, Human Health Risk, Ecological Risk, And Insect Resistance Management For *Bacillus thuringiensis* (*Bt*) Cotton Products

TO: James J. Jones, Director
Office of Pesticide Programs

FROM: Paul I. Lewis, Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

THRU: Larry C. Dorsey, Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Joseph J. Merenda, Jr., Director
Office of Science Coordination and Policy

Please find attached the minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, Virginia from June 8-10, 2004. These meeting minutes address a set of scientific issues being considered by the U.S. Environmental Protection Agency regarding Product Characterization, Human Health Risk, Ecological Risk, And Insect Resistance Management For *Bacillus thuringiensis* (*Bt*) Cotton Products

Attachment

cc:

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OPP Docket

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Mark Whalon, Ph.D.

SAP Report No. 2004-05

MEETING MINUTES

FIFRA Scientific Advisory Panel Meeting, June 8-10, 2004, held at the Holiday Inn Arlington , Arlington, Virginia

A Set of Scientific Issues Being Considered by the U.S. Environmental Protection Agency Regarding:

Product Characterization, Human Health Risk, Ecological Risk, And Insect Resistance Management For *Bacillus thuringiensis* (*Bt*) Cotton Products

NOTICE

These meeting minutes have been written as part of the activities of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP). This report has not been reviewed for approval by the United States Environmental Protection Agency (Agency) and, hence, the contents of this report do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or commercial products constitute a recommendation for use.

The FIFRA SAP was established under the provisions of FIFRA, as amended by the Food Quality Protection Act (FQPA) of 1996, to provide advice, information, and recommendations to the Agency Administrator on pesticides and pesticide-related issues regarding the impact of regulatory actions on health and the environment. The Panel serves as the primary scientific peer review mechanism of the EPA, Office of Pesticide Programs (OPP) and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. Food Quality Protection Act Science Review Board members serve the FIFRA SAP on an ad hoc basis to assist in reviews conducted by the FIFRA SAP. Further information about FIFRA SAP reports and activities can be obtained from its website at http://www.epa.gov/scipoly/sap/ or the OPP Docket at (703) 305-5805. Interested persons are invited to contact Paul Lewis, Designated Federal Official, via e-mail at lewis.paul@epa.gov.

In preparing these meeting minutes, the Panel carefully considered all information provided and presented by the Agency presenters, as well as information presented by public commenters. This document addresses the information provided and presented within the structure of the charge by the Agency.

SAP Report No. 2004-05

MEETING MINUTES:

FIFRA Scientific Advisory Panel Meeting, June 8-10, 2004, held at the Holiday Inn Arlington, Arlington, Virginia

A Set of Scientific Issues Being Considered by the U.S. Environmental Protection Agency Regarding:

Product Characterization, Human Health Risk, Ecological Risk, And Insect Resistance Management For *Bacillus thuringiensis* (*Bt*) Cotton Products

Mr. Paul Lewis
Designated Federal Official
FIFRA Scientific Advisory Panel
Date: August 17, 2004

Gary Isom, Ph.D.
FIFRA SAP Session Chair
FIFRA Scientific Advisory Panel
Date: August 17, 2004

Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
June 8-10, 2004

Product Characterization, Human Health Risk,
Ecological Risk, And Insect Resistance
Management For *Bacillus thuringiensis* (*Bt*) Cotton Products

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PUBLIC COMMENTERS

Oral statements were made by:
Rod Herman, Ph.D., Dow Agrosciences
Monte Mayes, Ph.D., Dow Agrosciences
Larry Sernyk, Ph.D., Dow Agrosciences
Nick Storer, Ph.D., Dow Agrosciences
Laura Tagliani, Ph.D., Dow Agrosciences
Ray Layton, Ph.D., Dupont Company, on behalf of the Agricultural Biotechnology Stewardship Technical Committee, Non-target Organism Subcommittee
Jane Rissler, Ph.D., Union of Concerned Scientists
Graham Head, Ph.D., Monsanto

Written statements were received from:
National Cotton Council
Monsanto
JR Bradley, North Carolina State University

INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed its review of the set of scientific issues being considered by the Agency pertaining to its review of product characterization, human health risk, ecological risk, and insect resistance management for Ba*cillus thuringiensis* (*Bt*) cotton products. Advance notice of the meeting was published in the *Federal Register* on May 12, 2004.

The review was conducted in an open Panel meeting held in Arlington, Virginia, from June 8-10, 2004. The meeting was chaired by Gary Isom, Ph.D. Mr. Paul Lewis served as the Designated Federal Official. Mr. Joseph J. Merenda, Jr. (Director, Office of Science Coordination and Policy, EPA) and Janet Andersen, Ph.D. (Director, Biopesticides and Pollution Prevention Division, Office of Pesticide Programs, EPA) offered opening remarks at the meeting. Mr. Leonard Cole (Office of Pesticide Programs, EPA) provided an introduction and highlighted the goals and objectives of the meeting. The Agency's product characterization and human health safety assessment for stacked plant-incorporated protectants, environmental effects assessment for WideStrike cotton, and issues related to establishing an insect resistance management plan for WideStrike cotton were presented by John Kough, Ph.D. (Office of Pesticide Programs, EPA), Zigfridas Vaituzis, Ph.D. (Office of Pesticide Programs, EPA) and Sharlene Matten, Ph.D. (Office of Pesticide Programs, EPA), respectively. Sharlene Matten, Ph.D. (Office of Pesticide Programs, EPA) concluded the Agency's presentations by discussing Bollgard and Bollgard II cotton bollworm insect resistance management.

SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

Human Health Risk

The Panel concluded that toxicity studies showed no adverse effects at the highest levels tested, either for the individual Cry proteins or for the two proteins together. The Panel agreed with the Agency that combined oral toxicity studies for multiple proteins is not necessary if each protein had been previously independently tested, unless such testing would be intended to detect any effects produced by interactions between the proteins.

Based on the evidence presented in the safety assessment of the single *Bt* containing strain and the more recent extensive testing of the stacked Cry1F/Cry1Ac material, the Panel concluded that there is little to suggest that the stacked variety poses significantly more risk or unanticipated consequences to agronomic performance than do varieties with either the Cry1F or Cry1Ac genes introduced singly.

Ecological Risk

Direct hazards to vertebrates due to exposure to the Cry1F and Cry1Ac proteins should be minimal or non existent, making consideration of synergy in vertebrates unwarranted. Thus, the Panel concluded that toxicity testing on the combinations of Cry proteins is not necessary. While synergism is not considered to be important for the Cry1 proteins under consideration, the Panel believed that future testing of non-target effects of these types of toxins should proceed, with both toxins to be expressed by genetically engineered plants rather than with individual purified toxins. Testing mixtures of compounds would provide data that are more relevant to the proposed use scenarios and likely exposure scenarios.

The Panel recognized improvements in the quality of the Agency's analysis and the degree to which this reflected a positive response by the Agency to recommendations made by previous SAP reviews of plant-incorporated protectants (PIPs). The Panel also noted that the Tier I tests were generally of a higher quality than the tests that other SAPs have reviewed. Overall, however, detailed analysis of the field-based evaluations revealed that these did not meet current standards, and the Panel repeated requests made by other SAPs, that the Agency issue detailed guidelines for semi-field, and field-based procedures. The Panel recommended that the exposure pathway and diet used within the *Chrysoperla* test be examined further, to determine the rigor and repeatability of the test, and also suggested that *Orius* spp. be considered as a more appropriate test subject. The Panel also considered that a more appropriate parasitoid than *Nasonia* should be selected.

The Panel agreed that multi-year field studies to assess potential longer-term effects of WideStrike cultivation on persistence of toxins in the soil and on populations of non-target organisms are applicable to *Bt* cotton and should be considered. Overall, the likely reduction in the use of broader-spectrum insecticides afforded by the cultivation of *Bt* cotton in general is likely to have positive effects on the ecosystems. Nonetheless, longer-term and broader-scale evaluation of PIP crops will be necessary for improved ecological understanding. The Panel

believed strongly that long-term field studies should not proceed without some guidance relative to experimental protocols and clearly defined endpoints. Without such guidance, we are unlikely to resolve any unexpected detriments or benefits associated with the use of transgenic crops.

WideStrike Cotton Insect Resistance Management

The combined expression of *Bt* proteins in WideStrike cotton meets the Agency's definitions of high dose for pink bollworm (PBW) and tobacco budworm (TBW). In addition, reasonable doses of the combined protein were evident for control of cotton bollworm (CBW). Based on the high dose evidence, the Panel concluded that it is valid to assume that resistance occurring in PBW or TBW will likely be inherited as a recessive trait. However, CBW is more tolerant of both proteins and it seems possible that resistance will be less recessive. WideStrike cotton does appear to offer a high dose for TBW, a high dose of Cry1Ac for PBW, and reasonable doses of Cry1F and Cry1Ac for CBW. The same high dose/refuge strategy practiced thus far as a resistance management approach for Bollgard cotton should be applied for WideStrike.

While the Panel supported the Agency's conclusion that incomplete shared binding of Cry1Ac and Cry1F receptors in TBW and CBW is expected to lead to incomplete cross-resistance, differences were expressed on the molecular mechanism involved in the process. In addition the Panel raised the issue that another as yet unidentified major resistance mechanism may not occur. The Panel agreed with the Agency that there is no basis to believe that the occurrence of resistance in the field will be due to a mechanism other than binding site modification.

The Panel identified several areas of concern with the Dow Agrosciences CBW model that make its use problematic. These problems must be addressed if this model is to be used to assess the durability of WideStrike cotton. The Panel believed that use of the current model, once corrected of the identified errors, would be an appropriate vehicle to explore the parameter space with the goal of finding areas where resistance does occur in the 15 year time horizon and assessing whether it occurs within biologically plausible initial conditions and parameter values.

Since the dose of the Cry1Ac and Cry1F in WideStrike Cotton was demonstrated to be high against populations of TBW, the Panel believed that WideStrike will be more durable than that predicted by Peck (1999) for single Cry1Ac cotton.

The Panel agreed that the HOSTS database is insufficient to address the issue of CBW alternate hosts as natural refugia. The Panel agreed that there are insufficient empirical data in the registrant report to demonstrate that alternative hosts are producing susceptible, fit individuals in sufficient quantity, at the correct time and proximity to maximize the probability of matings between homozygous-susceptible individuals and individuals heterozygous for resistant traits.

Even though the Panel raised limitations with the model as described, the Panel was in

strong agreement that the proposed IRM plan by the registrant is sufficient for WideStrike cotton and supported the prediction of a delay in resistance of TBW, CBW and PBW to WideStrike cotton for 15 years. The overall consensus was that the existing IRM options that have been applied to the single-toxin Bollgard cotton will be equally or even more effective in protecting against resistance in the double-toxin WideStrike cotton.

Bollgard Insect Resistance Management

The Panel could not determine whether CBW reverse migration is expected to have any impact on CBW adaptation to *Bt* cotton or *Bt* corn.

While the Panel agreed that pyrethroid oversprays in Bollgard cotton improve the control of susceptible corn earworm (CEW), the effect of pyrethroid oversprays in delaying resistance in CEW is probably overstated. The Panel agreed that there is little need to include pyrethroid oversprays in Bollgard II plots in the models of Gustafson et al. There is some evidence of greater tolerance in larvae originating from Bollgard fields relative to those coming from non-Bollgard fields.

The Panel agreed that sufficient data were provided to establish that C_3 and C_4 alternate hosts function to some degree as unstructured refugia. However, the Panel expressed concern on the methodologies used to assess adult productivity in the alternate hosts. The Panel agreed that CBW production should be measured at a larger scale than the local farm, or field level because of the high mobility of adult CBW. In response to the request for methods on quantitatively calculating "effective refuge size," the Panel provided techniques for quantifying CBW populations in the identified alternate hosts that were identified as natural refugia.

The Panel agreed with the Agency that a weighted average is an appropriate choice for determining the contribution of alternate hosts to the refuge size. The Panel believed, however, that exploring detailed questions about time to resistance and the effect of alternate hosts on resistance would benefit from the development of a more detailed model.

PANEL DELIBERATIONS AND RESPONSE TO THE CHARGE

The specific issues to be addressed by the Panel are keyed to the Agency's background documents, references and Agency's charge questions.

Agency Charge

Human Health Risk

1. The Agency examines the safety of proteins based on the source of the protein, the protein's pesticidal mode of action, comparisons of the amino acid sequence to toxins and allergens and the results of acute oral toxicity testing. The company provided numerous mammalian oral toxicity studies to demonstrate the safety of the introduced Cry 1Ac and Cry1F protein insecticidal toxins. The toxins were tested both separately and in

combination. The Agency believes tests with combinations of pure proteins may address possible synergistic interactions between introduced proteins. However, the Agency believes that unless there is an indication that the two proteins would interact, such as being parts of a binary toxin or attaching to the same receptor, there is little to justify testing the two proteins together when separate oral toxicity tests indicate a lack of toxicity for the individual proteins.

Does the Panel have additional comments on this position including identifying instances where it would be justified to require the toxicity testing of two proteins in combination?

Panel Response

The Panel's comments to this question are specific to expressed proteins. Comments on unintended effects are presented by the Panel in their response to question 2. The relevant human health issue is related to consumption of these proteins. A search of the literature regarding these substances failed to find any indication that there are any other significant issues directly related to human health.

The Agency has been asked to evaluate a line of cotton containing two Bt Cry proteins intended for insect control. This line was produced by cross breeding two lines containing independent transformation events. The pesticide registrant provided data characterizing the two transformation events, showing that the structures of the inserts were not altered during crossing, and demonstrating stable Mendelian inheritance of each insert. In terms of human health, the pesticide registrant provided sequence analysis data for all the proteins involved (the Cry proteins and the markers), stability data, and the results of oral toxicity studies using mice. The Panel concluded that these toxicity studies showed no adverse effects at the highest levels tested, either for the individual Cry proteins or for the two proteins together. It should be noted that the testing was done with material that was highly equivalent (but not identical) to the proteins expressed in plants. In addition, because cotton proteins make only a very low contribution to the human diet, exposure to these proteins is expected to be very low.

The fact that the cotton line involved expressed two insect control proteins raised the question of whether, or when, it is necessary to carry out combined oral toxicity studies for multiple proteins if each protein has been independently tested previously. This testing would be intended to detect any effects produced by interactions between the proteins. The Agency has stated the belief that such testing is not justified unless there is specific evidence for such interactions. In general, the Panel agreed with this statement.

The basic principles for assessing the safety of transferred proteins in bioengineered plants have been well developed. A good summary of these principles can be found in the CODEX document "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." In general, these principles suggest that, for proteins, safety assessment should focus on source, function, similarity to known toxins, anti-nutrients and allergens, and stability. This guidance suggested that "appropriate oral toxicity studies may need to be carried out in cases where the protein … is not similar to proteins have that previously been

consumed safely." These are essentially the same criteria specified by the Agency.

First, previous evaluations of potential human health risks associated with (PIPs) plant-incorporated protectant proteins may be considered as having set a precedent for this approach. In earlier cases where only a single PIP protein was expressed in a transformed plant, it was in fact accompanied by a second protein that was not active insecticidally, but instead served as a marker to identify a transformation event. In the present consideration of WideStrike cotton, synthetic genes coding for the insecticidal proteins Cry1F and Cry1Ac were integrated independently into separate cotton breeding lines and accompanied, in both cases, by the *pat* gene encoding for the marker protein phosphinothricin acetyltransferase (PAT). In effect, the individual transformation events for each PIP protein involved expression of two new proteins in a target plant—the marker and the pesticidal protein. In these cases, each protein was evaluated independently. The classical breeding of the two transformed cotton lines eventually produced WideStrike cotton that expresses the pyramided PIP proteins Cry1Ac and Cry1F along with the intact marker protein PAT and an incomplete version of PAT. Whereas the insert from the Cry1Ac event (3006-210-23) contains one intact copy of the insect resistance gene *cry*1Ac and one intact copy of the plant selectable marker gene *pat*, the insert from the Cry1F event (281-24-236) contains one intact copy of the insect resistance gene Cry1Ac, one intact copy of the marker gene *pat*, plus an additional hybridizing fragment of the marker gene *pat* (Dow AgroSciences Study 010075.01). The presence of the partial *pat* fragment results in a 4th product expressed in WideStrike cotton that has a potential amino acid sequence 90% identical to PAT and generates the same homology profile as the full length PAT sequence (Dow AgroSciences Study ID: GH-C 5573). Despite its truncated resemblance to the intact PAT protein and reduced expression, the 4th product must still be considered along with Cry1Ac, Cry1F and PAT for potential effects on human health. The question remains whether testing of two or more proteins together is necessary when separate toxicity tests for individual proteins indicate a lack of toxicity. Since the Panel agreed with the Agency that testing for interactions between the proteins is not justified unless specific evidence points to the contrary, the Panel sees no basis why, if independent evaluation is considered appropriate for two proteins, it should not also be considered appropriate for four or more proteins.

Second, although there are many examples of binary (or multimeric) protein toxins, the interactions between the proteins involved are specific among members of a toxin family. The Panel is not aware of any instances where a "new" toxin has been created by unexpected interaction between two known proteins. Conversely, it seems unlikely that two proteins that interact to produce a biological effect would independently have unexpected activity, except in well-defined situations (for example as blockers of transport or digestion pathways). Again, it appears unlikely that unexpected interactions will occur between two unrelated proteins. The only examples would be if physical interactions between two unstable proteins inhibited gastric digestion or increased stability during processing. Therefore, it is reasonable to treat each protein independently in situations such as this. It is possible that simple biochemical tests could be used to determine whether such physical interactions occur between independent proteins, in which case the combination can be targeted for digestion studies.

Third, the principle of independent assessment has been used for many years for food

additives and is implicit in microbial risk assessments. There is no reason to believe that the issues involved with these proteins should affect the use of this principle.

Fourth, the relatively low expression levels for the proteins involved make it unlikely that they would be able to interact extensively and maintain their biological function. Unanticipated interactions seem more likely to result in sequestration and reduced activity for the proteins involved.

Fifth, given that each of these proteins has previously been used independently in food plants, the Panel concluded that they would fit the criteria of being similar to proteins that have previously been consumed safely. Therefore, they do not rise to the level of concern that would suggest the need for combined toxicity testing.

In the charge to the Panel, the Agency also raised the possibility that having two proteins attached to the same receptor could trigger a need for combined toxicity testing. It appears that, in this context, this means binding to the same receptor in humans. Any proteins that have known biological effects in humans need to be evaluated, regardless of mechanism.

There is one point of terminology that needs clarification by the Agency - the definition of the term "stacked" and "pyramidal" such as when used for stacked traits or stacked proteins. There are different ways to "stack" or "pyramid" traits (multiple transformation or cross breeding) and it is unclear to the Panel whether the Agency considers these differences to be significant.

Agency Charge

2. When traits are introduced into crop plants using the transformation techniques of modern biotechnology or even traditional breeding, one of the areas of concern is the possibility of unintentional changes. There is a general difficulty in screening for these unforeseen changes since it is a conceptual leap to anticipate the unexpected. However, in general the approach has been to examine general performance of the new cultivars like agronomic performance and compositional analysis to detect unintentional effects. PIP products can be both transformed lines and the result of traditional breeding of two transformed lines to yield a new product with combined traits like WideStrike cotton. In both cases, the new PIP product must be registered just as other new combinations of pesticide active ingredients must be registered.

For PIP products resulting from traditionally bred transformed lines, under what circumstances, if any, would it be appropriate to examine agronomic performance and compositional analysis to provide a screen for unintentional changes in the crop? Please describe other ways EPA might consider screening for potential unintentional changes in a crop.

Panel Response

Agronomic Performance and Composition Analysis

Based on the evidence presented in the safety assessment of the single *Bt* containing strain and the more recent extensive testing of the stacked Cry1F/Cry1Ac material, the Panel concluded that there is little to suggest that the stacked variety poses significantly more risk or unanticipated consequences to agronomic performance than do varieties with either the Cry1F or Cry1Ac genes introduced singly. The Panel was unaware of any reason to believe that previously undetected and unanticipated changes would have occurred in the plant breeding beyond the uncertainties normally associated with plant breeding and the original transformation process in parental GM lines.

The food safety of the two individual Cry proteins present in WideStrike has already been established. It is worth noting that cotton fiber is not a food and does not contain residual Cry proteins. Cotton meal, which may contain small amounts of Cry proteins, is usually only fed at low levels to ruminants because they cannot tolerate larger amounts of gossypol which is relatively toxic to non-ruminant animals. Cottonseed oil is a bleached and purified product that contains only trace amounts of protein. Cottonseed oil is one of several vegetable oils consumed by humans and thus is a relatively minor dietary component.

Composition, phenotypic traits and agronomic performance can be powerful tools for revealing unintended effects and can be viewed as screening methods since they are the end result of the balanced and coordinated expression of numerous multigenic traits. Plant breeders normally eliminate from further development plants with visible or compositional defects or undesirable alterations. Compositional or agronomic studies are normally not required on plants resulting from traditional breeding.

Suggested Additional Procedures To Screen For Potential Unintentional Changes In the Crop

For the Panel's response to the question, the Panel considered the screening for unintended changes to WideStrike and, on a broader perspective, other plant-incorporated protectants (PIP) and genetically modified crops. Screening for unintended effects is encouraged. Such a screening could use profiling methods for unintended effects, e.g. expression microarrays or metabolomic studies (e.g., as discussed in SAFOTEST **(http://www.entransfood.nl/RTDprojects/SAFOTEST/safotest.html).** However, as these tests are still under development, toxicology studies in rodents, looking for effects of unintended effects, although unlikely, could be appropriate. While the targeted safety assessment system in place today has apparently been effective in risk characterization, it cannot eliminate the chance that unintended or unexpected consequences of plant breeding will escape detection. It would be extremely useful to have comparative data regarding the frequency and magnitude of unintended effects associated with conventional plant breeding with or without the use of genetic engineering techniques upon which a quantitative risk assessment could be based. The Agency could play an active role in advancing comparative risk assessment by supporting research, perhaps in cooperation with FDA and USDA, on unintended effects associated with plant breeding.

Technologies such as metabolic profiling, gene expression systems and proteomics might be useful for evaluating unintended effects. These methods need to be more fully developed and their utility documented before they can be used to inform the scientific review. Previous reports such as The US General Accounting Office (GAO), in its assessment of the FDA's safety testing of GM foods (GAO 1992) and several European entities (http://www.entransfood.com) have concluded that non-targeted profiling methods might be useful in assessing the risks associated with unintended effects. The Panel recommended that the EPA, in partnership with FDA and USDA, support research on the development and validation of profiling methods and provide support for the further development of site-specific gene insertion techniques. The Panel's detailed comments are presented below.

Potential unintended effects of GM foods on human health

Unintended effects of traditional breeding methods on levels of anti-nutritional or toxic constituents in food organisms have been characterized in conventional organisms. Organisms derived from conventional breeding methods including tissue cultures may have a somewhat enhanced possibility for genetic (and epigenetic) instabilities, such as the activity of mobile elements and gene-silencing effects (Bhat and Srinivasan 2002). These effects could result in an increased possibility of pleiotropic unintended effects, e.g., increased or decreased expression of constituents or possibly modifications in expressed proteins as well as epistasis, (the interaction of the inserted gene with other genes).

In general, the compositional analysis should be performed on the basis of validated scientific methods. Strategies for the compositional analysis in food products derived from GM plants have been established, where key substances are identified and analyzed per species. Furthermore, in order to be able to interpret the data from the compositional analysis of individual animal products adequately, insight into the natural variation in the relevant macro- and micronutrients and antinutrients, if present, will be required.

In the future, compositional analysis may also be based on unbiased profiling of the GM food product and the conventional counterpart. Techniques for the profiling approach are now under development and can be divided into three subsections: genomics, proteomics and metabolomics to screen for differences in the GM plants in relation to the gene transcription products, proteins and metabolites, respectively. At the moment, however, none of these techniques is yet validated and ready for routine use in risk assessment (WHO/FAO expert consultation, 2003).

The Molecular Biology of Unintended Effects

The problem of assessing unintended effects has been a matter for scientific discussion for a long time and the principles of assessment by international scientific organizations have changed considerably. For example, the use of the concept of the substantial equivalent has been criticised extensively, and the idea of the principle has been reduced to its use as a starting point of a risk assessment.

In principle, traditionally bred transformed lines should have the same characteristics as the parent organisms. However, analyses of events have pointed to potential different outcomes in some cases where the basis of these events is not presently fully understood.

Two specific issues for consideration are:

(1) A molecular characterization of the new product should show that the recombinant traits/ sequences in the new product are identical to the insertion /traits in their parental lines. In principle, the method of breeding the two parental lines could affect the genetic characteristics of the inserted traits. Therefore, evidence is needed that no such effect has occurred. For these products, a risk assessment combining evidence from parental lines and the product should be requested.

(2) Stability is another concern. For other similar products, it is uncertain how many generations need to be observed to establish stability of inserted traits.

Sites of Insertion of Foreign Genes and Potential Effects

One of the concerns with any genetically modified organism is the precise location point(s) of the inserted gene or genes. Single copy insertions of the genes at locations other than in functional gene regions or in regulatory areas is the goal but with current technologies insertion is typically relatively random. If significant anomalies occur they will surely be picked out and discarded through breeding programs but more subtle effects might possibly slip through. In the specific case at hand, the utilization of Cry1F and Cry1Ac genes incorporated into the cotton genome, it is noteworthy that both for the single insertions via typical recombinant DNA techniques and then the construction of the Cry1F/CRY1Ac stacked strain by traditional breeding involved only single insertion events in each case.

Potential health or environmental risks of genetically combining T-DNA transgenes in WideStrike cotton through traditional breeding are unlikely to differ from those of the parental transgenic lines. However, our present knowledge concerning unique or special risks associated with GM crops is somewhat limited and presently under study (NAS, 2002). One ongoing concern is that T-DNA insertion into the genome is a mutagenic event, which could trigger unintended changes in nutritional or toxicological properties of a transformed crop (Schubert, 2002). T-DNA insertions that disrupt agronomic performance or gross chemical composition can be detected by current methods of screening, whereas other insertions that alter biosynthesis of molecules which are toxic, allergenic or carcinogenic would likely be overlooked. Given that there are no *a priori* means to predict these different outcomes, additional information should be sought on potential genetic effects of T-DNA insertion, as outlined below.

T-DNA inserts and effect on genome organization

Assessments of risks posed by GM crops rely on comparisons with conventionally bred (non-transformed) counterparts having a history of safe use. Apart from comparing agricultural

characteristics and chemical composition, GM plants are also routinely analyzed for the number of T-DNA insertion sites, the number of gene copies at these sites, and the organization of DNA within inserts. In addition, the effect of T-DNA on proximal open reading frames of cellular proteins is also considered. However, additional measures outlined below would better inform us of potential downstream genetic effects associated with transgenic insertion of foreign genes.

For instance, routine Southern hybridization analysis of WideStrike cotton and its parental transformed lines suggest that T-DNA inserts coding for individual insecticidal proteins are present as single copies in the cotton genome. The importance of this observation is that single transgene inserts breed true and show fewer unwanted effects (i.e., transgene silencing or mutations) than multi-copy transgene lines. However, single-copy T-DNA inserts are known to trigger large-scale chromosomal rearrangements, including translocations (Forsbach et al, 2003). In such cases, large portions of flanking DNA at the T-DNA insert site may also be duplicated and translocated to multiple chromosome locations (Nacry et al, 1998, Tax and Vernon, 2001). These types of chromosomal rearrangements would likely complicate interpretations of Southern hybridization data, by suggesting that a single-copy of the transgene was present, when in fact duplication and translocation of the T-DNA resulted in an additional chromosomal copy with identical flanking sequences. Therefore to complement and extend current strategies for assigning transgene copy number, the chromosome location of each T-DNA insert should be routinely mapped through use of Mendelian segregation results or molecular markers for cotton (Tomkins et al, 2001; www.genome.clemson.edu/projects/cotton/bac and www.cottoninc.com/Agriculture/homepage.cfm?page=3157).

A related concern is whether T-DNA-induced mutations are qualitatively or quantitatively similar to those which occur as a consequence of traditional crop breeding. It is known that cross-breeding of genetically different cultivars can result in major chromosomal rearrangements. In addition, mobile DNA elements (transposons) also induce genetic changes in plants. At present, it is far from clear whether plant transformation differs from these processes and poses unique or greater genetic risks.

T-DNA inserts and effect on gene expression

Several hundred base pairs of genomic sequence were identified by sequencing of regions flanking T-DNAs bearing the Cry1Ac and Cry1F transgenes in WideStrike and parental cotton lines. When used as *in silico* probes, BLAST searches failed to reveal significant homology between these sequences and those previously deposited in the GenBank database. Moreover, analysis of 1032 bp (534 bp from 5' border and deleted 16 bp + 482 bp from the 3' border) of cotton sequences flanking the *Bt* Cry1Ac cotton 3006-210-23 insertion site revealed no significant (<450 bp, 150 aa) open reading frames at this cloned locus. Similarly, analysis of 5028 bp (2073 bp from 5' border and deleted 53 bp + 2902 bp from the 3' border) of cotton sequences flanking the *Bt* Cry1F cotton 281-24-236 insertion site revealed no significant (>450 bp, 150 aa) open reading frames at this cloned locus. Although cotton genes were thus not computationally identified at the T-DNA insertion sites, it is unclear why attempts were not made to determine whether these flanking sequences correspond to one or more transcriptionally-expressed cellular genes.

In this regard, it is noteworthy to consider that T-DNA is widely used by plant biologists as a mutagenic agent to study the biological roles of specific genes (Forsbach, 2003). Moreover, T-DNAs in the present application contain strong agrobacterial enhancer sequences (4*ocs*) which are known to drive expression of genes that are proximal to the insertion site [this process, known as "activation tagging" (Tani H, 2004), is widely used in modern plant biology research to identify novel genes.] Thus to more fully investigate the possibility that T-DNA inserts are in active genes, Northern blot hybridization to detect cognate mRNA transcripts should be done using 5' and 3' sequences that flank each T-DNA as probes. Comparing the size of transcripts detected by Northern blot in fractionated RNA from nontransformed and transformed lines will assess whether the T-DNA insertion physically disrupted or significantly (2 SD from the norm) affected expression of the cellular gene. It is also recommended that due to potential "enhancer effects" by multiple *ocs* promoter sequences, additional flanking sequences 5' and 3' from the T-DNA should be identified to characterize genes that are distal to the insert site. Expression of these distal genes would be monitored by Northern blot analysis or through more systematic investigations using DNA microarrays.

Unintended effects that may result from the insertion of DNA into the plant genome represent hypothetical hazards that, as noted previously, have yet to be demonstrated to occur in products presented to the Agency for review. The challenge is to define specific biochemical or metabolic changes that represent risks that warrant rejection or management. This requires specific evidence of a molecular or compositional change that would trigger further investigation.

It is impossible to quantify and characterize the risk associated with unintended effects without defining it in terms of molecular or metabolic changes. It bears repeating that while unintended changes may be possible, it is both not clear that they present any new or different risks that are significant (meaningful to human and animal health). To date no evidence has been presented that significant risks occur (Beachy, 2002, Kuiper 2001).

Conventional plant breeding itself produces unintended effects. There is a long history of safety associated with plant breeding. Historically, plant breeders have depended on phenotype and performance to detect unintended effects and reject plants harboring undesired characteristics. In some cases, compositional studies may inform the selection of individuals worthy of further development. One is reminded of the fact that plant breeders have successfully employed strong mutagenic techniques such as chemical mutagens, UV light, and radiation to produce genetic diversity and altered traits.

New Possibilities for Gene Insertion

Major advances in techniques for precise placement of foreign genes into host organisms should very soon render these particular concerns about unintended consequences of foreign gene insertion relatively insignificant. Recent research has shown that highly precise implanting of foreign genes into specific sites in a host genome is not only possible, but can be accomplished fairly readily. The work of Lambowitz and his colleagues at the Institute for

Cellular and Molecular Biology at the University of Texas at Austin on site-specific DNA insertion through the use of autocatalytic group II introns is notable in this regard (Karlberg et al., 2001, Mohr and Lambowitz, 2003). While such positional gene insertion will certainly serve to greatly ameliorate unintended effects, it will not eliminate them entirely, As Schubert (2001) and others have pointed out, genes can act at a distance in unexpected ways. New compounds or pathways might be created or activated and unexpected associations between molecules may occur.

Animal Feeding Studies for Unintended Effects

It is often suggested that animal studies should be used as a non-targeted screen for undetected changes for PIPs. There are several limitations to the use of animal studies on whole foods. The first challenge is that it is often difficult to formulate a diet that is nutritionally adequate and well-accepted by the test animal which also contains a large percentage of a whole food (FAO/WHO, 2000; Chassy, 2004). Animal studies have proven useful for the evaluation of pharmaceuticals, food additives, industrial and agricultural chemicals, and environmental contaminants because these compounds have biological activity at fairly low levels of exposure. Carefully performed toxicology studies employing various concentrations of pure chemicals can be used to determine a lowest observed adverse effect level (LOAEL) in a particular animal species, typically a 90 day rodent study. Such studies allow the setting of maximum permissible exposure levels that provide an acceptable safety margin. It is often impossible to devise an animal diet with a whole food such that it contains a sufficient quantity of a toxicant that will elicit a biological response. Chassy et al. (2004) indicated:

"A review (Munro and others, 1996b) of 120 rat bioassays (each of 90 day duration) of chemicals of diverse structure including food additives, pesticides, and industrial chemicals found LOAELs to range from 0.2 to 5000 mg/kg body weight with a median of 100 mg/kg and a 5th percentile of 2 mg/kg. To achieve the 5th percentile of exposure from a toxic constituent present in, say, a food crop in a rodent bioassay (at a food incorporation rate of 30%) the toxin would have to be present at a level of 80 ppm. To achieve the median exposure of 100 mg/kg it would have to be present at 5000 ppm. These concentrations fall well within the range of existing analytical techniques for detection of inherent toxicants in food. The concentrations should also be readily detected during compositional analysis of the known toxicants in the host organism used to generate the improved nutrition crop."

It is clear that analytical techniques have far greater power than animal studies for the detection of individual compounds. Moreover, animal studies on whole foods suffer from the lack of a specific targeted hypothesis as well as the possible presence of a variety of confounders. It is much easier to design a study to investigate an effect on a specific target organ, enzyme level or serum metabolite concentration than it is to compare the health outcomes of two diets. Although several whole food studies have been reported (Chassy 2004), it may be more appropriate to use animal studies to probe for suspected metabolic or toxic effects on a case-by-case basis than it would be to require them in every case. It is also not clear that whole food animal studies would extrapolate very well to human populations that have great genetic and dietary diversity. Animal studies are therefore not likely to be an effective screen for

unintended effects.

Targeted Compositional Analysis

Phenotype and functionality are highly complex traits. Expression of literally thousands of genes must be coordinated in both extent and timing. Expression is also modulated by extrinsic environmental signals, availability of nutrients, pests, diseases and a host of other factors. Phenotype and function are therefore highly sensitive indicators that should not be regarded lightly.

Compositional studies are also powerful indicators that no significant unintended effects have occurred in the breeding and development process. There are three important points to make about composition. The first is that "we are what we eat." It follows that all we need to know is the safety of the components of the food we eat. The only thing that really matters in a safety assessment of a food or ingredient derived from a transgenic crop is the composition of the product that consumers will eat. Levels of transcription and translation as well as varying rates of turnover of protein and mRNA normally occur in all organisms. If these natural oscillations do not result in compositional changes that have health or safety implications, such changes are of little consequence.

The second important point to be made about composition that is often misunderstood is that there is a natural range in concentrations observed for many metabolites in a plant. It is misleading to look in a food composition database and conclude that maize is composed of 9.5% protein. The number in the database represents an average value that may be reasonably used for calculations of, for example, protein content of a diet. It does not, however, reveal the wide range of concentration of proteins (and all other metabolites) that may be found in individual samples of maize. It is not possible to draw conclusions about unintended effects related to changes in composition without understanding the natural variability in natural product composition. Until recently, very little information was available about natural variability in composition. ILSI has recently placed a free and easily accessible crop composition database online (http://www.cropcomposition.org/). The utility of the database has been described in a recent publication (Ridley et al., 2004).

In many instances, the content of secondary metabolites that are of interest for their nutritional, health beneficial or health protective effects may be even more variable than macronutrients. A health claim has recently been approved for soy protein consumption by the FDA. Many consumers are interested in increasing the soy and isoflavone contents of their diets in response to reports of various potential health benefits. The data presented in Table 1 demonstrate that soybeans vary greatly in isoflavone content. The same cultivar grown at 4 different sites gave rise to a nearly 4-fold difference in isoflavone content. Almost 3-fold differences were observed between four commercial varieties of soybean.

The third point to be made about the value of composition studies is that they are powerful sensing probes for the concentration of almost all the remaining cellular metabolites. This is because many of the analytes that are evaluated in composition studies (i.e., amino acids,

fatty acids, vitamins, toxicants such as gossypol, etc.) are products or intermediates in one or more metabolic pathways. Cell metabolism is a highly integrated and interconnected set of highly regulated enzymatically catalyzed pathways. Knowledge that the concentration of dozens if not hundreds of metabolites is within normal range is strong evidence that most all metabolites are within normal ranges. It is therefore not necessary to measure each individual metabolite. Perhaps more importantly, the currently used targeted composition studies often account for over 99% of the total composition and it specifically targets micro-components that are of health or safety significance (i.e. toxicants, vitamins, bioactive phytochemicals, allergens, antinutrients, etc.). It is worth repeating that there is no scientific evidence that targeted composition studies that evaluate over 100 analytes from diverse metabolic pathways have failed to detect significant risks. Moreover, there is also no evidence that the kinds of hypothetical unintended effects that might arise from gene insertion do not also occur in conventional breeding. Several unintended effects of this kind have been reported to occur as a result of conventional breeding programs (Kuiper et al., 2001).

It is important to stress that not only does content of specific metabolites vary over a broad range in plants, but also that changes in the composition of a plant may be beneficial. In fact, numerous development projects are in progress that seek to alter the content of macro- or micronutrients, eliminate allergens or toxicants, or introduce other compositional changes that may be health beneficial. Golden Rice is a well-known example of this kind of strategy (Potrykus 2001). A new risk assessment paradigm may need to be created in order to assess the unintended changes that might accompany large changes in composition.

Almost all of the first wave of biotechnology-derived crops that have been approved and commercialized were designed to be no different than conventional varieties of that crop except for the addition of one or two additional traits—often intended to enhance the agronomic properties. While the Panel indicated that GMO crops were considered as safe since being shown to be "substantially equivalent" to their conventional counterparts, but this is a misunderstanding of the concept of substantial equivalence (FAO/WHO, 2000). Substantial equivalence uses compositional analysis to identify *differences* that merit further risk characterization so it is in effect a comparative assessment paradigm. The finding of differences *per se*, does not demonstrate an increased risk. As a consequence of misunderstanding of the substantial equivalence paradigm, it has been suggested that it should be evolved into the *Comparative Assessment* paradigm (Kok and Kuiper 2003). The use of the comparative assessment concept also lends itself to safety assessment of compositionally-modified novel foods (Chassy, 2004). The Agency can reasonably expect to be asked to review crop plants that have PIP(s) and nutritional enhancements so it is essentially to accept that composition difference is now and will increasingly be in the future a fact.

It should also be noted that human diets vary by an even greater range than the composition of the individual components in the diet. This has enormous consequences for public health. The comparative safety of any unintended changes must always be related to exposure in the context of the whole diet and the range of dietary intakes represented in the particular subject population (Chassy et al., 2004).

Metabolome

An excellent recent review by Goodacre (2004) described the current state of art of technology and understanding of metabolism and metabolomics.

The Panel commented that translation (proteome) and transcription (transcriptome) may provide useful insights into cell function and metabolism, and the composition of the food derived from a plant that determines fitness for human or animal consumption. It is instructive to investigate methods that might shed additional insight into what has now become referred to as the metabolome. One approach would be to conduct a comprehensive analysis of the concentration of each cellular metabolite. One could envision a rapid parallel analyzer that would provide a complete blueprint of the metabolome. The short-coming of this approach is that databases that establish norms for each metabolite would need to be constructed, the normally observed ranges in concentration documented, and concentrations of each metabolite that has safety implications determined. There is at present little data that would relate nutrition, development, performance and health of animals to more than a few dozen compounds, primarily the nutrients. The development of non-targeted metabolic screening methodology as well as paradigms for understanding the implications of specific concentrations is a subject of research. We may be some years away from this kind of metabolic profiling, although in the near term some metabolite screening technologies may prove useful in identifying changes that could then be evaluated by more targeted methods in order to understand their significance.

In the meanwhile, there is another approach to metabolome screening that might be called metabolic-fingerprinting. It is a logical extension of the composition analysis that is now used to assess the safety of transgenic crops. It should be possible to look at the metabolic pathways in a plant cell and select a dozen or two dozen key metabolic intermediates that strongly correlate with overall cell composition. The information required to do this is in the literature and expert physiologists and biochemists could probably reach a consensus on which molecules to select for studies aimed at validating this intelligent targeted fingerprinting.

There is, however, no way that such studies can cope with totally novel metabolites that arise from newly formed pathways or activation of silent genes. This would be in effect, "knowing the unknowable." Fortunately, the multi-pronged safety assessment process employs several distinct types of tests (as noted above) in order to screen for significant unintended effects that would be undetected by compositional analysis.

Metabolic Profiling

Plants produce an extraordinary array of small compounds and these can affect all stages of development as well as the properties of the final materials from these plants that are used in commerce. In the past, analysis of such metabolites has essentially been on a case by case basis and has been limited to only a few chemical entities. Nutritional and compositional assays of GM plants examine such properties as the levels of proteins, sugars, fats and other materials. These targeted assays are certainly extremely useful. However, when screening for potential unanticipated consequences of foreign gene introduction, it would be very desirable to have very

broad yet precise indicators of what is going on in the modified and non-modified plants as far as metabolites are concerned. New tools for looking at issues such as metabolic profiles are coming into widespread use and these could provide a much more broad-band examination of modified and non-modified plant materials in a cost-effective manner.

The recent techniques that have become available can detect and quantify several hundreds of plant metabolites in a single analysis (thus the term metabolic profiling), using techniques such as gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS). Such procedures lend themselves well to providing a more detailed understanding of the potential effects of foreign gene introduction into plants. From the evidence available to date, it is very likely that few differences will be seen between the already heavily studied GM crops and their non-GM counterparts. It would be very reassuring that at a basic level, little metabolic disturbance was occurring because of the transgenic alterations. Finally, testing of new genetically altered materials in this manner, early on in the development process, would serve to provide an early warning of additional concern.

Proteomics

Future studies of genetically engineered plants using proteomic approaches look very favorable for comparison of transformed and non-transformed varieties as well as for traditionally bred variants of transformed species. As has been expressed by Voet and Voet (2004) proteomics investigates "all proteins expressed by an organism, with an emphasis on their quantification, localization, modifications, interactions and activities. " Tools used to gather such information include 2-D gel electrophoresis combined with mass spectrometry, amino acid composition and sequence analysis, peptide mass fingerprinting and matrix-assisted laser desorption ionization (MALDI)- time of flight (TOF) mass spectrometry for identification of post translational modifications. While these processes can be highly precise and definitive in ascertaining differences between samples, for full utilization they do depend upon the availability of adequate databases, which are still being assembled for many plant species.

Gene Microarrays

The use of gene microarrays to assess, for example, which genes are being transcribed into messenger RNA at a particular time, in a particular organ, tissue type or even sub-cellular organelle has been transforming molecular biology in recent years. This same technology can potentially be employed to answer questions about how plant metabolism might be altered by the incorporation of foreign genes, for example those of the Bt toxins. Of course the use of such microarrays in a meaningful manner requires knowledge about the genome of the plant in question. In this connection, it is encouraging to note that sequencing of the cotton genome is proceeding apace, that approximately 50,000 EST sequences are available and that microarrays covering about 30% of the cotton genome are already available (http://cottongenomecenter.ucdavis.edu/microarrays.asp).

These newer techniques of metabolic profiling, proteomics and gene chips are indeed in

their infancy as far as application to plant systems is concerned and clearly need database collection, validation, etc. before they are put into regulatory use. Additionally, when this is accomplished, they should be used as adjuncts to present test methodologies, which have served well. What these new tools, with their broad span analysis do offer is great potential in the future for allowing recognition of unintended consequences of genetic manipulation early in the process. This is true whether the genetic changes are made by traditional or recombinant methodologies.

Agency Charge

Ecological Risk

1. WideStrike cotton is a product expressing pyramided Cry1F and Cry1Ac Bt proteins. The submitted non-target effects studies examined the effects of the Cry1F and Cry1Ac proteins separately and in combination to detect any synergistic effects on non-target wildlife. No synergistic effects or increase in non-target host range were seen as a result of combining these two proteins in the same product.

The Panel is requested to comment on the need for non-target hazard data development on the combinations of Cry proteins being considered for registration when data on the effects of the individual Cry proteins are readily available and show no adverse effects.

Panel Response

The Agency has done a good job of preparing documents presenting the available data regarding control of TBW, CBW and PBW in cotton. Cry1Ac and Cry1F seem to be rather specific. Thus direct hazards to vertebrates due to exposure to the Cry1F and Cry1Ac proteins should be minimal or non existent, making consideration of synergy in vertebrates unwarranted. Hazards to invertebrates have a higher likelihood of showing effect. In fact, synergy has been observed for *Bt* toxins and spores in various lepidoptera (Moar et al. 1989, Dubois and Dean 1995, Johnson and McGaughey 1996), and between different CryIV and CytA toxins in *Aedes aegypti* (Chilcutt and Ellars 1988). In contrast, other studies have failed to demonstrate any synergistic interactions between Cry1A toxins in various lepidopteran insects (Moar et al. 2002, by Tabashnik 1992). One citation (Chakrabarti et al. 1998) indicated a synergistic interaction between Cry1Ac and Cry1F in *Helicoverpa armigera*. Access to the article would have assisted the Panel to fully examine the experimental methods and analytical techniques. Although interactions between toxins and spores would not be an issue with transgenic plants that express only the toxins, potential interactions among different Cry toxins may be important in resistance management (e.g., Tabashnik et al. 1997). Considering all data presented and information from one literature review, the predominance of data demonstrated no synergy for Cry1 proteins. Therefore, the Panel concluded that synergistic effects are unlikely to occur when plants produce Cry1Ac and Cry1F at the concentrations that are currently needed to control TBW, CBW and PBW.

When each toxin was tested independently and in combination at environmentally

relevant concentrations, none of the data indicated any synergistic or additive effects on the target or non-target organisms tested. Evaluations of non-target organisms were generally done at rates far in excess of what these organisms would be exposed to in the field.

Based on the written and verbal information provided by the Agency, the Panel believed that non-target hazard data can be treated similarly to those data in the Human Health Risk Assessment, for which a similar question, as noted previously was raised. Indeed, this is a very similar issue, since mammals are also non-target organisms. Therefore, the Panel concurs with the opinions the Panel expressed in the answer to Question 1 from the Product Characterization/Human Health Risk Assessment, that toxicity testing on the combinations of Cry proteins is not necessary. The Panel's opinion that Cry proteins do not require further synergistic testing will not necessarily extend to other types of *Bt* toxins.

While synergism is not considered to be important for the Cry1 proteins under consideration, the Panel believed that future testing of non-target effects of these types of toxins should proceed, with both toxins to be expressed by genetically engineered plants rather than with individual purified toxins. Testing mixtures of compounds would provide data that are more relevant to the proposed use scenarios and likely exposure scenarios. Testing the toxicity of mixtures for several non-target insect species would also provide data that could refine field evaluations to determine any effects on insect species diversity. This could be a critical parameter controlling food resources for, and thus impacts on avian and mammalian species. Such an evaluation will ultimately provide data that could be used in ecological risk assessments where the question that should be asked is: What is the combined effect of Cyr1Ac and Cry1F on species elimination from ecosystems?

Some concern was raised regarding the small amount of Cry1F data found through a CAB search of the literature since 1973. A single non-target study of monarch butterflies (Helmich et al. 2001) and 11 studies involving target species were found. The Panel suggested: a) USDA has compiled more Cry1F data than is available in the materials prepared for this review or in the peer reviewed literature; b) it would be useful if all non-target data from the Agency presentation, the USDA evaluations, and peer reviewed literature be compiled into one summary table or a series of tables for reference purposes in future evaluations of this and similar products.

Agency Charge

2. The weight of evidence from the reviewed data indicates that there will not be a hazard to wildlife from the commercialization of WideStrike cotton. Although the Bt proteins expressed by WideStrike are known to affect only lepidopteran insect species, the Agency evaluated studies of potential effects on a wide variety of non-target organisms that might be exposed to the Cry1F and Cry1Ac protein, i.e., wild mammals, birds, invertebrates, and aquatic species. EPA concluded that aquatic and terrestrial wildlife was not likely to be harmed and that WideStrike cotton was not likely to threaten the long-term survival of any non-target wildlife populations.

The Panel is requested to comment on the Agency's analysis of the currently available data on the potential impacts of WideStrike cotton on non-target species.

Panel Response

The Panel recognized improvements in the quality of the Agency's analysis and the degree to which this reflected a positive response by the Agency to recommendations made by previous SAP reviews of PIPs. The Panel also noted that the Tier I tests were generally of a higher quality than the tests that other SAPs have reviewed. Overall, however, detailed analysis of the field-based evaluations revealed that these did not meet current standards, and the Panel repeated requests made by other SAPs, that the Agency issue detailed guidelines for semi-field, and field-based procedures. The Panel recommended that the exposure pathway and diet used within the *Chrysoperla* test be examined further to determine the rigor and repeatability of the test and also suggested that *Orius* spp. be considered as a more appropriate test subject. The Panel also considered that a more appropriate parasitoid than *Nasonia* should be selected. Finally, the Panel expanded the table of suggested arthropod test organisms, first developed by the August 2002 SAP, to include aquatic species. A detailed response to the question by the Panel is provided below.

The Panel noted improvements in the quality of the Agency analysis and assessment since the Panel last met on this subject approximately two years ago. Given that some of these improvements appear to have been in direct response to recommendations made by previous SAPs, the current Panel argued that this highlighted the value of the public review process and that it also provided evidence of a positive response to SAP comments by the Agency.

With respect to the Tier I screening procedures reported for WideStrike cotton, the general quality of the submissions was considered to be better than in previous cases, particularly with regard to characterization of test material in the bioassay procedures, the selection of test organisms and the quality and clarity of the reporting. The Agency review was found to be more informative of its logic and reasoning, and more explicit about the limitations of the tests. This made it easier for the Panel to be constructively critical.

The Panel had general comments concerning maximum hazard dose evaluation. The Panel did not find the protocols for Tier 1 testing appropriate. The duration of many tests were determined by control mortality exceeding 20%. The Panel suggested that control mortalities as high as 20% raise a concern of an error with the protocol and that tests either need to be repeated or that some adjustments in protocols are required. Sample sizes also seem small, providing little power to discern differences that may exist.

The greatest challenge raised by the Panel mainly arose through the lack of progress with development of EPA-approved protocols for field-based evaluations, clarity regarding their role relative to laboratory-based evaluations, and the availability of intermediate options that might mitigate the need for long-term, open-ended, field studies, that are not designed to test clearly stated hypotheses. The Agency acknowledged that specific protocols are lacking and that science needs to advance in order for protocols to be developed. Thus, field data are treated as

supplementary by the EPA, and procedures are not subjected to the critical scrutiny that is the norm for required tests.

With regard to the maximum hazard approach, the effectiveness of this approach is dependent upon species selection (where a range of relevant physiologies should be evaluated, relative to the mode of action of the test material), and rigor in the conduct of tests, which should follow detailed protocols. The Panel noted that the tests reported meet many of the criteria listed by previous Panels, but, it argued that in order to affect permanent advances in the standards of testing, criteria for the conduct of tests need to be built into new written requirements or test guidelines.

Of particular importance for inclusion in these new guidelines are criteria noted by two previous SAPs (in 1999 and Aug 2002). These include: 1) verification of exposure levels of test organisms to proteins throughout the bioassay; 2) detailed quantification of EEC in the field; 3) clearly stated endpoints; 4) a clear statement that tests which fail to reach the designated endpoint are not eligible for consideration; 5) use, where possible, of foods used by test species in their relevant habitat; 6) verification that the food offered to the species actually contained the administered material, at the intended dose, throughout the investigation; 7) verification that all life stages of the species are exposed appropriately to the transgene product (i.e. actually contact the toxin in relevant ways); and 8) that there is sufficient replication and that sufficient numbers of insects were screened based on statistical power.

The Agency stated that field testing was "recommended by the August 2002 SAP". Ultimately, scientific confidence in Tier I screening should be sufficient to limit further tiers of testing when no effects are detected at the maximum hazard dose. This would mean that no open field investigations would be undertaken with some products. Confidence in this approach would increase if:

- The armory of guidelines were to be expanded to include extended laboratory and semi-field approaches as an intermediate step, before full field testing was requested. No effects or failure to exceed a trigger or threshold value in these tests would cause the testing to cease at that point. Widely used intermediate testing methods include extended laboratory tests (use of more realistic substrates and exposure pathways within the laboratory) or semi-field tests (confinement of individual or multiple species of test organisms within microcosms, mesocosms, field cages or barriered arenas). The Agency is encouraged to avail itself of opportunities to discuss method development in the international arena, for example in relevant working groups of such organizations as the International Standards Organization, OECD, International Organization of Biological Control, and SETAC, among others.

- Bioassessment protocols were developed for surveys in whole fields to help answer concerns about the possible detection of rare events, effects resulting from combinations of treatments that do not occur within conventional experimental designs or concerns about long-term, unforeseen effects. Such protocols could then be employed on the health of agroecosystems, or as a component of post-release product stewardship.

- The field evaluation guidelines presented were greatly improved with respect to experimental design, sampling procedures, taxonomic focus, statistics and interpretation.

The Panel reiterated its recommendations from the August 2002 FIFRA SAP meeting regarding:

- The requirement that registrants evaluate and select test sites from a number of candidates, in the season before testing begins, to determine whether the organisms of concern are present and sufficiently abundant to provide a basis for statistical discrimination of small but significant effects.

- Use of sampling methods of known efficiency and precision with consideration to within-plot variability when determining the intensity and frequency of sampling.

- Use of a scale and layout of the experiment that minimizes the risks of edge effects and reinvasion from untreated control plots, and which takes into account the dispersal rates and phenology of the organisms of concern.

The Panel recognized the limitations of laboratory-based testing, and it may be argued that the requirement for field testing will continue until a dataset has been generated that effectively validates the maximum hazard method. The most important limitations to laboratory testing, that can be compensated for by field tests, were noted by the August 2002 FIFRA SAP and included the statements that:

- Levels and routes of test material exposure that may not be realistic in the laboratory.

- Effects assessments are made after short-term exposure of organisms, not lifetime exposures as might occur in the field.

- Organisms in the field are subject to supplementary stresses that have additive effects, including sub-optimal temperatures and humidity, and starvation and parasitism, that amplify impacts that occur under the optimal physical and biological conditions of laboratory tests.

- Laboratory tests may evaluate an appropriate category of organism, but they inevitably fail to evaluate species that are actually exposed to test substances in the field.

Despite these limitations, the Panel argued that the Agency could already be striving for a more structured approach to risk assessment for PIPs, guided by more detailed protocols, that expand the availability of replicated intermediate field test data, which increase the potential for long-term, multi-field surveys after release, and which probably decrease the expectation for open-ended field census studies with their various methodological challenges (as noted in the August 2002 meeting minutes).

With respect to the avian hazard assessment, the Panel was asked to consider the effects of a reduced food base for birds utilizing corn fields that have a high non-target insect mortality. Are nestlings any more susceptible to Cry proteins after they have been biotransformed by the insect and fed to the nestling? Do birds lack the bioactivating enzyme or the actual receptor site for the toxin? Could songbird or game bird nesting success change with WideStrike cultivation relative to conventional cotton cultivation? Perhaps insect prey availability would be sufficiently different to produce a difference in nesting success. This question emerged from UK work with grey partridge and other species impacted indirectly by agrochemicals.

With respect to aquatic species, the Panel noted that it was correctly stated by the Agency that the August 2002 SAP recommended a list consisting only of terrestrial taxa for Tier 1 testing. The Panel noted however, that this does not mean that aquatic taxa were excluded as being potentially affected in some circumstances, even if not in the present case for cotton. A proposal was made to extend the table of proposed test organisms in the 2002 FIFRA SAP meeting minutes to indicate where aquatic taxa may be incorporated. It was also suggested from the August 2002 FIFRA SAP meeting minutes that *Daphnia* might be substituted by a member of the shredder functional feeding group (e.g., some Diptera or Trichoptera species might be more appropriate) to increase the opportunity for uptake of the toxin. Precipitate formation in the reported *Daphnia* test was difficult to interpret, but may imply that the test organisms were not exposed to the test substance.

The table of recommended test taxa from the August 2002 FIFRA SAP meeting minutes for selection of test taxa has been edited as follows (underlined):

Functional Group	Examples
Anthropocentric Functions	
Secondary pests	-Sporadic pests, induced pests
Natural enemies	-Predators, parasitoids, parasites, competitors, ants, and weed-eating herbivores
Rare or endangered species	-Red list species or species of value for biodiversity conservation
Species that generate income	-Honey bees, silk moths
Species of social or cultural value	-e.g. Monarch butterflies, honey bees, organisms conferring health of lotic or lentic aquatic systems associated with crop or near crop habitats
Ecological Functional Groups	
Non-target herbivores	-Plant eating species that are not the target of the transgene (including aquatic species if appropriate)
Secondary consumers	-Species that eat herbivores; predators, parasitoids, parasites
Pollinators	-Bees, selected Diptera (e.g. Syrphidae) and Coleoptera, etc.
Decomposers	-Scavengers, ants, Collembola, micro-organisms, earthworms, mites, nematodes, and aquatic taxa, particularly those within the shredder functional feeding group
Seed dispersers	-Birds, small mammals, ants

Concerning possible long-term impacts on soil invertebrates, the hazardous outcome of impacts on soil taxa was narrowly defined as crop residue build up in the field. This should be expanded to acknowledge the need to protect soil health and ecological function. Residue build up may be one unintended negative consequence of impact on soil organisms that could reveal more significant harm to biogeochemical processes.

Previous Panels have recommended changes to the list of non-target insects to be tested and those same concerns are raised again here. *Nasonia* is a poor model as a parasitoid in the cotton system and there would be many other more suitable models (*Cotesia, Trichogramma*, etc.). *Chrysoperla* is a good model for the cotton system, but others are just as good and maybe better from the standpoint of greater exposure to toxins via plant feeding. *Orius insidiosus* has been suggested in past Panels and would be a good model because it is very common and abundant in cotton and also feeds on pollen and plant sap. (The Panel noted that the minute pirate bug is *O. tristicolor* not *insidiosus*). *Geocoris punctipes* would be another very good

model for the cotton system. This species occurs in high abundance throughout the cotton belt. It is also a well-known plant sap feeder and feeds on small caterpillar larvae. Both *Orius* and *Geocoris* are available commercially and easy to rear in the laboratory.

The registrant was congratulated for addressing the issue of toxin decay in the presented diets via ELISA and SDS-PAGE. However, the Panel commented that with a small amount of additional testing, the detection of toxins in the non-targets themselves would be possible and prudent.

Concerns were raised by the August 2002 FIFRA SAP on *Bt* corn relative to diet exposure in *Chrysoperla*. It is not clear that this issue has been satisfactorily addressed here. The toxin is still present only on the surface of the moth eggs. Are the insects ingesting the toxin? Could this be tested by ELISA? Is the registrant confident that the other insect models tested are actually ingesting the toxins? Could this be verified with ELISA? Previous panels have suggested artificial diets for testing purposes. This would seem to alleviate many of the problems associated with definitive exposure. Artificial diets have been developed for *Chrysoperla, Orius* and *Geocoris*. Could these be explored as better models for toxin delivery? One Panel member suggested that while this may be a useful method for exposure, some care needs to be taken. Many diets contain enzymes, pro-oxidants and other substances that may affect incorporated toxins. The high level of nutrition may also mask more subtle effects of the toxin or may even enhance their effects. The Panel member suggested that special precautions may be needed to avoid these issues. Several companies hold ARS patents for these diets including Buena Biosystems and Beneficial Insectary and would likely be able to supply the diets. Even if these diets don't permit insect reproduction they may be sufficient for shorter-term longevity/survival studies.

The Panel noted that the Hymenopteran parasitoid test was incorrectly described as a larval test in the Agency's review. It also noted enhanced toxicity in the treatment with both proteins at the maximum hazard dose.

The post-emergence mortality in all bee treatments detracted slightly from the confidence one has in the honey bee test conclusions. More confidence would have been warranted if post-emergence mortality of controls had not indicated that something other than the dosed toxins was influencing bee viability.

Regarding non-target organism susceptibility in general, one Panel member considered an analysis for Cry 1f and/or Cry 1Ac receptors in non-target organisms. Perhaps the standard non-target representatives could be characterized for presence of Cry1F and Cry1Ac receptors? The scientific literature indicated that lepidopterans are the ONLY group of organisms that are capable of being affected by *Bt* toxins. However, the Panel member had seen no publications which actually attempted to detect *Bt* receptors in the spectrum of representative non-target organisms – other than mammals. Physical confirmation of receptor absence in non-target gut epithelial cells would reduce the level of uncertainty and reinforce the indication that Cry proteins, alone and in combination, exhibit little to no toxicity. In addition, the absence of

receptors would negate any concerns for food-chain transfer of bioactivated *Bt* toxins by lepidopterans which may be taken as a food source.

With regard to the field studies that were submitted, the Panel noted EUP restrictions in the first year that limit the area that can be planted. Thus, a series of concerns arise that could reasonably have been considered by the Agency and the registrant in planning, execution, analysis and interpretation of these investigations. Although guidelines are not available, the Panel expected standards of current science to be met.

The Panel noted that page 28 of the registrant review stated that plot sizes in these studies were 1,000 square meters. In fact the scale was far less than this, namely 2,667 square feet in the Arizona experiment and 6,400 square feet in the Louisiana experiment, which were only 50 feet and 40 feet long respectively, with mustard and pig weed, or bare earth barriers to separate plots. Invertebrate sampling was undertaken from the central 40 feet and 60 feet, respectively, in each study, but this still means that samples in neighboring plots include points that are within a few feet of each other. The consequences of this are that predatory invertebrates traverse plots to find prey within the scale of the whole experiment. If these animals survive pesticide or PIP treatments, then the pattern of invertebrate abundance across the study reflects treatments to a degree, but also reflects this redistribution of animals between plots. The data therefore contribute very little towards answering the study objectives, which require treatments to be independent of each other.

The Panel noted that if toxic chemicals are used in a non-*B*t treatment, organisms may move between plots and succumb to these chemicals within the period of toxicity, if they are susceptible. Reinvasion from neighboring plots then occurs and, once toxicity has declined, effects that would persist on an agriculturally relevant scale are obliterated. These scale and reinvasion effects reduce abundance across the study as a whole and limit radically the ability to discriminate between treatments. It is impossible to determine from small-scale field tests, how toxic pesticides are to beneficials, and it is also not possible to detect impacts or even positive attributes of unsprayed *Bt* plots.

To confirm that scale and reinvasion effects may have compromised the WideStrike field test data package, at least in Arizona, the Panel noted that chlorpyrifos was used in the non-transgenic control. This material is very toxic to natural enemies, including spiders, and the Agency should reasonably have expected significant reductions in the non-*Bt* plots as a check that the experiment had sufficient sensitivity to discriminate treatment effects.

The Panel noted that the numbers of organisms in the Louisiana study seemed to be extremely low, and this requires explanation if data are submitted for review in the form of a final report. The Panel suggested that the numbers were too low to discriminate treatment effects.

The Panel also noted that the current codification of the risk assessment approach does not allow some important positive issues to be highlighted. An important advantage of the use of WideStrike cotton is much lower application rates of chemical pesticides. The early field trial

showed, as might have been predicted, that the number of species was higher in the WideStrike field than in the conventional (non-transgenic) cotton culture involving heavier application of chemical pesticides.

With respect to soil degradation, the Panel noted that, strictly speaking, the protein half life study quantifies the temporal change in activity, not of intact protein. If there was direct evidence, then the protein concentration was decreasing. Otherwise, a short sentence qualifying the results would be helpful. Although unlikely, some protein might still be intact but bound or sequestered in some manner such that its activity is not expressed.

Agency Charge

3. The Agency has sufficient information to conclude that there is no hazard from the proposed uses of WideStrike cotton to non-target wildlife, aquatic and soil organisms. However, the Agency is requesting additional, primarily long term effects data that were recommended by previous Panels for PIP corn. The supplementary studies would provide additional weight to support the Agency's conclusions.

The Panel is asked to comment on (a) the scientific value of the proposed additional studies that are identified at the end of the Environmental Assessment section, including avian chronic exposure testing and multi-year field and soil persistence/terrestrial expression studies, and (b) the applicability of these data to PIP cotton.

Panel Response

The Panel was asked to comment on the scientific value of additional studies to assess longer-term effects associated with the commercial cultivation of WideStrike cotton. Three aspects were considered: (1) additional avian chronic exposure testing; (2) multi-year field studies to assess potential longer-term effects of WideStrike cultivation on persistence of toxins in the soil; and (3) populations of non-target organisms. The Panel agreed that such studies are of considerable scientific value and finds that with the exception of avian chronic exposure testing, that such studies are applicable to *Bt* cotton and should be considered. While the Panel is aware of limiting its comments to risk assessment, and not risk management issues, the Panel did not find that such long-term studies should necessarily be required as a condition for registration of WideStrike cotton. Overall, the likely reduction in the use of broader-spectrum insecticides afforded by the cultivation of *Bt* cotton in general is likely to have positive effects on the ecosystems. Nonetheless, longer-term and broader-scale evaluation of PIP crops will be necessary for improved ecological understanding. The Panel believed strongly that long-term field studies should not proceed without some guidance relative to experimental protocols and clearly defined endpoints. Without such guidance, we are unlikely to resolve any unexpected detriments or benefits associated with the use of transgenic crops.

Avian Chronic Exposure Testing

The Panel found that testing of multi-year field effects on non-target vertebrates is

generally essential. It can readily be seen from the numerous publications Brewer et al. (1989, 1989a, 1990, 1992; Tank et al. 1992, 1992a) that significant annual differences are apparent with the same pesticide treatments by the same applicators in the same fields. However, Tier 1 testing of representative vertebrate models shows that the *Bt* toxins found in WideStrike cotton have no measurable short-term adverse effects. Furthermore, direct exposure to *Bt* toxins through direct ingestion of seed cotton or through ingestion of intoxicated arthropod prey by avian species in the field is likely to be minimal because birds rarely forage in cotton fields (September 21-24, 1999 FIFRA SAP Meeting). Thus, the probability of direct chronic exposure to *Bt* toxins in cotton fields is unlikely. Indirect effects on avian populations are possible through the elimination of caterpillar prey. However, since foraging in cotton is minimal, this effect is unlikely to be important. As a result, the Panel finds little scientific merit in conducting additional chronic exposure testing.

Soil Persistence

Bt toxins find their way into the soil either through root exudates or the breakdown of crop residues (shoots and roots). The Panel agreed that data submitted relative to soil persistence suggest that Cry1 proteins are quickly degraded. However, persistence of the Cry proteins goes hand in hand with persistence of crop residues in which they reside. The registrant conducted tests on toxin persistence in two ways; (1) by incorporating purified Cry1Ac and Cry1F alone and in combination into soil and (2) by incorporating finely ground WideStrike plant material into a cotton soil. In both cases, the ability to detect the toxin was monitored as was the decline over time of the toxicity to a target insect.

The Panel had several comments about this approach. First, the Panel found that incorporation of purified toxins serves only to demonstrate that the toxins are biodegradable. It does not reflect what is likely to happen in the field, nor are half-life values derived from these data meaningful. The Panel agreed that, provided it is accessible, the toxin will be broken down.

Second, use of finely ground plant material does not reflect actual field situations where residues are incorporated in large 'chunks' that require shredding to initiate decomposition. This discrepancy may be mitigated somewhat by the fact that defoliant use and mechanical harvesting in cotton production does in fact generate reasonably pulverized material after mechanical harvesting is complete. Finely ground plant material obviates the role and possible effects on shredders and also provides a vast surface area for microbial attack thus leading to enhanced rates of decomposition. Access to the toxin by proteases is key to its decomposition. If the toxin is bound in residues, sequestered in soil aggregates or bound to clays or humic material, it is not accessible and hence will accumulate or at least enjoy longer term persistence in the soil. This is likely to be ecologically unimportant as a toxin or toxicant bound in such a manner to resist biodegradation is unlikely to be bioavailable to most soil organisms (Anhault et al. 2000; Awata et al., 2000; Gevano et al. 2000, 2001; Morrison et al. 2000).

A number of studies have shown that Cry toxins from *Bt* resist microbial degradation by binding to clay and humic acid fractions in soil, but retain insecticidal activity despite being bound (Venkateswerlu and Stotzky, 1992; Tapp and Stotzky, 1995; Koskella and Stotzky, 1997;

Crecchio and Stotzky, 1998; Tapp and Stotzky, 1998; and Saxena et al., 1999). Further, Saxena and Stotzky (2000) showed that larvicidal activity increased with the length of time *Bt* corn plants were grown, suggesting that microbial degradation may not have kept pace with the increased root exudation of the toxin into soil with time. An alternative explanation may be that the toxin was unavailable for degradation as it was bound, and concentrated with time on the surface of clays and humic materials.

The Panel found that the fate of the toxin in different soil types with different charge characteristics is needed. Studies to understand the mechanism of toxin binding in relation to access of proteases is also important. There is little information on the fate and persistence of *Bt* toxins in the field, with the exception of one field study reporting that while the levels of Cry1Ab toxin in no-till corn decreased to 0.3% of the initial concentration after 200 days, degradation was initially delayed and lower in a conventionally tilled system (Zwahlen et al., 2003a, b). However, Head et al. (2002) did not detect Cry1Ac toxin in soil samples taken about 90 days after the last planting from fields planted with *Bt* cotton for several consecutive years. The Panel agreed that more data are clearly needed here as this is also a point of ecological as well as public concern. These findings are consistent with and largely echo the concerns expressed by the August 27, 2002 SAP and the current Panel fully agree with the recommendations forwarded by this prior SAP for future multi-year testing. The current Panel further suggested that additional studies should consider the role of residue degradation, residue dry matter partitioning and major pools of structural carbon, and measurement of effects on residue shredding organisms.

Non-Target Effects

Not withstanding some of the flaws outlined under Question 2 to this Panel, Tier 1 testing under current guidelines has demonstrated no measurable short-term hazard from the two Cry toxins expressed in WideStrike cotton to representative organisms from terrestrial, soil and aquatic systems. In addition, *Bt* Cry toxin proteins are susceptible to metabolic, microbial, and abiotic degradation once they are ingested or excreted into the environment. As a result, unlike many insecticides that persist and accumulate through the food chain, Cry proteins are not expected to have the potential for bioaccumulation.

Thus, the Panel agreed with the Agency's conclusion that the Cry toxins in WideStrike cotton pose no short-term hazards. However, the Panel also agreed with the Agency that Tier 1 testing is insufficient for assessing the potential for long-term environmental effects. Assessing this level of hazard will require carefully designed field studies conducted over longer periods of time and at relevant spatial scales. The bulk of evidence (Groot and Dicke 2002, Conner et al. 2003) suggested that most of the effects, if any, from *Bt* toxins in the field will be either sublethal and/or result largely from indirect effects via complex community interactions. Tier 1 is limited to direct exposures and largely measures only lethal effects. Sublethal effects are usually subtle but may have significant effects on longer-term population dynamics and community level interactions (Elzen 1989; Croft 1990). Examples include reductions in adult longevity, reductions in reproductive output, increased susceptibility to other environmental factors, and altered searching behavior in the case of predators or parasitoids. Although some of

these factors could be examined in laboratory studies, their implications and impacts can only be realistically evaluated with longer-term field studies. Indirect effects are more complex and likely to be more pervasive due to the large number of interactions. As an example, the soil food web represents an intricate set of feeding relationships.

To evaluate the risk to these organisms of adverse effects of the stacked Cry1Ac - Cry1F genes requires a consideration of the hazard these toxins present and the level of exposure the organisms are likely to have. Extant research has shown that there is no direct toxicity to soil bacteria and fungi, rather, these are the organisms responsible for active degradation of the Cry toxins in soil. Higher order feeders such as protozoa, nematodes, collembolans, mites and earthworms have no receptors for toxin binding and hence are unlikely to be adversely affected by the presence of the toxin in the plant tissues being broken down and thus no hazard. Field evidence with *Bt* corn supports this point (Devare et al. 2004).

In general, the evidence presented by the registrant and evaluated by the Agency for collembolan and earthworms also indicated a lack of direct toxicity. However, the Panel questioned the veracity of the collembola data, because the first two tests on the Cry1Ac pure toxin indicate an effect on collembola fecundity, which was reduced by close to 50%. When the two purified toxins, Cry1Ac and Cry1F, were tested together or plant tissue expressing the Cry1Ac toxin were fed to collembolans, no adverse effects were detected. The report submitted to the Agency for review suggested the Cry1Ac toxin may have been contaminated in some way. If the Cry1Ac preparation used was compromised, why was the reduced fecundity not also observed in the combined test? Long-term adverse effects are not precluded by these results. Changes in the biological quality of soil accrue slowly. Even in conservation agriculture, no-till systems and legume rotations, benefits may not be detectable for 5-10 years. In order to have confidence that WideStrike does not have adverse ecological effects, long-term, post-release monitoring is needed. As already noted, effects of the toxin may be sublethal or cumulative and the possibility of this needs to be ascertained.

One aspect of crop assessment that has not been dealt with adequately in the current analysis was whether there are changes in dry matter partitioning or carbon allocation within the WideStrike cotton plant tissues that could influence the decomposition dynamics of the crop residues after harvest. The soil microbial community relies on fixed C in plant material for energy and nutrients for metabolism, growth and reproduction. Different forms of C are more or less readily decomposed. Simple sugars and amino acids represent very labile C pools, whereas cellulose and hemi-cellulose are more stable pools and lignin the more recalcitrant pool. Should the incorporation of the Cry genes lead to unintended changes in the way that structural C is allocated within the plant, then decomposition dynamics of the material could be altered. An increase in lignin, for example, would result in a slower rate of decomposition. This was a result reported by Stotzky (2000).

Stotzky (2000) reported that CO_2 evolution from soil amended with ground *Bt* corn biomass expressing the Cry1Ab gene was significantly lower than that from soil containing unmodified, isogenic corn biomass. While the mechanism by which Cry1Ab might depress microbial activity is unclear, unintended changes in structural C allocation within the plant is a

possibility and Saxena and Stotzky (2001) went on to conclude that increased lignin content in the corn was responsible for their observations. Stotzky has also reported that finely-ground *Bt* rice, potato, and tobacco residues degrade more slowly than their non-*Bt* counterparts in laboratory studies for reasons that are unclear (Stotzky 2002), as no differences in lignin content were detected for these other crops. Analyzing transgenic crop residues for proportions of structural C in different forms may be useful for addressing concerns for potential differences in the rates of residue decomposition between *Bt* crops and their non-*Bt* isolines. The relevant comparisons would be: (1) insect affected non-transgenic cotton, as C partitioning is grossly affected by insect feeding; and (2) insecticide protected non-transgenic cotton, the commercial standard. Once structural C forms have been quantified, a soil organic matter dynamics model such as Roth C or the Century model could be used to predict if any change in decomposition dynamics would be expected. The Panel noted that finely ground plant material is not an appropriate means to assess decomposition dynamics that might be operating in the field, nor is it the best means by which to evaluate toxin persistence (as discussed above). This is another reason why long-term post-release monitoring is important and why it should include an analysis of the fate of the cotton residues under commercial field conditions.

In and of itself, slower decomposition of residues is not a bad thing. Conservation agriculture strives to increase soil organic matter content and slower decomposition might serve to be beneficial for overall soil quality. However, a build-up of crop residues may also be indicative of non-target toxicity to a key group of soil decomposers called the shredders. These are micro-arthropods for the most part, mites being a prime example. Adverse effects on mites could be quite destabilizing to the soil food web, and an effect such as this might not be picked up without longer-term field monitoring. Some consideration should be given to using mites as a target group for toxicity testing due to their important role in communination.

Overall, long-term testing on relevant scales will only be possible once registration is granted and WideStrike can be grown commercially. The Panel recommendations for long-term evaluation are consistent with the spirit of recommendations made by NRC (2002) and several previous FIFRA SAPs (e.g., December 1999, August 2002). The NRC, for example, envisioned long-term field testing of non-target effects and other environmental and pest management concerns as part of what they call “postcommercialization validation testing”. The main thrust of such studies would be to conduct appropriate, hypothesis-driven, and adequately replicated field studies in large commercial-scale plots for the purpose of determining the accuracy and adequacy of pre-commercialization testing. This same general reasoning was expressed by the August 2002 SAP. A critical, and still unresolved issue that has been put forward by both the NRC (2002) and prior SAPs is the lack of guidelines for conducting long-term field studies such that these studies will provide meaningful and robust results. For example, (1) which methods and types of data should be collected; (2) what spatial scale should be examined; (3) which experimental designs are most appropriate; and (4) most importantly, what criteria will be used to determine impact or the lack thereof? The Panel believes that long-term field studies should not proceed without some guidance relative to protocols and clearly defined endpoints.

The Panel recognized that field studies have been and will continue to be conducted to examine non-target effects. A number of field-level studies have been published on transgenic

corn and cotton expressing various Cry proteins in the last few years (e.g., Candolfi et al. 2004; Musser and Shelton 2003; Men et al. 2003; Al-Deeb et al. 2003; Al-Deeb and Wilde 2003; Dively and Rose 2002; Moar et al. 2002; Naranjo 2002 and Devare et al. 2004). In general, these studies largely support Tier I hazard testing results reported to the Panel and demonstrate no measurable adverse effects. Several of these studies have been relatively long term ($\geq$ 3 years). However, in light of the issues raised by Jepson (2002) and previous FIFRA SAPs regarding scale, design and sampling issues, it remains unclear whether these studies would be considered sufficiently robust to definitively address population and community level effects if such effects exist. Nonetheless, scientists are using the growing body of data to address some of the issue of concern to the Agency and many of these are being conducted independent of registrant needs. It seems likely that these types of studies will continue to be conducted with or without the support of industry and it would be prudent if the scientific community could provide guidelines that would ensure and perhaps improve the quality and value of the results for both risk assessment and broader ecological understanding. Issues include plot sizes, replication, sampling methods, sampling intensity, appropriate positive and negative controls, taxonomic coverage and clearly specified endpoints. The Panel suggested that some of this guidance may be extractable from existing and ongoing studies.

One of the more significant aspects of field testing that needs to be addressed is spatial scale. Jepson and colleagues (Jepson and Thacker 1990; Sherratt and Jepson 1993; and Jepson 2002) provide compelling evidence that spatial and temporal components must be considered in evaluating toxicological effects of insecticides and that this is largely driven by the mobility of the species under consideration and their phenology and ecological requirements. While the Panel believed that it would be desirable to conduct studies at spatial scales mimicking commercial production practices, it may not always be feasible to do so based on economic and logistical considerations. Directly working in commercial fields often removes an important level of control necessary to implement a consistent experimental design. Insecticides needed for other pests and other production constraints may interfere. On the other hand, establishing independent commercial-scale research plots may be cost-prohibitive, especially if studies are to be conducted with sufficient replication. Very large plots may provide the necessary independence, but also introduce significant within-plot variation thereby requiring additional sampling for precise estimation (Jepson 1994). Although more commercial-scale study plots may be desirable, the issue of independence versus heterogeneity will need to be assessed relative to plot size. How large do plots really need to be? This will likely be system specific and depend on the taxa being assessed. For example, relatively small plots (< 0.5 acres) may be sufficient for studying some predatory arthropods. Naranjo (2002) found that 0.25 acre plots were sufficient to determine differences in a positive control of broad-spectrum insecticides for a complex of 20 arthropod predator taxa, indicating that there is a fairly high degree of fidelity within plots of this size. Prasifka et al. (2004) has shown that *Hippodamia convergens* readily move from sorghum to cotton on a commercial scale, but they also showed fidelity of this beetle in cotton even in the absence of its preferred aphid prey. Many of the predatory arthropods found in cotton are generalist feeders and are relatively insensitive to the densities of any one prey. In the western US, pink bollworm, the main target of *Bt* cotton, is essentially invulnerable to natural enemies. Thus, the removal of this insect from the system has little effect on natural enemy populations. Other Lepidoptera in this area are relatively rare and their presence is

inconsistent. Abundant prey in the form of thrips, whiteflies and *Lygus* bugs are unaffected by *Bt* cotton. Thus, predators may not be affected by changes in prey density that may cause them to disperse.

Replication is another critical component of the experimental design as it bears directly on the issue of statistical power, or the ability to detect differences that may be relatively small between *Bt* and non-*Bt* plots. This aspect will clearly be system-specific. Perry et al. (2003) estimated that a sample size of 60 may be necessary to measure environmental effects of herbicide-tolerant crops in the UK, and higher sample sizes were suggested by NCR and the August 2002 SAP. In a recent meta-analysis of a 5-year study, a sample size of 40 was only sufficient to discern a 20% significant difference between densities of arthropod predators in non-*Bt* versus *Bt* cotton expressing Cry1A. In the long run, there will likely be a trade-off relative to plot size and replication and this will need to be determined on a case by case basis depending on the taxa to be examined and the ecological setting. In addition, it may be possible to perform retrospective power analyses on many of the past and ongoing non-target studies that would be useful in developing guidelines for future studies.

Sampling methods are another important consideration. For example, sweep nets and beat clothes are a common method of measuring relative abundance of foliar-dwelling arthropods in row crops such as cotton, and these methods are probably sufficient for comparative studies. Absolute sampling methods such as whole plant counts would be desirable, but such methods are likely to be much more costly due to aggregated arthropod distributions. In the supplemental multi-year field studies being requested by the Agency, protocol suggests the use of sweep nets, sticky traps and pitfall traps to assess effects on non-target arthropods. These methods will likely produce useful data, but careful consideration will need to be given to the number of samples collected in each experimental unit. The interplay of plot size, replication, sampling method, and sample size for these methods will ultimately determine the statistical power and the ability of the assessment to discern relevant differences.

Taxonomic resolution is another aspect that is critical to address. Many of the existing non-target field studies have focused on selected taxa. Such an approach is generally necessary as there are well over 500 species of arthropods alone inhabiting cotton across the southern tier of the US, and the soil invertebrate community is large and diverse as well. Predaceous and parasitic natural enemies have been the focus in many studies of *Bt* crops. Such species are consistently present in the cotton system; many occur at moderate to high densities, and further, their standing in higher trophic levels may provide insight into effects in lower trophic levels and may act as sensitive indicators of community change. The Panel suggested that assessment of such taxa may be useful for multi-year testing. The supplemental studies by the registrant provided relatively broad taxonomic coverage. In particular, the study in Maricopa, Arizona attempted to examine all above-ground taxa. Such studies complicate analyses, and careful thought will need to be applied in interpreting the meaning of any observed differences. The resolution of a large percentage of the taxa will likely be too poor to enable definitive conclusions. However, the Panel recognized that studies like this may be helpful in narrowing the taxonomic coverage of future, more-targeted evaluations. The choice of taxa to examine may be system specific and should include consideration of likely exposure to *Bt* toxins through either direct or indirect means.

Final considerations include the inter-related issues of study length and desired end-points for assessment. With clear focus on short-term hazard, Tier 1 testing generally uses direct mortality within a short period of time as the designated endpoint. Such simple endpoints are not likely to be useful, or even measurable, in long-term field assessments for environmental effects. Instead, effects (either positive or negative) are likely to be manifested as reductions or increases in population densities of specific taxa, changes in diversity, or changes in community organization and function. As indicated above for the soil system, such changes may be slow and take many years to shape observable changes. However, even when changes do occur, what criteria should be used to judge a positive, negative or neutral effect? For example, a five-year study on the non-target effects of Cry1A-expressing cotton on 20 arthropod predator taxa (Naranjo 2002, in part) showed a general pattern of population reductions in *Bt* cotton. Changes in this select community in *Bt* cotton varied from a negative change of 19% in one year to a positive change of 15% in another year when compared to non-Bt cotton. Irrespective of statistical significance, the predator community declined by 8% averaged over the entire 5 years. This average value would have been different after 2, 3 or 4 years and would likely be different if the study proceeds another 5 years. In contrast, the positive control treatments based on use of broad-spectrum insecticides caused an average reduction of 62%. During this same period, functional studies on the natural enemy community showed no statistically significant differences in rates of predation and parasitism between unsprayed *Bt* and non-*Bt* cotton on two different pests. Irrespective of statistical significance, rates of predation on pink bollworm eggs and pupae declined 4-6% on average in *Bt* cotton; parasitism and predation of whitefly nymphs increased 5-8% on average in *Bt* cotton. Has this system been impacted either positively or negatively by the use of *Bt* cotton?

Horizontal Gene Transfer

Some concern has been expressed over the potential for transgenes to move from *Bt* crops to soil microorganisms. In this regard, we should consider the likelihood of this occurring in the 'best-case' scenario – that is between closely related bacteria in the soil and contrast this with the probability presented by genes located in the plant genome. There is sufficient genetic similarity within this clade to question whether *B. cereus* and *B. thuringiensis* are distinct species because what defines *B. thuringiensis* as distinct from *B. cereus* is the presence of the plasmid in *Bt* that contains the Cry toxin and associated cytotoxic genes. If the plasmid is transferred to *B. cereus*, it becomes indistinguishable from *Bt*. How frequently this plasmid is exchanged among members of this clade in soil is unknown. *B. anthrasis* is closely related, but distinguished by carrying a different plasmid with a different set of toxin genes. Recombination or lateral gene transfer between *Bt* and *B. anthracis* has not been reported. Thus in nature such gene flow appears to be restricted even between very closely related species. Given this, what is the likelihood that a full length toxin gene will be transferred intact to a recipient bacterium? First, the needed flanking insertion regions would need to be present in the recipient, which in itself, is highly unlikely. Second, there would need to be sufficient selective pressure operating to drive the acquisition of the genes by the recipient bacterium. Such selective pressure is highly unlikely in the soil environment is. Whilst it cannot be completely ruled out, the likelihood of *Bt* genes transfer from a transgenic crop to soil is exceedingly small.

WideStrike Cotton Insect Resistance Management

Agency Charge

1. *Dose.* Three methods (two laboratory and one field) outlined by USEPA's Scientific Advisory Panel (1998) were used to demonstrate that WideStrike cotton expresses a high dose of Cry1Ac and Cry1F against tobacco budworm (*Heliothis virescens*, TBW). Dow AgroSciences (Dow) employed one laboratory-based and one field-based method to demonstrate that WideStrike cotton has a high dose (Cry1Ac only, Cry1F is non-toxic) against pink bollworm (*Pectinophora gossypiella*, PBW) Results of two field studies indicate that WideStrike cotton produces a moderate dose against cotton bollworm (*Helicoverpa zea*, CBW), but a very high level of control (94%). The Agency concluded that WideStrike cotton expresses a high dose of Cry1F and Cry1Ac against TBW (Cry1Ac alone expresses a high dose and Cry1F a nearly high dose); a moderate dose of Cry1F and Cry1Ac against CBW, and a high dose of Cry1Ac against pink bollworm.

The Agency asks the SAP to comment on the Agency's analysis of dose for TBW, CBW, and PBW, the likelihood that resistance will be inherited as a recessive trait, and its impact on insect resistance management for WideStrike cotton.

Panel Response

The Panel addressed this question with an understanding that a combination of field and laboratory studies performed by the registrant had evaluated the toxicities of the Cry1Ac and Cry1F expressed in WideStrike cotton against TBW, CBW, and PBW. The Panel also provided a detailed response of field efficacy and high dose considerations.

The combination of the laboratory and field approaches clearly demonstrated that the combined expression of *Bt* proteins in WideStrike cotton meet the Agency's definitions of high dose for PBW and TBW. Based on existing knowledge of the reduced susceptibility of CBW to Cry1Ac, the registrant did not pursue the full spectrum of high dose studies on CBW, but still managed to show good field control relative to non-Bt cotton. In general, there was good replication within trials, and with some tests, replication across states, altogether building a robust data set in support of the contention by the registrant that toxin expression in WideStrike cotton constitutes a high dose for PBW and TBW.

One caveat with the field efficacy trials was that 9 of 19 trials were conducted using artificial infestation techniques in the trial plots. While the success of this approach was gauged in some trials by substantial infestations that resulted in the unsprayed control plots, and is often required to obtain a sufficient infestation in the field, the general practice of using laboratory colonies in field studies should be avoided when possible due to concerns about reduced vigor and fitness in frequently inbred lab strains. The potential risk is that genetically compromised lab strains may not provide the full challenge to a breeding line as do natural field populations, and therefore may not represent a full test of the resistance in the breeding line. In future considerations of such data, the Agency may want to consider guidelines for use of laboratory

colonies to better guarantee quality control. Poorly managed colonies have a greater probability of producing results consistent with a high dose.

Having established the high dose trait of WideStrike cotton against PBW and TBW, and given the broad history to date of resistance being recessive wherever resistant larvae can survive on *Bt* transgenic hosts (Tabashnik et al. 2003), the Panel concluded that it is valid to assume that resistance will likely be inherited as a recessive trait. CBW is more tolerant of both proteins and it seems possible that resistance will be less recessive. Without abundant alternate host plants that do not express these same *Bt* genes, CBW would be more prone to resistance.

Based on these assumptions and on the criteria to determine functional dominance established by previous FIFRA SAPs, the combined expression of *Bt* proteins in WideStrike cotton meets the Agency's definitions of high dose for PBW and TBW. In addition, reasonable doses of the combined protein were evident for control of CBW. Based on the high dose evidence, the Panel concluded that it is valid to assume that resistance occurring in PBW or TBW will likely be inherited as a recessive trait. However, CBW is more tolerant of both proteins and it seems possible that resistance will be less recessive. These studies support the position for durable sprayable *Bt*s in organic cotton production and other crops, such as tomatoes. The same high dose/refuge strategy practiced thus far as a resistance management approach for Bollgard cotton should be applied for WideStrike.

WideStrike cotton does appear to offer a high dose for TBW, a high dose of Cry1Ac for PBW, and reasonable doses of Cry1F and Cry1Ac for CBW. The same high dose/refuge strategy practiced thus far as a resistance management approach for Bollgard cotton should be applied for WideStrike.

Field Efficacy

Five field efficacy trials each were completed for TBW and CBW while a single trial was run for PBW in Arizona. In addition to these three principal pests, field efficacy data was also collected for 5 other cotton pests (Dow Agrosciences submission: "Efficacy of Cry1F/Cry1Ac Cotton Against a Wide Range of Lepidopterous Pests", 2002). By measuring cotton boll damage and infestations, the collective field data were conclusive in establishing that WideStrike cotton provided superior control of TBW and PBW relative to either sprayed or untreated plots of the conventional cotton line PSC355. In the case of CBW, boll damage/infestation levels in MXB-13 were equal to or less than the unsprayed control in 96% of the comparisons (53% significantly less damaged) while 58% of the comparisons showed equal or less damage than the sprayed control. Although some trials were conducted in 2001 of two and one gene plants that showed lower efficacy, these trials were believed to be from early selections that likely had some non-expressing plants.

High Dose

To determine if the two *Bt* proteins expressed in WideStrike cotton meet one of the Agency's definitions of high dose, i.e. 25x the dose required to kill 99% of susceptibles,

laboratory bioassays that incorporated a 25-fold dilution of carpal wall tissue or leaf tissue were used against neonates of PBW and TBW, respectively. Extremely high mortalities and growth inhibition were observed against both species. An additional test using 2- day old TBW larvae determined to be approximately 25-fold more tolerant than neonate larvae was conducted using fresh WideStrike leaf tissue grown in Mississippi or California, as well as 2 different laboratory colonies of TBW. The leaves grown in Mississippi proved to be more toxic than those grown in California, but across both studies mortality was considered to be extremely high. The field component of the high dose testing was performed for both PBW and TBW to determine if field mortality on WideStrike met the other Agency definition of high dose, i.e. mortality >99.99%. For PBW, only one third instar was found in a cotton boll out of >12,000 infested. Inspections of squares and bolls using three different methods revealed three live TBW neonates out of more than >270,000 fruit infested, thus demonstrating field mortality meeting the high dose definition. No specific studies were attempted in order to satisfy the high dose criterion for WideStrike against CBW.

Also of interest for the future are the doses for other species of Lepidoptera, which have not been of concern to date for resistance management due to their broad host range:

Spodoptera frugiperda (Fall armyworm): Average survival was 8.1% in field test. Exposed neonate larvae did not develop. Therefore a high level of control of this species, but not a high dose.

S. exigua (Beet armyworm): Controlled in the field. However, the Panel questioned whether this was a moderate dose?

S. eridania (Southern Armyworm): Excellent control with less than 0.8% defoliation observed in field studies.

Pseudoplusia includens (Soybean looper): 91-98% controlled in the field. A high level of control but not a high dose.

While not of concern unless and until more crops are transformed with *Bt* genes, these are important background data for considering resistance management strategies for these species.

Agency Charge

2. *Cross-resistance.* Resistance to *Bt* proteins can occur through several different mechanisms. Alteration of binding receptors has been the most common mechanism observed. The binding patterns of the Cry1F and Cry1Ac proteins in CBW and TBW indicate there are shared and unique binding sites. In TBW, Cry1Ac binds to at least three receptors, while Cry1F binds to at least two, only one of which binds Cry1Ac. In CBW, Cry1Ac and Cry1F each bind to at least four receptors, of which two are shared. For CBW, approximately 60% of Cry1Ac binding is to receptors that also bind Cry1F, and the remaining 40% of Cry1Ac binding is to receptors that do not bind Cry1F. Incomplete

shared binding is expected to lead to incomplete cross-resistance when resistance is mediated by receptor changes. Thus, a mutation in a gene that codes for a receptor that binds both insecticidal control proteins (ICPs) will not prevent all binding of either ICP and thus alone will not allow high survival of the insect bearing even two copies of it, on WideStrike (Cry1F/Cry1Ac) cotton plants.

The Agency asks the SAP to comment on EPA's conclusion that incomplete shared binding of Cry1Ac and Cry1F receptors, in TBW and CBW, is expected to lead to incomplete cross-resistance and thus the likelihood of enhanced survival on WideStrike cotton is expected to be small. Please comment on EPA's conclusion that resistance is more likely to be associated with receptor binding modifications rather than other mechanisms of resistance such as detoxification in the midgut lumen by proteases that cleave the insecticidal control protein(s), metabolic adaptations, protease inhibition, gut recovery, and behavioral adaptations.

Panel Response

Apart from the known protoxin solubility and proteolytic processing factors governing *Bt* toxin specificity, the insect host range of *Bt* toxins is due to the recognition of target receptors on larval gut epithelial cells. As resistance management plans are based on data characterizing these molecules, it is relevant to determine which toxin classes compete for larval gut receptor binding sites. Intimate knowledge of the target insect's *Bt* receptors are crucial to predict and avoid the possibility of cross-resistance occurring. This forms the subject of the first part of the question dealing with the Agency's conclusion that 'incomplete shared binding of Cry1Ac and Cry1F receptors, in TBW and CBW, is expected to lead to incomplete cross-resistance and thus the likelihood of enhanced survival on WideStrike cotton is expected to be small.'

While the Panel supported the Agency's conclusion that incomplete shared binding of Cry1Ac and Cry1F receptors in TBW and CBW is expected to lead to incomplete cross-resistance, differences were expressed on the molecular mechanism involved in the process. In addition the Panel raised the issue that another as yet unidentified major resistance mechanism may not occur. The results presented by the registrant on incomplete shared binding of Cry1Ac and Cry1F receptors are encouraging, but given our still modest understanding of the relationship between membrane binding assays and actual toxicity to the target insects, it would be premature to conclude that there will be no significant cross-resistance between the two toxins. Cross-resistance does not seem likely, but cannot be completely dismissed as a possibility. The Panel cited, as the basis for this cautionary, the altered cadherin genes described for the YHD2 strain of *H. virescens* and a strain of pink bollworm *Pectinophora gossypiella* from Arizona. These alleles seem to be characterized by high fitness costs and relatively poor survival on *Bt* plants. In contrast, resistant strains of diamondback moth, for which efforts to find either altered cadherins or altered aminopeptidases have been unsuccessful, prosper on *Bt* plants and seem to have relatively low fitness costs. This suggests that a major mechanism of resistance, already widespread in field populations of diamondback moth, remains to be discovered. That being said, given the limited or incomplete complementarity between shared Cry toxin binding sites in the principle lepidopterans selected so far (TBW and CBW), it is

unlikely in the absence of another mechanism (as described above) imparting broad cross resistance that either of these pests will develop resistance to either Bollgard II or WideStrike pyramided cotton.

The Panel was in agreement that the issue of insect binding proteins is a complex one as it generally involves more than a simple 'one toxin-one binding protein' scenario. Current data indicated that any one toxin may potentially have one to six insect binding molecules which in turn may share a binding site with a different toxin.

With regards to CBW, the Agency review based its assessment on competition binding data on larval brush border membrane vesicles (BBMVs) demonstrating that Cry1F possessed a low affinity for a receptor subset shared with Cry1Ac. The data also showed that in the presence of high saturating levels of Cry1F, approximately 40% of the Cry1Ac total binding capacity was still available, suggesting that 40% of the total Cry1Ac-specific sites are unique to Cry1Ac and not shared with Cry1F. Since CBW is not sensitive to Cry1F, the Panel was uncertain if the idea of Cry1F/Cry1Ac cross-resistance in the conventional sense was applicable in this case but concluded that the presence of Cry1F would not affect Cry1Ac toxicity. If the shared receptor lost its ability to bind to Cry1F (or Cry1Ac), survival on WideStrike cotton would not be expected since an additional mutation would also have to occur in order to produce a homozygous resistant insect. However, for an insect that is sensitive to both Cry1F and Cry1Ac toxins, which is the case for the TBW, the molecular details are less certain.

A general comment was made about toxin-receptor binding methodology field in that it contained a number of inherent weaknesses. The Panel believed it was important to outline these binding weaknesses as IRM decisions are based on models generated by these data. The Panel then noted the differences between receptor binding and functional receptor binding. To illustrate this point, the Panel mentioned that toxicity to insect larvae have been correlated with the existence of high affinity binding sites on BBMVs of susceptible insects. However the opposite may not be true. There are examples of toxins that bind to BBMVs with high affinity but are non-toxic to the insect. The most relevant were data showing that Cry1J, a protein nontoxic to TBW, possessed a five-fold higher affinity for the *H. virescens* receptor A than Cry1F which is toxic to CBW (Jurat-Fuentes and Adang 2001). A similar example was also mentioned for the non-toxic Cry1Ac for *Spodoptera frugiperda* (Luo et al. 1999). The key point the Panel wished to make was that some predicted high affinity binding sites may not directly play a role in toxicity i.e., they are non-functional. In reference to earlier sessions on Ecological Risk Assessments where the possibility of looking for receptors in non-target animals was discussed, the Panel stressed their concern about the potential to generate spurious binding results with toxins like Cry1Ac which is known to have lectin-like activity (Cry1Ac binds to N-acetylgalactosamine residues). This could result in interactions with binding potential molecules in other non-target species but yet would represent non-functional binding and consequently have no toxicological impact. So, unless the receptors are individually purified and tested, competition binding patterns and what constitutes functional binding, are subject to interpretation of indirect data or deductive reasoning.

It was originally shown over ten years ago (Van Rie et al. 1989) that CBW had three

receptor populations: receptor A which binds all three Cry1A proteins, receptor B which binds only two (Cry1Ab and Cry1Ac) and receptor C which is a Cry1Ac only receptor. In the case of CBW, the EPA's assessment is based on newer data that confirmed the earlier work and expanded it to include Cry1F and Cry1J. This inclusion helped the Panel to underline a second weakness in binding assays which is, not all toxins can be labeled. In this specific case, radioiodination of Cry1F resulted in loss of binding capacity and subsequently, toxicity. Although with unlabelled Cry1F one can demonstrate a shared receptor site with Cry1Ac, one cannot, however, assess whether Cry1F itself has its own unique (i.e., unshared with Cry1Ac) high affinity receptor binding site. This is where the concept of incomplete cross-resistance as initially put forth by the Agency becomes uncertain as it is based on the existence of three receptor populations. One population is shared between Cry1F and Cry1Ac and two unshared populations, one for Cry1Ac and one for Cry1F. However, based on current methodology limitations, the Panel could not conclude if Cry1F has such an unshared population.

A final weakness in current binding assays discussed by the Panel involved the use of ligand blot assays. Looking at the model presented for CBW showing four receptor groups (Jurat-Fuents and Adang 2001), there was a separate binding protein for Cry1F independent of Cry1Ac. The existence of this binding protein was deduced from ligand blot experiments. Ligand binding assays are assays where BBMV binding proteins are denatured by boiling in anionic detergent (SDS) and separated based on their molecular weights in SDS-PAGE gels. These gels are then blotted onto a membrane and probed with a labeled toxin. Unfortunately, this is a difficult, highly variable technique, subject to labeling artifacts and differential receptor processing. There are numerous instances in the literature where these ligand blots vary substantially from lab to lab. However, in this case, the Panel was reassured to see that the data also included biotinylated toxins and anti-Cry toxin antibodies to ensure the data was as robust as it could be. If one considered only the competition binding data, only three receptor populations would remain of which one would contain a shared Cry1F /Cry1Ac receptor. The Panel noted that ligand blots indicated the presence of two Cry1F binding proteins from one study (Fuents and Adang 2001) but another study showed the existence of four Cry1F binding proteins (Banks et al. 2001) and that they are of similar size as the Cry1Ac binding molecules. The Panel recommended the Agency exercise caution as ligand blot data can occasionally give spurious results and to take that into consideration in any IRM decision.

The Panel raised the issue concerning the lab derived Cry1Ac resistant strain of *H. irescens* (YHD2) which is cross-resistant to Cry1Aa and Cry1Ab but can bind Cry1Ab and Cry1Ac in a fashion similar to wildtype (Lee at al. 1995). The conclusion from that study showed that the mutation occurred in Receptor A. As the current model for TBW shows that Receptor A binds all three Cry1A toxins as well as Cry1F, the Panel believed that it was important to note that this strain was also cross-resistant to Cry1F, thus illustrating two points:
1) the presence of high affinity binding sites in *H. virescens* BBMVs that do not appear to be involved in toxicity;
2) the possibility that resistance to Cry1Ac could potentially result in cross-resistance to Cry1F as shown in a lab-derived resistant strain (Gould et al. 1995).

In presenting the question of incomplete cross-resistance, the Agency indicated that the

mutation of a shared receptor, when resulting in the loss of one Cry toxin, causes the cessation of binding of the second toxin. The Panel noted exceptions to this assumption by stating various examples in the literature (e.g., *P. xylostella* and *H. virescens*) where the loss of binding to one toxin in a shared receptor did not result in the loss of binding of the second toxin (Ferre and Van Rie 2002). This simply means that the toxins bound to different epitopes on the binding molecules. The Panel further noted that in the case of the 120 kDa aminopeptidase (APN) purified from *Manduca sexta*, these epitopes are situated far enough apart so that two Cry1Ac toxin molecules could actually bind to the APN at the same time (Masson et al. 1995).

In regards to the original hypothesis that incomplete shared binding may lead to incomplete cross resistance, the Panel was not convinced by the newer four receptor site model for TBW since it was unclear that Cry1F had its own unique binding site (unshared with Cry1Ac). The Panel did recognize that the literature indicated a plethora of shared Cry1F/Cry1Ac binding sites in TBW which was presumably a result of similarities in both toxin's domain II loops (Jurat-Fuentes and Adang 2001). These shared sites presumably represent a mixture of different functional genes (i.e. aminopeptidases, cadherins or possibly large glycoconjugates). Therefore, under the assumption of functional binding, the same rules for incomplete cross resistance would still apply, that a mutation occurring in one gene causing resistance through loss of binding to one of the stacked toxins will result in incomplete cross-resistance as the second toxin can bind to another site.

The Panel believed that in addition to incomplete receptor sharing hypothesis, it is important to consider the cost in fitness that would occur with receptor mutation/decreased toxin binding. Resistance via the altered toxin binding mechanism is accompanied by loss of binding function, and therefore presumably imparts a "cost" to resistant individuals. This view is strengthened when both principle binding sites in Lepidoptera involve cadherin and aminopeptidase receptors because each of these receptors are important cellular components required by the insect having significant physiological cell adhesion or membrane binding and transport functions. Apart from having a second different active toxin to bypass the inactivated receptor, the added fitness cost would also contribute to the prevention of cross-resistance under this scenario.

The Panel maintained that broad receptor-based cross resistance is an improbable outcome, especially with refugia, and the emerging information documenting cotton recruitment of unselected CBW from surrounding alternate hosts and regions. Therefore, even dramatic selection with Cry1Ac alone in the absence of refugia is not likely to result in a reduced field efficacy of either Bollgard II or WideStrike cotton against TBW and CBW. The hypothesis of incomplete cross-resistance as stated should only be considered on a case by case basis as what applies to one species may be inappropriate for a second.

The Panel also recommended that the Agency be aware of, and take into consideration, the problems generated with current receptor binding methodologies and urged them to investigate ways to circumvent these problems such as alternate labeling strategies for recalcitrant toxins (S^{35}, C^{14} methylation, etc.) or non-destructive real-time optical measurements of protein binding.

Part 2. Resistance is more likely to be associated with receptor binding modifications rather than other mechanisms of resistance.

Alternate resistant mechanisms do have the potential to occur but to date these have only been demonstrated in the laboratory. Throughout the history of resistance management, lab selection experiments have given results that were inconsistent with those observed in the field, both because laboratory populations are less likely to include rare single major genes (due to an inevitably limited sample size in the initial field collections) and because laboratory selection is more likely to "save" resistance genes that have very large fitness costs in the field. Selection in the field, in contrast, will screen far more rare alleles, and alleles with large fitness costs are unlikely to increase very much. The Panel agreed with the Agency that there is no basis to believe that the occurrence of resistance in the field will be due to a mechanism other than binding site modification.

To put the question into perspective, a summary of the known mode of toxin action occurring in the insect after toxin ingestion is needed. After ingestion in a crystallized protoxin form, solubilization must first occur in the gut of a susceptible insect where gut proteases then activate the protoxin producing a protease resistant core. This activated toxin then attaches to a specific docking protein or receptor on the surface of gut epithelial cells. Functional binding to the receptor is followed by oligomerization into a tetrameric structure and subsequent insertion into the membrane causing a disruption in the cellular ion balance, and eventual cell death through a colloid osmotic lysis process. In theory, toxin resistance can occur at any of these steps.

It was stipulated that the most important type of resistance would be that found under actual field conditions. To date, only one major Lepidopteran resistance mechanism has been reported which was a reduction in toxin binding on gut brush border target sites in the pest *P. xylostella*, the diamondback moth (Ferre and Van Rie 2002). Despite the possibility of resistance through other mechanisms described below, the history of resistance evolution to *Bt* sprays in *P. xylostella*, strongly implies that reduced binding to receptors is likely to be the most common and significant mechanism of resistance, even if the specific details of the reduced binding mechanism varies between species. In principle, all of the other possible mechanisms of resistance are also available in *P. xylostella*, but resistance has repeatedly evolved in the field through receptor binding as at least the main if not sole mechanism of resistance.

Switching from field-derived resistance cases to those produced in the laboratory setting, the Panel pointed out that for the majority of different insect species showing an altered binding resistance phenotype, there also exist resistance strains from the same species showing three major resistance mechanisms other than reduced binding. For example it has been shown that Cry proteins can be detoxified in the midgut through a decreased rate of protoxin activation and an increased rate of toxin degradation in the resistant CP-73 line of TBW (Forcada et al. 1996). In contrast to this, it has been shown in *Choristoneura fumiferana*, even though this insect is highly susceptible to Cry1A toxins, increasing concentrations of gut juice increases the proteolytic degradation of purified activated toxin (Bah et al. 2004). Decreased susceptibility to

Cry1Ac in *Plodia interpunctella* was shown to be due to the absence of a major gut protease (Oppert et al. 1997).

A second mechanism, enhanced epithelial recovery, was evolved after selection with sublethal doses of Cry1Ac resulted in moderate resistance in TBW while similar selection using Cry3A toxin in Colorado potato beetle (CPB) resulted in high (>500fold) resistance via this putative mechanism. Selection with one toxin, Cry1Ac, has also been demonstrated to result in more than one resistance mode of action, strong cross resistance to Cry1Fa and low resistance to Cry2A toxin in TBW. In that Cry1Ac resistant strain CP73-3, midgut epithelial cell damage had occurred in resistant TBW leading to the conclusion that enhanced gut recovery had occurred. In terms of cost, resistance via altered proteolytic processing may or may not impart a cost to the resistant individuals while putative enhanced epithelial recovery, at least in CPB, was accompanied by reproductive and developmental costs. Therefore, if these resistance mechanisms surfaced and they were recessive, as in lab strains and in the absence of *Bt* cotton selection in the field, resistance reversion may occur in field populations.

A third mechanism, behavioral resistance, was also considered by the Panel to be theoretically possible, but most scenarios would depend on detection of the Cry toxin in the transgenic plant. Although anecdotal accounts of these phenomena have been reported from some conventional *Bt* spray formulations, to date, no credible data supporting this phenomenon in Lepidoptera has been reported. In principle, insects could evolve to selectively feed on those parts of transgenic plants that have lower expression. However, feeding on more hidden or lower parts of plants has always been a potential method of avoiding pesticide sprays and therefore of broad cross-resistance across the last 50 years but has yet to be described for any pest. Although there are examples of behavioral resistance in insects like house flies, they have generally been rare. A probable explanation is that the impacts of environmental influences are often high on the expression of a wide range of behavioral traits; this in turn lowers the heritability of such traits and the rate at which they can be selected. In contrast, physiological traits (such as reduced binding) are less influenced by the environment and are thus more likely to be selected rapidly. The general consensus of the Panel was that behavioral resistance seems unlikely to become a major mechanism of resistance on transgenic plants.

The ability of aminopeptidases, cadherins as well as some relatively uncharacterized glycoconjugates capacity to bind *Bt* toxins with high affinity has been known for many years, yet determination of the functional nature of the genes genetically linked to resistance has been difficult. To date, no linkage of resistance to aminopeptidases or glycoconjugates has been published. Recently one publication demonstrated a tight linkage between a cadherin gene known to bind Cry genes and resistance in the YHD2 strain of TBW (Gahan et al. 2001). Although this resistant allele has not been found in TBW in the field, another publication recently came out showing that field derived strains of the *P. gossypiella* did possess mutated alleles of the cadherin gene associated with resistance to Cry1Ac (Morin et al. 2003). All resistant *P. gossypiella* screened for three different mutations in this gene were homozygous for the resistance genes and all the susceptible larvae were heterozygous. This makes cadherin the leading target for DNA based resistance screening. Although the evidence for altered Cry receptors (cadherin) being the major mechanism of Cry toxin resistance in the field is

compelling, the Panel cautioned that due to fitness costs observed with this mutation, it is highly likely that another major resistance mechanism existing remains high.

Agency Charge

3. *CBW modeling.* Dow's CBW modeling efforts show that EPA can have high confidence that there will not be a significant change in population fitness of CBW on WideStrike cotton in a 15-year time horizon even without a high dose for either Cry1Ac or Cry1F and incomplete cross-resistance (20 to 60% maximum shared binding). Market share analysis of WideStrike cotton versus other *Bt* cotton products had little effect on the rate at which CBW may adapt in either the North Carolina or Mississippi Delta agroecosystem. Refuge size, whether sprayed or unsprayed, had no significant impact on CBW population fitness on WideStrike cotton after 15 years. In the Delta, the immigrating non-selected population from alternate hosts further reduces the local rate of adaptation. The local structured refuge only supplies a small proportion of the non-selected insects in the Delta. The availability of CBW alternate hosts, coupled with a non- *Bt* cotton refuge are additional levels of assurance for WideStrike cotton product durability. Additional empirical information is needed on the function and effectiveness of alternate hosts on the rate of CBW adaptation.

The Agency asks the SAP to comment on the predictions made by the DAS CBW model, i.e., the likelihood that the population fitness of CBW on WideStrike cotton in a 15-year time horizon will remain unchanged, even without a high dose for either Cry1Ac or Cry1F and incomplete cross-resistance (60% of Cry1Ac binds to the Cry1F receptor).

Panel Response

The model used by the registrant is a spatially explicit simulation model based on Storer (2003). The model was extended to explore a system of three transgenic products, two stacks (partially sharing one receptor) and a single gene product. Together, the three products shared a total of three protein receptors. It explores scenarios using crop mixtures from two agroecosystems, North Carolina and the Mississippi Delta.

The Panel identified several areas of concern with the registrant's CBW model that make its use problematic. These problems must be addressed if this model is to be used to assess the durability of WideStrike cotton in these areas. The Panel believed that use of the current model, once corrected of the identified errors, would be an appropriate vehicle to explore the parameter space with the goal of finding areas where resistance does occur in the 15-year time horizon and assessing whether it occurs within biologically plausible initial conditions and parameter values.

The results from the model were presented as population fitness (or changes thereof). Population fitness, measured as the frequency weighted average of the genotype fitness, is a nonlinear function of both time and log gene frequency. In short, population fitness changes little until the resistance allele is close to one, thus masking many of the important gene frequency changes that occur early in the evolution of resistance. The Panel agreed that this

property of population fitness makes it difficult to understand the results presented. The Panel recommended that resistance allele frequencies to the individual receptors be presented instead, as this would be much more informative and enable Panel members to better understand differences between runs of the model with different parameters.

The Panel identified a potentially serious problem in the manner in which the fitnesses of the receptor genotypes were estimated. The fitness of the nine receptor genotypes for binding by Cry1Ac to receptors A and B are based on partitioning W_2, the fitness of the doubly homozygous susceptible genotype on Cry1Ac cotton into $W_{2A} = 1\text{-}x\cdot Z$, fitness in the face of $x\cdot 100\%$ binding to receptor A, and $W_{2B} = [1\text{-}(1\text{-}x)\cdot Z]$, fitness in the face of $(1\text{-}x)\cdot 100\%$ binding to receptor B, as follows: $W_2 = W_{2A}\cdot W_{2B}$. This can be expressed in terms of survival rates [keeping in mind that these rates are relative to survival of the corresponding homozygous resistant genotype(s)] as follows: $S_2 = S_{2A}\cdot S_{2B}$ for $S_{2A} = (1\text{-}x\cdot Z)$ and $S_{2B} = [1\text{-}(1\text{-}x)\cdot Z]$. Thus, Z can be interpreted as the probability of mortality if all of the binding is to receptor A or if all of the binding is to receptor B, but this leads to a contradiction. If the effect of a toxin molecule binding to receptor A is identical to that for binding to receptor B, then the resulting survival will be independent of x: $S_2 = (1\text{-}Z)$; however, the formulation of the DAS model results in a dependence on x: $S_2 = (1\text{-}Z)+x\cdot(1\text{-}x)\cdot Z^2$. Moreover, Z can exceed a value of 1.0—clearly an invalid result for a probability: e.g. for $S_2 = 0.2$ and $x = 0.6$ (the best empirical estimates for these variables), $Z = 1.08$. Expressing the problem in another way, survival in the face of $x\cdot 100\%$ binding to receptor A and survival in the face of $(1\text{-}x)\cdot 100\%$ binding to receptor B are not independent events so that the joint survival rate can not be set equal to the product of individual survival rates. An alternative formulation is as follows: $S_2 = \{1\text{-}[x\cdot Z_A+(1\text{-}x)\cdot Z_B]\}$ for Z_A, the probability of mortality if all binding is to receptor A, and Z_B, the corresponding probability for receptor B. For this model, mortality due to Cry1Ac ranges from Z_A to Z_B as a linear function of x; and, for $Z_A = Z_B = Z$, $S_2 = (1\text{-}Z)$ as expected. The fitnesses of the nine receptor genotypes (p. 30) can all be expressed in terms of Z_A and Z_B, but it is not possible to estimate Z_A and Z_B given the available data. With one empirically-derived datum—W_2—one cannot estimate two, empirically independent quantities. In other words, one cannot solve for more unknowns than the number of equations relating those unknowns. The survival of one of the other genotypes on Cry1Ac cotton is also needed.

Following this FIFRA SAP meeting, Panel member John Schneider provided additional comments on the biological interpretation of Z. Such comments were not considered or reviewed by the Panel and are being provided as an appendix to these meeting minutes (Appendix A).

The sensitivity analysis of the model showed that the model was sensitive to several biological parameters about which we have limited information undermining the confidence of using the model to determine the durability of WideStrike. For example, the model was sensitive to immigration, initial gene frequency, fitness of the R-alleles, flowering dates and the use of alternate hosts. We know little about these parameters in the field (except flowering dates) and so the model outcomes, as run, are hard to interpret. In particular the model's claim that resistance will not occur in the 15- year period cannot be verified without understanding the biological parameters in the model.

The parameters used, and initial starting conditions, were biologically appropriate. They were, however, uninformative as to under what conditions the model may predict the appearance of resistance. Simpler, deterministic models often predict that evolution of resistance will occur in a shorter time frame than suggested by the model presented. In such cases, it should be incumbent upon the users of spatially explicit models to provide an explanation for the differences. The discrepancy could be caused by subtle differences in model formulation that may or may not be realistic (e.g., many of us essentially left refuges in one place until Peck et al. (1999) identified this as a problem), or it could be an important phenomenon that needs further exploration (as in the Peck model). An alternative approach to the problem would be to explore under what conditions of initial conditions and parameter values resistance does appear. Looking at worse-case scenarios for resistant development allows the user of the model to assess how likely the parameter combinations, in which resistance occurs, will be biologically plausible. Just because there are model outcomes using biological realistic parameters where resistance is delayed for 15 years, it does not follow that there might not yet be cases where the model suggests that resistance will occur within the 15-year window that also uses biologically realistic parameters.

The Panel also suggested that, as the product was not a high dose product for cotton bollworm, the possibility of a single, additive, resistance mechanism that provided low levels of resistance (5-50 fold) to all receptors should be explored. While such a mechanism is unlikely to provide complete resistance, it could compromise the product if it increased population fitness (survivorship in the field) to unacceptable levels.

The Panel also noted that while it is clear that immigration of bollworm adults into the Delta agroecosystem does occur, it is a subject of debate how important those moths are relative to populations that overwinter locally. The model also appeared to be constructed as a continent-island model, with no impact of reverse migration of selected individuals into the southern overwintering population. This may or may not be a reasonable assumption, but no data was given to support it. We know that in a high-dose refuge system that resistance evolves by the slow contamination of the refuges, it is unclear how much impact reverse migration and contamination of the overwintering refuges might have on the rate of the evolution of resistance.

Agency Charge

4. TBW modeling. **For TBW, durability is expected to be greater than that predicted using the TBW model by Peck et al. (1999) where the worst case (structured refuge is moved each year) is 17 years. TBW exhibits similar patterns in binding studies as does CBW and WideStrike cotton expresses a high dose against TBW. The Cry1Ac component alone is a high dose and the Cry1F component alone is not quite a high dose.**

The Agency asks the SAP to comment on the relative WideStrike cotton durability against TBW using the Peck et al. (1999) model.

Panel Response

Since the dose of the Cry1Ac and Cry1F in WideStrike cotton was demonstrated to be high against populations of TBW, the Panel believed that WideStrike will be more durable than that predicted by Peck (1999) for single Cry1Ac cotton. In most models with which the Panel is aware, two stacked products outperform single toxin products in delaying resistance. This will be true for WideStrike cotton as well.

Much weight is given to the 1993 result of a frequency of $1.5x10^{-3}$ Gould et al. (1997). Since it has been 11 years since that original work was done, assumptions about initial frequency should be conservative. It does not seem unreasonable that under continued selection over the last several years for *Bt* resistance, the potential for higher frequencies should be considered. One feature of the Peck (1999) model that seems relevant is that resistance first appeared in small foci. Unless one was fortunate to be monitoring that particular focus of resistance development when control failures had begun, it may be too late to stop resistance development.

While looking at the parameter space of the model is challenging, it is important to understand under what conditions we can expect resistance. An important aspect of modeling is sensitivity modeling, but it's also important to look at worst case scenarios, and to explore under what conditions the models predict shortened times to resistance.

Agency Charge

5. *Alternate hosts.* Dow utilizes its CBW model that simulates two agroecosystems that consist of CBW crops hosts soybean, maize, and cotton in varying amounts, three insecticidal control proteins (Cry1Ac, Cry1F, and Cry2Ab), and three protein receptors. Dow also uses the HOSTS data base, and carbon isotope work by Gould et al. (2002) to support the use of CBW alternate hosts as an effective means of reducing the population-wide selection pressure to the two ICPs expressed in WideStrike cotton (metapopulation dynamics effects). To support the effectiveness of alternate hosts as natural refugia, data are needed on the larval and adult production of CBW on each alternate host for each generation relative to cotton and WideStrike cotton and the spatial scale and source of moth production.

The Agency asks the SAP to comment on:
a) the sufficiency of the WideStrike cotton database to address the issue of CBW alternate hosts as natural refugia, and,

b) whether additional data are needed on the larval and adult production of CBW on each alternate host for each generation relative to cotton and WideStrike cotton and the spatial scale and source of moth production to confirm the effectiveness of CBW alternate hosts as natural refugia.

Panel Response

5a) The Panel agreed that the HOSTS database is insufficient to address the issue of CBW alternate hosts as natural refugia. The registrant references an online data base for Lepidoptera where researchers can search for host plants by family, genera, and/or species. The database is a good research tool, but in the context of the current discussions, it should be considered as a starting point for empirical research to better understand how CBW utilizes its hosts. The database may contain errors and does not provide empirical data about the numbers of larvae feeding on each host or the timing of host utilization.

5b) There is potential for the utilization of alternative hosts, in combination with the long-range dispersal ability of CBW, to diminish the community-wide responses to selection for adaptation to WideStrike cotton. However, the Panel agreed that there are insufficient empirical data in the registrant report to demonstrate that alternative hosts are producing susceptible, fit individuals in sufficient quantity, at the correct time and proximity to maximize the probability of matings between homozygous-susceptible individuals and individuals heterozygous for resistant traits.

In order to develop a rational IRM plan for CBW on WideStrike cotton, the effect of alternate host plants on the proportion of each CBW population that is subjected to selection pressure and the effect of migration by CBW among these populations on resistance frequencies must be considered.

The registrant neither presents nor cites any empirical data concerning the relative numbers of CBW larvae on various host plants during the generations that selection is occurring (except Gould et al. 2002, as discussed below). To the Panel's understanding, no such data exist for wild host plants due to the difficulty of spatially quantifying the abundances of these hosts and due to the absence of any unique, host plant-specific tags in the adults. Due to this lack of data and given the fact that these host plants do not exert selection pressure for resistance, the conservative approach would be to ignore them. Unfortunately, the model presented by the registrant includes wild host plants, and the importance of these hosts relative to other host plants is unspecified. For cultivated host plants, the occurrence of CBW on *Bt* and non-*B*t hosts is modeled as resulting from host plant preference coefficients and dispersal characteristics of CBW and from the overall abundances and spatial distributions of the hosts. However, as has been presented earlier, some of these key assumptions in the model are unspecified; and no model output showing the densities of CBW on the various cultivated host plants is presented. The registrant refers to data on the relative importance of C_3 and C_4 host plants for CBW (published in Gould et al. 2002) to support their general position that alternate hosts are important. Gould et al (2002) report that >40 % of the CBW collected from pheromone traps in Texas and Louisiana in August developed on C_4 larval host plants rather than on cotton or other C_3 larval host plants. Because the crop phenologies are such that these individuals did not develop on local corn, they may have developed on other, as yet unidentified, local, non-Bt host plants. This interpretation of these data does provide qualitative support for the conclusion that resistance to WideStrike cotton is likely to develop slowly.

Gould et al. (2002) made a different interpretation of their late season data (> 1 August). They suggested that most of the observed late-season moths from C_4 plants developed on corn at higher latitudes and migrated into Texas and Louisiana. Given the presence of *Bt* corn in the

Midwest, an understanding of the development of resistance to *B*t crops in the Midsouth would require an understanding of the effect of the contribution of these immigrants to local resistance frequencies. The registrant CBW model did not include late season immigration, but it did incorporate early season immigration (presumably from lower latitudes) into the Midsouth location of the Mississippi Delta agricultural area. Gould et al. (2002) reported that >40% of the CBW moths from 14/15 samples collected in May or earlier in Texas and Louisiana developed on C_4 larval hosts. Assuming that there were no important, unidentified, late-season, Midsouth C_4 hosts, these data also suggest that early season immigration occurs. However, the percentage contribution of the immigrants to the local, reproducing population, how this percentage varied among years, and the source of the immigrants, which determined their history of exposure to *Bt* crops, would need to be known in order to understand the potential for resistance in CBW in the Midsouth.

Further evidence of the lacking of empirical data/studies in the registrant report concerns the long distance dispersal of CBW adults in a short period of time. This has been shown by previous migration studies from southern Texas to Central Texas and into Oklahoma. Thus, it is obvious that we must examine moth production on a regional and possibly even state-by-state basis. Although migrants do not meet the registrant's definition of adults produced in refugia, the Panel does not believe that discounting the influence of migrants into a production system is the issue. Instead, the problem will be quantifying the migrants and their effect on the system.

Agency Charge

6. *IRM Plan.* The WideStrike cotton IRM plan has the following proposed refuge requirements:

a. 5% external unsprayed refuge option. Five percent of the cotton fields must be planted to non-*Bt* cotton and not be treated with any lepidopteran-control technology. The refuge must be at least 150 ft. wide (preferably 300 ft.) and within ½ mile (preferably adjacent or within 1/4 mile or closer) of the *Bt* cotton.

b. 20% external sprayable refuge option. Twenty percent of the cotton fields must be planted to non-*Bt* cotton and may be treated with lepidopteran-active insecticides (or other control technology) except for microbial *Bt* formulations. The refuge must be within 1 mile (preferably within ½ mile or closer) of the *Bt* cotton fields.

c. 5% embedded refuge option for TBW and CBW. Five percent of a cotton field (or fields) must be planted with non-*Bt* cotton as a block within a single field, at least 150 ft. wide (preferably 300 ft. wide) or single-field blocks within a one mile squared field unit. The refuge may be treated with lepidopteran-active insecticides (or other control technology) only if the entire field or field unit is treated at the same time.

d. Embedded (in-field strip) refuge option for PBW. One single row of a non-*Bt* cotton variety must be planted for every 6 to 10 rows of *Bt* cotton. This can be treated with lepidopteran-active insecticides (or other control technology) only if the entire field is

treated at the same time.

e. <u>Community refuge option.</u> Farmers can combine neighboring fields within a one-mile squared field unit that act as a 20% sprayable refuge or the 5% unsprayed refuge. Participants in the community refuge option must have a community refuge coordinator, and appropriate documentation is required. It also includes the requirements for annual resistance monitoring, annual compliance assurance program, grower education, remedial action plans, and annual reporting. Any plan that focuses on TBW, CBW, and PBW should be adequate to maintain susceptibility in secondary pests, such as fall armyworm, beet armyworm, southern armyworm, cabbage looper, and soybean looper. A market mix of different *Bt* cottons and other control technologies further reduces the expected selection pressure for resistance from the Cry1F and Cry1Ac proteins expressed in WideStrike cotton.

The Agency asks the SAP to comment on the scientific data available to support the proposed IRM plan and whether that data support a delay in resistance of TBW, CBW, and PBW resistance to the Cry1F and Cry1Ac proteins expressed in WideStrike cotton for at least 15 years.

<u>Panel Response</u>

The refuge requirements proposed in the WideStrike cotton IRM plan represent a continuation of refuge options that have been available to growers of Bollgard cotton. The apparent success of the high dose/refuge strategy in avoiding resistance to Cry1Ac is perhaps the strongest argument for maintaining the status quo, even if uncertainty exists about whether the IRM plan is indeed responsible (Fox 2003) for the lack of identifiable resistance in the field. While earlier arguments for >16% untreated refuges were not adopted as regulatory policy at a time when no commercial outcomes were known, the accumulated data 8 years later suggest that the gap between theory and practice (Denholm and Rowland 1992) remains substantial in terms of our ability to make fine-scale predictions. On the other hand, theory has provided the basis for adopting the current IRM strategy by predicting that resistance at a single locus would be significantly delayed if there was a low initial frequency of the resistance allele, mating between resistant and susceptible adults occurred extensively, and that R was inherited recessively (Comins 1977, Curtis 1985, Carriere and Tabashnik 2001). The outcome thus far of 8 years of commercial use suggests that the basic theory is correct, but that our ability to precisely define the parameters within the theory remains limited.

The Panel was in strong agreement that the proposed IRM plan by the registrant is sufficient for WideStrike cotton and supported the prediction of a delay in resistance of TBW, CBW and PBW to WideStrike cotton for 15 years. The proposed theory is supportive of the principle of delaying resistance by confronting adaptable genomes with two or more toxins. In particular, model simulations performed by Curtis (1985) of the time to resistance when target populations are confronted with two toxins instead of one show that resistance will be substantially delayed so long as certain conditions are met. The goal of each of the proposed refuge options is to ensure that these theoretical conditions are met in the real world of IRM for

Bt cotton. The overall consensus was that the existing IRM options that have been applied to the single-toxin Bollgard cotton will be equally or even more effective in protecting against resistance in the double-toxin WideStrike cotton.

Their strategy seems sufficiently robust for the CBW, the pest with the perceived greatest threat for the development of resistance. Previous SAPs determined the placement of refugia described in the registrant's document were sufficient for cotton cultivars expressing single Cry1 toxins. Because WideStrike expresses two Cry1 proteins at reasonable levels during the entire season, the registrant's recommendations for refugia placement are relatively more conservative than those recommended for cottons expressing single Cry1 toxins. In addition, the consistency in refugia requirements across different Cry products will reinforce grower education relative to IRM and refugia placement in cotton, increasing the probability of grower compliance.

The Panel recommended that there should be an inspection of a random sample of the 5% external refuges to compare the quality of the plants (nodes, fruiting structure) in the refuge and the *Bt* crops for which they are matched. Plant quality alone should be a fairly easy and low cost measure of the overall quality of the refuges. It should also be easy to check spray records for the site and make cursory inspections of whether insects and spiders are roughly as common as should be expected in an unsprayed field. In addition, for a sample of some of the crops and refuges, there should also be a comparison of egg densities of the key targeted pests, at least for TBW and CBW, on the refuge and neighboring *Bt* crops. Some states, such as Mississippi, already have a field sampling system for so-called "index fields". It may be possible to extend this system to include sampling targeted and recorded for *Bt* and refuge fields.

The registrant made certain assumptions that have been built-in to each of the refuge options, and the role of the refuges in supplying susceptible genotypes in the management of resistance was too critical to allow the assumptions to go untested. Empirical testing of the performance of the various refuge options should be pursued with the goal of determining if susceptible moths are being generated and dispersed into *Bt* fields at a rate high enough to satisfy the theoretical ratio of 500:1 susceptible moths to resistant moths. The following questions were posed as a heuristic device for critically examining how refuges are contributing to managing resistance of *Bt* cotton:

- Are embedded refuges as efficient as external refuges at recruiting moths relative to the rate of recruitment that occurs in the parent block of *Bt*?
- Will a greater relative proportion of moths visit the Bt cotton before treatment thresholds are reached in the non-*Bt* embedded refuge?
- Do embedded refuges have the potential of reducing regional densities of target Lepidoptera by avoiding the sometimes large buildups that occur in refuges?

Empirical answers to these questions and others could help to evaluate the veracity of assumptions underlying each of the refuge options and would also help with the parameterization of simulation models.

Implementation of the 5% external refuges should include measures of their effectiveness

in attracting egg deposition and generating susceptible moths. For some years, there has been concern that many of these external refuges are not grown in the same manner as the *Bt* crops they are intended to serve (i.e., since growers believe that these refuges will likely suffer yield losses, the untreated external refuges tend to be planted late on the poorer land and with less attention to irrigation, fertilizer, weed management, etc., and some may in fact be sprayed through oversight or perhaps intentionally).

The Agency could not address how often the 5% external refuge is used or what have been the results of the compliance assessments to date. There are some indications that this is not a popular refuge strategy and may be declining in use. However, external comments, including written comments from the National Cotton Council, have supported its continued availability.

The 20% external sprayable refuge option may often be the best from the standpoint of resistance management for *Bt* crops. A 5% refuge can never be more than 5%. On the other hand, although a 20% refuge may be sprayed (perhaps reducing it to a 4% effective refuge when spray mortality is 80%, which has historically been close to the average), whenever the refuge is not sprayed (as it often won't be at various times during the season due to low pest density), it is a 20% refuge.

Implementation of embedded refuges began some years after the initial commercialization of *Bt* cotton in 1996. A point was made that one of the motivations for embedded refuges was to have the source of susceptible moths within the *Bt* cotton field to promote random mating between susceptible moths generated in the refuge cotton with any resistant genotypes that might arise in the Bt cotton. The within-*Bt* field proximity of the refuge cotton would help to overcome potential weaknesses of the external refuges that require dispersal over a longer distance. One Panel member commented that the principal reason for the embedded refuges was to generate more susceptible moths by having a wide enough refuge blocks within the *Bt* cotton to retain susceptible moths to produce even higher numbers within the refuge. There are contrasting requirements of embedded refuges for PBW and CBW, and that concern in part about adequate dispersal of the weak flyer PBW from external refuges into *Bt* cotton prompted the advent of embedded refuges in Arizona *Bt* cotton production.

Bollgard and Bollgard II Insect Resistance Management

Agency Charge

As a condition of the Bollgard and Bollgard II registrations, EPA required that the Monsanto Company conduct CBW alternate host research studies and pyrethroid overspray studies to support the adequacy of the 5% external, unsprayed, structured refuge. In addition, EPA required that the Monsanto Company conduct research on the north-south movement, i.e., reverse migration, of CBW and its impact on *Bt* corn and cotton insect resistance management.

1. ***North-south movement.*** **Based on the modeling studies submitted using the data in**

Gould et al. (2002), CBW (also called corn earworm in corn) reverse migration has no significant impact (0.05<*P*) on CBW adaptation to *Bt* corn and cotton.

The Agency requests that the SAP comment on whether CBW reverse migration is expected to have any significant impact on CBW adaptation to *Bt* crops.

Panel Response

The Panel believed that the spatially explicit, two patch model presented by Agricultural Biotechnology Stewardship Technical Committee (ABSTC) provided conclusions that are overly optimistic when compared to more simple models that are in the literature. However, there is too little detail in the ABSTC report to examine what factors are favoring the slow build up of resistance alleles. Storer et al. (2003) does provide more detail relative to the variables and their effect on resistance evolution. The Panel believed that if spatially explicit models will be used, more detailed modeling is necessary, especially with regard to the seven prerequisites the ABSTC report states are necessary for reverse migration to influence the development of resistance in *Bt* corn and *Bt* cotton. Thus, the Panel could not determine whether CBW reverse migration is expected to have any impact on CBW adaption to *Bt* cotton or *Bt* corn.

Agency Charge

2. *Pyrethroid oversprays.* Pyrethroid oversprays in Bollgard cotton fields will increase the level of control of CBW, delay the evolution of resistance, and increase the relative effectiveness of the 5% external, unsprayed, structured refuge. These findings support the general predictions of the Gustafson et al. (2001/2004) model. Pyrethroid sprays on Bollgard II plots do not provide a statistically significant difference in reduction of CBW infestation or damage from untreated Bollgard II cotton fields or from treated Bollgard cotton fields, and should not be included as a parameter in the Gustafson et al. (2004) model.

a. The Agency requests that the SAP comment on whether pyrethroid oversprays in Bollgard cotton fields are likely to increase the level of control of CBW, delay the evolution of resistance, and increase the relative effectiveness of the 5% external, unsprayed, structured refuge.

b. The Agency also requests that the SAP comment on EPA's recommendation that pyrethroid oversprays not be included as a parameter in the Gustafson et al. (2004) model for Bollgard II.

c. Marcus et al. (2004) found that CBW larvae (late instars) in North Carolina Bollgard plots were half as susceptible to Cry1Ac (i.e., more tolerant) as were populations from non-Bollgard cotton survivors in the F1 generation.

The Agency requests the SAP comment on whether the cotton bollworm larvae coming from Bollgard fields are more tolerant to the Cry1Ac protein than those larvae

coming from the non-Bollgard fields. What, if any, additional genetic work should be conducted to better understand the nature of this Cry1Ac tolerance.

d. The Agency requests the SAP to comment on the value of using a Cry1Ac-resistant CBW colony to investigate the genetic basis for CBW survival on Bollgard cotton.

Panel Response

2a) The Panel agreed that the data provided by the registrant show significantly fewer larvae and less damage to the cotton plant in pyrethroid-treated Bollgard cotton relative to Bollgard cotton that was not sprayed, and this a consistent pattern at all four locations. Based on these data, it appears sound that pyrethroid oversprays in Bollgard cotton improve the control of susceptible CEW.

The Panel agreed that the effect of pyrethroid oversprays in delaying resistance in CEW is probably overstated. In their document, the registrant stated that "Pyrethroids may be relatively more effective in Bollgard cotton because Bollgard cotton survivors may be compromised in some way, or there may be an increased probability of Bollgard cotton survivors contacting pyrethroid residues on Bollgard cotton". The field studies relied on natural infestations of CEW, presumably susceptible individuals. Gustafson et al. (2004) assume that RR genotypes on Bollgard cotton experience the same 17% survival in the face of insecticide applications as observed for SS genotypes on Bollgard cotton. If instead one assumes that RR genotypes on Bollgard cotton experience the same 35% survival as observed for SS genotypes on conventional cotton [averages reported by Greenplate (2004)], then RR genotypes would enjoy a survival twice and RS genotypes a survival up to twice that for SS genotypes on sprayed Bollgard cotton. Consequently, resistance should develop faster (but perhaps only slightly) when Bollgard cotton is sprayed than estimated by Gustafson et al. (2004b). Especially because the Bollgard product is not a high dose for CEW, it will be important to consider differing susceptibilities to pyrethroids as it relates to the general differences among resistant genotypes in the larval stress they encounter when feeding on Bollgard cotton.

The magnitude of the delay to resistance in CBW given insecticidal sprays on Bollgard cotton will depend on whether the sprays are performed by producers as effectively as in the reported studies. For example, Jackson et al. (2001, 2002, 2003) reported making two insecticide applications 6-19 days apart during mid-July to mid-August. Is this typical for NC or for other regions of the cotton belt? The insecticidal effect on differential survival of RR and SS genotypes could be greater if two generations of CBW were treated.

2b) The Panel discussed two points of view concerning the inclusion of pyrethroid oversprays of Bollgard II fields in the Gustafson et al. (2004) model. The first was the use of oversprays within a resistance management context. If resistance begins to evolve, oversprays could potentially help to control resistant individuals. Reductions in the numbers of resistant adults emerging from pyrethroid oversprays targeting larvae feeding on Bollgard II cotton would have

the potential to diminish the numbers of resistant adults emerging from Bollgard II fields relative to those coming from refuges. As a consequence, the probability of matings between resistant individuals from the Bollgard II cotton and the refuge would be enhanced. Inclusion of oversprays into the Gustafson model would evaluate the benefits of pyrethroids as a resistance management tool.

The second point, and the focus of the question from the perspective of the Agency, refers to the likelihood that growers will use pyrethroid oversprays in Bollgard II plots. The data supplied by the registrant indicated that there were no significant differences in the control of CBW in Bollgard II and Bollgard II oversprayed with a pyrethroid. As a consequence, growers are less likely to use pyrethroids in Bollgard II fields due to the excellent control of CBW provided by the product. However, pyrethroids and other insecticides could be used in Bollgard II fields to control pests such as stink bugs and plant bugs that would incidentally impose mortality on any CBW in the fields; and Bt cotton fields are generally treated mid to late season more frequently for such pests than are conventional cotton fields. Nevertheless, at least in the midsouth, the incidence of insecticide applications to Bollgard II cotton that would impose significant mortality on CBW is likely to be low. The Panel agreed that there is little need to include pyrethroid oversprays in Bollgard II plots in the models of Gustafson et al. (2004) from this perspective. However, the Agency may want to consider that as resistance to Bollgard II evolves, more larvae will be present in Bollgard II fields and the management practices used by growers may include oversprays.

2c) Based on the data submitted by the registrant, the Panel concluded that there is some evidence of greater tolerance in larvae originating from Bollgard fields relative to those coming from non-Bollgard fields. Non-overlap of fiducial limits is a conservative measure of a statistically significant difference between population means: i.e., fiducial limits may overlap and the population means still be significantly different. Given that the overlap in their fiducial limits was small, the LC_{50}s for the offspring of the CBW strain collected from Bollgard cotton (BGF1) and from non-Bollgard cotton (NBTF1) observed by Marcus et al. (2004) might well be significantly different ($P<0.05$). Also, inadvertent selection for genetic change of insect populations under laboratory conditions can occur very rapidly—especially for small populations and when mating is communal. Of course, maternal effects may also be operating.

Although it is most likely that these survivors are significantly resistant, these populations need to be further characterized and an understanding of the nature of resistance developed beyond the F2. For instance, vigor tolerance needs to be eliminated. If the R-trend holds up, then pyrethroid oversprays may be warranted and the High dose/refugia strategy may need to be modified to combat the situation. After the registrant answers these questions, they may develop a thorough follow up study thereafter. In this way, actual resistance evolution in a high dose/refugia system could be documented and the factors contributing to this evolution understood. If the individuals derived from this field situation were found to be resistant after scientific scrutiny, then appropriate culture, selection and mechanism determination studies should be pursued. A good method to accomplish this is via paired matings, family analyses resulting in isolines with varying tolerances to Cry1Ac. Follow-up studies should include the mechanism of resistance inheritance as well as comparison with other lab selected strains. Use

of Bollgard plant material in assays would also greatly enhance the research.

2d) Greater knowledge of how RR individuals respond to pyrethroid treatments would improve the pyrethroid-survival parameter of Cry1Ac-resistant genotypes in the Gustafson et al. (2004) model mentioned above. The Panel discussed two methods to conduct such experiments: (1) long-term selection in the laboratory with field-collected individuals and; (2) screening a great number families from the field. This would lead to characterizing their abilities to perform on Cry1Ac media, with the subsequent creation and maintenance of isolines with varying levels of resistance to Cry1Ac.

Mass selections in the laboratory require the maintenance of large colonies over long periods of time. If resistance alleles are rare, there is a reasonable likelihood that the resistant individuals will not be "seen" in the assays until after many generations due to their representation only in RS individuals. To maintain any resistant alleles, it will be necessary to use unrealistically low concentrations of Cry1Ac to allow the survival of some RS individuals. In the mass mated arenas it will take many generations before sufficient numbers of RS x RS matings occur. The concern of using such low doses is that resistance mechanisms created via mass selections will be artificial with respect to the type of resistance that would be selected for Bollgard plants in the field. The laboratory artifact of using low concentrations of Cry1Ac would not provide useful data for inclusion in the Gustafson et al. model.

Screens of isolines from field-collected individuals using plant tissue or concentrations of Cry1Ac comparable to expression in Bollgard cotton will provide better information. The initial investment in research time when resistance is rare will be great since thousands of families need to be evaluated in at least the F2 generation. If any isolines exhibit great survival on a medium containing Cry1Ac then these isolines may be maintained and used for further characterization of resistant genotypes. If none of the initial families exhibit resistance, they can be discarded and therefore eliminate the need for long-term rearing for a major resistant trait that is not present.

Research of this nature would make an excellent addition to the model, but finding a single major gene will be difficult and unlikely.

Agency Charge

3. *Alternate hosts.* Based on the two-year, studies in five states, both C_3 and C_4 alternate hosts serve as unstructured refugia. Data show that CBW moths are produced on alternate hosts throughout the landscape (spatial scale is greater than 10 miles) in sufficient numbers throughout the cotton growing season to mate with any putative resistant CBW moths emerging in Bollgard or Bollgard II cotton fields and dilute resistance. That is, the susceptible CBW moths coming from alternate hosts will reduce the intensity of Cry1Ac and Cry2Ab2 resistance selection in CBW and lower the likelihood of resistance evolution. The contribution of susceptible CBW adults from alternate hosts is greater than that from the 5% external, unsprayed, structured non-*Bt* cotton refuge. Despite the limitations EPA has identified associated with the Gustafson et al. (2001/2004) model, the CBW alternate

host data support the model's predictions that alternate hosts will substantially delay resistance.

a. Based on the larval productivity analyses, adult productivity analyses, and satellite imaging analysis, the Agency asks the SAP to comment on the relative contribution of the C_3 and C_4 alternate hosts as unstructured refugia to dilute CBW resistance.

b. Based on the data, the Agency also asks the SAP to comment on the spatial and temporal scale across the landscape, e.g., 1 mile, 10 mile etc., in which CBW adult production should be evaluated.

c. EPA concludes that "effective refuge size" should be a weighted average of the proportion of moths coming from each alternate host for each CBW generation (5 to 6 generations) in each cotton production system (geography).

The Agency asks the SAP to comment on how to quantitatively or semi-quantitatively calculate "effective refuge size" locally and regionally using available data (see above).

Panel Response

3a) The Panel agreed that sufficient data were provided to establish that C_3 and C_4 alternate hosts function to some degree as unstructured refugia. However, the Panel expressed concern on the methodologies used to assess adult productivity in the alternate hosts. The primary concern was the use of pheromone traps, and to a lesser extent the use of larval counts, to quantify adult CBW productivity in alternate hosts. The Panel indicated that pheromone traps typically only indicate adult male CBW activity in a given area. Thus, traps do not provide a meaningful measurement of adult productivity. The Panel also addressed the need to examine the temporal and spatial availability of alternate hosts in relation to CBW populations produced in *Bt* cotton. One Panel member indicated that behavioral and mating data are necessary to confirm the dilution of resistance; that is, susceptible insects from alternate hosts are in fact mating with adults from *Bt* crops.

Corn has long been recognized as a primary producer of CBW moths and undoubtedly contributes susceptible moths to the system. Also, it has long been accepted that a CBW population emerging from corn will infest cotton and other alternate hosts, as described by the registrant. Thus, the temporal occurrence of the adult populations between these hosts and cotton are crucial for mating to occur between susceptible adults reared in alternate hosts and resistant adults surviving transgenic cotton. However, the larval and adult productivity data provided, in addition to C_3 and C_4 analyses, only indicates the *occurrence* of the production of bollworm moths in the C_3 and C_4 alternate hosts. Head and Voth (2004) provide data on alternate hosts defined as soybeans, corn, sorghum, peanuts, and non-*Bt* cotton. The methodologies for estimating larval productivity and adult productivity are probably overestimates and underestimates, respectively, of adult CBW production. The larval productivity measurements based on counting of late instars assumes that all counted larvae will reach adulthood. Pheromone traps only indicate adult male activity in a given area, not a

measurement of adult productivity. Further, the traps do not discriminate between immigrants and insects produced locally, and must compete with other insect behaviors (i.e., mating/calling). Without more definitive data quantifying temporal and spatial production of susceptible CBW moths from each of the C_3 and C_4 hosts, and confirmed mating behavior of subsequent adults, the current refuge requirement(s) should continue.

The number of adults emerging from alternate hosts may also be an incomplete description of the impact of those hosts on the evolution of resistance. As the Peck et al. (1999) model demonstrated for between years, moving refuges over time can reduce their effectiveness. Data was presented demonstrating in several versions and revisions of the Caprio et al. (1998) model, that refuges that are temporally unstable over time during the season (such as early and late soybean fields, different wild hosts) will be less effective per unit area than are refuges that are temporally stable over time (e.g., persist for at least 2 generations). The model suggested that an individual adult moving from corn into a cotton refuge (a refuge that would persist for the next two generations) would have a realized fecundity 3-fold greater than a similar adult moving into early soybeans (a refuge that only persisted for the ensuing generation). In this case, the Panel defines realized fecundity as the number of offspring that are descendants of the adult that enter diapause. Those offspring would be G2 insects (second generation from the initial adults).

Following this FIFRA SAP meeting, Panel member Michael Caprio provided additional comments on C_3 and C_4 alternate hosts. Such comments were not considered or reviewed by the Panel and are being provided as an appendix to these meeting minutes (Appendix B).

3b) The Panel agreed that CBW production should be measured at a larger scale than the local farm, or field level because of the high mobility of adult CBW. The Panel expressed concern that even the 10 mile range may be relatively short in some cases based on previously observed migratory movement within a very short time frame, but local phenomena may also be important. The Panel also provided one example (Raulston et al. 1992) of previous large-scale field studies for measuring CBW production over a large production region.

Jackson et al. (2003) indicated "the average cotton field in the area has been estimated at 15 acres…" in North Carolina. These are relatively small field sizes for cotton and it is not surprising that CBW moth movement across the landscape between C_3 and C_4 plants would be observed. After all, we know that adult CBW can move approximately 400 km in just under 8 hours of migratory flight. In these situations, it would seem that spatial and temporal scales for assessing CBW should likely be evaluated in an area that encompasses a representative sample of all suspected C_3 and C_4 hosts within an agroecosystem and perhaps other cotton production regions. Again, there is the problem of quantifying the migrants and their effect on the system.

As previously mentioned, field studies for assessing CBW development are not simple tasks but can be done. For example, in a large-scale study for assessing CBW population development over a large production area, Raulston et al. (1992) used pupal digs to determine the number of moths produced from fruiting corn in northeastern Mexico and South Texas. Night-time observations have also been used to assess adult abundance in a region. These types of studies provide more accuracy in terms of spatial and temporal production of CBW adults but

labor requirements and logistics will probably be the limiting factor in determining the extent of future spatial and temporal assessments.

3c) The Panel agreed that "effective refuge size" should be a weighted average of the proportion of moths produced from each alternate host. The Panel commented that the term "effective refuge size" implies evaluation of varying plot sizes of all alternate hosts and suggested that some clarification by the Agency may be in order. The Panel provided additional input pertinent to this issue in response to Question 4a posed by the Agency for Bollgard and Bollgard II. In response to the request for methods on quantitatively calculating "effective refuge size", the Panel provided techniques for quantifying CBW populations in the identified alternate hosts that were identified as natural refugia.

The term "effective refuge size" implies the evaluation of various "refuge sizes" and sources (i.e. C_3 and C_4) to identify that which is best suited for providing the necessary numbers of susceptible insects. Therefore, the techniques described below for measuring adult production would need to be replicated over a range of "refuge sizes" and suspected sources. These data, in addition to the described biological data, would also assist in refining the Gustafson et al. (2001) model especially since it would include corn, the preferred CBW host, as a potential source of susceptible insects. Gustafson et al. (2001) did not include corn as a source of susceptible insects.

One Panel member was of the opinion that an "effective refuge size" cannot be calculated, based on the data provided, for two reasons: 1) the methodologies with which larval and adult productivity estimates were obtained; and 2) lack of definitive biological data (i.e. temporal and spatial adult emergence from various C_3 and C_4 plants, and behavioral or mating observations).

Thus how does one obtain these biological data? The C_3/C_4 data presented here provides a starting point in terms of relative measurements of the population, but as previously mentioned these are probably inadequate for providing precise population numbers. Other possibilities for obtaining quantitative data have been previously implemented (Gore et al. 2004; Jackson et al. 2002, 2003; Raulston et al. 1992). Some of these methods included:

1. Deployment of emergence cages (possibly a square meter in size but this does not preclude other sizes) throughout all suspected C_3 and C_4 sources, under varying soil types, irrigation, etc., to provide a more accurate picture of spatial adult productivity in each of the suspected sources. This would address questions regarding whether late instars reached adulthood, whether males captured in pheromone traps were produced locally or moved in from adjacent sources, and would provide a measure of sex ratios. Additionally, moths emerging from these sources could undergo genetic analyses to provide data on the genetic composition of the emerging adult population. This latter would be very informative given the assumption that susceptible moths mate with resistant (homozygous and heterozygous) moths. Gore et al. (2004) used similar caging techniques to estimate temporal emergence of CBW adults from field corn.

2. Harvesting of late instars on developing fruit and subsequent monitoring of insect development to adulthood on the same larval food source. Jackson et al. (2002, 2003) estimated production of CBW in Bollgard and Bollgard II cotton under differing insecticide treatment regimes.
3. Digging for pupae and holding pupae in surrogate cells in the soil to estimate emergence based on Raulston et al. (1992).
4. Night-time observations to assess insect activity in an area and obtain information such as occurrence and timing of mating behavior, mating frequency based on dissections of adults, and a general idea of the effect of the host crop on the insect behaviors.

Based on previous studies (Gore et al. 2004; Jackson et al. 2002, 2003; Raulston et al. 1992), there are some biological data on corn and cotton, two of the identified "alternative hosts," but additional data are needed for the remaining hosts.

Agency Charge

4. *Gustafson et al. CBW model.* Monsanto modified Caprio's (1998a) two-patch, deterministic, non-random, population genetics model (publically available) to create a new CBW model, Gustafson et al. (2004, originally submitted to the Agency in September 2001 as part of the *Bt* Crops Reassessment) that included alternate hosts and synthetic pyrethroid oversprays as parameters. Sensitivity analyses showed that the model output (years to resistance) was sensitive to both of these parameters. Gustafson et al. (2004) have calculated "effective refuge size" as the sum of the total acres by county represented by the four alternate crop hosts – corn, sorghum, peanuts, and soybeans, and wild hosts (defaulted as 10% of the cotton acreage) as a percent of cotton acres. This model predicts that the 5% external, unsprayed, structured refuge option is adequately protective to delay CBW resistance if effective refuge size (alternate hosts) and typical use practices for Bollgard cotton, i.e., synthetic pyrethroid oversprays, are included. When this model was submitted to the Agency in 2001, empirical data to support the use of alternate hosts and synthetic pyrethroid were lacking.

a. The Agency asks the SAP to comment on the "effective refuge size" calculation. Does the SAP agree with the Agency's conclusion that "effective refuge size" is a weighted average of the proportion of moths coming from each alternate host for each CBW generation (5 to 6 generations) in each cotton production system (geography)?

b. The Agency requests the SAP to comment on the strengths and weaknesses of the Gustafson et al. (2004) model and its utility with regard to the effective contribution of alternate hosts as natural refuge per generation. How would the model output be altered if the calculation of "effective refuge size" is changed (see a. above). What are the SAP's recommendations for refining the Gustafson et al. (2004) CBW resistance management model or using a different CBW resistance management model to more appropriately consider the spatial and temporal dynamics of CBW utilization of alternative hosts by generation based on the data in Head and Voth (2004)?

c. The Agency requests the SAP to comment on validity of using the average pyrethroid efficacy value against CBW based on all the field studies conducted in all four states (North Carolina, Louisiana, Mississippi, and South Carolina) as the parameter value in the Gustafson et al. (2004) model rather than just the Brickle et al. (2001) data from South Carolina.

Panel Response

a.) The Panel agreed with the Agency that a weighted average is an appropriate choice for determining the contribution of alternate hosts to the refuge size. The Panel noted that the estimation of the total refuge proportion also requires an estimate of the emergence from transgenic crops (e.g., the proportion in refuge is relative to the total number of adults, including those emerging from transgenics). When this estimate of total adult emergence is made, the numbers emerging from transgenic crops (where the gene(s) of interest are utilized) should be corrected for losses due to selection (see Appendix B). Other transgenics, assuming there is not an interest in resistance to transgenetics and other pesticides, should be assumed to be additional mortality factors and no correction applied.

b.) The Gustafson et al. model is a deterministic, two patch, generational model which will be useful for exploring broad general questions about the use of alternate hosts and pyrethroid oversprays in Bollgard and Bollgard II transgenic cotton. Continued use of the model would certainly require incorporation in a detailed fashion of the data acquired through the alternate host plant study. The Panel believed, however, that exploring detailed questions about time to resistance and the effect of alternate hosts on resistance would benefit from the development of a more detailed model. Specifically, a spatially explicit model that can pick up the more nuanced structure and timing of insect emergence in alternate hosts will be necessary to address the questions posed by the Agency.

c.) The Panel concluded that it would have been prudent to explore the impact of the data developed as a result of the Agency's request. The additional data should impact the mean or mid-value used, and perhaps also have some impact on the extreme values. It would seem that the additional data might alter the relative position of the extreme values (e.g., are they symmetric about the mid-value), as well as the breadth of those values (e.g., as additional data has been collected, should we not have more confidence in the mid-values?) As noted by the Panel's response to question 2(a), the Panel believed it is likely that the impact of pyrethroid oversprays might vary with genotype, and this must be incorporated into the model.

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APPENDIX A

Public commenter Nicholas Storer, Ph.D. (Dow Agrosciences) provided supplemental comments on the biological interpretation of Z: "a pool of Cry1Ac activity (which in turn is a function of concentration and specific activity)." Based on this information and further analysis, Panel member John Schneider provided additional comments. Such comments were provided by Dr. Schneider after the meeting, thus they do not reflect the Panel's position.

The formulation of Dow's CBW model is consistent with the following interpretation of *Z*: $Z = M_{2A}+M_{2B}$, for $M_A \equiv$ "mortality due to binding receptor A sites" $= (1-S_{2A})$ and $M_{2B} \equiv$ "mortality due to binding receptor B sites" $= (1-S_{2B})$. In addition, as Storer points out, this formulation assumes that the survival rates S_{2A} and S_{2B} are independent and that the effect of a molecule of Cry1Ac binding a receptor A site is identical to the effect of a molecule binding a receptor B site. The characteristics of this model can be discerned by considering the following results. For $W_2 = 0$ (i.e. 0% mortality), at $x = 0$ (i.e. no binding to receptor A sites), $M_{2A} = 0$ and $M_{2B} = 1$ as expected; but at $x = 0.5$ (i.e. 50% of binding by Cry1Ac is to receptor A sites and 50% to receptor B sites), $M_{2A} = M_{2B} = 1$. In this case, one observes that the binding capacity available for each of the two receptors is two-fold that necessary to cause 100% larval mortality. For $W_2 = 0.2$ (the value assumed in the model), at $x = 0$, $M_{2A} = 0$ and $M_{2B} = 0.8$ as expected; but at $x = 0.5$ (close to the best estimate of 0.6), $M_{2A} = M_{2B} = 0.55$. As in the first case, one observes that excess binding capacity is present—although considerably less than that observed for $W_2 = 0$. In addition, the behavior of the model with the latter set of parameter values is perhaps more revealingly expressed by noting that mortality due to binding at a given receptor increases more rapidly per unit increase in binding at low levels of binding than at high levels. This would appear to be biologically unrealistic given the possibility of threshold effects in the consequences of toxin binding. The ramifications of the characteristics of this particular formulation on the sensitivity analysis of variation in binding parameter *x* presented by Dow are unclear.

APPENDIX B

Panel member Michael Caprio provided additional comments concerning interpretation of C_3 and C_4 ratios as a response to the Bollgard cotton insect resistance management question on alternate hosts (question #3). Such comments were provided by Dr. Caprio after the meeting, thus they do not reflect the Panel's position.

The data from the C_3/C_4 isotope studies have raised questions since they suggest that there is an excess of C_4 individuals over what one might expect from our knowledge of the distribution of potential host plants. The data have been interpreted to mean that there is a source of C_4 individuals that has not been identified, whether this be wild hosts or southward migration of CEW from the corn belt. The question might be rephrased to ask not where the excess of C_4 individuals come from, but rather why there is a paucity of C_3 individuals.

The interpretation of the C_3/C_4 isotope data is complicated because it cannot be immediately utilized to estimate the proportion of the population exposed to a C_3 transgenic crop (in other words, what is the contribution of C_4 plants as a refuge to C_3 transgenic crops). A second, related question, is what is the overall proportion of C_4 plants in the environment? The difficulty with the first question is that the C_3/C_4 ratios represent post-selection numbers, that is, the numbers emerging from habitats after selection has occurred in the transgenic habitat. As such, the preselection number of individuals, the number of individuals actually exposed to selection, must be corrected for the individuals that were lost in the transgenic fields as a result of selection.

As an example, Gould et al. (2002) found on one date that the ratio of C_3 to C_4 adults was 60:40. These are valid data and a good estimate of the production of adults from C_3 and C_4 plants. The problem is that selection by transgenic cotton has already removed individuals from the C_3 pool, so we cannot directly translate these numbers into refuge size, at least as we currently use the term: the proportion of oviposition on hosts without selection for Bt counteradaptation. In order to estimate the actual proportion of individuals that were exposed to selection, we must ask what habitat distribution, with the inclusion of selection, would have produced the observed post-selection numbers. This is most easily done by correcting the number of C_3 individuals observed by the expected number lost to selection on the transgenic crop. We assume for the sake of simplicity that the data above came from a trap in which one captured 60 moths identified as C_3 and 40 as coming from C_4. We must take the C_3 portion and replace the numbers removed by selection. In order to do this, we need to know the proportions of C_3 transgenic and non-transgenic habitats as provided by the registrant. If we assume that the overall habitat consisted of 95% transgenic habitat and 5% unsprayed refuge, we know that

$60 = 0.05x + .95*M*x$

where x is the initial number of eggs laid, and M is the survivorship of bollworm on Bt-cotton

Assuming M = 0.1, we can estimate that x=414 (i.e., 354 C_3 adults are missing due to selection in the transgenic cotton). Thus, the actual refuge size due to C_4 plants is closer to

40/(413+40)*100 = 9.1%.

This correction is incomplete depending upon the selection model used. If the Gustafson model incorporates sprays of refuges in the 80-20 option, then these individuals should be added back in by our calculations because they will subsequently be removed by the model. Similarly, if sprays in the Bollgard fields are incorporated into the model, then those individuals should be added back into the equation (otherwise they would be removed twice). The same goes for productivity of alternate hosts, though in that case we should not add them now because they will be added by the model later. All this suggests that we should perhaps give more thought to what exactly we mean by "refuge %" and how it must be calculated. Of course, some of the refuge is also C_3, so the calculation that follows only estimates the ratio of C_3:C_4 assuming all the C_3 are unselected.

This addresses the second general question - where are all these C_4 hosts, when in fact many of the expected C_3 individuals are removed by sprays or transgenic toxins. If we assume that the C_4 hosts are unsprayed, then the following calculations will tell us what proportion of eggs were laid on C_3 versus C_4 hosts prior to any sprays or mortality due to transgenics. The other question, what is the actual refuge size, would incorporate both C_3 and C_4 refuges (easily done) and depend on specifics of the selection model utilized.

The correction may get more complicated as additional habitats are included, and a more general solution is required. For example, we now assume that there are sprayed refuges, unsprayed refuges as well as transgenic cotton. We must now add in a correction factor to account for the fact that the production of moths from the sprayed refuges is less than the production from the unsprayed refuges. This can probably be estimated reasonably accurately from the registrant's late larval sampling, assuming that there are no large differences between habitats in mortality after this sample date. We can standardize on unsprayed cotton as a relative one (i.e., production in all other habitats is expressed as relative to the number produced per area of unsprayed cotton). This factor was implicitly included above, but since *Bt* is unsprayed (in our imaginary case), the factor in both cases was 1. In the case of sprayed cotton, sprays might reduce production of adults by perhaps a specific mortality rate (assuming 80% here). This could also be directly estimated from the Monsanto larval data if data exist on untreated refuges, or less preferably by estimation from the number that one would expect to emerge from unsprayed Bollgard plots (sprayed plots)/(M*observed BG larvae). We can call this factor P_i (productivity of habitat i relative to unsprayed cotton). We can now write a more generalized equation:

$$C_3 = \sum_{1}^{nhabitats} S_i M_i P_i x$$

S_i is proportion of the i^{th} habitat of the total C_3 producing habitat
M_i is the survivorship in the transgenic crop
P_i is the productivity of the i^{th} habitat relative to unsprayed non-Bt cotton
x is the corrected C_3 component
C_3 is the observed number of C_3 moths

Let us assume now that the habitat producing C_3 individuals can be assigned into 90% of the area that uses the 80-20 option (sprayed refuge), while the remaining cotton acreage uses a 95-5 option (unsprayed refuge). We can calculate the total portion of each habitat as follows

Bollgard	Unsprayed refuge	Sprayed refuge	
95	5	0	x 10%
80	0	20	x 90%
or			
9.5	72.0	18	
81.5	0.5	18	= 100%

These would be the S_i in the equation above (once divided by 100 to represent proportions), and we can now expand our equation

60 = .815* .1 * 1 * x [Bollgard component] + .005 * 1 * 1* x [unsprayed refuge option] + .18 * 1 * 0.2 * x [sprayed refuge component]

60 = .1225x
or
x = 489.8 and the initial C_4 contribution would be estimated as
40/(489+40) = 7.4%

This is the proportion of eggs oviposited on C_4 hosts. It is not an estimate of refuge size which would include oviposition on non-transgenic C_3 hosts as well.

We could include highly productive refuges (as an example, chickpea produces many more larvae/unit area), and in such cases the P_i factor might exceed 1. We can also accommodate sprayed Bt-cotton by changing P_i for the *Bt* cotton by an appropriate amount. If *Bt* cotton is sprayed an average of once per generation, we could set P_1 in the previous equation to

0.2. This would account for the fact that some of the survivors on the Bt-cotton were then removed by pyrethroid sprays. This value may underestimate the number produced, as multiple sprays are used to reduce populations in conventional cotton, but only one is used in Bt-cotton. Of course, data exist that the pyrethroid sprays are more effective in Bt-cotton. The result would change to:

60 = .815* .1 * 0.2 * x [bollgard component] + .005 * 1 * 1* x [unsprayed refuge option] + .18 * 1 * 0.2 * x [sprayed refuge component]

.

60 = .0736x

or

x = 815.2 and the initial C_4 contribution would be estimated as

40/(815.2+40) = 4.7%

We should be able to work forward from a specified configuration of fields to generate a C_3:C_4 ratio, and then using that figure, the known field distributions, and productivity, to work backwards and regenerate the initial field distribution. We assume that 60% of the farms in an area use the 80-20 option, 38% use the 95-5 option, and 2% of the area is planted to chickpeas (to demonstrate incorporating of highly productive hosts). Assuming chickpeas provide a 3-fold greater production factor, it would be easiest if we assume an arbitrary number of moths (say 100) are produced in unsprayed cotton (this is actually the number of C_3 moths we would catch if all the C_3 producing habitats were unsprayed cotton). We can now calculate the number of moths we would collect in our traps, assuming trap efficiency is density independent . A question remains whether this matters as C_4 capture would also be reduced. Thus

100 *	0.6 *	0.8 *	0.1 *	1	=	4.8	unsprayed Bt cotton in 80-20
100 *	0.6 *	0.2 *	1 *	0.2	=	2.4	sprayed refuge in 80-20
100 *	0.38 *	0.95 *	0.1 *	1	=	3.6	unsprayed Bt cotton in 95-5
100 *	0.38 *	0.05 *	1 *	1	=	1.9	unsprayed refuge in 95-5
100 *	0.02 *	1 *	1 *	3	=	6.0	area planted to chickpeas.

This is a good example of where moths come from in a low dose situation. The refuges only account for slightly more than half of the adults, but they do it on 16% of the area.

The predicted total C_3 moths sampled would then be X=18.7.

To work backwards, we must know X, the S_i (which is the product of columns 2 and 3), the M_i (column 4) and P_i (the relative productivity given in column 5). Estimates of all these parameters could be derived from the data collected by the registrant.

We then apply equation 1 from above:

X = 0.6*0.8*0.1*x+0.6*0.2*0.2*x+0.38*0.95*0.1*x+0.38*0.05*x+0.02*3*x

18.7 = 0.1851x

x= 101 a reasonable estimate given rounding error.

Again, this is not an estimate of refuge size, but it does give an estimate of the initial proportion of eggs that were laid on C_3 crops. If our selection model does not account for sprays in the 20% refuges nor the oversprays, then it is correct to add these numbers back into the C_3 number. If the model does not include differences in productivity, those individuals should be accounted for here. It would be more interesting to isolate the effects of the selection for *Bt* resistance from all these other components, based on an interest in refuge size. If you want to account for all these C_4 individuals, then the complete calculation is most appropriate (and would make your job easier).

The question of most general biological importance would be to estimate the actual proportion of C_3 and C_4 hosts, and this would require adjustments of all C_3 hosts, as well as any C_4 hosts that might be contributing and have their productivity impaired by anthropogenic means (*Bt*-corn, perhaps sorghum). Although Gould et al. (2001) suggested that whatever the source, something must be producing many C_4 moths, it may be instead that something is removing C_3 moths. The actual ratio of eggs laid on the two types of hosts may be quite different than the ratio of adults produced, perhaps significantly so. In this specific case, the ratio might be less than the observed 40%, perhaps as little as 4-5%. Given that the registrant data indicates observed ratios as low as 10%, the corrected ratios could be as low as 1-2%, and it is this number which should be incorporated into most models. One might then begin to seriously suggest that wild hosts or corn regrowth could account for a significant portion of the C_4 moths, and it might also make us rethink the importance of North-South migration. It should be the job of the selection model to properly account for points such as sprays and productivity differences between crops (as the both the Gustafson and Storer models attempt to). Of course, as long as the data is there, it would make sense to take the data for all the crops as is, adjust the *Bt*-cotton data as we are interested in selection for resistance to it, and then properly allocate all the habitats, both C_3 and C_4, to refuge that are not selected. This would give us a reasonably good estimate of the actual refuge size, rather than just mentioning C_4 hosts as a sort of minimal refuge size. Such an approach would better utilize the registrant data and may be workable since the data had already been collected by the registrant.