**UNITED STATES ENVIRONMENTAL PROTECTION AGENCY**
WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES, AND
TOXIC SUBSTANCES

September 8, 2006

**MEMORANDUM**

**SUBJECT:** Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting Held June 13 - 15, 2006 on the Analysis of a Natural Refuge of Non-Cotton Hosts for Monsanto's Bollgard II Cotton.

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

**FROM:** Myrta R. Christian, Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

**THRU:** Steven Knott, Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Clifford J. Gabriel, Ph.D., Director
Office of Science Coordination and Policy

Attached, please find the meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, Virginia on June 13 - 15, 2006. This report addresses a set of scientific issues being considered by the Environmental Protection Agency pertaining to the Analysis of a Natural Refuge of Non-Cotton Hosts for Monsanto's Bollgard II Cotton.

Attachment

**cc:**

James B. Gulliford
Susan Hazen
Margaret Schneider
Amy Farrell
Anne Lindsay
Margie Fehrenbach
Janet Andersen
Debbie Edwards
Steven Bradbury
William Diamond
Arnold Layne
Tina Levine
Lois Rossi
Frank Sanders
Richard Keigwin
William Jordan
Douglas Parsons
Enesta Jones
Vanessa Vu (SAB)
Alan Reynolds
Sharlene Matten
Leonard Cole
Betty Shackleford
OPP Docket

**FIFRA Scientific Advisory Panel Members**

Steven G. Heeringa, Ph.D. (Chair of the FIFRA SAP)
Kenneth M. Portier, Ph.D.
Janice E. Chambers, Ph.D.
Gary Isom, Ph.D.

**FQPA Science Review Board Members**

Gary P. Fitt, Ph.D.
Fred L. Gould, Ph.D.
Charles A. Guse, M.S.
David G. Heckel, Ph.D.
Terrance M. Hurley, Ph.D.
Anthony R. Ives, Ph.D.
Michael J. Livingston, Ph.D.
Thomas W. Sappington, Ph.D.
John C. Schneider, Ph.D.

# SAP Minutes No. 2006-03

## A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

# ANALYSIS OF A NATURAL REFUGE OF NON-COTTON HOSTS FOR MONSANTO'S BOLLGARD II COTTON

**JUNE 13 - 15, 2006**
**FIFRA Scientific Advisory Panel Meeting,**
**held at the Holiday Inn - National Airport,**
**Arlington, Virginia**

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). The meeting minutes represent the views and recommendations of the FIFRA SAP, not the United States Environmental Protection Agency (Agency). The content of the meeting minutes does not represent information approved or disseminated by the Agency. The meeting minutes have not been reviewed for approval by the Agency and, hence, the contents of these meeting minutes 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 is a Federal advisory committee operating in accordance with the Federal Advisory Committee Act and established under the provisions of FIFRA as amended by the Food Quality Protection Act (FQPA) of 1996. The FIFRA SAP provides 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 Myrta R. Christian, SAP Designated Federal Official, via e-mail at christian.myrta@epa.gov.

In preparing the 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 by the Agency within the structure of the charge.

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# SAP Minutes No. 2006-03

## A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

## ANALYSIS OF A NATURAL REFUGE OF NON-COTTON HOSTS FOR MONSANTO'S BOLLGARD II COTTON

## JUNE 13 - 15, 2006
## FIFRA Scientific Advisory Panel Meeting, held at the Holiday Inn - National Airport, Arlington, Virginia

**Steven G. Heeringa, Ph.D.**
**FIFRA SAP Chair**
**FIFRA Scientific Advisory Panel**
**Date: September 8, 2006**

**Myrta R. Christian, M.S**
**Designated Federal Official**
**FIFRA Scientific Advisory Panel**
**Date: September 8. 2006**

**Federal Insecticide, Fungicide, and Rodenticide Act**
**Scientific Advisory Panel Meeting**
**June 13 - 15, 2006**

# ANALYSIS OF A NATURAL REFUGE OF NON-COTTON HOSTS FOR MONSANTO'S BOLLGARD II COTTON

## PARTICIPANTS

### FIFRA SAP Chair

**Steven G. Heeringa, Ph.D.**, Research Scientist & Director for Statistical Design, University of Michigan, Institute for Social Research, Ann Arbor, MI

### Designated Federal Official

**Myrta R. Christian, M.S.**, FIFRA Scientific Advisory Panel, Office of Science Coordination and Policy, EPA

### FIFRA Scientific Advisory Panel Members

**Janice E. Chambers, Ph.D.**, **D.A.B.T.**, William L. Giles Distinguished Professor & Director, Center for Environmental Health Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS

**Gary E. Isom, Ph.D.**, Professor of Toxicology, School of Pharmacy & Pharmacal Sciences, Purdue University, West Lafayette, IN

**Kenneth M. Portier, Ph.D.**, Program Director, Statistics, American Cancer Society, Statistics and Evaluation Center, Atlanta, GA

### FQPA Science Review Board Members

**Gary P. Fitt, Ph.D.**, Assistant Chief, CSIRO Entomology, Australian Cotton CRC Indooroopilly, Queensland, Australia

**Fred L. Gould, Ph.D.**, William Neal Reynolds Professor of Agriculture, North Carolina State University, Raleigh, NC

**Charles A. Guse, M.S.**, Research Associate, Department of Crop Sciences University of Illinois, Urbana, IL

**David G. Heckel, Ph.D.**, Director, Max-Planck-Institute of Chemical Ecology, Beutenberg Campus, Jena, Germany

**Terrance M. Hurley, Ph.D.**, Associate Professor, Department of Applied Economics University of Minnesota, St. Paul, MN

**Anthony R. Ives, Ph.D.**, Professor, Department of Zoology, University of Wisconsin-Madison, Madison, WI

**Michael J. Livingston, Ph.D.**, Agricultural Economist, U.S.D.A., Economic Research Service, Washington, D.C.

**Thomas W. Sappington, Ph.D.**, Research Entomologist, U.S.D.A., Agricultural Research Service, Corn Insects & Crop Genetics Research Unit, Iowa State University, Ames, IA

**John C. Schneider, Ph.D.**, Professor/Research Entomologist, Department of Entomology & Plant Pathology, Mississippi State University, Mississippi State, MS

## INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed their review of the Analysis of a Natural Refuge of Non-Cotton Hosts for Monsanto's Bollgard II Cotton. Advance notice of the meeting was published in the *Federal Register* on March 31, 2006. The review was conducted in an open Panel meeting held in Arlington, Virginia, from June 13 to June 15, 2006. Dr. Steven G. Heeringa chaired the meeting. Myrta R. Christian served as the Designated Federal Official.

The FIFRA SAP met to consider and review the Analysis of a Natural Refuge of Non-Cotton Hosts for Monsanto's Bollgard II Cotton. The Agency was seeking input from the Scientific Advisory Panel on whether a natural refuge of non cotton hosts is an effective refuge to delay the potential for tobacco budworm resistance to the proteins (Cry1Ac and Cry2Ab2) expressed in Bollgard II® cotton. Monsanto Company had submitted an application for the extension of the FIFRA section 3 registration of the plant-incorporated protectants (PIP) *Bacillus thuringiensis* Cry2Ab2 protein and the genetic material necessary for their production [PV-GHBK11] in event MON 15985 cotton and *Bacillus thuringiensis* Cry1Ac protein and the genetic material necessary for their production [PV-GHBK04] in event MON 15985 cotton. This product is intended to provide protection against tobacco budworm, cotton bollworm, pink bollworm, loopers, armyworms, and other lepidopteran insects. The data submitted included the productivity of tobacco budworm on each alternative host, timing and synchrony of production on each alternative host, the spatial and temporal scale of alternative hosts, and modeling efforts to simulate the likelihood of resistance under different regional scenarios.

The agenda for this SAP meeting included an introduction of the issues under consideration provided by Mr. Leonard Cole (Biopesticides and Pollution Prevention Division (BPPD), OPP). Issues related to the tobacco budworm sampling and gossypol analysis were provided by Mr. Alan Reynolds (BPPD, OPP). Issues related to effective refuge calculations and modeling for tobacco budworm and cotton bollworm were presented by Dr. Sharlene Matten (BPPD, OPP).

Dr. Janet Andersen (Director, BPPD, OPP) offered opening remarks at the meeting.

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 at the meeting, especially the response to the Agency's charge.

## PUBLIC COMMENTERS

**Oral statements were presented as follows:**

Graham Head, Ph.D., on behalf of Monsanto Corporation
Mr. Kenneth B. Hood on behalf of Perthshire Farms in Gunnison, MS
B. Roger Leonard, Ph.D., on behalf of Louisiana State University, LSU Ag Center, and selected cotton organizations in LA
Phillip Roberts, Ph.D., on his own behalf
Richard T. Roush, Ph.D., on his own behalf
Nicholas P. Storer, Ph.D., on behalf of Dow AgroSciences LLC
Mr. Mike Tate on behalf of National Cotton Council
Michael F. Treacy, Ph.D., and Sidney W. Hopkins, Ph.D., on behalf of Hopkins Agricultural Services, Inc.
Mr. Ray Young on behalf of Louisiana Ag Consultant's Organization

**Written statements were provided by:**

Craig A. Abel, Ph.D., Agricultural Research Service, U.S. Department of Agriculture
Michael Adang, Ph.D., University of Georgia
Michael A. Caprio, Ph.D., Mississippi State University
Mr. David Dunlow, President, North Carolina Cotton Producers Association, Inc.
Mr. Allen B. Helms, Jr., National Cotton Council of America
James C. Jennings, Ph.D., U.S. Biotechnology Regulatory Affairs
Michele C. Mara, Ph.D., and Nicholas E. Piggott, Ph.D., North Carolina State University
Mr. Bruce Niderhauser, President, North Carolina Crop Consultants Association
Richard T. Roush, Ph.D., University of California Division of Agriculture and Natural Resources, Davis, CA
Nicholas P. Storer, Ph.D., Dow AgroSciences

## SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

### Sampling and Methodology

Monsanto relied entirely on pheromone traps to sample populations of tobacco budworm (TBW), and therefore based all conclusions on analyses of males only. The Panel recognizes that both male and female movement affects resistance evolution, and that our knowledge of TBW movement patterns and behavior is incomplete. While the Panel agreed that sampling TBW populations with pheromone traps was a logical and valid approach in principle, caution must be exercised in interpreting data based on pheromone trap sampling, because there are numerous uncertainties arising from our limited knowledge of TBW movement and trapability. For example, the geographic scale to which the trap captures can be extrapolated remains unknown. Furthermore, the captures provide no information about the proportion of moths that leave their natal habitat, which has a large effect on the rate of resistance development.

The Panel identified a number of uncertainties and potential biases associated with the sampling design itself. The distribution of traps among production regions and counties was highly variable and non-random, with sampling intensity in some regions being very low. The sequential subsampling strategy biased estimates of the proportion of non-cotton-fed males upward. Of substantial concern to the Panel is that the novel gossypol analysis technique itself has not been validated by other laboratories, the threshold of detectability was not reported, and several key assumptions associated with its use and data interpretation have not been tested. Given that the gossypol data are the foundation of Monsanto's petition, it is critical that EPA scrutinize the technique and its assumptions thoroughly.

Annual variation in effectiveness of unstructured refuge can arise from annual variation in per-plant insect production from alternative hosts, density of wild hosts, and percent of total acreage planted to various non-Bt host crops. Variable weather can have a significant effect on temporal and spatial availability and quality of wild host plants. The Panel agrees that one year of data in Texas and Tennessee are insufficient to assess the stability and adequacy of unstructured refuge in those areas. The Panel is concerned that the proportion of natural refuges and alternative hosts may be generally low in parts of the ”MidSouth” region (Arkansas, Louisiana, and Mississippi, in addition to Tennessee and East Texas). Some Panel members agreed that two years of data from North Carolina and Georgia indicate that natural refuge and continued cultivation of a high proportion of non-Bt corn in these states could maintain resistance management in the absence of structured refuge.[1]

The Panel noted several sampling biases apparent in the estimation of the proportion of TBW natural refuge, which were generated by low trap captures and Monsanto's handling of those situations. Exclusion of dates and locations with zero captures introduces a downward bias

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[1] Following this FIFRA SAP meeting, a Panel member provided additional analysis and comments regarding the validity of extrapolating data from areas sampled by Monsanto in North Carolina and Georgia to areas that were not sampled. Such comments were not considered or reviewed by the Panel during the meeting, and are being provided as an appendix to these meeting minutes (Appendix 5).

in estimates of proportion natural refuge ($R_{nat}$) for the MidSouth region. Although the "worst-case" counties targeted by Monsanto for sampling as a group have relatively complete data, they in fact may not be worst case, since the true worst cases cannot be identified due to lack of data.

**Statistical Analyses**

Monsanto's approach to pooling the gossypol data was to use a simple multiple-test method. The decision to pool was a global one in that every sample date within a month for a sample location was pooled, or every sample location within a county was pooled. While there are some advantages to this method, it has some significant disadvantages as well, and the Panel concluded that there are better, more powerful statistical methods available for determining the appropriateness of pooling. The Panel emphasized the need to incorporate biological justifications into decisions to pool, where to pool, and when to pool, rather than relying on geopolitical spatial boundaries or calendar month temporal boundaries. The Panel suggested an approach to analysis where two or more alternative generalized linear mixed effects models are created about plausible alternative hypotheses and to use formal statistical tests to determine whether any of these alternative models is significantly better at fitting the data in hand than the null hypothesis model. Benefits to this approach include the ability to 1) estimate the variance components for the deviations from mean percent; 2) determine if these variance components can be related to other covariates, and 3) incorporate and test for the presence of correlation in responses one might expect from repeated measurements in time. None of these three are possible with the multiple-testing approach. The analysis of gossypol fraction differences among counties and months using the linear logistic model also could be formulated more appropriately as a generalized linear mixed effects model. The results may suggest less pooling, different spatial pooling, and/or different temporal pooling than Monsanto's multiple-test analysis. The Panel agreed that the investment in this more-complex and formal analysis is warranted, because the estimates of gossypol fraction produced from the analysis form the basis for subsequent refuge size estimates.

Although Monsanto supplied only data pooled across dates within month for each trap and then across traps within county, the Panel performed a preliminary generalized linear mixed effects model analysis on these data to address EPA's charge to "describe" statistical analyses quantifying variation in the natural refuge across locations and time. This analysis was intended to be illustrative of the linear mixed effects model approach recommended by the Panel, but not intended to be definitive. It is not definitive because of problems with the data (selection biases and spatially inadequate sampling) and because alternative, equally well-justified, ecologically-relevant schemes for pooling states into regions other than the East and MidSouth regions used in this analysis can be envisioned. In spite of these limitations, several results are relevant to EPA's charge to the Panel: $R_{nat}$ declined with Month (June, July, August), and intensity of agricultural activity ("Hills" vs. "Flats", see below) and Year (2004, 2005) were not statistically significant effects in the model.

**Effective Refuge Calculation and Modeling**

In its calculation of $R_{nat}$ for CBW, Monsanto removed non-Bt cotton moth production from both the numerator and denominator of the effective refuge calculation. The Panel

determined that this equation instead should omit this parameter only from the numerator, and consequently Monsanto's equation for $R_{nat}$ leads to overestimates that can be substantial. Based on data reported in Monsanto's petition, overestimates of the natural refuge for CBW were largest for Georgia (37%) and East Texas (44%).

Furthermore, although Monsanto included the effects of insecticide sprays in its model for CBW at the request of the 2004 SAP, it did not do so for the TBW model. Without correction for spraying in non-Bt cotton, Monsanto's equations can substantially overestimate the amount of $R_{nat}$ for TBW, with the magnitude of the correction depending on the proportions of the three refuge options used. Monsanto provided insufficient data to make these corrections. Estimates of $R_{nat}$ were below 0.05 for some "worst-case" counties in Mississippi and Louisiana, even without adjusting for insecticide-induced mortality in Bt-cotton refuges. With this adjustment, estimates of $R_{nat}$ could be even lower.

For both CBW and TBW, the calculations assume that males are well-mixed at the spatial scale of variation in habitat types. Strong circumstantial evidence suggests this probably is not true for TBW. $R_{nat}$ will be overestimated in counties with extensive cotton production, because underestimates of the proportion of trapped male TBW originating from non-Bt cotton will lead to overestimates of $R_{nat}$. This could be an important source of overestimation if the gossypol assay gives false negatives, a concern of the Panel given Monsanto's description of this technique.

The estimates of $R_{eff}$ and $R_{nat}$ are imprecise, due to uncertainty in the estimates of the parameters in the equations. Imprecision is possibly large for TBW in those counties used as scenarios for modeling, because the estimates of $R_{eff}$ and $R_{nat}$ for these counties are low. Thus the Panel notes the necessity of calculating confidence intervals for estimates of $R_{eff}$ and $R_{nat}$.

The Panel agrees that Monsanto's simple deterministic model has identified the geographic regions where there is very little risk of resistance developing (e.g., Georgia). It also, therefore, identifies the regions where the risk of resistance developing is greater (e.g., the MidSouth region). However, the model as executed cannot adequately assess these risks because years to resistance and product efficacy are insufficient for risk evaluation. Proper assessment of risk in these regions requires acquisition of more data, a more robust statistical analysis of the data, and a more detailed approach to modeling that includes both spatial and temporal variability in natural refuge. In predicting resistance evolution to Bollgard II, there is not only uncertainty in the estimation of parameters used in models, but also "model uncertainty." Structural or model uncertainty is difficult to assess, because it may depend on subtle assumptions made in modeling that have large impacts on model predictions. The only way to address model uncertainty is to analyze multiple models that as a group encompass a range of assumptions about resistance evolution. Three principle assumptions of Monsanto's model were challenged, specifically widespread dispersal of the pests, socio-economic factors affecting the market share of the products, and the single–locus resistance per toxin receptor with no cross resistance. These assumptions will likely mean that Monsanto is overestimating the time to resistance.. On the other hand, two broad assumptions made by Monsanto – that there are no fitness costs associated with resistance, and that resistance corresponds to a single locus per toxin – might lead Monsanto to underestimate the time to resistance. Determining the net effect of the

numerous simplifying assumptions made in the Monsanto model requires a more rigorous modeling effort.

Two important dimensions to the question of mosaics of single and dual gene products are product market share and temporal variability of the market share. Monsanto addressed the product market share dimension but not the temporal changes that should be expected as individual products lose efficacy. The Panel conducted a controlled experiment in which the temporal variability of the mosaic was held constant while varying product market shares. The results revealed an opportunity cost of increasing the market share of the pyramided product in order to reduce selection for the shared toxin. This opportunity cost is an increase in selection pressure for the pyramided toxin. In a second experiment where total market share was held constant with increasing temporal variability in the adoption of the pyramided product, the single toxin product is on the market longer, which speeds the evolution of resistance to the shared toxin. While these experiments suggest that a quick transition to pyramided toxin products may not always be the best strategy due to the opportunity cost of increasing selection for the pyramided toxin, slow transition rates can only be supported by relatively heavy selection pressure on the pyramided toxin. The Panel agreed that the majority of papers published over the past decade using a variety of modeling strategies have found that quicker transitions to pyramided toxin products are much preferable in terms of resistance management.

**Overall Data/Results Interpretation**

The Panel cautions EPA that Monsanto's model is a non-spatial model implemented deterministically and that this technique may be applicable to a very limited set of specific geographic situations. The Panel's acceptance of the results for a particular cropping system and pest species should not be interpreted as a precedent for future registrations.

There are many uncertainties, caveats, and assumptions evident throughout the modeling and analyses presented by Monsanto and revealed by the Panel discussion around the questions posed by EPA. Most of these by themselves might not in fact prove dangerous to IRM for CBW and TBW, but collectively they represent unacceptably high levels of uncertainty, especially for the MidSouth region.

The key data that Monsanto uses to assess natural refuges comes from the gossypol assay for identifying the non-cotton fraction of the TBW population. While this technique is innovative and potentially very valuable, the Panel had concerns about its validity, accuracy and repeatability. Although the Panel received a description of the analytical technique, it was not complete and important questions concerning the validity of the technique were identified. The Panel thus recommends additional review of the technique by EPA staff, publication of the assay method in a peer-reviewed journal, performance and publication of experiments to mimic the conditions experienced by trapped males prior to analysis, and validation of the methodology by independent laboratories. If the gossypol technique withstands critical scrutiny, the following comments will apply.

The Panel has noted some potentially serious errors and biases in Monsanto's calculation of natural refuge for TBW and CBW which must be addressed. In addition, the Panel would

prefer a more integrated and comprehensive statistical analysis of the spatial and temporal variability of refuge estimates and moth trap data to tease out crucial details regarding the appropriate spatial regions and the critical temporal periods that pose the most risk. Despite the potential biases, some Panel members concluded that for North Carolina and Georgia there are significant and reliable non-cotton refuges present that should be adequate to manage Bt resistance in TBW associated with cotton systems involving Bollgard II cotton.[2] Current evidence for adequate natural refuge in the MidSouth region is not convincing. Because resistance likely will evolve in areas with little effective refuge, such areas are of particular concern. When the estimated proportion of natural refuge for CBW and TBW is low (5-10%), higher levels of uncertainty attach to a number of assumptions and calculations for the proportion of effective refuge. Estimates of natural refuge below 5% or even (depending on refuge option) below 1% are not uncommon in the MidSouth. Alabama was not sampled at all and, as a transitional state between the East and MidSouth regions, must be sampled before a recommendation can be made. Tennessee and East Texas require additional sampling because both were sampled only one year. Other ecologically distinct production areas in Texas likewise must be sampled. Only with additional information can an informed judgment be made regarding the stability and adequacy of the natural refuge in these areas.

## PANEL DISCUSSION AND RECOMMENDATIONS

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

### Sampling and Methodology

### Agency Charge

**1. The Panel is asked to comment on the pheromone sampling strategy employed by Monsanto in which only male tobacco budworm (TBW) were trapped.**

**Is this an appropriate sampling strategy? Can inferences about female TBW be derived from data gathered exclusively with males?**

### Panel Response

**Summary**

[2] Following this FIFRA SAP meeting, a Panel member provided additional analysis and comments regarding the validity of extrapolating data from areas sampled by Monsanto in North Carolina and Georgia to areas that were not sampled. Such comments were not considered or reviewed by the Panel during the meeting, and are being provided as an appendix to these meeting minutes (Appendix 5).

After a review of current knowledge of dispersal behavior of TBW and other heliothines, the Panel considers the appropriateness of pheromone sampling for making inferences about the availability of susceptible insects from natural and managed refuges. The Panel points out that despite intensive sampling in some areas, other cotton-producing areas of equal interest were under-represented and thus the overall picture is incomplete. For the better-sampled areas, the Panel feels that despite the existence of certain biases that remain because females were not sampled, it is the abundance and distribution of the refuge-generated male population that is of greater importance, and so trapping males is an appropriate strategy. The evidence for an adequate supply of refuge-generated males does not depend on the total trap counts as much as it does on the fraction of the trapped sample that tested negative in the gossypol assay. There is a bias inherent in the subsampling method used by Monsanto to estimate this fraction from traps that produced too many moths to test individually. Moreover, from the standpoint of determining the fraction of a trapped sample that originated from cotton, there are additional potentially serious biases that have not been adequately addressed. Extrapolating the results of the gossypol analysis from the trapped sample to the population as a whole depends on the validity of assumptions that have not been sufficiently tested, according to the information provided by Monsanto.

**Background**

Pheromone trapping uses chemically-synthesized components of the female-produced male-attraction pheromone to lure and trap males of a given species. It enables the collection of male moths with a relatively low effort as traps can be left at the same locations for prolonged periods of time. Males are usually trapped at night when they are actively seeking females for mating. Females are not attracted to the traps. When the traps are visited and contents removed, the total number of males since the last visit can be recorded, and the moths are available for analysis, although these can be in poor condition or even dead for several days. Many factors, including weather conditions and trap location in the landscape, influence how many moths are caught in a given night by a particular trap. The actual number trapped also depends on the effective area from which moths are attracted; on how attractive the trap is (i.e., the per-moth probability of being trapped given that they are in that area); and on the number of moths in that area. Although large fluctuations in trap catch probably reflect large fluctuations in the number of moths in the average effective trapped area, a more precise statement is usually not possible because of the large variability of other conditions. It is not generally accepted by entomologists that trap catches provide a robust and accurate measure of absolute abundance in a given area.

The question is whether this is an appropriate sampling strategy for the purposes of evaluating the durability of transgenic cotton to resistance development in the pest. Trap catches were used by Monsanto to infer two different things:

1) *Abundance of the pest population over time.* These include comparisons over the course of the season as well as between different counties and states. A high local abundance and/or high dispersal is inferred from a high trap count.

2) *The composition of the sample.* Among the trapped moths, the ratio of two different types, classified according to some physical or chemical analysis, is used to infer the type of

plant the moth consumed when it was a larva. These include carbon isotopes reflecting the C3 or C4 photosynthetic pathway, presence of gossypol reflecting consumption of cotton, or presence of cotinine reflecting consumption of tobacco.

In the following sections, the Panel addresses these two issues as they relate to current knowledge of moth abundance and dispersal patterns, and how reliably and over what scale these are indicated by pheromone trap counts. The Panel then considers whether the sampling intensity and frequency employed by Monsanto was adequate. Finally, the appropriateness of this sampling strategy to estimating the likely contribution of males originating from non-cotton host plants to the refuge population is considered.

**Overall dispersal patterns of adult moths.**

The appropriateness of male pheromone trapping as a sampling technique should be evaluated in light of what is known about dispersal. Male and female dispersion across landscapes are related, at least to the extent that males having arrived in a cotton field from elsewhere may indicate that females have moved there as well. However, Monsanto's categorical statement that dispersal behavior of males and females is similar for TBW (Gustafson et al. 2005) is inaccurate because this remains an open question. In most heliothine species both sexes can undertake extensive pre-mating movements (Fitt 1991). Females usually do not reach sexual maturity and "call" by releasing pheromone until the second or third night after emergence. An assessment of the local area and available crops encountered by newly emerged moths as they search for nectar sources and potential oviposition sites probably plays a significant part in how far moths disperse (Fitt 1991) and whether they undertake a truly migratory movement out of the region altogether (Fitt 1989, Fitt et al 1995). Nonetheless, the scale of local movements will usually allow moths to "sample" the local environment much more broadly than the natal field where they emerge. Likewise these species typically display a period of nectar feeding, oviposition and short-range flights immediately after dusk each night of their lives which will further re-distribute moths outside of fields to adjacent habitats or other fields.

Differential mobility of males and females might be envisaged since males actively search for stationary females. During mating, which occurs from 1-2 hours after dusk until 3-4:00 am, females are inactive, releasing pheromone from near the tops of plants, while males engage in characteristic high-speed, directed flights in search of pheromone plumes (Fitt 1989). However, the characteristic mate searching flights displayed by males when casting for pheromone plumes is almost always constrained within a field or habitat/crop patch, at least for some heliothines. For example, in a field with a large strip of corn embedded in cotton, males of *Helicoverpa armigera* cast back and forth above the corn. When they crossed the transition between corn and cotton they flew 5-15 m into the cotton before quickly rebounding and flying back above the corn. This phenomenon was observed simultaneously on both edges of a 2-m tall 5-ha block of corn in a 20-ha block of cotton (Fitt unpublished).

Mark-recapture studies have been conducted to characterize the pattern of adult TBW dispersal and to compare dispersal of males and females. Schneider et al. (1989) estimated that about 70% of emerged marked males moved greater than 18 km (i.e., out of the sampling arena) without being trapped. Extrapolation of dispersal curves suggested that all released males would

be within 50 km of the edge of the study area. The same study compared the movement of males and females in a large-scale, mark-release-capture experiment in the Mississippi Delta region. Progeny of released females were distributed at least as far from the release area as were released/pheromone trap-captured males. Thus, it appears that female TBW can move as much or more than males under at least some conditions. Schneider (1999) used mark-recapture data from four different years to estimate median movement of males ranging from 9.3 – 23.2 km per generation, and calculated an effective sampling area of about 20 ha/trap. They observed patchiness in trap captures at the scale of several km, but a mostly uniform distribution over a scale of tens of km.

F-statistics based on allozyme variation led Korman et al. (1993) to conclude that the average diameter of a local TBW population was only 8 km or less. Data from genetic markers in general indicate low genetic structuring (and therefore high gene flow) across wide geographic areas. At the same time, "typical" gene flow seems to be temporally dynamic, highest in the spring, and restricted later in the season as evidenced by increasing $F_{ST}$'s (genetic differentiation) as the season progresses (Han and Caprio 2004). This pattern of apparent decreased gene flow during the summer is supported by observations of increasing pyrethroid resistance as the season progresses, with a drop in resistance at the beginning of the following year (Luttrell et al. 1991, Sparks et al. 1993, Leonard et al. 1995, Bagwell et al. 2000). The difference in per-generation movement observed by Schneider et al. (1989) and Schneider (1999), where releases were made early in the season, and that deduced by Korman et al. (1993), where moths were collected in late June and early July, could be explained by this phenomenon as well (Schneider 1999, Han and Caprio 2004).

**Is pheromone trapping an appropriate sampling strategy?**

The Panel generally agreed that the use of pheromone traps was a logical and valid way to sample from extensive populations of CBW and TBW. Pheromone traps are widely used for broad scale population monitoring of heliothines, but as noted by many Panel members pheromone traps are not utilized for making management decisions in the immediately adjacent crop, because of the inconsistent relationship between males captured in a pheromone trap and the number and reproductive activity of females in adjacent fields. Monsanto has relied on Leonard et al (1989) to justify the use of pheromone traps on the basis that there is a positive relationship between trap catches and egg densities in nearby cotton. However, the positive relationship found in that study applied not on a trap-by-trap, field-by-field basis; but rather over a large local region (5-6.5 km radius), when TBW and CBW species, 8-10 pairs of traps, and 30-40 fields were all pooled. The same conclusion can be drawn in Australia where pheromone traps are poorly related to egg densities in adjacent fields. The Panel agreed that the main utility of pheromone trapping for insect pest and resistance management is to follow general trends and to obtain an idea of relative population levels for an area. In essence the validity of Monsanto's data collection is not reliant on a tight coupling of pheromone trap catches to local population dynamics. The key point is that analysis of moths from traps placed adjacent to cotton is to demonstrate that a significant proportion of those moths have developed on non-cotton hosts (based on C3/C4 analysis for CBW and gossypol analysis for TBW). But the general lack of relationship between trap captures and local dynamics does indicate that conclusions drawn about host history are valid only at some geographic scale above that of the trapping radius, a

scale that remains undefined. Thus the Panel agreed that the use of traps to collect males as an indicator of the host source, but not the geographic origin, of local populations is appropriate, unless host source somehow affects trapability (considered below).

However, the distribution of traps among production regions and counties was highly variable and non-random. Monsanto deliberately sampled in regions of interest with high cotton production. In spite of this intended emphasis, the intensity of sampling in some regions is very low. For example, there is no sampling in Alabama. Louisiana, which grows as much cotton as the whole of Australia and has a "cotton intensity" score similar to or greater than North Carolina (Monsanto Report 1, Table 3), was sampled with only four traps in one year and five in the next. Trapping intensities used in North Carolina, Arkansas, and Mississippi (about 40 trap locations in each) seem more reasonable and reliable. Since the western parts of the Cotton Belt (Louisiana, Texas) appear to have the lowest percentage of natural refuge and the greatest month to month variation (e.g., July versus August 2004 in Bossier County) we need more sampling points for more certainty about the estimates of refuge.

Three additional points apply to the conclusion about the general appropriateness of the male pheromone-trapping strategy:

1) *Movement of both males and females affects resistance evolution*, and it must be acknowledged that until additional experimental work is devoted to tracking females, our knowledge remains incomplete. The Panel felt that with a modest amount of additional effort, Monsanto could have obtained data to determine whether the host-plant history of males captured in pheromone traps is representative of females in the same area sampled by the trap. Females could be caught with a light trap or by hand, and gossypol content compared to that of males captured at the same time. Such a test would not have to be replicated as extensively as the pheromone trapping, although the comparisons should be replicated over time at each location during the season. A few paired comparisons in a single year across the different regions should be sufficient to address this question. The presence of differences at this point would indicate more extensive experiments are needed to characterize the female-specific patterns further, find out why they differ from the males, and assess the consequences to interpretation of the data and to resistance development.

   Mitigating this concern to some extent, the Panel considered that male dispersal is more important than that of females from the standpoint of evaluating the distribution of moths relative to refuges. Given the efficacy of Bollgard and Bollgard II for TBW it is reasonable to assume that any moths managing to emerge from a Bt cotton field are likely to be resistant. Emergent adults are likely to remain concentrated in the vicinity of the natal field throughout most of the cotton season while the crop remains acceptable for oviposition (Farrow and Daly 1987, Fitt et al. 1989, Schneider 1999, 2003, Han and Caprio 2004, Zalucki and Furlong 2005). What is thus required is an abundance of unselected males moving from the refuges into the Bt cotton area, to be chosen by resistant females for mating in preference to a resistant male. Thus, given what is known about TBW adult behavior, it is the mobility of males originating in refuges the Panel is most concerned about.

2) *The data collected on males give no information about the proportion that leaves their natal habitat*, which has a large effect on the rate of resistance in all of the models. Based on his literature review for Monsanto, Benedict (2005) comes to the conclusion that "pheromone traps are unlikely to accurately represent the absolute densities of males emerging from the crop they are placed in...". Monsanto purposely placed most of its traps next to a cotton field. Therefore it is impossible to know whether and what proportion of captured males with a cotton host-history emerged from the adjacent cotton field or were immigrants from unknown distances. If the Monsanto estimate of insects coming from non-cotton sources is biased, it is likely to be biased towards showing a proportion coming from cotton that is higher than the true proportion.

3) *Even if the proportion of males coming from non-Bt cotton vs. non-cotton is the same at the scale of a county, this does not imply that males are moving at the scale of the county.* Instead, this pattern could arise even if males show limited dispersal distances, provided the distribution of habitat types is relatively uniform across the county.

**Use of pheromone traps results to infer composition of the sample**.

The question here is whether the analytical method for detecting gossypol used on a subsample of trapped males provides an accurate estimate of the fraction of the total TBW in the sampled area that completed their larval development on cotton. There are at least three plausible sources of bias in the estimation:

1) *The sequential subsampling strategy itself, as described by Monsanto, exhibits a bias.* From the traps producing many males, the analysis was done in batches of ten. If the initial results indicated a fraction of cotton-feeding males was greater than 90%, another batch was analyzed. Thus the number of males analyzed from a given trap is not independent of the frequency of cotton-feeding males. This produces a biased estimate in favor of the fraction of non-cotton-fed males.

2) *There could be a difference between males and females with respect to the analytical method for detecting gossypol.* The reproductive tissues of males and females are quite different and may accumulate or retain gossypol in different amounts. The two sexes have different activity patterns and this might also affect retention of gossypol and a differential rate of change with the aging of the moth. The amount of gossypol available to the analytical method may differ between the sexes. Monsanto provided no data on the sexes of the individuals used in the laboratory validation experiments. If these are primarily females, they may not be appropriate for calibrating measurements on field-collected males.

3) *There could be other sources of variation in the amount of detectable gossypol in field-collected males* of different ages, collected in different weather conditions, and dead for different lengths of time. These could bias the estimation of cotton-feeders among non-trapped females as well as non-trapped males in the sampled areas. This is the most significant potential bias, as it would affect all of the estimates of the non-cotton-source

males and hence the estimates of the natural refuge. For example, if the amount of gossypol in a freshly-collected, cotton-fed male was high enough to be detected by the method, but the amount in a cotton-fed male that had been dead in the trap for five days was not high enough, the degree of bias would depend on the unknown distribution of arrival times of moths into the trap.

This last potential source of bias is particularly troubling because the supplementary information provided by Monsanto (draft manuscript by Orth, Head and Mierkowski, EPA-HQ-OPP-2006-0217-0013) raises several questions about the suitability of the analytical method to detect gossypol in moths. This is not a quantitative method. It does not permit the estimation of the absolute amount of gossypol in a sample by means of a standard curve. The detection method depends on the rate of fragmentation of the Schiff base in the electrospray apparatus, which in general is not a controllable process and furthermore depends on the body mass of the moth. The authors acknowledge problems with the internal standard as well. Since quantification is not possible with this method, the authors use classification criteria that they state are dependent on the actual apparatus used as well as the absolute amount of internal standard. These criteria are evaluated using laboratory-reared insects that are freeze-dried immediately upon emerging as adults, and kept frozen until analysis. These preservation conditions are very different from those experienced by the field-collected males from the pheromone traps. No data are provided to test whether comparable levels of gossypol detected in the laboratory reared insects would be present and detectable in such field-collected samples.

The Panel recommends that EPA ensure that the appropriate technical expertise is enlisted to evaluate whether the analytical method for gossypol could be biased, and to determine what sort of supporting evidence would need to be provided for its validation.

**Agency Charge**

**2. Monsanto's TBW sampling and gossypol analyses were conducted over a two year period (2004 and 2005). For several states (Tennessee and E. Texas) data were collected in only one year. The trends between seasons were generally consistent, although no statistical/correlation analysis was performed.**

**The Panel is asked to comment on what uncertainties exist from using data collected from this time period (i.e., 2 years for North Carolina and Georgia and 1 year for Tennessee and E. Texas) to adequately assess the potential of natural refuge (i.e., non-cotton hosts) as a substitute for structured refuge (i.e., non-Bt cotton)?**

**Panel Response**

**Summary**

1) The structured non-Bt cotton refuge gives some assurance that a minimum % of refuge is present and most importantly that it is interspersed among the Bt cotton fields.

2) In determining the adequacy of an unstructured refuge we must consider that the variation in the refuge provided by wild hosts and non-cotton crops can be influenced by the following factors:
    A) Year to year variation in quality and insect production from the wild hosts on a per plant basis.
    B) Year to year variation in the density of these wild hosts.
    C) Year to year variation in percent of total acreage planted to Bt and non-Bt cultivars of other TBW and CBW host crops.

3) The evidence from the eastern parts of the Cotton Belt (North Carolina and Georgia) combined with other published or submitted studies on host use and relative productivity of different crops (Jackson et al. submitted, EPA-HQ-OPP-2006-0217-0013) indicates that both species utilize a broad range of non-cotton hosts in this area, and more importantly that the relative proportion of moths generated in natural refuges or non-cotton crops is significant.

4) Data on non-cotton hosts of TBW in the MidSouth indicate that the size of the non-cotton refuge may be small in some areas and, because of potential spatial and year-to-year variation in TBW production from these hosts, there is a need for more years of data from many MidSouth locations.

**Discussion**

Monsanto has presented data from gossypol analysis of adult budworms over a two year period for most locations and one year for other locations. There is some variation from year to year but the differences are relatively small. The question arises of whether the relative numbers of budworms produced in the unstructured refuges can be expected to be similar in future years. There are a number of factors that must be considered. Some of them fall into the following categories:

1) Year to year variation in quality and insect production from the wild hosts on a per plant basis.
2) Year to year variation in the density of these wild hosts.
3) Year to year variation in percent acreage planted to Bt and non-Bt cultivars of cotton and other host crops.

Each of these will be discussed:

**1) <u>Year to year variation in quality and insect production from the wild hosts on a per plant basis.</u>**

There is a considerable amount of historical information on the wild plant species that serve as hosts for the TBW. However, most of these studies have been qualitative in nature or restricted to a single year of sampling, as pointed out by Benedict (2005) (e.g., Neunzig 1969). One recent study in Mississippi examined TBW larval and pupal production from Velvetleaf over a series of years (Carlos Blanco, unpublished data, Figs. 2-1, 2-2). The year-to-year variation revealed in this study is dramatic. If the results of this study are indicative of

expectations for variation found in TBW production from other wild hosts then we expect enough variation among single wild hosts to influence the overall number of TBW from the total set of wild hosts. Mueller and Phillips (1983) provide data on larval numbers for TBW on 4 wild hosts over a two-year period, but the two sites (one each year) were about 70 miles from each other. Their data show substantial variation as in the Blanco study. It is important to recognize that a high proportion of larvae on wild hosts can be parasitized and that this varies spatially and temporally (Mueller and Phillips 1983, Norris and Kogan 2005), so density of larvae may be a poor indicator of adult production. Stadelbacher (1981) examined wild host use by TBW over a 12 year period. Unfortunately, he only reported average numbers of larvae over all years.

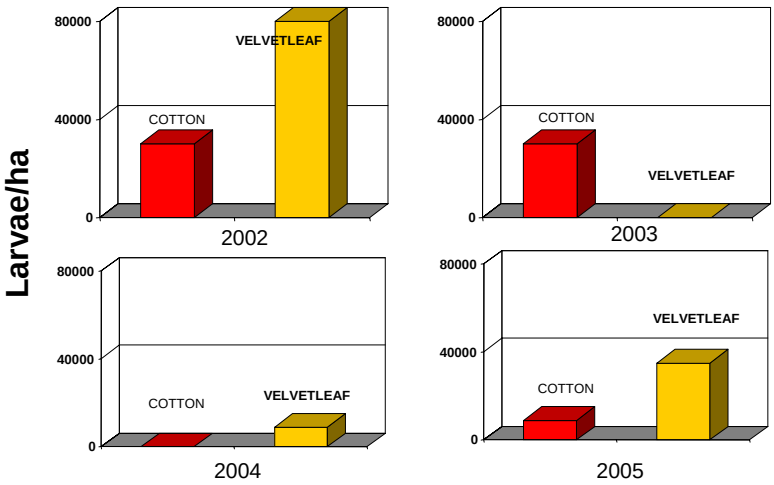


**Fig. 2-1.** Tobacco budworm larval production in cotton and velvetleaf, Washington Co., MS. Data from Carlos Blanco (unpublished).

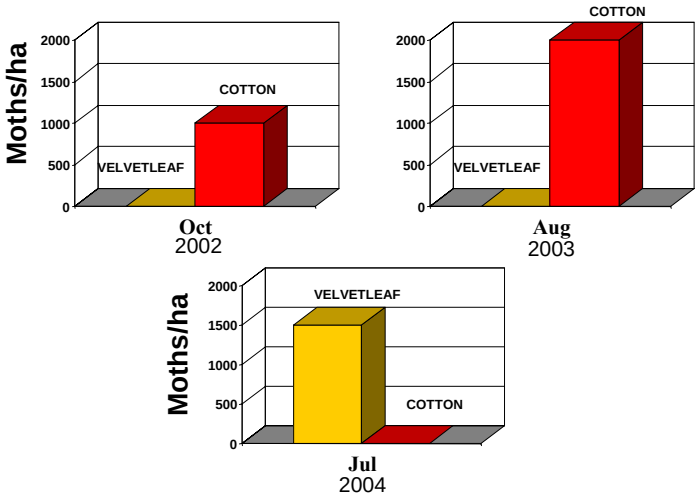


**Fig. 2-2.** Tobacco budworm moth production in cotton and velvetleaf, Washington Co., MS. Data from Carlos Blanco (unpublished).

**2) <u>Year to year variation in the density of these wild hosts.</u>**

Data on year-to-year variation in abundance of wild hosts typically have not been collected for pest management decisions. However, many wild hosts of TBW are known to grow in roadside areas and in Conservation Reserve Program (CRP) lands. Research in the 1980s in the MidSouth identified the primary early season hosts of TBW, and a program was proposed to decimate populations of these species by changes in management of roadside areas and other weed control measures (see Mueller et al. 1984). This brings up the fact that general changes in the management of roadside vegetation not directed at wild host management could impact the densities of wild hosts.

**3) <u>Year to year variation in percent acreage planted to Bt and non-Bt cultivars of cotton and other host crops.</u>**

Monsanto presents data from Federal Government analyses showing that the proportion of crop types grown in the Southeast and MidSouth have been relatively stable since 1995. It is worth examining cropping patterns over a longer period.

Kennedy and Storer (2000) examined variation in the number of hectares grown to wheat, corn, soybean, and cotton over a 15-year period from 1980 through 1995. The acreage planted to some crops more than doubled or were halved in a single year-to-year period (Tables 2-1, 2-2). Over the 15-year period, the number of hectares of cotton in North Carolina and Georgia increased more than 10 fold. In Mississippi, there was a more than six-fold change in corn acreage.

**Table 2-1.** Maximum year-to-year changes in production area (hectares X 1000) of four agronomic crops in each of three states during the period 1980 through 1995. (Modified from Kennedy and Storer 2000.)

| State | Wheat | Maize | Soybean | Cotton |
|---|---|---|---|---|
| N. Carolina | 65 | 20 | 119 | 128 |
| Georgia | 74 | 44 | 133 | 70 |
| Mississippi | 73 | 84 | 118 | 52 |

**Table 2-2.** Minimum/Maximum number of hectares (X 1000) planted with four agronomic crops in each of three states during the period 1980 through 1995. (Modified from Kennedy and Storer 2000.)

| State | Wheat | Maize | Soybean | Cotton |
|---|---|---|---|---|
| N. Carolina | 130 / 325 | 325 / 810 | 465 / 870 | 20 / 325 |
| Georgia | 140/ 595 | 160 / 650 | 130 / 850 | 49 / 610 |
| Mississippi | 73 / 445 | 40 / 250 | 730 / 1340 | 278 / 565 |

Although relative suitability of crop plants to pests is not expected to vary as much as that of wild hosts because of genetic uniformity and cultural farming practices, there can be substantial variation in year-to-year TBW production from cotton. Schneider (2003) estimated the percentage contribution of cotton to the overwintering population of TBW over 7 years (1996-2002) in northwest Mississippi. Annual variation in the contribution of cotton was high: (mean $\pm$ SD 9.1 $\pm$ 10.6%; range 0-29%; n = 7). If variability in host use during the period of selection for adaptation to Bt cotton (June-August) were similar to that observed for September, then confidence in the one to two years of data that is currently available for the MidSouth is insufficient.

If there is an increase in profitability of one of the crop hosts of TBW or CBW, it is expected that the acreage of this crop will increase and this could affect the size of the refuge. Data from China shows that as Bt cotton increased the profitability of growing cotton, the acreage planted to cotton in certain provinces increased (Table 2-3) (Kongming Wu, unpublished data).

**Table 2-3.** The planting history of Bt cotton and other host crops of the bollworm during 1998-2005 in Anci County, Hebei Province and Xiajin County, Shandong Province, China. (From K. Wu, unpublished.)

| Location | Year | Conventional Cotton (%) | Bt Cotton (%) | Maize (%) | Peanut (%) | Soybean (%) | Total area planted (ha) |
|---|---|---|---|---|---|---|---|
| Anci County | 1998 | 1.62 | 0.81 | 73.55 | 10.31 | 13.71 | 34,890 |
| | 1999 | 1.47 | 1.47 | 73.52 | 10.27 | 13.27 | 34,580 |
| | 2000 | 0.00 | 7.22 | 67.97 | 11.90 | 12.90 | 32,870 |
| | 2001 | 0.00 | 13.14 | 66.46 | 10.35 | 10.03 | 34,420 |
| | 2002 | 0.00 | 11.46 | 67.33 | 11.15 | 10.05 | 33,817 |
| | 2003 | 0.00 | 5.25 | 71.23 | 14.81 | 8.70 | 30,800 |
| | 2004 | 0.00 | 10.53 | 68.22 | 13.32 | 7.93 | 31,013 |
| | 2005 | 0.00 | 9.53 | 70.11 | 12.68 | 7.68 | 31,886 |
| Xiajin County | 1998 | 35.02 | 8.75 | 46.46 | 4.71 | 5.05 | 39,601 |
| | 1999 | 3.83 | 34.44 | 46.75 | 10.65 | 4.33 | 36,933 |
| | 2000 | 0.00 | 71.41 | 21.98 | 4.16 | 2.44 | 46,400 |
| | 2001 | 0.00 | 64.11 | 31.07 | 2.96 | 1.85 | 54,067 |
| | 2002 | 0.00 | 69.36 | 25.07 | 3.84 | 1.72 | 50,266 |
| | 2003 | 0.00 | 74.30 | 21.56 | 2.92 | 1.22 | 54,733 |
| | 2004 | 0.00 | 76.65 | 21.23 | 1.77 | 0.35 | 56,533 |
| | 2005 | 0.00 | 72.69 | 22.75 | 3.35 | 1.20 | 55,667 |

Stable isotope data from Monsanto's application and from Gould et al. (2002) indicate that a large proportion of CBW are developing as larvae on corn during specific periods of the growing season. Therefore, non-Bt corn is serving as the major refuge for CBW. It is important to note that some corn in the South and in the Midwest is Bt corn that has a single Bt toxin, Cry1Ab. Currently, the proportion of Bt corn in the Midwest and especially in the South is low,

but this may change as new cultivars are produced that have stacked herbicide tolerance, Cry1Ab, and another Bt toxin for corn rootworm. Instead of serving as a refuge, corn could be a selection agent for Bt resistance. Data in Gould et al. (2002) as well as from other sources (Sparks et al. 1975, Hartstack et al. 1982, Hendrix et al. 1987) indicate that CBW is migrating into the MidSouth from corn and other hosts in Mexico early in the growing season. Late in the season CBW seems to be migrating from the Midwest to the South. There is a need for more data on this phenomenon because such migration means that calculations of refuge must include the proportion of Bt corn in the Midwest and in Mexico.

Even with all of the uncertainties above, some Panel members believe that the data from North Carolina and Georgia offer substantial evidence that an adequate unstructured refuge currently exists because of the overwhelming percentage of TBW and CBW moths with a non-cotton history.[3] Furthermore, this outcome is easily explained, indeed expected, because of the current presence of large acreages of alternative preferred hosts in these regions, namely tobacco and peanuts. These two crops make up a substantial proportion of the acreage, but this may change with recent federal buy-out programs and changes in crop stabilization programs. Additionally, the proportion of Bt corn in the Carolinas and Georgia is currently small. If that proportion increases, the non-cotton refuge in these areas could decrease substantially.

A single year of data from areas of Texas and Tennessee are problematic, and given the low readings in several areas of the Delta, more extensive spatial and temporal data would be in order there as well. Unpredictable weather events or patterns can influence many variables including host crop planting date, timing of alternative host availability progressively through the season because of effects on host maturation, length of generations, dispersal (including proportion engaging in facultative long-distance migration), and local flight behavior. Monsanto is rightly concerned to use "worst case" scenarios for its model, but one year of data cannot represent a typical range of weather scenarios, much less worst case weather scenarios. BPPD (2006) expressed concern about the adequacy of two years of sampling, mainly because land use patterns could change, but temporal availability of certain alternative hosts due to weather is also important.

The Texas data seem particularly inadequate. Four of the five counties sampled are near one another from the same area of East Texas, and the other is from the Coastal Bend area. Other ecological areas of Texas should be sampled, such as the Lower Rio Grande Valley and the Texas High Plains. It is especially important that the latter be tested because it is such a dry environment, and it is hard to imagine that alternative local hosts could play much role there for TBW. Benedict's (2005) review of the literature indicates that from mid-June on, cotton is the main host, and the only abundant host, in the Delta region of Mississippi. As pointed out by Benedict (citing Sparks et al. 1993), pyrethroid resistance has been slow to develop in North Carolina, presumably because there are abundant alternative hosts that provide refuge from these

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[3] Following this FIFRA SAP meeting, a Panel member provided additional analysis and comments regarding the validity of extrapolating data from areas sampled by Monsanto in North Carolina and Georgia to areas that were not sampled. Such comments were not considered or reviewed by the Panel during the meeting, and are being provided as an appendix to these meeting minutes (Appendix 5).

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insecticides. In contrast, the biggest problems with pyrethroid resistance in TBW are in the Mississippi Delta, which implies there is little natural refuge available. In volume 3 of Monsanto's petition (Head et al. 2005), it is pointed out that pyrethroid tolerance in CBW may be building in Texas and Louisiana as well, again bringing into question the effectiveness of natural refuges in these areas.

**Agency Charge**

**3. In some counties/states, extremely low numbers of TBW were trapped, with some traps collecting only one insect. In Tennessee, TBW numbers were so low that data were not reported at all for 2004. In addition, cotton monitoring efforts have been recently hampered by low availability of TBW samples (possibly due to a suppressive effect of Bt cotton).**

**Do low overall numbers of TBW trap captures in some areas affect the ability to assess the effectiveness of natural refuge for IRM? What conclusions, if any, should be drawn from the failure to capture Bt-susceptible TBW at particular sites?**

**Panel Response**

EPA (BPPD 2006) expressed a concern that low numbers of TBW could mean that insufficient numbers of susceptible moths would be available to mate with resistant moths emerging from a Bt cotton field. The absolute numbers of susceptible moths emerging in an area by itself is not what matters, only the proportion of the local population that is emerging from Bt vs. refuge. If the global population is low, then few eggs are going to be laid in Bt cotton, and correspondingly fewer resistant insects will emerge. What is important is that those that do emerge from Bt cotton are still overwhelmed proportionally by susceptible individuals, even though their absolute numbers may be low. The primary problem with a low TBW population density is that it prevents assessment of the relative contribution of the natural refuge compared to non-Bt cotton.

The causes of low trap catch are uncertain. At least 5% to 20% of cotton in all counties growing Bt cotton is non-Bt cotton. Hence, a population of TBW sufficiently large to be detected with pheromone traps would be expected in counties with historically detectable TBW populations. The rarity of TBW in some of these counties brings into question the efficacy of currently required structured refuge – at least under some circumstances. Alternatively, although TBW pheromone traps are fairly sensitive measures of the presence of males seeking mates in a region, absence of catches does not necessarily indicate a lack of moth population (see Panel response to Charge 1). It is also possible that there has been a regionwide decline in TBW numbers due to the overall replacement rate decreasing below 1.0 owing to a high percent of the population dying on Bt cotton.

Using the generalized linear mixed model described in the Panel response to Charge 5, multiple analyses of $R_{nat}$ were performed with minimum sample size (*MinSS*) set to 1, 2, 5, 10, and 20 adults. [The Panel notes that similar results are obtained using sample size (i.e., number of moths tested) as a continuous factor in SAS PROC GLIMMIX analysis of $R_{nat}$.]. The

resulting least square mean estimates of $R_{nat}$ for each County by Month combination were used in turn as the dependent variable in a SAS PROC GLIMMIX analysis with Class variables Region and Month, Continuous variable Ln[*MinSS*], and interaction terms Region X Month and Ln[*MinSS*] X Region(Month). For the data divided into three regions (East, "HillsMidSouth", and "FlatsMidSouth"; see Panel response to Charge 5), Region and Ln[*MinSS*] X Region contributed significantly to variation in $R_{nat}$ because of differences between the East region and the two MidSouth regions but not because of any difference between the two MidSouth regions. The results of a two region (East and MidSouth) analysis are given in Fig. 3-1. This analysis shows that $R_{nat}$ declines at a significantly faster rate with Ln[*MinSS*] for the MidSouth region than the East region, and $R_{nat}$ for the East region does not vary significantly with Ln[*MinSS*]. In the MidSouth, the higher estimates of proportion natural refuge at lower minimum sample sizes suggests that small sample sizes cause a net upwards bias in the estimates of proportion natural refuge.

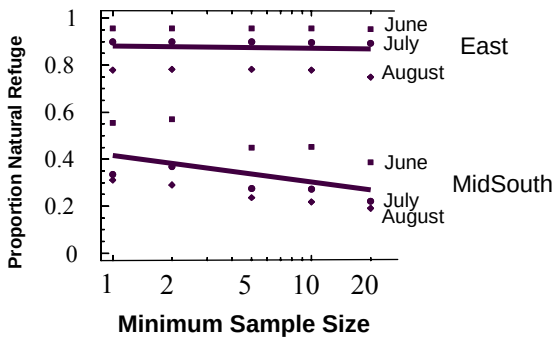


**Fig. 3-1.** Effect of minimum sample size (Ln scale) on least square mean estimates of the proportion of pheromone-trapped TBW originating from natural refuge by oviposition month and region.

The Panel notes several sampling biases apparent in estimation of the proportion of TBW natural refuge, which were generated by low trap captures and Monsanto's handling of those situations: an upward bias for $R_{nat}$ estimates in the East region due to the conditional sampling protocol used and a net bias of indeterminate sign in the MidSouth region due to the existence of both upward and downward biases of unknown relative magnitudes.

1) *Sites or counties with extremely low abundance of TBW (as measured by pheromone trap catches) were excluded from subsequent analyses.* Given that $R_{nat}$ June-August, the period of greatest TBW reproduction in cotton, is higher for the MidSouth region the lower the minimum sample size used in the estimation process (Fig. 3-1), it appears that $R_{nat}$ is higher in areas where the population density of TBW is lower. 10.6% (22/207) of the county X month sampling combinations were excluded from Monsanto's data set because 0 moths were captured. Thus, these exclusions introduce a downward bias in estimates of $R_{nat}$ for the MidSouth region.

2) *Sampling bias for counties and traps within counties with the "highest and most consistent" catches.* The Applicant states the following (p. 12, Head and Gustafson 2005): "Analyses [for gossypol in TBW] were focused upon those counties, and trap locations within counties, where numbers were highest and most consistent throughout the season." Given the negative correlation between numbers of pheromone-trapped moths caught and estimated $R_{nat}$, sampling bias for counties and for traps within counties with the "highest and most consistent" catches introduces a downward bias in estimates of $R_{nat}$ in the MidSouth region.

3) *Additional sets of moths analyzed where the non-cotton contribution was 10% or less.* The Applicant states the following (p. 12, Head and Gustafson 2005): "For the locations analyzed, moths were analyzed in sets of 10 for each trap-date combination. Where the non-cotton contribution was 10% or less for any trap-date combination, additional sets of 10 moths were analyzed, if available." This protocol makes sample size conditional on the observations and thus introduces an upward bias in estimates of $R_{nat}$ for both the East and MidSouth regions.

## Statistical Analyses

### Agency Charge

**4. Monsanto used the Fisher's Exact Test to determine whether the gossypol data could be pooled. Data were pooled for individual traps (i.e., for multiple collection dates for each month) and for counties (i.e., including all traps within a county for each month).**

**The Panel is asked to comment on Monsanto's approach to pooling the gossypol data.**

### Panel Response

In assessing Monsanto's approach to pooling the gossypol data, the Panel felt that more could have been done in the data analysis to assess the appropriateness of pooling. The analysis approach used by Monsanto consisted of a large number of independently performed Chi Square tests in which the P-value associated with the test was computed using an exact-enumeration technique (Fisher's exact method). A number on the Panel felt that while this approach was appropriate for the specific pooling task it was inadequate for addressing some of the other issues that came up in the pooling discussion.

Of importance to understanding the conclusions of the Panel's discussion is a clear understanding of the overall goals of the statistical analysis related to pooling. One goal was the determination of whether count data from multiple dates within a month for a sample location could be pooled, and, conditional on a decision that pooling was appropriate for all locations, the subsequent determination of whether to pool count data for sampling locations within a county (for a given month). The decision to pool was a global one in that every sample date within a month for a sample location was pooled or every sample location within a county was pooled. The decision by Monsanto based on their analysis was to pool both sample days and sample

locations, resulting in a dataset for subsequent analysis that consisted of counts of gossypol-positive and gossypol-negative sample results for each county for each sampling month.

The goals of the statistical analysis can be formalized in a set of hypotheses. The null hypothesis of the overall analysis is that for each county/month combination there is one gossypol distribution, indexed by its average gossypol fraction. A number of alternative hypotheses are conceptually considered, one being that locations within counties (after pooling within sample days within locations for a month) have varying gossypol distributions (or varying average gossypol fractions) and the other being that sample days within sampling locations have significantly different gossypol distributions (or varying average gossypol fractions).

The multiple-test method used by Monsanto has the following favorable properties with regard to the issue of pooling:

1) *The individual tests are simple to perform and easy to understand.*
2) *County/month tests are examined individually* and hence one can easily identify conditions where there are strongly significant differences.
3) *The two alternative hypotheses identified above can be examined in two separate sets of multiple tests* allowing different decisions on each issue and allowing consideration of the issue of pooling locations within county/month combinations to be conditional on the decision to pool sample days within sample locations.
4) *The level at which a difference is considered to be statistically significant can be adjusted to take into account the potential for Type II errors.* One Panel member suggested that setting the threshold for a significant difference at P = 0.20, for example, while resulting in more significant differences would also reduce the chances that sites within counties or sample days within sites that are truly different are missed and hence are pooled.

The multiple-test method used by Monsanto has the following unfavorable properties with regard to the issue of pooling:

1) *The method does not take into account experiment-wise error* (most statistical methods texts have a chapter on multiple comparisons in which experiment-wise error is discussed, see for example Ott and Longnecker 2001, Chapter 9 or Zar 1996, Chapter 11). Consider that each of the individual tests (in this case each Chi Square test) has a certain probability of resulting in a wrong conclusion (say each is performed at the Type I error probability of 0.05). When a large number of these tests are performed on a population where in fact there are no real differences (the null hypothesis of the pooling analysis) then we would expect to see a fraction of the tests being statistically significant. So if the Type I error for each individual test was 0.05, we would expect to see about 5% of the multiple test results to be significant. Statistical analysis methods that account for experiment-wise error typically do one of the following: i) reduce the Type I error of the individual tests so that only a few very significant test results are considered important, or ii) change the structure of the individual tests so only the larger differences are considered significant.
2) *The method does not provide a formal way of directly testing the two alternative hypotheses.* The conclusion to pool is subjective, determined primarily by looking at the

number of significant tests. Different individuals might make different assessments from the same data.

3) *Second level hypotheses cannot easily be tested.* For example it is not easy to determine if pooling over years or some spatial pooling other than at the county level is appropriate.
4) *The method does not take into account all of the data collected in the decision making process* since sample days with very low counts (including zero counts) and sample locations with low counts were not included in the analysis.

If the results of the multiple tests are examined with consideration of experiment-wise error, it is clear that there is little evidence for widespread differences in gossypol fractions among a month's sample days within sample location, or pooling these, among sampling locations within county by month. Statistical theory tells us to expect 5% of these tests to have P-values below 0.05 and 20% of these tests to have P-values below 0.20. With the comparison-wise Type I error rate set at $\alpha = 0.05$, for 2004, 5 of 102 (4.9%) tests of dates within months for individual traps were statistically significant, and at $\alpha = 0.20$, 21 of 105 (20%) tests were statistically significant. For 2005, at $\alpha = 0.05$, 9 of 170 (5.3%) tests of dates within months for individual traps were statistically significant and at $\alpha = 0.20$, 34 of 170 (20%) tests were statistically significant. These combined results suggest that pooling sample days within months at individual sites may not be a problem. But, for pooling across traps within each county, at $\alpha = 0.05$ for 2004, 3 of 38 (7.9%) tests of dates within months for individual traps were statistically significant and at $\alpha = 0.20$, 12 of 38 (31.5%) tests were statistically significant. For 2005, at $\alpha = 0.05$, 7 of 60 (11.7%) tests of dates within months for individual traps were statistically significant and at $\alpha = 0.20$, 15 of 60 (25%) tests were statistically significant. These combined results provide much less support for pooling across traps within a county.

A number of Panel members pointed out the need for biological relevance to support pooling, and that pooling to county level or state level (i.e., to geopolitical boundaries) may not be appropriate. There was concern that some localized (temporal or spatial) differences that could be important might be lost in a decision to pool to the county by month level. Some argued that the county level was too large, based on gene flow estimates of $\leq$ 8 km for TBW during the time when cotton is the favored host (Korman et al. 1993), and therefore the decision to pool, when to pool, and where to pool requires much more analysis than that provided in the Monsanto report. It was also pointed out that the question of whether unstructured refuges can safely replace structured refuges has little to do with what the data collectively say about pooling, or for that matter what they say on average for the Cotton Belt. The issue is what can be extracted from these data to address the question of unstructured refuges at appropriate temporal and spatial scales. Finally, it was recommended that Monsanto provide a biological justification for pooling that goes beyond simply a data analysis.

One Panel member suggested the use of a moving fixed-length time window (say four or six weeks) to allow assessment of different temporal moving period lengths on the tests results (e.g., either simple smoothing of within sample site temporal data, possibly with non-parametric methods, or incorporation of autocorrelation into the error structure of the generalized linear model). This could allow determination of appropriate pooling time periods. Another Panel member suggested that more appropriate spatial pooling might result from incorporating the distances among sample locations as a factor in the statistical analysis models (e.g., a model that

incorporates geospatial components, spatial autocorrelations, kriging, and possibly discrete zonation). In this way, appropriate spatial pooling boundaries could be determined and/or changes in spatial pooling boundaries could be determined.

The Panel concluded that there are better, more powerful statistical methods that are available for examining the alternative hypotheses related to pooling. The sampling plan described is hierarchical with states and months specified, then counties within states that were specifically selected for their large cotton acreage. Within sampled counties a number of sample trap locations were identified and these traps were monitored and sample moths collected on a weekly basis. The null hypothesis described above can be the basis of a model that assumes counts of gossypol moths in each month by state by county combination has its own (Binomial) distribution, indexed by the average gossypol percent (the Binomial success fraction), and that this distribution is the same for all locations within the county and all sample days within sample locations in that county. This can be further formulated in a fixed effects generalized linear model (McCullagh and Nelder 1989, Dobson 2001). The alternative hypotheses also can be formulated as a Binomial model, but in this case it is assumed that counts at sample locations within counties have gossypol fractions that vary about the expected county mean gossypol fraction, or that sampling dates also have gossypol fractions that vary from the average for that sampling location. These hypotheses can be formulated as extensions of the generalized linear model in which the two additional variance components are viewed as potential covariates explanatory to the observed (Binomial) fractions. The model that addresses the alternative hypotheses is a mixed effects generalized linear model (Lee et al. 2006). Thus the analysis approach suggested by multiple Panel members was to create two or more alternative generalized linear mixed effects models about plausible alternative hypotheses and use formal statistical tests to determine whether any of these alternative models is significantly better at fitting the data in hand than the null hypothesis model. It is important to the success of this analysis that the choices made regarding the various model features (e.g., using a logit link with a Binomial distribution assuming extra dispersion to account for the data being of counts of successes (gossypol positive) assuming Poisson arrivals at the trap) match features of the sampling as well as the biology of the moths. Alternate model formulations (e.g., via GEE methods, Dobson 2001) were also discussed by the Panel but specific details were not given.

While the models suggested above can be complex, the approach is one that is at the foundation of most statistical tests. In addition, there are some benefits to this model fitting/testing approach that cannot be obtained from the separate test approach, the primary ones being 1) the ability to estimate the variance components for the deviations from mean percent; 2) the ability to determine if these variance components can be related to other covariates (e.g., comparison across years, the topic of Charge 5); and finally 3) these models have the ability to incorporate and test for the presence of correlation in responses one might expect from repeated measurements in time. None of these three are possible with the multiple-testing approach.

The analysis of gossypol fraction differences among counties and months using the linear logistic model (page 122, Head and Gustafson 2005) could also be formulated more appropriately as a generalized linear mixed effects model. To a certain extent, the county and month effects should be viewed as random effects in the model which would lead to slightly different statistical tests.

There were some additional comments regarding the use of mixed effects generalized linear models:

1) *The protocol for choosing sample moths for the gossypol analysis involved the sequential addition of sample moths in batches of 10 in situations where the fraction of gossypol positive moths was small.* There was concern that this process had the potential of biasing the results of the individual Chi Square test. As the overall sample size increases for a given difference in gossypol fraction between the two or more groups (sample days within a location or sample locations within a county), the Chi Square test is more likely to reject the result. In addition, once the decision is made to pool, those sample days having more overall samples will have larger weight in the final pooled sample, essentially resulting in lower average gossypol fraction estimates. These same data used in the generalized linear mixed effects model will not have that same effect because of the way the model performs estimation and statistical tests.

2) *Without the sample day count data it was not possible to actually fit the proposed models* and hence it was not possible to determine if this model comparison approach would work for these data. It is possible that the model comparison approach might not be successful for some alternative models, primarily due to lack of balance in the achieved sampling plan. In particular, the methods used to estimate the variance components of the mixed effects general linear model might not. In this case, the multiple-testing approach used by Monsanto would be the basis for the analysis.

3) *A number of Panel members mentioned concerns with potential lack of power for any statistical analysis*, primarily due to the low numbers of moths measured for many sample days and sample locations. Power addresses the ability of the sampling design to identify significant differences, in this case significant time-to-time or location-to-location variability in gossypol fraction when it actually exists. Low sampling counts work against power in this case. Much more sampling would have been needed to have high power for this study. The generalized linear model analysis will have higher power for the pooling-related tests than can be achieved with the multiple-test approach.

4) *The generalized linear model approach, being based on a Binomial distribution for gossypol counts, allows appropriate computing of the uncertainty related to the gossypol fractions.* If the model related to the null hypothesis is accepted as the best description of the data (i.e., the decision is to pool across sampling dates and sampling locations within county and month) the resulting pooled estimates will have appropriately computed standard errors. This approach will result in slightly different confidence bounds on the gossypol fraction.

5) *All observed data can be included in the generalized linear model approach*, including zero counts of gossypol individuals on a sample day and counts of one. These data will not have a large influence on the model results but any information available collectively from these sites can be extracted through the model.

6) *Annual variability cannot be measured with only two years.* While the difference between the two years can be tested directly using the model-based approach, the relative importance of variability from year-to-year cannot be compared to county-to-county or site-to-site variability without additional years of sample data.

7) *There was little discussion of how to handle issues of missing data in the statistical analysis.* Since the original trap data were not available it was not clear how zero counts (trap actually checked and no moths observed) were handled differently from missing data (trap not checked). There are more complex statistical models available that incorporate missing data information into the generalized linear model (see Helsel 2005 for an introduction to this topic), but these were not discussed in detail.

The more formal statistical analysis suggested by the Panel represents a significant amount of additional work for Monsanto, but it was the consensus of the Panel that this investment is warranted. It was noted by the Panel that the gossypol fraction estimates produced from this analysis form the basis for subsequent refuge size estimates; namely, the $P_{NBTC}$ parameter used to estimate $E_{NC}$ in equation 8 (Gustafson and Head 2005), that is subsequently used to estimate $R_{nat}$ in equations 9 and 10 (Gustafson and Head 2005). The $R_{nat}$ parameter forms the basis for the request to eliminate structured refuges for Bollgard II cotton. The formal statistical analysis may suggest less pooling and/or a different spatial and/or temporal pooling plan resulting in estimates of $P_{NBTC}$ that are not necessarily county and month estimates. If the decision is not to pool, the $P_{NBTC}$ estimates across sampling dates and sample locations could be formulated as realizations of a spatial/temporal random process with distribution estimated via the generalized mixed effects linear model. These estimates would form the basis of a probabilistic (stochastic) approach to the modeling of refuges and substantially change how the overall refuge analysis results are considered.

Finally, the Panel suggested that if these types of sampling studies are to become more common and used as the basis of future decisions for resource management of genetically modified crops, EPA might wish to develop and publish recommendations for appropriate sampling protocols.

**Agency Charge**

**5. Monsanto did not conduct any statistical analyses comparing the two sampling years (2004 and 2005). The Panel is asked to comment on whether valid comparisons (on a qualitative basis) can be made between the two years without statistical analyses? Please describe any meta-statistical analysis that could improve the overall understanding of the effectiveness of natural refuge across locations and across time.**

**Panel Response**

**Summary**

The Panel concluded that valid comparisons between the two years is not possible via a meta-analysis. However, it is possible using a more formal statistical testing model based on a

generalized linear mixed effects model that takes into account ecologically relevant spatial scales and incorporates temporal autocorrelation. A preliminary analysis using the data available to the Panel concluded the following:

1. Analysis of variation in $R_{nat}$ including spatial variation at an ecologically relevant, regional scale shows that Region ("East" and "MidSouth"), Month (June, July, and August), and County(Year X Region) made statistically significant contributions. $R_{nat}$ for the East region was higher than that for the MidSouth region and declined for both regions with oviposition month. Intensity of agricultural activity within the MidSouth region did not affect $R_{nat}$.

2. Analysis of variation in $R_{nat}$ including spatial variation at an ecologically relevant, regional scale shows that Year (2004 and 2005) and Year X Region did not make statistically significant contributions.

**Discussion**

As described in the Panel response to Charge 4, Monsanto's count data for TBW host plant use are best analyzed using a generalized linear mixed effects statistical model (e.g., as implemented in SAS PROC GLIMMIX, SAS Institute, Inc. 2006). Which function of the count data should serve as the dependent variable depends on the question to be addressed. Whether to pool the data across potential sources of variation should be based on an analysis of $P_C$, the binomially-distributed proportion of cotton host use. In contrast, the question of which sources of variation significantly affect $R_{nat}$ should be based on an analysis of $R_{nat}$ rather than $P_C$. Although $R_{nat}$ is a function of $P_C$, calculation of estimates of $R_{nat}$ and its standard errors from least square mean estimates and standard errors of $P_C$ is not possible because the latter are not available under the assumption that County is a Random effect (see below). Ideally, the pooling issue should be resolved in a statistically rigorous fashion before an analysis of $R_{nat}$ is performed, but Monsanto supplied only data pooled across dates within month for each trap and then across traps within county. Fortunately, these pooled data appear to be adequate to address EPA's charges to the Panel (see Panel response to Charge 6).

Instead of retaining the spatially arbitrary division of the county data among the seven states represented in the data set, the county data were divided into three, ecologically more relevant regions: all counties in North Carolina and Georgia ["East", highly suitable non-cotton crop hosts available (tobacco and peanuts) and variable degrees of agricultural intensity]; selected counties in Texas, Tennessee, Arkansas, Mississippi, and Louisiana ["MidSouth Hills", less suitable non-cotton crop hosts available (soybean) and a lower degree of agricultural intensity]; and selected counties in Texas, Tennessee, Arkansas, Mississippi, and Louisiana ["MidSouth Flats", less suitable non-cotton crop hosts available (soybean) and a higher degree of agricultural intensity]. "Higher degree of agricultural intensity" is defined as at least 15% of landscape cropped. The counties included in MidSouth Hills are as follows: 2004 Arkansas (Little River), Louisiana (Bossier), Mississippi (Carroll, Chickasaw, Clay, Grenada, Lee, Lowndes, Madison, Monroe, Noxubee)]; and 2005 [Arkansas (none), Louisiana (Bossier, Rapides), Mississippi (Carroll, Chickasaw, Clay, Itawamba, Lee, Lowndes, Monroe, Noxubee, Prentiss), Tennessee (Haywood, Carroll, Fayette, Gibson), Texas (Austin, Burleson, Fort Bend)].

All other counties in these states were included in MidSouth Flats. Note that while there are good ecological reasons for the geographic division described above, other geographic divisions could be conjectured based on slightly different ecological assumptions, and this conjectured division could also be analyzed in the manner described below.

$P_C$ was calculated for all available Month X County combinations, including those for which the host use of only a single adult was determined. $R_{nat}$ was calculated under the following restrictions: (1) cotton was present in the county (three exclusions—all for Little River County in Arkansas), (2) the fractions of the landscape area planted to Bt ($A_{BtC}$) and non-Bt ($A_{NBtC}$) cotton were reported (five exclusions—all in Tennessee), and (3) $P_C \neq A_{NBtC}/A_{BtC}$ (no exclusions).

SAS PROC GLIMMIX (SAS version 9.1 for Windows, © 2002-2003, SAS Institute Inc, Cary, NC, USA) was used to analyze $R_{nat}$. The variable $R_{nat}$ is binomially distributed (range 0-1), so the conditional probability distribution of the data was set to binomial. County within Region X Year was treated as a Random effect in the analysis model. A first order, autoregressive covariance structure was specified to deal with the lack of independence of observations within County X Month. Linear and quadratic Month trends were tested using contrasts. The results are given in Fig. 5-1. Because interaction terms involving Region were not statistically significant and the MidSouth Hills and MidSouth Flats regions were not significantly different, the least square mean estimates for a MidSouth region combining the two are also shown in Fig. 5-1.

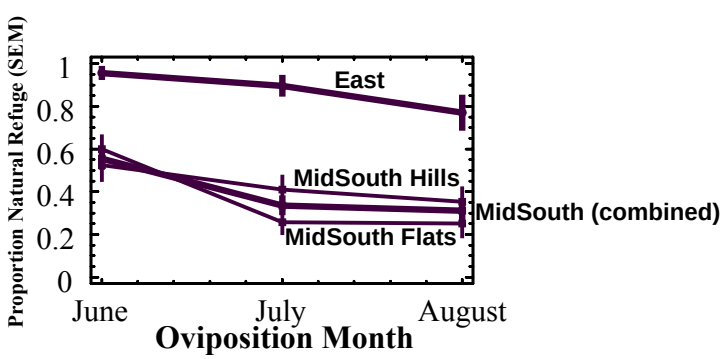


**Fig. 5-1.** Proportion of natural refuge for different regions estimated from pheromone-trapped TBW by month. Estimates for MidSouth (combined) uses estimates for MidSouth Hills and MidSouth Flats.

For the period of time during which TBW is subject to selection for counteradaptation to Bt cotton, the average level of $R_{nat}$ is much higher for the East region than for the MidSouth region. This difference may result from the presence of peanut and/or tobacco production in the East and the absence of correspondingly suitable non-cotton crop hosts in the MidSouth. During July and August in the MidSouth region, $R_{nat}$ averages ca. 0.3, which is considerably greater than 0.05, the non-Bt cotton structured refuge requirement. However, as discussed in the Panel response to Charge 7, counteradaptation to Bt cotton may develop locally and spread from

so-called "hotspots". $R_{nat}$ estimates for the counties in the MidSouth region that were singled out by Monsanto as "worst-case" examples (i.e., lowest $R_{nat}$ levels) are frequently below 0.05 (see Panel response to Charge 6). Consequently, the observed regional mean estimates of $R_{nat}$ are inadequate to demonstrate Monsanto's contention that natural refuges alone can prevent counteradaptation of the TBW to Bt cotton in the MidSouth region.

None of the models using Year (2004 and 2005) or Year X Region variation in $R_{nat}$ were statistically significant when the models included spatial variation at an ecologically relevant regional scale. This suggests that, at least for this limited data set, annual variation was small compared to other sources of variation. This analysis can only be considered preliminary due to the small number of years considered, and these results should not detract from the broader Panel recommendation that more years of data should be required for evaluating questions of this gravity.

## Effective Refuge Calculation and Modeling

### Agency Charge

**6. Monsanto has corrected their calculation of effective refuge size presented in Gustafson and Head, 2004 based on the Agency's (BPPD, 2004) and June 2004's Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel's (SAP) recommendations (SAP, 2004). Modifications to the calculation of the effective refuge size involved removing the assumption of constant effective refuge size and explicitly accounting for the lower production of CBW and TBW in cotton where survival of these insects is reduced. Estimation of the effective refuge now assumes a regionally specific annual cycle of effective refuge size, according to data collected in alternative host studies of CBW (Head and Voth, 2004) and TBW (Head and Gustafson, 2005). These data were combined with corn planting estimates on either the regional scale for CBW, or county-scale for TBW, to estimate effective (i.e., current (structured non-Bt cotton + non-cotton) and natural (non-cotton only) refuge sizes for each of what were conservatively assumed to be six annual generations for each pest.**

**a)** Estimation of the relative number of CBW adult moths produced by each of the five sub-compartments is given by the following equation: $\boldsymbol{M_{ij} = A_{ij}\, E_{ij}\, LB_{ij}\, LS_{ij}}$ **(Equation 1)**.

*[M* is the number of adult moths produced per unit area of the region; *A* is the proportion of the region occupied by the crop type of interest; *E* is the relative (to cotton, i.e., $E_{cotton}$=1) number of effective eggs (eggs that would produce adults in the absence of *B.t.* or pyrethroid sprays) laid in the crop type; *LB* is the fraction of larvae surviving in the presence of the *B.t.* crop; *LS* is the fraction of larvae surviving a pyrethroid insecticide spray on the crop; the subscript *i* refers to the compartment (*B* for *B.t.* or *R* for refuge); and the subscript *j* refers to the particular crop type within the compartment (1 = cotton, 2 = corn, 3 = other C3 host crop).]

The effective refuge, $R_{eff}$, is defined as the proportion of adult moths that would have been produced in the refuge compartment (non-Bt cotton, non-Bt corn, non-cotton C3 crops) in the absence of any induced larval mortality:

$$R_{eff} = \frac{M_{R1} + M_{R2} + M_{R3}}{M_{R1} + M_{R2} + M_{R3} + M_{B1} + M_{B2}}$$ (**Equation 5**; used when CBW populations were actively feedings in cotton, Generations 3-5)

Effective refuge estimations for all of the "non-cotton" generations are given by**:**

$$R_{eff}^{NC} = \frac{M_{R2} + M_{R3}}{M_{R2} + M_{R3} + M_{B2}}$$ **(Equation 6)**

The natural refuge component (i.e., non-cotton C3 crops + non-Bt corn components) of the total effective refuge is as follows:

$$R_{nat}^{CBW} = \frac{M_{R2} + M_{R3}}{M_{R2} + M_{R3} + M_{B1} + M_{B2}}$$ **(Equation 7)**

**The Agency asks the SAP to comment on the estimated CBW effective and natural refuge calculations.**

**b)** Pooled, county-level estimates of the percent cotton-reared TBW moths were combined with county-level landcover information to estimate the current effective refuge and natural refuge for each county per month. The relative TBW productivity of non-cotton areas within a county for a specific month is given as:

$$E_{NC} = \frac{(A_{NBTC} / P_{NBTC}) - A_{NBTC}}{A_{NC}}$$ **(Equation 8)**

The current effective refuge (non-Bt cotton + non-cotton hosts) for TBW is defined as the proportion of TBW moths actually produced in the effective refuge compartment prior to selection by Bt cotton:

$$R_{eff}^{TBW} = \frac{A_{NBTC} + (A_{NC}E_{NC})}{A_{BTC} + A_{NBTC} + (A_{NC}E_{NC})}$$ **(Equation 9)**

The estimated natural refuge (non-cotton hosts) for TBW is given by the following equation:

$$R_{nat}^{TBW} = \frac{A_{NC}E_{NC}}{A_{BTC} + A_{NBTC} + (A_{NC}E_{NC})}$$ **(Equation 10)**

**The Agency asks the SAP to comment on the estimated TBW effective and natural refuge calculations.**

**Panel Response**

**Summary**

1) Equation 7 overestimates the amount of natural refuge for CBW, $R_{nat}$, with the overestimates largest for Georgia (37%) and East Texas (44%).

2) Equations 8 and 9 overestimate the amount of effective refuge, $R_{eff}$, and natural refuge, $R_{nat}$, for TBW by not accounting for possible insecticide application in structured refuges. Generally, the size of the overestimation is roughly equal to $LS_{NBtC}$, the ratio of survival of TBW in non-Bt cotton (including from insecticide spraying) to their survival on non-cotton hosts. Thus, if $LS_{NBtC}$ = 20%, the true estimates of $R_{eff}$ and $R_{nat}$ are roughly 20% of the values given by Monsanto. The overestimation can be considerably less severe when $A_{NBtC}/A_{BtC} << P_{NBtC}$ (e.g., Lenoir Co., North Carolina, and Mississippi Co., Arkansas).

3) Underestimates of $P_{NBtC}$ will lead to overestimates of $R_{nat}$. This could be an important source of overestimation if the gossypol assay gives false negatives (see Panel response to Charge 1) and hence underestimates of $P_{NBtC}$.

4) The estimates of $R_{eff}$ and $R_{nat}$ are imprecise, due to uncertainty in the estimates of the parameters in the equations. Imprecision is possibly large for TBW in those counties used as scenarios for modeling (see Panel response to Charge 7), because the estimates of $R_{eff}$ and $R_{nat}$ for these counties are low (see Panel response to Charge 3). Monsanto does not provide enough information to assess the level of uncertainty, and it is unclear whether the sampling intensity from the present study is sufficient to estimate this uncertainty.

**Discussion**

Specific issues regarding the calculations for (a) CBW and (b) TBW are presented below followed by (c) a discussion of Panel concerns regarding parameter uncertainty.

**a) CBW**

The Panel had several specific concerns about the equations used to calculate $R_{eff}$ and $R_{nat}$ for CBW.

*Equation 1*

This equation assumes that movement rate of males from all habitat types is the same. If males are more likely to disperse from a given type of habitat, this will have the same consequences for the estimates of $R_{eff}$ and $R_{nat}$ as increasing the number of eggs laid in these habitats, E. The consequences of bias in estimates of E are described below.

*Equation 2*

Monsanto provides evidence that two pyrethroid applications are common for cotton regardless of whether or not it is Bt cotton. However, conventional and Bt cotton differ in the proportion of acres that receive pyrethroid applications. Based on this information, Monsanto assumes the survival rate of CBW on cotton is $LS_{i1} = [T_{i1}\ ((1/3) + (2(1-K_{i1})/3))] + (1 - T_{i1})$. Equation 2 can be interpreted as a weighted average of the survival rate over three generations of CBW when two out of the three generations receive pyrethroid applications. Alternatively, Monsanto could have employed the assumptions $LS_{i1}^{1} = 1$ for the first generation of CBW on cotton, and $LS_{i1}^{23} = T_{i1}(1-K_{i1}) + (1 - T_{i1})$ for the second and third generations. These alternative assumptions are more consistent with what is being described in the field. Note that $LS_{i1}^{1} > LS_{i1} > LS_{i1}^{23}$, which means Monsanto's methodology will underestimate production of CBW moths for the first cotton generation and overestimate production for the second and third generations. Therefore, Monsanto's methodology will underestimate the temporal variability of CBW moth production and effective refuge calculations. Simulations conducted by one Panel member in preparation for this SAP meeting, using the model reported in Hurley et al. (2006), suggest this type of increased temporal variation can speed the evolution of resistance (see also Ives and Andow 2002). These results are not detailed in this report due to the modest differences that were observed. These modest differences suggest Monsanto's simplifying assumption may be a reasonable, although not necessarily conservative, approximation. Still, Monsanto could strengthen the credibility of their results by incorporating the type of temporal variability in pyrethroid applications observed in the field into their model.

*Equation 5*

Biases in the estimates of $A_{ij}$ , $E_{ij}$ , $LB_{ij}$ , and $LS_{ij}$ have different consequences depending on the habitat considered. Specifically, underestimates of all of the parameters for refuges (in cotton, corn, and other C3 host crops) will underestimate $R_{eff}$ and $R_{nat}$. Conversely, underestimates of all of the parameters for Bt cotton and Bt corn will overestimate $R_{eff}$ and $R_{nat}$.

*Equation 7*

Monsanto (Gustafson and Head 2005) defines the natural refuge as

$$R_{nat}^{CBW} = \frac{M_{R2} + M_{R3}}{M_{B1} + M_{B2} + M_{R2} + M_{R3}}, \qquad \text{(Equation 6-1)}$$

which removes non-Bt cotton moth production from both the numerator and denominator of the effective refuge calculation. This equation instead should omit $M_{R1}$ only from the numerator to give

$$R_{nat}^{CBW} = \frac{M_{R2} + M_{R3}}{M_{B1} + M_{B2} + M_{R1} + M_{R2} + M_{R3}} \qquad \text{(Equation 6-2)}$$

Monsanto's equation for $R_{nat}$ leads to overestimation that can be substantial. Based on the data reported in Tables 2 and 5 (Gustafson and Head 2005), Monsanto's estimate of the natural refuge ($R_{nat}^{CBW}$) will be 37, 12, 6, and 44% higher than the natural refuge estimate given by Equation 6-

2 for Georgia, Mississippi, North Carolina, and Texas. Appendix 1 gives a correct derivation of Equation 7.

*Additional concerns*

These equations are applied at the scale of entire states. This assumes either that the population of CBW is completely mixed at the regional scale (i.e., a given trapped insect has the same probability of coming from any location in the region), or that the distribution of habitats is uniform across the region at the scale of insect movement (i.e., for a trapped insect, the distribution of habitat types in the specific area from which it was produced is the same as the distribution of habitat types throughout the entire region). Data are presented to show that CBW often travel long distances. Nonetheless, it is very difficult to determine from these data whether the CBW population is completely mixed at the scale assumed by equations 1-7; this is discussed further under Charge 1. We note, however, that Bollgard II is not strongly high-dose for CBW, and this may affect the impact of incomplete mixing on the rate of resistance evolution.

A related concern is brought up by EPA's review (p. 14, BPPD 2006). No information is given about whether traps were located next to Bt or non-Bt fields. The basic assumption behind equations 1-7 is that the CBW population is completely mixed with respect to habitat types. Therefore, under this assumption there should be no effect of trap placement on the habitat of origination of the moths. This concern of the EPA reviewers suggests that they do not believe that CBW males are completely mixed at the regional scale.

**b) TBW**

*Equations 9-10*

Calculations for TBW are similar to CBW, but assume that the survival of TBW from Bt crops is zero. Also, the effect of differential survival in non-cotton hosts and non-Bt cotton including that due to insecticide spraying (which was included for CBW after a request by the SAP 2004) was not included in the case of TBW. Including differential survival and expressing $E_{NC}$ in terms of measured parameters (e.g., $P_{NBTC}$) gives

$$R_{eff}^{TBW} = \frac{LS_{NBTC} A_{NBTC}}{P_{NBTC} A_{BTC} + LS_{NBTC} A_{NBTC}} \qquad \text{(Equation 6-3)}$$

and

$$R_{nat}^{TBW} = \frac{(1 - P_{NBTC}) LS_{NBTC} A_{NBTC}}{P_{NBTC} A_{BTC} + LS_{NBTC} A_{NBTC}} \qquad \text{(Equation 6-4)}$$

where $LS_{NBTC}$ is the survival of TBW from pesticide application and other mortality factors in non-Bt cotton relative to survival in non-cotton hosts.

The effect of including insecticide application in non-Bt cotton can be large and the resulting values for $R_{nat}$ quite low; for example, 20% survivorship from insecticide spraying in non-Bt cotton results in a decrease in the estimate of $R_{eff}$ and $R_{nat}$ to roughly 20% of its original value when the area is dominated by Bt cotton and the natural refuge is small ($P_{NBTC}\,A_{BTC} >> LS_{NBTC}\,A_{NBTC}$), as occurs in the counties used as scenarios in the modeling. Recalculation of $R_{nat}^{TBW}$ for Monsanto's seven "worst-case" counties for the oviposition months June, July, and August, shows that $R_{nat}$ in the MidSouth region is not infrequently below 0.05 for $LS_{NBtC} = 1$, and is generally below 0.05 for $LS_{NBtC} = 0.2$ and 0.1 (Figs. 6-1, 6-2, and 6-3, respectively; Gibson

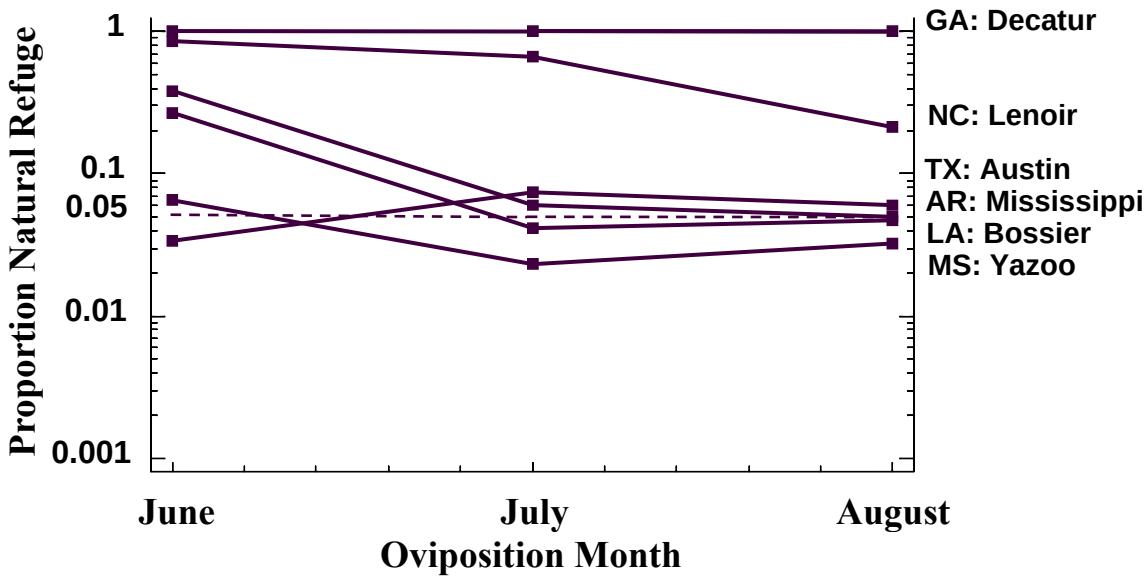


**Fig. 6-1.** Proportion of natural refuge, $R_{nat}$, calculated by month from pheromone trap-captured TBW for worst-case counties in six states, under the condition $LS_{NBtC} = 1$.

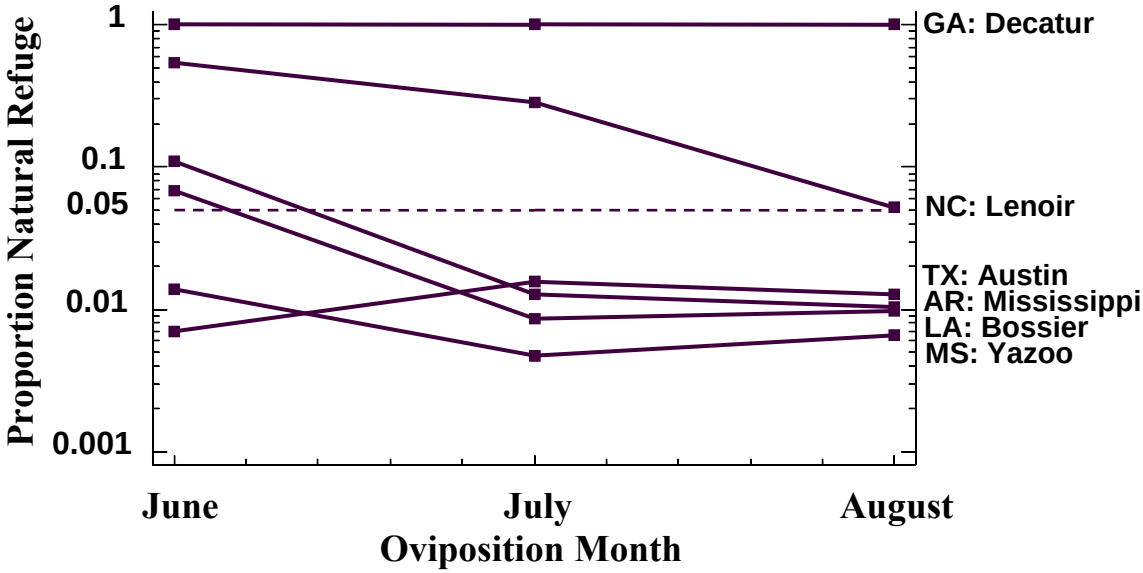

**Fig. 6-2.** Proportion of natural refuge, $R_{nat}$, calculated by month from pheromone trap-captured TBW for worst-case counties in six states, under the condition $LS_{NBtC} = 0.2$.

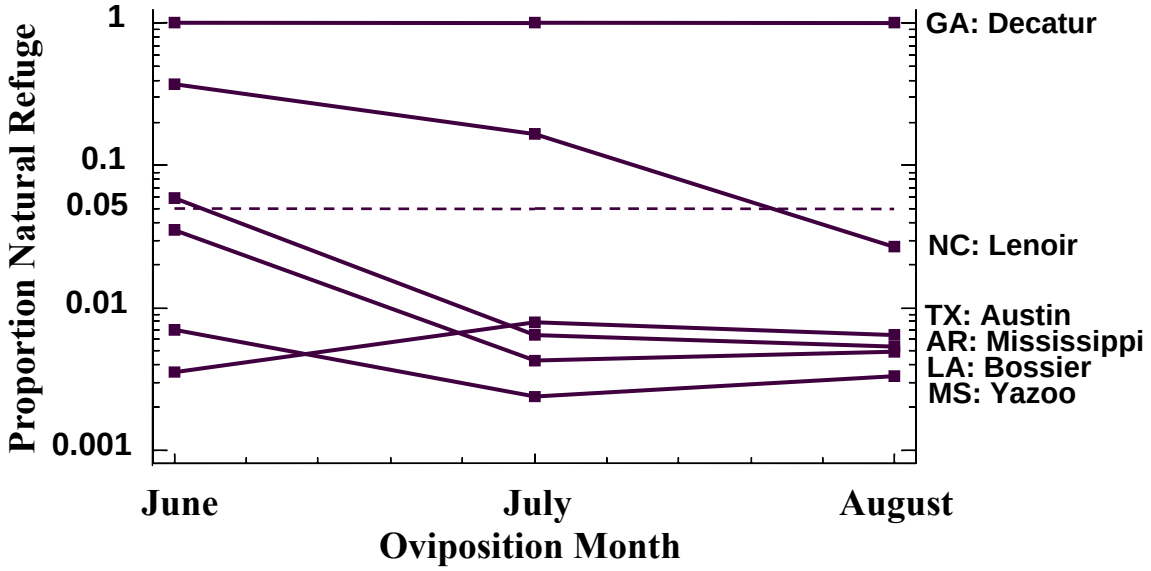


**Fig. 6-3.** Proportion of natural refuge, $R_{nat}$, calculated by month from pheromone trap-captured TBW for worst-case counties in six states, under the condition $LS_{NBtC} = 0.1$.

County, Tennessee, excluded due to lack of data for June and August). Even for counties in the East, $R_{nat}$ can be below 0.05 for $LS_{NBtC} = 0.1$ (Fig. 6-3). Also, it is apparent that $R_{nat}$ was unusually low in Austin County, Texas, relative to other MidSouth counties in June 2005. Whether this is a difference peculiar to East Texas or represents year to year variability that may also occur in other areas is an open question.

These calculations, of course, depend on the differential level of mortality caused by spraying, $LS_{NBtC}$. If, for example, most of the structured refuge is in the form of 5% external unsprayed refuge, then $LS_{NBtC}$ will be close to 1, and the incorrect formulas used by Monsanto will not grossly overestimate $R_{eff}$ and $R_{nat}$. As a result, detailed information is needed about the deployment of refuges and the intensity of insecticide use to properly calculate $R_{eff}$ and $R_{nat}$.

Some discussion arose amongst Panelists about how to incorporate insecticide spraying in refuges into equations 9-10, and the Panelists agreed on the following argument. The correction for spraying in non-Bt cotton affects the calculations for $R_{eff}$ and $R_{nat}$ mainly through changing the productivity of TBW from non-Bt cotton relative to the potential productivity of TBW from Bt cotton that would occur in the absence of mortality from Bt. The importance of the contrast between productivity in non-Bt cotton vs. the potential productivity in Bt cotton is due to the manner in which the refuge reduces the rate of resistance evolution. While susceptible TBW can only reproduce in the refuge, resistant TBW can also reproduce in Bt cotton fields. Therefore, the definition of refuge (as correctly used by Monsanto) is the ratio of productivity of susceptible TBW from refuges to the potential productivity of resistant TBW from all habitats.

To make this explicit, consider the equation for $R_{eff}$ in terms of $M$, defined (as in Monsanto's equation 1 for CBW) as the production of TBW from different habitats:

$$R_{eff}^{TBW} = \frac{M_{NBTC} + M_{NC}}{M_{BTC} + M_{NBTC} + M_{NC}} = \frac{LS_{NBTC}E_{NBTC}A_{NBTC} + E_{NC}A_{NC}}{E_{BTC}A_{BTC} + LS_{NBTC}E_{NBTC}A_{NBTC} + E_{NC}A_{NC}} \qquad \text{(Equation 6-5)}$$

Here, values of $E_{BTC}$ and $E_{NBTC}$ are the densities of eggs oviposited in the different habitats, and mortality from Bt, $LB$, has been excluded because resistant TBW will not suffer from this mortality. Note that the term involving $A_{BTC}$ in the denominator, $E_{BTC}\,A_{BTC}$, does not include $LS_{NBTC}$. Therefore, including spraying mortality in non-Bt cotton refugia reduces the estimate of $R_{eff}$ by accounting for the reduction in production of susceptible TBW from non-Bt cotton relative to the potential production of resistant TBW from Bt cotton. Correcting the equations for spraying in non-Bt cotton also changes the estimates of $E_{NC}$, because spraying will decrease the production of TBW from non-Bt cotton relative to non-cotton refuge. Nonetheless, it is the contrast between reproduction of resistant TBW from Bt crops and susceptible TBW from non-Bt crops that is responsible for the overestimates of $R_{eff}$ and $R_{nat}$ in Monsanto's equations.

*Sensitivity of TBW calculations to parameter biases*

For TBW the effects of biases in parameter estimates on $R_{eff}$ and $R_{nat}$ can be summarized using Equations 6-3 and 6-4 above:

$A_{NBTC}$ – If the estimates of land-area cover of non-Bt cotton are high, then the estimates of $R_{eff}$ and $R_{nat}$ will be high as well.

$A_{BTC}$ – If the estimates of land-area cover of Bt cotton are high, then the estimates of $R_{eff}$ and $R_{nat}$ will be low.

$LS_{NBTC}$ – If the estimates of the survival from spraying insecticide in non-Bt cotton are high (or, as assumed by Monsanto, 1), then the estimates of $R_{eff}$ and $R_{nat}$ will be high.

$P_{NBTC}$ – If the estimates of the proportion of (non-Bt) cotton-reared insects as opposed to those originating from non-cotton wild hosts are high, then the estimates of $R_{eff}$ and $R_{nat}$ will be low. This effect on $R_{nat}$ deserves special attention. As discussed in Charge 1, the Panel is concerned that the gossypol assays are giving false negatives, and therefore underestimating $P_{NBTC}$. Underestimating $P_{NBTC}$ will overestimate $R_{nat}$. Therefore, until the gossypol assay is validated, the estimate of $R_{nat}$ should be considered an overestimate, although it is unclear how much of an overestimate it is. Note that this source of overestimation of $R_{nat}$ is separate from the source of overestimation caused by ignoring insecticide application in non-Bt cotton described above.

Like the calculations for CBW, the calculations for TBW assume that males are well-mixed at the spatial scale of variation in habitat types. In fact, it is unlikely that TBW males from non-Bt cotton and non-cotton hosts are well-mixed during the cotton growing season, at least in Louisiana. The seasonal pattern of variation in the frequency of resistance to pyrethroid

insecticides as this resistance became established in Louisiana (low at the beginning of the year and increasing as the growing season progressed only to return the next spring to a level nearly as low as the level of the previous spring) (Leonard et al.1995, Bagwell et al. 2000) is strong circumstantial evidence that the population of TBW selected for resistance to the insecticides in cotton was isolated during the cotton production season from a very large TBW population not under such selection, and that the populations were panmictic (unstructured or random-mating populations) in the fall and/or spring. This interpretation is supported by increased genetic structuring (decreased gene flow) of local TBW populations during the cotton-growing season in western Mississippi measured with random amplified polymorphic DNA markers (Han and Caprio 2004). In counties with extensive cotton production, this will tend to bias the estimates of $P_{NBtC}$ upwards, resulting in underestimation of $R_{nat}$.

**(c) Parameter uncertainty**

The calculations make numerous assumptions about the parameters *A*, *E*, *LS*, *LB*, and *P* that are used. They also assume that these parameters are known without error. To discuss the issue of parameter uncertainty, we will focus here on the calculations for TBW (corrected as described above), noting that similar issues arise for the calculations for CBW.

Uncertainty in the estimates is difficult to assess but could be large. Monsanto does not provide information about the uncertainty of some parameters. Although Monsanto provides confidence intervals for the estimates of *P* (see note below), these confidence intervals do not directly translate into confidence intervals for $R_{eff}$ or $R_{nat}$. It is possible, however, to estimate or approximate confidence intervals in $R_{eff}$ and $R_{nat}$ using the statistical approaches described in Charge 4 if uncertainty of all parameter estimates is known.

To illustrate the issue of parameter uncertainty, consider the case in which $LS_{NBTC} = 1$ (as assumed by Monsanto), $P_{NBtC} = 0.5$, $A_{NBTC} = 0.05$, and $A_{BTC} = 0.95$. Thus, $R_{nat}^{TBW} = 0.0476$. Assume that $A_{NBTC}$ and $A_{BTC}$ are all known without error, but that $P_{NBTC}$ is calculated from a single trap that collected 10 males; thus, for AVE[$P_{NBtC}$] = 0.5, the expected number of non-cotton males is 5, but there is binomial uncertainty around this value. Figure 6-4 gives the distribution of estimates of $R_{nat}^{TBW}$ computed from simulation. There is a 17% chance of estimating a refuge size greater than 0.1; in other words, there is a 17% chance that the estimate of $R_{nat}^{TBW}$ is twice the actual value of $R_{nat}^{TBW}$. If this occurred for the counties used in Monsanto's modeling scenarios, Monsanto would overestimate the size of the refuge by 100%. This example demonstrates that Monsanto needs to calculate confidence intervals for their estimates of $R_{eff}$ and $R_{nat}$ to account for the range of expected refuge sizes in risk calculations.

*Note on confidence intervals for $P_{NBtC}$:* The formula for the upper limit of the 95% CI for $R_{nat}$ (p. 121, Gustafson and Head 2005) is incorrect as presented, but the correct formula apparently was used in the calculations. The lower confidence limit given (p. 121, Gustafson and Head 2005) is an approximation that fails when all the TBW test negative for gossypol. For a total of *n* individuals tested, the correct formula is: $LL = 0.05^{1/n}$ (Louis 1981). This correction results in a small increase (≤ 7.1%) in *LL*.

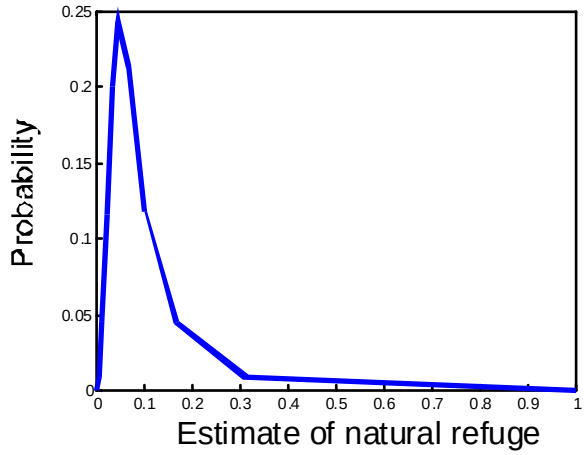


**Fig. 6-4.** Probability distribution of the estimates of $R_{nat}^{TBW}$ assuming AVE[$P_{NBtC}$] = 0.5, $A_{NBTC}$ = 0.05, and $A_{BTC}$ = 0.95; $P_{NBTC}$ is calculated assuming a binomial sample of 10 males.

**<u>Agency Charge</u>**

**7. Monsanto examined the durability of each of the three Bt cotton products (i.e., Bollgard, WideStrike, and Bollgard II) individually and together in the marketplace using a three-gene model. The Bt protein, Cry1Ac, is common to all three products. The presence of each of these products in the marketplace selects for potential resistance to Bollgard cotton, expressing only the Cry1Ac protein, and also selects for resistance to the other two products through the common selection for Cry1Ac resistance. The products vary greatly in the rate at which they select for resistance to Cry1Ac because of the presence of additional insecticidal proteins in Bollgard II (Cry2Ab2) and in WideStrike (Cry1F).**

**The three-gene model for insect resistance evolution used in this study is based on a conceptual model similar to that proposed by Dow AgroSciences (DAS) for its product, WideStrike cotton, and was reviewed by a recent U.S. EPA Scientific Advisory Panel (SAP) (SAP, 2004). However, the SAP questioned some of the mathematical details of the DAS model and Monsanto has made some changes to address the SAP's concerns. As shown in schematic form in Figure 3, Appendix 2, the three-gene model is based on the following assumptions concerning the mechanism of activity of the three commercial Bt cotton products (Bollgard, Bollgard II, and WideStrike cotton):**

- **The Cry1Ac toxin, present in all three products, binds to two receptors, 60% to receptor A and 40% to receptor B.**
- **The Cry1F toxin, present only in WideStrike cotton, binds exclusively to receptor A.**
- **The Cry2Ab2 toxin, present only in Bollgard II cotton, binds exclusively to receptor C.**

**a) <u>CBW.</u>** Based both on the intrinsic durability of each of the three *B.t.* cotton products (**Figure 4, Appendix 2**) and the three-gene modeling analyses for all three Bt cotton products together in the marketplace (**Table 14, Appendix 2),** Bollgard II retained the highest level of efficacy against CBW in all scenarios (all regions). Given the assumptions of the three-gene model and its limitations, there is likely enough effective natural refuge to be sufficient to delay the evolution of resistance to Bollgard II cotton for more than 25 years (not a precise number of years) under all plausible scenarios in all four regions (**Table 14, Appendix 2**). This is because of the relatively high mortality of individuals heterozygous to Cry1Ac resistance in the presence of Cry2Ab2, as compared to WideStrike. WideStrike is intermediate in many scenarios because of the shared binding receptor between Cry1F and Cry1Ac and the likelihood of cross-resistance is greater. Bollgard is weakest in all scenarios because there is no high dose for CBW and it is a single-gene product. Monsanto's models predict that CBW resistance to Bollgard cotton will evolve in less than the 30 year horizon in the Georgia, Mississippi, and E. Texas regions in most scenarios except for 2-C (Bollgard = 0.1; Bollgard II = 0.8; WideStrike = 0.1). Resistance always took at least 30 years to evolve to all three Bt cotton products in the North Carolina region in all scenarios, even the natural refuge scenarios. When Bollgard cotton acreage is minimized, Bollgard II and WideStrike longevity is maximized **(Table 14)**. Large amounts of Bollgard II cotton in the marketplace increased the durability of both Bollgard and WideStrike **(Table 14, Appendix 2)**. Uncertainties in the pheromone captures, estimation of adult productivity, carbon isotope analyses, spatial analysis, estimation of effective refuge calculation, degree of shared binding affinity of Cry1Ac to receptor A and B, genetics of resistance, resistance mechanism, initial resistance allele frequency, and other modeling assumptions affect the precision and accuracy of the modeling predictions. Monsanto's modeling also does not consider pre-selection for Cry1Ac resistance. Ten years of selection pressure (since 1996) for resistance to Cry1Ac has already occurred. Field resistance to Cry1Ac places additional selection pressure on the Cry2Ab2 component of Bollgard II cotton.

**Given the assumptions and uncertainties in Monsanto's CBW modeling efforts, the Agency asks the SAP to comment on the utility of the modeling to predict the effectiveness of natural (non-cotton C3 crops + non-Bt corn) vs. current effective refuge (non-Bt cotton + non-Bt corn + non-cotton C3 crops) to manage CBW resistance to the toxins expressed in Bollgard II. Discuss the impact of pre-selection for Cry1Ac resistance on the modeling output.**

**b) <u>TBW.</u>** The intrinsic durability of all three Bt cotton products is much greater for TBW than for CBW because of the "high dose" of Cry1Ac for TBW expressed in all three products. In virtually all cases, all three products retained their efficacy (i.e., no resistance) for more than 30 years (maximum time for the simulation) even if all cotton in a region is planted to that product and no structured refuge is required (i.e., all natural refuge). The only exceptions occur for Bollgard cotton in Tennessee and Mississippi. Given the assumptions of the three-gene model and its limitations, there is likely enough effective natural refuge to be sufficient to delay the evolution of resistance to Bollgard II cotton for more than 30 years (i.e., the time horizon of the model, not to be interpreted as a precise number of years) under all plausible scenarios in all four regions. This is due to

the extremely high efficacy of Cry1Ac against TBW, and the fact that Cry1Ac is present in all three Bt cotton products. In the state with the lowest natural refuge for TBW, Mississippi (see **Table 13, Appendix 2**), resistance to Cry1Ac and Cry1F evolved after 21 years in scenario 1-N if the structured refuge requirements for Bollgard and WideStrike cotton were removed. Uncertainties in the pheromone captures, gossypol analyses, spatial analysis, estimation of effective refuge calculation, degree of shared binding affinity of Cry1Ac to receptor A and B, genetics of resistance, resistance mechanism, initial resistance allele frequency, and other modeling assumptions affect the precision and accuracy of the modeling predictions. Monsanto's modeling also does not consider pre-selection for Cry1Ac resistance. Ten years of selection pressure (since 1996) for resistance to Cry1Ac has already occurred. Field resistance to Cry1Ac places additional selection pressure on the Cry2Ab2 component of Bollgard II cotton.

**Given the assumptions and uncertainties in Monsanto's TBW modeling efforts, the Agency asks the SAP to comment on the utility of the modeling to predict the effectiveness of natural (non-cotton hosts) vs. current effective refuge (non-Bt cotton + non-cotton hosts) to manage TBW resistance to the Bt toxins expressed in Bollgard II cotton. Discuss the impact of pre-selection for Cry1Ac resistance on the modeling output.**

**Panel Response**

**Introduction**

The Panel cautions EPA that acceptance of a simple non-spatial model used deterministically in this registration package could be interpreted as a precedent for future registrations. The panel advises EPA to clearly state under what conditions use of such models is appropriate. The specific model developed by Monsanto and the method used for its analysis were intended to address a very specific modification to an existing registration. The results clearly identify geographic regions where resistance evolution poses minimal risk, but the model and analyses are inadequate for evaluating the risk in any of the other regions or scenarios.

This is a very simple analytical model intended to address a very specific question: Would the removal of structured refuge significantly increase the risk of developing resistance to the three Cry toxins currently used in the environment? As with any model, the quality of the output and the ability to analyze output for the purpose of answering a question depends on 1) the choice of parameters that dictates the structure of the model, 2) the quality of the parameter estimates used as inputs to the model, and 3) the validity of the assumptions represented by the structure, inputs and method of execution.

The Panel challenged all three aspects of the Monsanto model. Panel responses to Charges 1 – 6 examined many of these issues in greater detail. In this section we focus on evaluating Monsanto's claim that natural refuges are sufficient to protect against resistance evolution in the "worst case" scenarios for each of the regions they consider.

**Difficulties Interpreting Model Results**

Monsanto's model, as presented to the Panel, appears to have identified some geographic regions where there is very little risk of resistance developing. By identifying regions of little risk, it also identifies the regions where the risk of resistance developing is greater. However, the model (as executed) cannot adequately assess risk in these regions, such as in Mississippi and East Texas regions. Proper assessment of risk in these regions requires acquisition of more data, a more robust statistical analysis of the data, and a more detailed approach to modeling that includes both spatial and temporal variability.

Monsanto has taken a cafeteria approach to modeling this system. They have used a simple analytical model and truncated execution at 30 years. Truncating execution is a constraint typically applied to, and more appropriate for, stochastic simulation models that are prohibitively computer intensive. While 30 years may represent a reasonable (and perhaps long) product life, Monsanto's decision to truncate execution at 30 years renders all of the TBW and much of the CBW output inadequate for assessing the risk of resistance evolution if structured refuges were removed.

Tables 14 and 15 (Appendix 2 in Gustafson and Head 2005) present the years to resistance and the efficacy at 30 years for each product by region. Years-to-resistance is a relative index or qualitative method of assessing risk, a point made clear in the EPA presentation. A secondary perspective on risk is provided by the product efficacy. However, by truncating execution of the model at 30 years, product efficacy becomes the only perspective. The TBW results (Table 15) show no qualitative differences at all and cannot be evaluated. The CBW results (Table 14) show qualitative differences for some scenarios but the 30-year efficacies suggest that resistance would develop soon after the 30-year time horizon.

The efficacy of a product does not translate well to a resistance allele frequency, especially when the product contains more than one toxin, affects more than one locus or when cross resistance is not involved. The 30-year allele frequencies for each locus would provide a third perspective for assessing risk and aid in our overall understanding of the process of evolution to transgenic crops.

Given the simplicity of the analysis, this model should have been run until resistance developed regardless of the number of generations or years. If Monsanto wants to demonstrate that resistance never develops under a particular scenario, then allele frequencies for resistance for each locus need to be provided showing that there has been no change over some period of time (either from initial allele frequencies or some stable equilibrium allele frequency over an extended period of time).

In predicting resistance evolution to Bollgard II, there is not only uncertainty in the estimation of parameters used in models (as described in previous sections), but also "model uncertainty," as is always the case. Model uncertainty refers to very different predictions that can arise from different models. Unfortunately, while there are statistical methods to account for parameter uncertainty, it is much more difficult to formally assess model uncertainty before resistance evolution occurs. Of course, once resistance has arisen, it is possible to validate models against the data. For example, Livingston et al. (2002) show that a simple genetic model

can characterize the evolution of pyrethroid resistance in CBW and TBW field populations. Nonetheless, in the absence of such empirical data for Bt cotton, and in particular for a two-toxin product like Bollgard II, model uncertainty can only be addressed by careful mathematical examination of different models to understand why they give different results.

The model which Monsanto used for its analyses (Gustafson and Head 2005) is based on Caprio (1998). Like all models, this model makes numerous simplifying assumptions about the biology of TBW and the processes that affect resistance evolution. Exploration of this model (Appendix 2) demonstrates that seemingly subtle differences in the assumptions not only can have a large impact on predictions about the rate of resistance evolution, but can also change which of the parameters have the greatest influence on the rate of resistance evolution. This highlights the problem that model uncertainty affects not only the quantitative predictions about the rate of resistance evolution, but also the key factors that must be measured to make these predictions.

A suite of well-understood models of resistance to a pyramid Bt product does not yet exist. Furthermore, key issues like spatial structure, linkage disequilibrium, and differential movement of males and females have not been explored in detail. This makes model uncertainty high.

**Parameter Estimates**

Parameter uncertainty is a common problem in risk analysis that can often lead to incorrect analysis and inappropriate conclusions. However, there are several methodologies for incorporating parameter uncertainty and exploring the sensitivity of the results to differences in parameter estimates. The uncertainties associated with many of the parameter estimates have been discussed under Charges 1-6. In general, these uncertainties can be resolved with the collection of more years of data and better spatial sampling of the regions. Even without more data, a more appropriate statistical analysis should provide a better understanding of the hosts from which moths developed and the geographic and temporal distribution of moth abundance in each region. The most significant challenge and the greatest uncertainty involves the gossypol analysis. All estimates of moth production from non-cotton refuges are dependent on the validity of this technique (see Panel response to Charge 3). Confirmation of the gossypol analysis as a means of assessing the proportion of moth trap catches originating from cotton hosts would have the greatest impact on the confidence of risk assessment regardless of the modeling techniques used.

Both analytical and simulation models have been used in the past to assess the risk of resistance to plant-incorporated protectant compounds. Deterministic and stochastic approaches have been used with both kinds of models. Monsanto's model is a simple analytical model using perhaps the simplest deterministic approach. This technique makes broad assumptions concerning the year-to-year stability of the landscape characteristics. Monsanto explicitly defines the proportions of each product as constant over the 30-year time horizon (see socio-economic factors below). Implicitly, this model also assumes year-to-year stability in the proportions of crop types, the proportions of continuous and rotated crops, and the spatial

distribution of various hosts throughout the landscape. Similarly, the biology and behavior of the insect in response to the landscape is assumed to be stable from year-to-year.

An alternative to an analytical model is to simulate the system in detail. Risk can be assessed using either modeling technique by varying the parameter estimates across a range of values. Using a deterministic approach, inputs can be chosen as fixed values representing the mean (or median as appropriate) as well as the high and low values representing some confidence interval (assumed 95% CI). Monsanto used this basic approach assuming that the lowest moth trap catches in each region represents the greatest risk of refuge failure.

Conceptually this is an appropriate methodology but (i) the statistical analysis of the trap data, (ii) uncertainty about the similarity of male and female dispersal behavior, (iii) uncertainty about the accuracy of the gossypol analysis, and (iv) unknown uncertainty in other parameters, does not permit us to assess the lower bounds of the confidence interval with any accuracy. If these issues are resolved, we recommend a multi-pronged approach involving several modeling strategies, including simple deterministic models and more-complex simulation models. Mississippi and East Texas are regions that require particular attention in these modeling efforts.

**Assumptions**

Three principle assumptions of the model were challenged by the Panel, specifically, widespread dispersal of the pests, socio-economic factors affecting the market share of the products, and the single locus per toxin receptor without cross-resistance. The expected changes to these assumptions have the potential for resistance to evolve more rapidly than Monsanto's results suggest. Detailed evaluation of these assumptions follow.

The Panel also notes that two of the assumptions may be considered conservative because the literature suggests that resistance to transgenics involves multiple loci and may be associated with fitness costs. The anticipated effects of incorporating less conservative estimates are described after the challenges.

***Spatial and Temporal Variability of Dispersal***

Figures 1 and 2 in Benedict (2005) illustrate the magnitude of differences in moth trap catches and host origin throughout the season and between regions. Confidence in the assumption that TBW is dispersing widely across the landscape varies throughout the season. If there are periods when dispersal is limited and mating moths are derived from the local landscape, resistance can evolve in a local population as a "hotspot." Single-locus spatially explicit models of resistance evolution (e.g., Peck et al. 1999, Storer et al. 2003, Sisterson et al. 2004) show that when refuges become rare, resistance is much more likely to occur in hotspots of Bt fields that are relatively isolated from refuges. Generations of low dispersal can play a disproportionate role in the rate of resistance evolution. The very low $F_{ST}$ values reported in the literature for population differentiation (based on neutral genetic markers), indicating high gene flow in general for TBW (Han and Caprio 2004, Benedict 2005), could exist even in the face of the oscillating temporal dispersal patterns over the season in which a resistance allele is increasing in frequency. While low $F_{ST}$ values can reflect the effects of high levels of gene flow

at neutral loci caused by migration over a long period of time, something very different can be happening to allele frequencies at loci under strong selection pressure.

The structure of the Monsanto model is non-spatial and therefore assumes that the distribution of refuge and Bt fields and the dispersal behavior of the adult insect are similar throughout the landscape. These are not realistic assumptions. A model incorporating a spatially explicit landscape and requiring 2-loci for resistance was developed for this report by one Panel member (see Appendices 3 and 4). As found with single-locus models (Peck et al. 1999, Storer et al. 2003, Sisterson et al. 2004), in this 2-locus model hotspots can occur when refuges (either natural or structured) are rare. When hotspots occur, resistance evolution can be much more rapid than in the absence of hotspots. This poses a challenge for IRM, because strategies must be designed to eliminate all possible areas where hotspots might arise; a hotspot in a single county might cause resistance that spreads throughout a region. The model shows that hotspots can occur even when males are broadly dispersive provided females have limited dispersal; therefore, direct information about female movement is needed to assess the risk of hotspots. Finally, in the model hotspots seem to overwhelm the effects of a fitness cost of resistance. Although fitness costs to resistant genotypes (i.e., reduced fitness in the absence of selection from Bt) generally slow resistance evolution (see "Fitness costs" below), this effect was minimal when the model produced hotspots.

The Panel includes this model to emphasize several points: (1) Model structure is critically important; Monsanto's non-spatial model cannot produce hotspots and therefore might ignore an important component of resistance evolution. (2) Simple changes in model assumptions can lead to dramatically different outcomes. (3) Our ability to determine appropriate model structure is limited by the lack of basic information needed to understand resistance evolution, especially in a 2-loci system. Because the appropriate model structure cannot be determined, the Panel cautions against relying on any one model or modeling technique for evaluating resistance to Bollgard II.

***Socio-economic factors***

The assumption that the market share of each product would remain stable over a 30-year time horizon is unrealistic. If a particular product fails to control a pest, we should expect growers to change their choice of control measures. Monsanto's product market share scenarios do not account for this important socio-economic factor. For example, in scenario 3-N for Texas (see Table 14 in Gustafson and Head 2005) resistance to product A (WideStrike) evolves in 19.5 years, resistance to product B (Bollgard) evolves in 12.8 years, while resistance to product C (Bollgard II) does not evolve within the 30-year time horizon. The efficacy of Bollgard II at 30 years suggests that resistance will evolve soon after execution of the model was truncated. It is unrealistic to assume that growers would continue to use the two failed products for 17 (Bollgard) and 10 (WideStrike) years. Continued use of these products is equivalent to using these areas as refuge for product C (Bollgard II).

A more realistic assumption is that product market shares will decline for products A and B and that growers will replace these products with either an increase in the market share of product C or resort to some alternative control measure such as pesticides (another alternative

refuge). However, Monsanto assumes that agricultural producers plant the maximum allowable proportion of Bt cotton at the state level which, as time-series data on adoption show, is not likely to be the case over any 30-year period. This assumption leads to the simulation result that Cry1 and Cry2 resistance will evolve more rapidly than is likely to be the case in reality. TBW results are unlikely to be affected by including a grower behavioral response. However, from a quantitative perspective, including the grower's behavioral response (Livingston et al. 2006) could dramatically change the time to resistance for CBW results.

***Potential for Metabolic Cross-Resistance***

One Panel member challenged the assumption that resistance to each toxin is associated with an independent single locus.

The three-gene model for insect resistance evolution considered by Monsanto makes assumptions about the action and interaction of resistance genes that do not fully reflect the resistance mechanisms reported in the scientific literature. Thus modifications of the model to incorporate such resistance mechanisms might yield different predictions of the durability of Bt cotton. Three assumptions made by Monsanto are discussed below: 1) independent toxin action, 2) absence of minor resistance mechanisms, and 3) absence of a sequestering mechanism.

*1) Toxins may not act independently.* In the model presented by Monsanto, survivorship of an insect on a single toxin is represented as a logistic function of the toxin-receptor complex, and toxins are assumed to act independently so that the total survivorship is the product of the single-toxin survivorships. However, some studies show that toxins do not act independently, but instead synergistically or antagonistically (Tabashnik 1992). An example of antagonistic action, in which the mortality caused by a combination of toxins is lower than predicted by the mortality of each singly, is given by Liao et al. (2002). In this study, the median lethal concentration ($LC_{50}$) of Cry1Ac and Cry2Aa presented together in various ratios was considerably less than the $LC_{50}$ predicted by models of independent action of the separate toxins, for a susceptible strain of *Helicoverpa armigera*. This would imply that the multiplicative aspect of Monsanto's model could be underestimating the probability of survivorship on Bollgard II cotton of insects that have resistance to one of the two toxins.

*2) The evolution of "minor" resistance mechanisms may influence selection on "major" resistance genes.* In the model presented by Monsanto, the only mutations that affect resistance are in the receptors for the specific toxins. This "target-site" resistance has been firmly established as a major resistance mechanism, and mutations in one of the receptors (a 12-domain cadherin protein expressed in the midgut epithelium) have been identified that cause high Cry1Ac resistance in three lepidopteran species. (These are TBW (Gahan et al. 2001), pink bollworm *Pectinophora gossypiella* (Morin et al. 2003), and the old-world cotton bollworm *Helicoverpa armigera* (Xu et al. 2005)). However, additional resistance mechanisms are known, such as changes in proteases that activate the protoxin (Oppert et al. 1997, Li et al. 2004) or changes in abundances of other binding targets for Cry1Ac such as alkaline phosphatase (Jurat-Fuentes et al 2005). These are "minor" resistance mechanisms that acting alone would not permit survival on transgenic cotton. But by reducing the amount

of toxin that has access to the receptor, they may contribute to resistance, especially in individuals that are heterozygous for the mutation in the receptor itself.

*3) A novel, recently-proposed resistance mechanism of "toxin sequestration" could have profound effects on resistance development on single-gene and pyramided cotton varieties.* Gunning et al. (2005) claimed that overproduction of esterases in the "silver selected" strain of *Helicoverpa armigera* was responsible for Cry1Ac resistance in that strain. They proposed that esterases could bind to the toxin and keep it from interacting with its receptor in the midgut. Overproduction of esterases is well-established as a mechanism of resistance to organophosphorus insecticides in aphids and mosquitoes. Each esterase subunit binds a single molecule of the insecticide in its active site, sequestering the insecticide and keeping it from reaching acetylcholinesterase, its target in the nervous system. Gunning et al. (2005) proposed the same sort of sequestration was operating in the "silver selected" strain. Such a mechanism would be expected to be energetically costly, especially if a 1:1 ratio of sequestering esterase to Bt toxin were required. However, if the esterase-toxin complex aggregates with other toxin molecules, such a ratio might not be required. Instead, there might be a general precipitation of toxin, such as induced by elastase TPP-75 (a digestive protease) produced in the midgut of the spruce budworm *Choristoneura fumiferana*, which precipitates Cry1Aa at a 1% molar ratio (Milne et al. 1998).

The report by Gunning et al. (2005) is highly controversial, and although it was published under peer review in a respected journal, the results have not been independently confirmed by other workers to date. If they are, and if direct evidence is produced for an esterase present in the midgut lumen in sufficient quantities to prevent Cry1Ac from binding to its receptor(s) in the midgut, this information would have to be considered in formulating models of resistance development. In addition, if such an esterase could sequester other toxins, such as Cry1F or Cry2Ab, this single-resistance mechanism could protect the insect from pyramided varieties of Bt cotton as well as Bollgard.

The consequences of this putative new sequestration mechanism for the modeling would require addition of a term describing the esterase-toxin complex. If this complex could be formed for any Bt toxin, the three-gene model would probably reduce to a single-gene model where an increase in the resistance allele frequency would have equal impacts on the efficacy of Bollgard, WideStrike, and Bollgard II cotton. Such a resistance gene, if it existed, would be essentially immune to the benefits of pyramiding in reducing the threat of resistance. Moreover, in this case pre-selection with Bollgard could facilitate cross-resistance to the pyramided varieties.

### ***Single Locus per Toxin***

Here, “single locus per toxin” is defined to mean that it only takes an allele at a single locus to confer resistance to a single toxin, and there is no possible cross resistance from such alleles. Monsanto modeled resistance to each toxin as a single-locus problem affecting the independent binding sites. Therefore, the pyramided products containing two toxins require resistance to develop at two independent loci. Excluding the possibility of metabolic cross-resistance presented in the above scenario, single locus resistance for each toxin (as opposed to

resistance to each toxin requiring multiple resistance alleles at multiple loci) represents the fastest possible path to resistance evolution in the TBW where both Cry1Ac and Cry2Ab are considered to each be at a "high dose" (i.e., if phenotypic resistance is completely recessive at all loci involved in resistance). This is not the case with CBW where even Bollgard II is not a high dose and any resistance allele with a small effect on tolerance of one or both toxins is expected to be selected for. In some cases the forms of resistance that have been developed in laboratory colonies appear to involve multiple loci and the aggregation of several minor forms of resistance. Alves et al. (2006) assessed the number of loci involved in resistance to the Cry1Ab for two laboratory-derived resistant strains of *Ostrinia nubilalis*, the European corn borer. One method of assessment suggests that resistance involves at least 10 different loci while a second assessment technique suggests there are more than 20 loci involved. In contrast, the same techniques imply that resistance in the diamondback moth, *Plutella xylostella*, is related to a single locus (which may explain why this insect exhibits resistance in the field).

We cannot assume that the number of loci involved in Bt resistance in *O. nubilalis* is directly translatable to either CBW or TBW. However, field level resistance has not evolved in any of these three pests and allele frequencies for resistance remain at or below the levels observed when transgenics were first introduced. Obviously, we cannot assume that each toxin requires a specific combination of alleles at 10 completely independent loci. But, we can assume that resistance to each toxin involves more than one independent locus.

A two-toxin product with resistance conferred by a single independent locus for each toxin is probably comparable to a single toxin product with resistance conferred by two independent loci. Given this assumption, the direction of this effect is to delay resistance and the magnitude of the effect is probably exponentially related to the number of loci involved. By modeling resistance as a single locus for each toxin, the approach is both reasonable and conservative for TBW. If a multi-locus resistance were modeled for each toxin the effect would delay resistance in TBW and the magnitude of the delay would overwhelm most if not all of the uncertainties mentioned. This would not be the case for CBW.

***Fitness Costs***

The model was run without fitness costs. This is also a conservative assumption, perhaps very conservative (except possibly if hotspots occur; see Appendix 3). The first paragraph of Gustafson and Head (2005), Section 4.4 summarizes the literature evidence that Cry1Ac resistance is associated with fitness costs.

Fitness costs can take many forms, reduced fecundity, delayed development, and reduced overwintering survival to mention but a few of the possibilities. Fitness costs are effectively selection against the trait of interest once accounting for the direct fitness benefits of the trait under selection (i.e., factoring out the benefits of resistance to Bt toxins in Bt fields). The consequences of including fitness costs in models of the evolution of resistance dramatically change the possible outcomes. If fitness costs are included there are four potential outcomes:

1) Resistance could be delayed.

2) The allele frequency could reach a stable equilibrium. This type of system operates as a cline, a gradient of opposing forces that reaches a balance point with fitness costs selecting against resistance while toxins select for resistance. .
3) The population can become extinct.
4) The population can persist but the allele is extirpated from the population.

With some notable exceptions like the diamondback moth (*Plutella xylostella*), resistance to the Cry complex of toxins has been developed in laboratory colonies. Resistant individuals from these colonies exhibit developmental delays and a reduction in biomass when compared to susceptible individuals of comparable age not exposed to the toxin. These are qualitative descriptions that imply metabolic fitness costs.

Peck et al. (1999) simulated Bt resistance evolution for TBW in North Carolina. Developmental delays during the growing season caused a temporal separation of the adults emerging from refuge and Bt fields. Although this kind of separation may not fit the typical definition of a fitness cost, it resulted in positive assortative mating among resistant individuals and increased the rate of resistance evolution. However, as the magnitude of the developmental delays was increased, the time to resistance evolution became less predictable. Differences in the synchrony of the diapausing life-stage with the end of the growing season often resulted in selection against the individuals that had been selected for resistance earlier in the growing season. When these relationships were combined with allele frequencies below 0.03 the allele was often extirpated from the population.

Carrière et al. (2001) attempted to estimate the effect of diapause asynchrony quantitatively in *Pectinophora gossypiella*. Three different laboratory colonies exhibiting different levels of Bt resistance were reared without exposure to the toxin under photoperiods that should induce diapause. Individuals that pupated were not in diapause and would not survive the winter. Individuals that did not pupate were assumed to be in diapause. Alternative host availability for this pest in Arizona is so limited that cotton is the only viable host. So, individuals that emerge prior to spring planting are unlikely to find hosts for their offspring. Homozygous resistant individuals suffered a 71% reduction in spring emergence when compared to homozygous susceptible individuals. In addition, the strains with the greatest level of resistance suffered the greatest overwintering mortality.

Caprio (2006) submitted a public comment to the Panel presenting a cursory evaluation of the effects of fitness costs for the MidSouth region (Mississippi). Figure 7 in that submission shows that 25% of the model runs led to resistance evolution in less than 15 years in the absence of structured refuge, while Figure 8 shows that approximately 5% of the model runs led to resistance evolution with a structured refuge. Fitness costs in this model were implemented as a reduction in fecundity ranging from 0.0 to 0.5 with a most likely value of 0.1 but the mechanism of selection against resistance is irrelevant to the question. These fitness costs are still well below the losses reported by Carrière et al. (2001). Also submitted to EPA for the Panel was a second model (Gould et al. 2006) showing that when there is a high dose of each of two toxins, the resistance alleles to the 2 toxins never reach a problematic level as long as there is a recessive fitness cost of 0.05 and a 10% refuge. Even if selection with a single toxin cultivar initially raises the frequency of alleles for one toxin above 0.90, after introduction of the two toxin

cultivar the frequency of both alleles reach a very low equilibrium level. However, the Gould et al. model is deterministic and must be viewed with caution.

Model runs that result in extinction have been reported since IRM modeling began (Onstad and Guse 1999, Peck et al. 1999, Sisterson and Tabashnik 2005). Peck's model includes trivial fitness costs as an offset for the increase in allele frequency due to mutation. The Onstad and Guse model includes no explicit fitness costs. The scenarios in which the population is driven to extinction result from fitness costs that are an emergent property of the complex interactions that occur between the definition of the landscape, the biological and behavioral characteristics of the target pest and the rarity of the resistance allele. Resistance management strategies include an assumption that 1) extinction is not a probable outcome for chronic pest populations and 2) resistance will evolve in response to the widespread use of transgenic crops. Modelers have therefore focused on the situations and scenarios that lead to resistance and discounted the conditions that result in extinction and extirpation of the allele for resistance. Caprio's (2006) submission is important because it illustrates the proportion of model runs that do not conform to these two assumptions about the outcome. Nonetheless, Monsanto does not argue that Bollgard II is likely to cause the extinction of either CBW or TBW, and given this, the relevance of simulations leading to extinction is uncertain.

Monsanto references several other papers suggesting that fitness costs are associated with resistance but these three examples illustrate that fitness costs can delay and perhaps even prohibit the evolution of resistance to Cry toxins. There are many potential mechanisms for fitness costs but regardless of the mechanism, fitness costs represent one of the best hypotheses for explaining why field populations have not developed resistance in the past decade. By not including fitness costs, Monsanto has taken the most conservative approach to modeling this system. If fitness costs were included in the model, the magnitude of the delay effect could overwhelm the opposing effect of most of the uncertainties presented. On the other hand, one Panel member indicated that the effect of hotspots can easily overwhelm a fitness cost of resistance and lead to rapid resistance evolution despite high fitness costs as described in Appendix 3 (see also Table A3-1).

**Pre-Selection**

Given initial allele frequencies for resistance that are very low, in a range approximately from 0.001 to 0.01, the time until resistance can be observed in the field population is an inverse exponential function of the initial allele frequency. That is, when the initial allele frequency is very low, minor increases in the initial allele frequency result in significantly shorter times to resistance. Selection for resistance that may have occurred prior to removal of structured refuge would result in an increase in the allele frequency for resistance in the field population. So, the most conservative answer to the question is that in this range of allele frequencies any increase shortens the time to resistance dramatically.

The model uses an initial allele frequency of 0.002 based on Gould et al. (1997). In this study, male TBW moths were collected in the MidSouth region (Mississippi, Louisiana and East Texas) prior to the introduction of transgenic crops. The estimated resistance allele frequency was actually lower than the value used in this model (0.0015 with a 95% CI ranging from 0.0004

to 0.0041). The study employed a resistant laboratory colony derived from TBW collected earlier in North Carolina. A best approximation of the allele frequency for resistance in the North Carolina population is 0.001 (Gould et al. 1997). Uncertainty about this value was strictly qualitative.

Burd et al. (2003) estimated the allele frequency for resistance in CBW collected in North Carolina in 2000, several years after transgenics were in use. Allele frequencies for resistance to the Cry1Ac toxin averaged 0.00043 with a 95% CI ranging from 0.00001 to 0.00239. The frequency of the resistance allele for the Cry2Aa toxin averaged 0.00039 with a 95% CI ranging from 0.00001 to 0.00216.

It must be noted that the rarity of the allele makes it extraordinarily difficult to detect. The 95% confidence intervals reflect this uncertainty. While the introduction of transgenics has significantly changed the spatial and temporal properties of the landscape there is little evidence that any increase in resistance allele frequency has occurred in field populations in the US over the past decade. There is evidence of increasing resistance to Cry1Ac for *H. armigera* in China where there are no structured refuges (Kongming Wu, F. Gould, et al., unpublished; Table 7-1). Monsanto's model was run conservatively at the mid to high range for allele frequencies for both pests.

**Table 7-1.** Relative average development ratings for six-day old larvae of *Helicoverpa armigera* $F_1$ and $F_2$ generation female lines in populations from Anci, Hebei Province and Xiajin, Shandong Province, China. (From K. Wu, unpublished.)

| | | Relative average development rating per line | |
|---|---|---|---|
| Location | Year[a] | $F_1$ generation | $F_2$ generation |
| Anci | LF-R | 0.91±0.008 A | |
| | 2005 | 0.50±0.005 B a | 0.53±0.024 a |
| | 2004 | 0.42±0.005 C a | 0.44±0.009 a |
| | 2002 | 0.30±0.006 D a | 0.26±0.037 a |
| | SS1 | 0.30±0.003 D | |
| | SS2 | 0.29±0.003 D | |
| Xiajin | LF-R | 0.91±0.008 A | |
| | 2005 | 0.56±0.005 B b | 0.61±0.019 a |
| | 2004 | 0.53±0.003 B b | 0.65±0.019 a |
| | 2003 | 0.50±0.010 C b | 0.66±0.027 a |
| | 2002 | 0.38±0.006 D b | 0.55±0.020 a |
| | SS1 | 0.30±0.003 E | |
| | SS2 | 0.29±0.003 E | |

Means (± SE) with different letters are significantly different ($P < 0.05$; LSD test). Capital letters in same column in same location indicate difference in different years. Small letters in same row indicate differences between generations in same year.

[a] LF-R is a Bt-resistant laboratory strain. SS1 and SS2 are Bt-susceptible laboratory strains.

**Agency Charge**

**8. Modeling suggests that the overall durability of Bollgard II cotton can be enhanced if Bollgard cotton is removed from the marketplace. This conclusion is supported by other researchers who examined the benefit of managing resistance evolution to two toxins with dissimilar modes of action using a pyramided approach (Zhao et al., 2005; Roush, 1998; Livingston et al., 2004; Hurley, 2000; Caprio, 2005). On the other hand, the concurrent use of single- and two-gene Bt plants can offer exposed populations a "stepping stone" to develop resistance to both proteins. Bollgard, Widestrike, and Bollgard II cotton exist in a mosaic in southeastern cotton growing regions, with Bollgard dominating the total acreage. In 2004, Bollgard cotton acreage accounted for >95% of all Bt cotton acreage in the U.S. (see Head et al., 2005, MRID# 467172-03). Encouraging the adoption of Bollgard II will increase the overall durability of all three Bt cotton products. From an insect management point of view, removal of Bollgard cotton from the marketplace would benefit the two-gene products, Bollgard II and WideStrike.**

**The Panel is asked to address the implications for selection for CBW and TBW resistance if the mosaic of single gene and dual gene products remains in the marketplace for a number of years. How would selection pressure be reduced if the single gene product is removed from the marketplace gradually (e.g., >3 years) or rapidly (e.g., ≤3 years) over a period of years?**

**Panel Response**

Two important dimensions to the question of mosaics of single and dual gene products are product market share and temporal variability. Monsanto addressed the product market share dimension by looking at how resistance evolved for seven scenarios characterized by different product market shares. Temporal variability was not explored by Monsanto.

To explore these two dimensions, it is useful to conduct a controlled experiment. In the first experiment, one can hold the temporal variability of the mosaic constant, while varying product market shares. For example, one can assume that the same amount of each product is planted every year, while allowing the proportion of each product's market share to vary from one treatment to the next (Monsanto's analysis). In the second experiment, one can hold the total market share constant for each product, while allowing the temporal variability of market shares to increase. For example, one can assume that the proportion of cotton acres represented by each product does not change over a specified period of time (e.g., 200 generations), while allowing the proportion of market shares for each product in each generation to vary from one treatment to the next. In preparation for this SAP meeting, these two experiments were conducted with the deterministic two gene/two product simulation model reported in Hurley et al. (2006) using the parameters reported in Table 8-1. In the second experiment, four examples of temporal variation in product market shares were explored, holding the total product market shares constant at 0.5 over 200 generations. These examples are reported in Figure 8-1. These simulations were designed to demonstrate the general effects of product market share and temporal variability in

product market share. As such, they were not calibrated to any specific pests, products, or agro-ecosystem.

**Table 8-1.** Controlled parameter values for simulation model examples.

| Parameter Name | Value |
|---|---|
| Proportion of Refuge | 0.05 |
| Refuge Survival Rate for all Genotypes | 1.0 |
| Recombination Factor | 0.50 |
| Proportion of Non-Random Mating | 0.0 |
| *Shared Toxin (e.g., Cry 1Ac)* | |
| Susceptible Homozygote Survival Rate | 0.0 |
| Heterozygote Survival Rate | 0.02 |
| Resistant Homozygote Survival Rate | 1.0 |
| Initial Frequency of Resistant Alleles | $1.0 \times 10^{-3}$ |
| *Pyramided Toxin (e.g., Cry 2Ab2)* | |
| Susceptible Homozygote Survival Rate | 0.0 |
| Heterozygote Survival Rate | 0.02 |
| Resistant Homozygote Survival Rate | 1.0 |
| Initial Frequency of Resistant Alleles | $1.0 \times 10^{-3}$ |

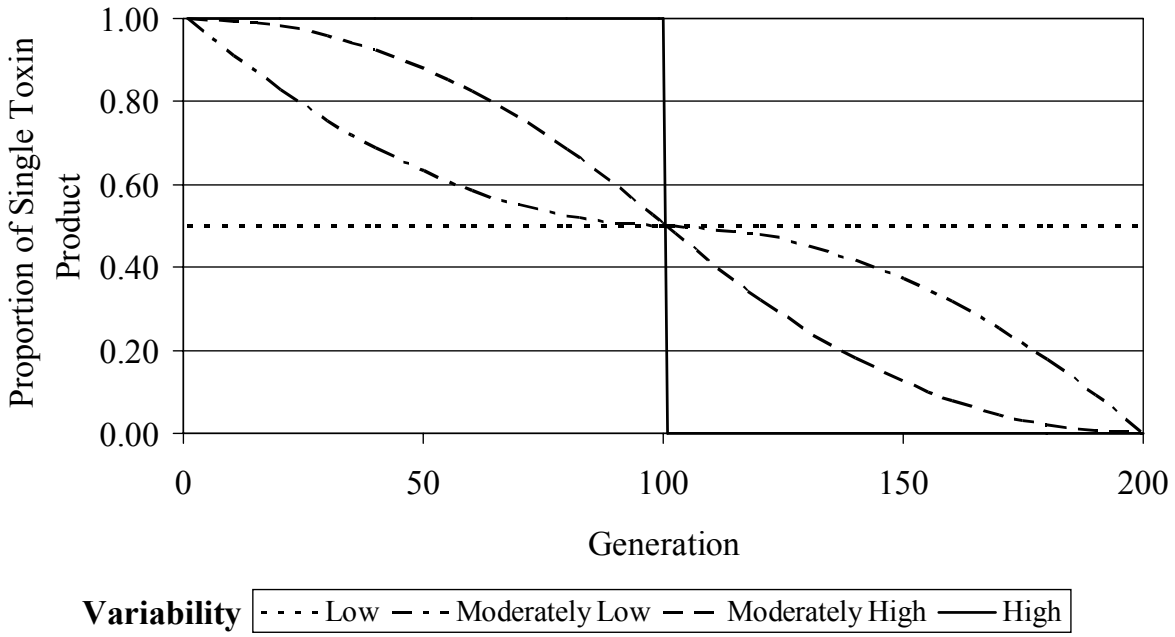


**Figure 8-1.** Example of temporally variable transition rates with equal market shares for single and pyramid toxin products (these transition rates were used for the analysis reported in Table 8-3).

In the first experiment, reducing the market share of the single toxin product slows the evolution of resistance to the pyramided toxin, which replicates Monsanto's results (see Table 8-2). For resistance to the pyramided toxin, there was not a monotonic relationship between market share and the evolution of resistance. Initially, increasing the market share of the pyramided product speeds the evolution of resistance. But once the market share of the pyramided product is high enough, further increases slow the evolution of resistance. The explanation of these results has to do with several countervailing effects. Increasing the market share of the pyramided product reduces selection for the shared toxin, which increases the time to resistance for the shared toxin. Increasing the time to resistance for shared toxin allows dual modes of action to operate for a longer period of time in the pyramided product, which is positive for resistance management. Increasing the market share of the pyramided product increases selection for the pyramided toxin because a greater proportion of the insect population is exposed to this toxin. This increased selection pressure reduces the time to resistance for the pyramided toxin, which is negative for resistance management. Increasing the market share of the pyramided product also means that there will be less refuge for the pyramided toxin once resistance evolves to the single toxin product because the single toxin product serves as refuge for the pyramided toxin after resistance to the shared toxin has evolved. This effect also reduces the time to resistance for the pyramided toxin, which is negative for resistance management. This simulation illustrates the opportunity cost of increasing the market share of the pyramided product in order to reduce selection for the shared toxin. This opportunity cost is an increase in selection pressure for the pyramided toxin.

**Table 8-2.** Example of the relationship between product market share and time to resistance.

| Market Share | | Time to Resistance (Generations) | |
|---|---|---|---|
| Single Toxin Variety (e.g., Bollgard) | Pyramided Variety (e.g., Bollgard II) | Shared Toxin (e.g., Cry 1Ac) | Pyramided Toxin (e.g., Cry 2Ab2) |
| 1.00 | 0.00 | 13 | > 200 (0.001) |
| 0.75 | 0.25 | 15 | > 200 (0.0036) |
| 0.50 | 0.50 | 21 | 197 |
| 0.25 | 0.75 | 38 | 104 |
| 0.10 | 0.90 | 87 | 117 |
| 0.00 | 1.00 | > 200 (0.001003) | > 200 (0.001003) |

Note: When resistance failed to emerge after 200 generations, the proportion of resistant alleles is reported in parentheses.

For the second experiment, high temporal variability speeds the evolution of resistance for both toxins (Table 8-3). This experiment provides one interpretation of what has been called the "stepping stone" effect. Holding total market share constant, increased temporal variability

in the adoption of the pyramided product means that the single toxin product is on the market longer, which speeds the evolution of resistance to the shared toxin. This means the pyramided product will effectively become a single toxin product sooner, which speeds the evolution of resistance to the pyramided toxin. This "stepping stone" effect is negative for resistance management and will be more pronounced when the market share of the pyramided product is relatively small.

**Table 8-3.** Example of the relationship between the variability of product market shares (see Figure 8-1) and time to resistance.

| | Time to Resistance (Generations) | |
|---|---|---|
| Product Market Share Variability | Shared Toxin (e.g., Cry 1Ac) | Pyramided Toxin (e.g., Cry 2Ab2) |
| Low | 21 | > 200 |
| Moderately Low | 13 (0.696) | 179 |
| Moderately High | 13 (0.868) | 156 |
| High | 13 (0.872) | 113 |

Note: When resistance failed to emerge after 200 generations, the proportion of resistant alleles is reported in parentheses.

While this experiment suggests that a quick transition to pyramided toxin products may not always be the best strategy due to the opportunity cost of increasing selection for the pyramided toxin, slow transition rates can only be supported by relatively heavy selection pressure on the pyramided toxin. The Panel agreed that the majority of papers published over the past decade using a variety of modeling strategies (from simple deterministic models to complex stochastic and spatially explicit models) have found that quicker transitions to pyramided toxin products is preferable in terms of resistance management.

The Panel noted that mosaics and slow transitions to pyramided products could negatively impact minor as well as major resistance mechanisms. Such mechanisms include alterations in midgut protease activity (Li et al. 2005) and changes in the abundances of proteins that bind Bt toxins such as aminopeptidases and alkaline phosphatase (Jurat-Fuentes and Adang, 2004). These minor mechanisms cannot by themselves enable survival of insects on transgenic cotton, but may supplement resistance levels in heterozygotes for target-site mutations. Products expressing a single Bt toxin are more likely to select for these minor mechanisms than pyramided toxin products because of the higher toxicity of the latter. These mechanisms may then boost resistance levels of insects carrying mutations for the receptor to the pyramided toxin. This would accelerate resistance development, relative to the models utilized by Monsanto that only consider target-site resistance.

While the majority of the literature that explores the effects of the transition from single to pyramided toxins finds slow transitions have a negative impact on resistance management,

there are counter examples. A stochastic, spatially explicit simulation model (Storer et al. 2003; Livingston, Storer, Gould, Kennedy, and Van Duyn, unpublished) shows that quicker transition rates to pyramided toxins slow resistance to the shared toxin, while increasing resistance to the pyramided toxin. This result is an example where there is strong selection for the pyramided toxin relative to the shared toxin, which is partly attributable to the resistance fitness costs assumed in the model. Livingston et al. (2006) used an extended version of this model that incorporates the behavioral response of a representative cotton producer and Cry2Ab2 and pyrethroid resistance evolution, and found that grower behavior can affect management of Cry1Ac and Cry2Ab2 resistance in CBW. This result is driven by two factors: economic and biological. In the model, the cotton producer chooses between cotton varieties based on profitability. When resistance to the shared toxin (Cry1Ac) evolves in the CBW metapopulations, the representative farmer switches from planting only Bollgard (the single toxin product) to planting only Bollgard II (the pyramided toxin product), which reduces Cry1Ac selection pressure. A 0.025 fitness cost of carrying the Cry1Ac resistance allele, conventional insecticide (pyrethroids) use, and the presence of non-Bt corn and soybean fields, which provide unstructured refuge, subsequently allows susceptibility in CBW to Cry1Ac to re-evolve. Furthermore, profit received by the representative eastern North Carolina producer is highest when the structured refuge requirement is removed for both Bollgard and Bollgard II, resistance to Cry1Ac evolves very slowly and the Cry1Ac resistance allele frequency declines when the producer switches to Bollgard II, and Cry2Ab2 resistance never evolves.

The Panel generally agreed that the weight of evidence supports a quick transition to pyramided products when there is no mechanism for resistance fitness cost. One Panel member pointed out that it is for this reason that the Australian cotton industry developed an agreed policy of rapid transition from single to pyramided Bt cottons. To further protect the future benefits of pyramided products, this policy imposed a cap on the area of single toxin cotton until pyramided varieties were ready for release. However, the Panel also noted that resistance fitness costs and economic factors that affect grower behavior could promote the transition from single to pyramided toxin products. One Panel member emphasized that it is important to incorporate grower behavior and what seed producers are doing with Monsanto's Cry toxins. For example, in eastern North Carolina, cottonseed varieties with the Cry1Ac gene that growers actually plant are available only when stacked with Monsanto's Roundup Ready gene; and cottonseed varieties expressing Cry1Ac and Cry2Ab2 that growers actually plant are available only when stacked with Monsanto's Roundup Ready Flex gene, the latter allowing growers to use Roundup longer during the growing season and more effectively. Livingston et al. (2006) incorporated these important aspects of the eastern North Carolina situation in their analysis, and the results strongly suggest that the behavior of cotton growers and seed companies likely will expedite transition from Bollgard to Bollgard II.

While Monsanto's analyses were consistent with the views of the panelists, Monsanto's analyses only contributed to the recommendations of the Panel and did not sway the Panel. This is important to note, because it illustrates the importance of having multiple, independent lines of evidence in making scientific recommendations.

## Overall Data/Results Interpretation

### Agency Charge

**9. There are three major variables to evaluating structured refuge for Bt crops: a) production of a sufficient number of susceptible insects relative to any resistant survivors of the Bt crop, b) proximity of the refuge to the transgenic crop to facilitate random mating between susceptible (from the refuge) and resistant (from the Bt crop) insects, and c) developmental synchrony of the refuge with the transgenic crop to promote random mating.**

**Given Monsanto's sampling, gossypol analysis, spatial and temporal analyses, and modeling evaluation, the Agency asks the panel to comment on whether Monsanto's analysis scientifically supports the conclusion that natural refuge will be comparable to the effectiveness of structured refuge for management of TBW resistance to the Bt proteins expressed in Bollgard II cotton for each of the four regions: the Carolinas, Georgia, Mississippi Delta, and Texas.**

### Panel Response

#### General Comments

Firstly, the Panel would like to compliment Monsanto on the extent and quality of information provided to support their case that natural refuge will be sufficient for resistance management for Bollgard II cotton. A number of aspects of the research significantly improve understanding of the ecology of Heliothine pests associated with Bt cotton systems.

Nonetheless, there are many uncertainties, caveats, and assumptions evident throughout the modeling and analyses presented by Monsanto and revealed by the Panel discussion around the questions posed by EPA. Given the magnitude of the potential consequences of the decision EPA must make, the Panel must apply the most rigorous scientific questioning to all aspects of the proposal, the data, and the conclusions.

#### The Gossypol Technique

One particular area of concern relates to the innovative gossypol analysis developed by Monsanto. The validity, accuracy and repeatability of this technique for identifying the non-cotton fraction of the TBW population is central to Monsanto's data presentation and argument for a natural refuge. Although the Panel received a description of the analytical technique, the description was incomplete. We thus highlight the following concerns:

- The proportion of non-cotton moths is determined by the inability of the analytical assay to detect gossypol in the bodies of field-collected male moths in pheromone traps. Since the assay does not provide a quantitative estimate of the amount of gossypol actually present, it is potentially liable to significant biases that have not yet been experimentally evaluated.

- For example, if the gossypol technique has a high detection threshold, it is quite possible that a number of samples would be classified as being from non-cotton sources when in fact they simply have gossypol values that are below the detection level of the method. We thus need more information about the sensitivity of the method.

- If gossypol concentrations decline in male moths from cotton that have remained dead for a week in a pheromone trap in the field, there could be a significant underestimation of the fraction of moths originating from cotton. This would project into a significant overestimation of the size of the natural refuge in the models, with a concomitant underestimate of the time taken to develop resistance in the pests. This bias factor could equal or exceed the other sources of variation of concern to the Panel, such as the variability between regions or on different dates.

Although Monsanto gave some verbal responses to these concerns during the public meeting, the Panel recommends a rigorous examination of the gossypol analysis technique. This should include at a minimum:

- A review by EPA staff with appropriate chemistry expertise;
- Publication of the assay method in a peer-reviewed journal;
- Performance and publication of experiments to mimic the conditions experienced by trapped males prior to analysis;
- Validation of the methodology by independent laboratories.

If the innovative gossypol analysis does stand rigorous examination, the Panel notes that it will be of considerable value for ongoing research by others working in this field.

In the remainder of our assessment we have assumed that the gossypol analysis is accurate and sensitive, and that the estimates of non-cotton moths are reliable.

**Estimates of Natural Refuge and Modeling of Resistance Risk**

Monsanto has clearly incorporated a number of elements of conservatism throughout their analysis, explicitly utilizing a "worst case" approach in many parameters in their modeling. The Panel questions whether collectively the data and modeling allow a sweeping conclusion that natural refuge is sufficient to delay resistance to Bollgard II across the four Cotton Belt regions outlined in the submission (Fig. 9-1) – North Carolina (NC), Georgia (GA), Mississippi Delta (MS), East Texas (TX) – at a spatial scale within these regions at which resistance is most likely to develop.

Monsanto's simulations indicate that with current estimates of natural refuge, development of Bt resistance in TBW to Bollgard II was unlikely within 30 years in any of the four regions. Monsanto does not argue that natural refuge will be strictly comparable to the effectiveness of structured refuge, except to the extent that it will delay resistance for at least 30 years. Because all simulations were truncated at 30 years, it is not possible for the Panel to judge the relative effectiveness of resistance management with or without structured refuge. That said, a predicted durability of at least 30 years would seem more than adequate for

biotechnology-based products. Monsanto's decision to use worst-case estimates for input parameters is conservative and may partially mitigate problems associated with parameter and model uncertainty. However, the sensitivity of model output to parameter values and model assumptions makes this difficult to assess.

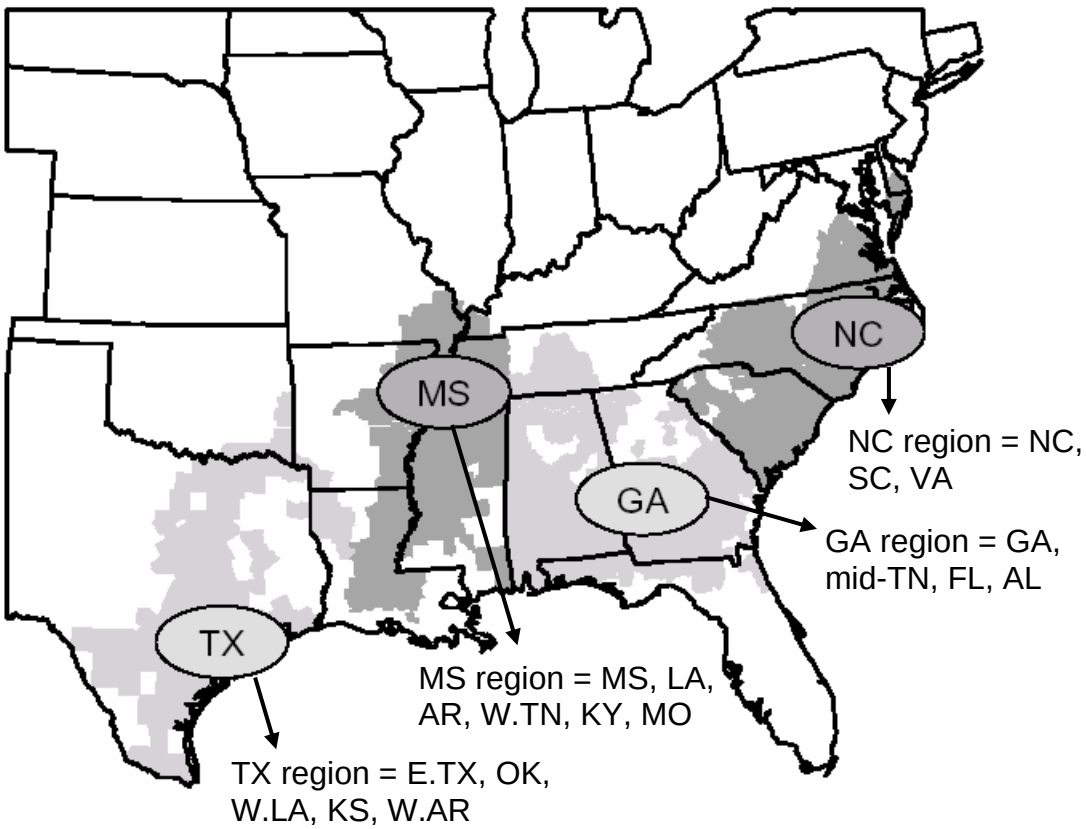


**Fig. 9-1.** Modified from Fig. 2 in Gustafson and Head (2005), incorporating modifications by Reynolds (2006). Caption in Gustafson and Head (2005) reads, "Definition of CBW modeling regions, which include all counties with some *Bt.* cotton sales (2003-5) and adjacent counties with at least one of the following crops according to USDA estimates for 2004: corn, cotton, peanuts, or soybeans."

The Panel noted some potentially serious biases in the calculation of natural refuge for TBW and CBW (see Panel response to Charge 6), which should be addressed by Monsanto. Despite the potential biases in estimates of natural refuge, some Panel members concluded that for Georgia and North Carolina, Monsanto has demonstrated that there are significant and reliable non-cotton refuges present as part of the cropping system and the wider environment, and this should be adequate to manage Bt resistance in TBW associated with cotton systems involving Bollgard II cotton.

Following this FIFRA SAP meeting, a Panel member provided additional analysis and comments regarding the validity of extrapolating data from areas sampled by Monsanto in North Carolina and Georgia to areas that were not sampled. Such comments were not considered or

reviewed by the Panel during the meeting, and are being provided as an appendix to these meeting minutes (Appendix 5).

Other regions are more problematic. While the simulations suggest long-term durability against TBW in all regions, the Panel does not believe that one year of data for East Texas and quite limited and variable sampling through parts of the MidSouth are sufficient to establish the reliability of adequate natural refuge for TBW. Texas especially must be sampled more thoroughly, both spatially and temporally. Areas representing different ecozones in that state must be sampled. One year of data does not provide coverage for the somewhat unpredictable rainfall in that region which may cause high variability in alternative host availability and phenology. For some parts of Texas, we have zero years of data, such as the Lower Rio Grande Valley and the High Plains. It was unclear to the Panel whether the Monsanto amendment covered all of Texas, given that west Texas and the High plains are currently part of a pink bollworm eradication program utilizing Bollgard cotton. Nonetheless, we highlight that the High Plains are very dry with most cotton being irrigated. The literature indicates (Benedict 2005) that there are almost no significant alternative hosts available to TBW in that area. Given the wide variety of agroecozones in Texas, it is not possible to extrapolate data on natural refuge from East Texas to the Texas High Plains, the Lower Rio Grande Valley, the Winter Garden Region, the Rolling Plains, etc.

The variability and uncertainties highlighted above in calculations of natural refuge and its utility in minimizing the risk of Bt resistance evolution indicate that Monsanto's data analysis could be much more comprehensive. As noted in its response to Charge 5, the Panel would prefer a more integrated and comprehensive analysis of the spatial and temporal variability of refuge estimates and moth trap data to tease out crucial details regarding the appropriate spatial regions and the critical temporal periods that pose the most risk.

**Worst-case scenarios**

Throughout its submission, Monsanto indicates that it has simulated the worst-case scenario with regard to the adequacy of natural refuge. One Panel member noted that resistance is likely to evolve in one area and then spread and that by picking counties with the lowest effective refuge, Monsanto is in fact not selecting the "worst-case" scenario, but instead the "most-likely" scenario for resistance development. Little re-assurance can be gained from a risk assessment perspective by picking the areas with lowest effective refuge. The true worst-case scenario involves first selecting the areas with lowest effective refuge and then using worst-case scenarios for other parameters applied for these areas.

**Assurance of Natural Refuge**

Current requirements for the registration of Bollgard and Bollgard II include monitoring for Bt resistance evolution (currently done by USDA Stoneville for Bt cotton). Resistance will be easier to prevent than remediate after it has arisen. By removing structured refuge, Monsanto will remove farmer control of effective refuges and transfer that "responsibility" to other farmers and the natural environment. How can Monsanto guarantee that natural refuge will always be there and be adequate? Who would be responsible for that assurance? The Panel suggests that if

the structured refuge requirement were removed, a comprehensive monitoring effort would have to extend beyond resistance monitoring per se to include monitoring levels of effective refuge through estimates of area, moth productivity, or ongoing gossypol analyses of moths trapped in cotton-growing areas.

This leaves open the residual issue of how areas of non-cotton host crops for TBW (tobacco/peanuts/soybean) or CBW (largely maize/soybean) might change in the future if refuge requirements are dropped for Bollgard II cotton. Will more of the maize/peanut/soybean acreage be switched to cotton with the result that natural refuge will decrease? The modeling analysis of Gustafson et al. (2005) includes simulations of significantly reduced soybean area, and the authors conclude that it would have little impact on effective refuge for CBW. However, they do not include the assumption that the area taken out of soybeans is replaced with Bollgard II cotton. Furthermore, no consideration is given to the possibility that the percent acreage of corn planted to Bt cultivars will not increase as stacked transgenic corn cultivars become more prevalent.

**Specific Comments on the three key components required for an effective refuge**

*a) Production of a sufficient number of susceptible insects relative to any resistant survivors of the Bt crop.*

A number of different modeling approaches suggest that pyramided plants like Bollgard II, including two highly efficacious proteins, require smaller effective refuges than a single gene product and those quite small refuges can delay resistance for considerable periods. Based on the use of the gossypol technique and carbon isotope ratios, and the various calculations of areas of potential refuge, Monsanto has clearly demonstrated that a significant proportion of moths across all regions are generated from non-cotton sources. This point is more fully discussed in the Panel's response to Charge 2. Setting aside for the moment the inherent uncertainties in all simple models and the lingering uncertainties about temporal and spatial variability of natural refuge, Monsanto's modeling of the durability of Bollgard II against TBW utilized worst case estimates of natural refuge from the data set and indicated resistance could be delayed for at least 30 years by reliance on natural refuge. Nonetheless the refuge data also demonstrate that the currently structured refuge crops of non-Bt cotton are contributing a significant proportion of both TBW and CBW moths to populations across the Cotton Belt and must thus be contributing to the delay of resistance over and above that provided by natural refuge. What is a sufficient amount of refuge? EPA's having already accepted a 5% structured refuge as adequate for resistance management for Bollgard cotton could suggest in simplistic terms that any credible estimates for natural refuge above this level would represent a comparable or at least acceptable protection against resistance.

Because resistance will likely evolve in areas with little effective refuge, these areas are of particular concern. When the estimated proportion of effective refuge for CBW and TBW is low (< 10%), higher levels of uncertainty attach to a number of assumptions and calculations for the proportion of effective refuge:

1) *The estimates of effective refuge become less certain as the estimates become smaller*, because in areas with high Bt cotton usage (small refuges), few insects are collected (see Panel response to Charge 3). Thus, when estimates of effective refuge are low, they are also less precise. This might be addressed to some extent by additional, more-intensive sampling as suggested under Charges 2 and 3.

2) *When the proportion of effective refuge is low, models of resistance evolution become more sensitive to these values*. For example, the difference in time to resistance between the cases of 25% and 20% refuge is often small compared to the difference between the cases of 10% and 5% refuge.

3) *Model uncertainty* (different predictions produced by structurally different models) *increases when the amount of refuge is small.*

4) *Resistance can evolve in "hotspots" where the proportion of refuge area is small.* This is shown by numerous models of resistance evolution that include spatially explicit landscapes (unlike the Monsanto model presented).

5) *Small amounts of effective refuge likely will coincide with greater variability in the amount of refuge through the growing season.* Therefore, depending on weather conditions, there could be generations that effectively experience no refuge.

6) *If there is little effective refuge, the amount of refuge could be highly variable* due to future changes in cropping patterns, non-agricultural land use, application of insecticides or other insect control measures in effective refuge, and environmental change as discussed above.

While any one of these areas of uncertainty might not in fact prove dangerous to IRM, as a group they represent unacceptably high levels of uncertainty. All of these areas of uncertainty increase and compound each other when the area of effective refuge is below 5-10%.

Spatially explicit modeling (Peck et al. 1999, Storer et al. 2003, and Sisterson et al. 2004, 2005) demonstrates that, with certain assumptions about pest mobility, resistance development can commence as hotspots in the landscape. In general the evidence for wide-scale mobility of TBW and CBW might mitigate that effect; however, we do not know the geographic scale of such hotspots for TBW. It might be the county scale, but possibly is smaller than that based on what we know from the literature about dispersal and gene flow during the cotton-host season (e.g., Korman et al. 1993, Sparks et al. 1993, Leonard et al. 1995, Schneider 1999, Han and Caprio 2002, 2004). The more comprehensive data analysis suggested under Charges 4 and 5 will assist in identifying the appropriate spatial and temporal scale for pooling of data and for identifying gaping holes in the provision of natural refuge that might serve to generate resistance hotspots. These "gaping holes" might reflect inadequate spatial sampling or temporal patterns driven by climate whereby natural refuge appears adequate in a region one year, but inadequate the next due to differences in the timing or intensity of rainfall. This might well be an issue for the MidSouth and Texas region where natural refuge is lower and climatic extremes may lead to significant change in the adequacy of natural refuge. For these reasons we argue that further data

are necessary to characterize the level of variability to be expected, so that an informed judgment can be made regarding the suitability of natural refuge. Although weather is also variable in North Carolina and Georgia, the contribution of alternative hosts is so great that this region may be well-buffered in the provision of natural refuge.

*b) Proximity of the refuge to the transgenic crop to facilitate random mating between susceptible (from the refuge) and resistant (from the Bt crop) insects.*

With structured refuge it is possible to specify and regulate the proximity of refuges to Bt crops. It is not possible to manage or to assure proximity when relying on natural refuge, which might derive from patches of alternative crops and wild hosts which are unlikely to be distributed uniformly. Spatial structure of the crop and refuge environment will interact with mobility of the pest. Both CBW and TBW are potentially highly mobile insects (Benedict 2005, Schneider et al. 1989) relative to the spatial patterning of hosts and non-hosts in the cropping regions where they occur. This does not necessarily mean that all moths move extensively every generation and in fact there is good evidence that they do not (Hayes 1991, Korman et al. 1993, Sparks et al. 1993, Leonard et al. 1995, Schneider 1999, 2003, Bagwell et al. 2000, Han and Caprio 2002, 2004). In some parts of the Cotton Belt there may be the potential for hotspots of resistance to develop where TBW populations are regularly associated with Bt cotton and lack localized refuge. Monsanto's trapping of male moths in pheromone traps adjacent to cotton crops indicates at least that those moths which had been generated on non-cotton hosts have moved into the vicinity of a cotton crop.

Monsanto has done a sound job in seeking to quantify land-use patterns in the counties where data were collected, and in most areas sampled the combined analyses clearly indicate that non-cotton hosts do contribute a proportion of moths to local populations. However, the counties sampled are not always representative of all counties within a given state. Also, with only 1 or 2 years of data it is difficult to assess the reliability of natural refuge in proximity to Bt cotton in some parts of the western end of the Cotton Belt.

*c) Developmental synchrony of the refuge with the transgenic crop to promote random mating*

There have always been questions about the synchrony of heliothine populations on Bt crops and refuges. For CBW and *H. armigera* in Australia and elsewhere, it appears that survivors on Bollgard cotton develop more slowly than those on conventional refuge crops. Development rates, and therefore adult emergence times, of both CBW and TBW can differ substantially depending on the host plant species (Lukefahr and Martin 1964, Nadguada and Pitre 1983, Hayes 1988). This immediately raises the specter of lack of synchrony of emergent refuge-generated moths with those from Bt crops. TBW displays quite distinct generational patterns across seasons in the Cotton Belt. Even so, generational peaks span 2-3 weeks and get progressively broader through the season due to partially overlapping generations. The temporal scale used in Monsanto's analysis (monthly) is probably appropriate for these species. The more comprehensive generalized linear modeling of the refuge estimates suggested by this Panel would provide a more acceptable assessment of the spatial and temporal variability in refuge adequacy.

**Conclusions**

The Panel understands that both Monsanto and cotton industry representatives would prefer a single management strategy to accompany Bollgard II cotton across the Cotton Belt, as is the case with Bollgard and Widestrike cotton. However, based on the data provided, the Panel does not believe a single conclusion regarding adequacy of natural refuge can be applied across the whole Cotton Belt "from Texas to North Carolina."

With the caveat that the accuracy of the gossypol technique must be validated, some Panel members support removal of the structured refuge requirement for Bollgard II cotton in North Carolina and Georgia.[4] The refuge requirement should not be removed in Alabama and parts of Texas because these areas were not sampled. The refuge requirement should not be removed in Tennessee and East Texas because of insufficient data. The refuge requirement should not be removed in Mississippi, Arkansas, and Louisiana because the data presented suggest that the natural refuge may be inadequate. A reassessment of these recommendations, based on additional data from the excluded areas/states, could be warranted.

[4] Following this FIFRA SAP meeting, a Panel member provided additional analysis and comments regarding the validity of extrapolating data from areas sampled by Monsanto in North Carolina and Georgia to areas that were not sampled. Such comments were not considered or reviewed by the Panel during the meeting, and are being provided as an appendix to these meeting minutes (Appendix 5).

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# APPENDIX 1

*Correct Derivation of Equation 7*

To understand the mistake that Monsanto made in their derivation of Equation 7, it is useful to reconstruct Monsanto's equations from a slightly different perspective. Consider a landscape of area $\alpha$. This area is divided into five distinct compartments: Bt cotton ($\alpha_{B1}$), Bt corn ($\alpha_{B2}$), non-Bt cotton ($\alpha_{R1}$), non-Bt corn ($\alpha_{R2}$), and non-cotton C3 ($\alpha_{R3}$) crops such that $\alpha = \alpha_{B1} + \alpha_{B2} + \alpha_{R1} + \alpha_{R2} + \alpha_{R3}$. Let moth production per unit area for each of these crops prior to Bt selection be $\eta_{ij}$ for $i = B, R$ and $j = 1, 2, 3$. Total moth production for this landscape can then be written as $m = \alpha_{B1}\eta_{B1} + \alpha_{B2}\eta_{B2} + \alpha_{R1}\eta_{R1} + \alpha_{R2}\eta_{R2} + \alpha_{R3}\eta_{R3}$. Average moth production per unit area is $M = m/\alpha$.

Effective refuge ($R_{eff}$) is defined as the proportion of moths produced by non-Bt and non-cotton C3 crops (i.e., $R1$, $R2$, and $R3$ crops),

$$R_{eff} = \frac{\alpha_{R1}\eta_{R1} + \alpha_{R2}\eta_{R2} + \alpha_{R3}\eta_{R3}}{\alpha_{B1}\eta_{B1} + \alpha_{B2}\eta_{B2} + \alpha_{R1}\eta_{R1} + \alpha_{R2}\eta_{R2} + \alpha_{R3}\eta_{R3}}$$

$$= \frac{\frac{\alpha_{R1}\eta_{R1}}{\alpha} + \frac{\alpha_{R2}\eta_{R2}}{\alpha} + \frac{\alpha_{R3}\eta_{R3}}{\alpha}}{\frac{\alpha_{B1}\eta_{B1}}{\alpha} + \frac{\alpha_{B2}\eta_{B2}}{\alpha} + \frac{\alpha_{R1}\eta_{R1}}{\alpha} + \frac{\alpha_{R2}\eta_{R2}}{\alpha} + \frac{\alpha_{R3}\eta_{R3}}{\alpha}}$$

where $\frac{\alpha_{ij}\eta_{ij}}{\alpha}$ is moth production for the $i$th and $j$th crop per unit area, or $M_{ij}$ as defined by Monsanto. Substitution implies $R_{eff} = \frac{M_{R1} + M_{R2} + M_{R3}}{M_{B1} + M_{B2} + M_{R1} + M_{R2} + M_{R3}}$, which is exactly the same as Monsanto's Equation (5).

Natural refuge ($R_{nat}$) consists of the proportion of moths produced on non-cotton, non-Bt hosts (i.e., $R2$ and $R3$ crops) such that

$$R_{nat} = \frac{\alpha_{R2}\eta_{R2} + \alpha_{R3}\eta_{R3}}{\alpha_{B1}\eta_{B1} + \alpha_{B2}\eta_{B2} + \alpha_{R1}\eta_{R1} + \alpha_{R2}\eta_{R2} + \alpha_{R3}\eta_{R3}}$$

$$= \frac{\frac{\alpha_{R2}\eta_{R2}}{\alpha} + \frac{\alpha_{R3}\eta_{R3}}{\alpha}}{\frac{\alpha_{B1}\eta_{B1}}{\alpha} + \frac{\alpha_{B2}\eta_{B2}}{\alpha} + \frac{\alpha_{R1}\eta_{R1}}{\alpha} + \frac{\alpha_{R2}\eta_{R2}}{\alpha} + \frac{\alpha_{R3}\eta_{R3}}{\alpha}}$$

$$= \frac{M_{R2} + M_{R3}}{M_{B1} + M_{B2} + M_{R1} + M_{R2} + M_{R3}}.$$

This expression for natural refuge does not match Monsanto's Equation (7). The relationship between $R_{nat}^{CBW}$ and $R_{nat}$ is $R_{nat}^{CBW} = \left(1 + \frac{M_{R1}}{M_{B1} + M_{B2} + M_{R2} + M_{R3}}\right) R_{nat}$, such that $R_{nat}^{CBW} > R_{nat}$.

Figure A1-1 illustrates the compartments used by Monsanto to calculate the effective refuge. When Monsanto calculates natural refuge using Equation (7), it effectively eliminates the non-Bt cotton compartment (Figure A1-2). By doing this Monsanto effectively assumes a reduction in the area of interest: $\alpha' = \alpha_{B1} + \alpha_{B2} + \alpha_{R2} + \alpha_{R3}$, instead of $\alpha$. This assumption can create a bias because $M_{ij}$ is calculated per unit area based on $A_{ij} = \alpha_{ij}/\alpha$ in Figure A1-1, not $\alpha_{ij}/\alpha'$ in Figure A1-2. How much bias can this assumption produce? Based on the data reported in

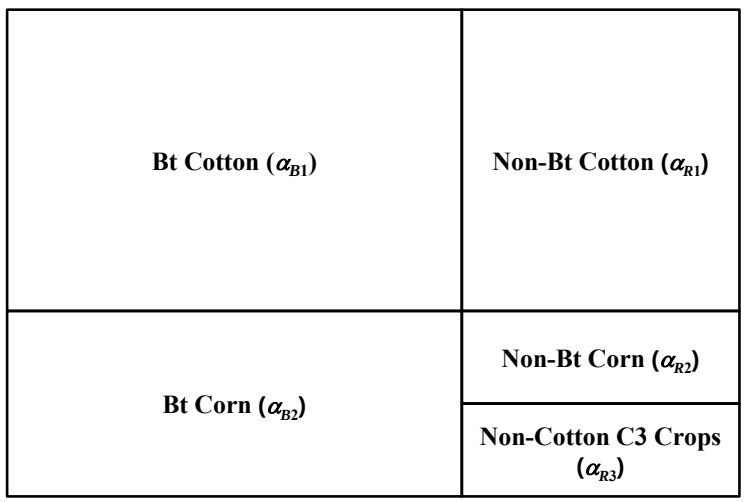


**Figure A1-1.** Crop acreage compartments for Monsanto's effective refuge calculation.

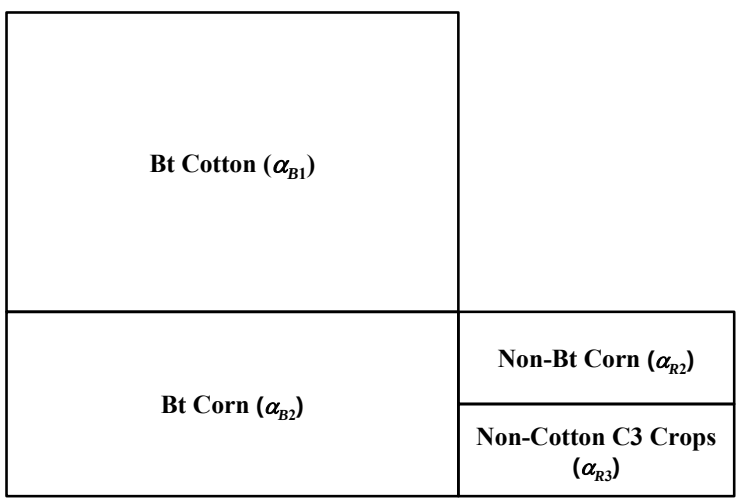


**Figure A1-2.** Crop acreage compartments for Monsanto's natural refuge calculation.

Tables 2 and 5 (Gustafson and Head 2005) assuming $LB_{B1} = LB_{B2} = 1$ prior to Bt selection, Monsanto's estimate of the effective refuge ($R_{nat}^{CBW}$) will be 37, 12, 6, and 44% higher than the natural refuge estimate given by $R_{nat}$ for Georgia, Mississippi, North Carolina, and Texas, respectively.

For the *current effective refuge*, $R_{eff}$, simulation, Monsanto only needs their correct estimate of the effective refuge, so their result should not be biased. This is not true for *natural refuge* simulations. For *natural refuge*, $R_{nat}$, simulations, Monsanto keeps the area of cotton fixed, while deleting structured refuge for Bollgard II for the simulations reported in Table 14 and all structured refuge for simulations reported in Figure 4. This effectively reduces non-Bt cotton acreage, while replacing it with Bt cotton acreage. If $\phi$ is the proportional reduction in non-Bt cotton acreage, effective and natural refuge become

$$R_{eff} = \frac{\alpha_{R1}(1-\phi)\eta_{R1} + \alpha_{R2}\eta_{R2} + \alpha_{R3}\eta_{R3}}{(\alpha_{B1} + \alpha_{R1}\phi)\eta_{B1} + \alpha_{B2}\eta_{B2} + \alpha_{R1}(1-\phi)\eta_{R1} + \alpha_{R2}\eta_{R2} + \alpha_{R3}\eta_{R3}}$$
$$= \frac{(1-\phi)M_{R1} + M_{R2}\eta_{R2} + M_{R3}\eta_{R3}}{M_{B1} + \phi\alpha_{R1}\eta_{B1} + M_{B2} + (1-\phi)M_{R1} + M_{R2} + M_{R3}}$$

and

$$R_{nat} = \frac{\alpha_{R2}\eta_{R2} + \alpha_{R3}\eta_{R3}}{(\alpha_{B1} + \alpha_{R1}\phi)\eta_{B1} + \alpha_{B2}\eta_{B2} + \alpha_{R1}(1-\phi)\eta_{R1} + \alpha_{R2}\eta_{R2} + \alpha_{R3}\eta_{R3}}$$
$$= \frac{M_{R2} + M_{R3}}{M_{B1} + \phi\alpha_{R1}\eta_{B1} + M_{B2} + (1-\phi)M_{R1} + M_{R2} + M_{R3}},$$

which imply $R_{nat}^{CBW} = \left(1 + \frac{\phi\alpha_{R1}(\eta_{B1} - \eta_{R1})}{M_{B1} + M_{B2} + M_{R2} + M_{R3}}\right) R_{nat}$ such that $R_{nat}^{CBW} > R_{nat}$ for $\phi \geq 0$. This result suggests the upward bias in Monsanto's natural refuge estimate will be either systematically increasing or decreasing in the market share of Bollgard II depending on the relative moth productivity of Bt and non-Bt cotton prior to Bt selection. The data in Tables 2 and 5 suggest $E_{B1}\ LB_{B1}\ LS_{B1} > E_{R1}\ LB_{R1}\ LS_{R1}$ for all regions such that increasing Bollgard II market share, increases the upward bias in Monsanto's natural refuge calculation.

# APPENDIX 2

## *Model Uncertainty*

The Caprio model (Caprio 1998) used by Monsanto does not explicitly incorporate population densities. Modifying the model to include population densities and density-dependent population growth rates creates a model that violates some reasonable assumptions about the biology and ecology of insects like TBW. This illustrates a difficulty in assessing models that, even though simple in structure, nonetheless have properties that are not transparent. This illustrates the problem of model uncertainty.

### *A general "2-patch" model of resistance evolution*

To explore this issue, we produced a model (code attached below) for single-locus resistance based on the following assumptions:

1. A fraction Q of the suitable habitat (in which females lay eggs) is refuge, and a fraction 1–Q is Bt.

2. Following emergence, males and females disperse randomly, so that the proportion of males and females in refuge and Bt fields are Q and 1 – Q.

3. Mating is random, and the sex ratio is 1:1.

4. Bt is high dose. Therefore, survival from Bt of homozygous resistant larvae is $s_{RR} = 1$, while $1 >> s_{RS} \geq s_{SS}$. Genotype does not affect survival of larvae in refuge.

5. The net reproductive rate of larvae in refuge (i.e., the survival of larvae times the number of female offspring produced per female larva that survives to adulthood) is given by a function $f_R(x_R)$, where $x_R$ is the density of larvae.

6. The net reproductive rate of larvae in Bt fields that survive the Bt toxin is given by a function $f_{Bt}(x_{Bt})$, where $x_{Bt}$ is the density of larvae that survive Bt toxin. $f_{Bt}(x_{Bt})$ is a continuous function (but not necessarily monotonically decreasing). For notational convenience, let $f_{Bt}(x_{Bt}) = F_{Bt}$ in the limit as $x_{Bt}$ approaches zero. Thus, $F_{Bt}$ is the net reproductive rate of larvae that survive Bt toxins when the density of surviving larvae in Bt fields is very low. $F_{Bt}$ is assumed to be a constant.

7. In the absence of a resistance allele (i.e., for a purely susceptible population), the mean density of insects approaches some fixed value X. Because the model does not include temporal environmental variability, this condition will be satisfied whenever a purely susceptible population is persistent (i.e., does not go extinct and does not increase in density to infinity).

Under these assumptions, the following mathematical result holds (see Ives and Andow 2002 for methods). Let p(t) denote the frequency of the resistance allele in generation t. Then

the asymptotic rate of increase of the frequency of the resistance allele in the limit as p(t) approaches zero is given by

$$p(t+1) = \left(F_{Bt}(1-Q)\left(s_{RR}\,p(t) + s_{RS}(1-p(t))\right)+1\right)p(t)$$

Here, "asymptotic rate of increase" means the rate of increase after transient fluctuations in the relative frequencies of the resistance allele in Bt and refuge fields have dampened out. Although this approximation strictly holds only in the limit as p(t) approaches zero, numerical studies show it generally to be accurate for $p(t) < 0.1$. For $p(t) > 0.1$, resistance normally occurs in only a few generations for most reasonable parameter values, so the breakdown in the approximation when $p(t) > 0.1$ does not greatly affect conclusions that can be drawn.

The interesting consequence of this result is that the asymptotic rate of resistance evolution does not depend on the function $f_R(x_R)$, the survival of larvae in the refuge and the fecundity of the surviving females. It also does not depend on the functional form of $f_{Bt}(x_{Bt})$, only on $f_{Bt}(x_{Bt} \to 0) = F_{Bt}$. Therefore, the theorem holds for a fairly broad class of models.

A key conclusion for models that conform to assumptions 1-7 is the importance of $F_{Bt}$, the net reproductive rate of larvae that survive Bt toxins when the density of surviving larvae in Bt fields is very low. The higher $F_{Bt}$, the more rapid resistance evolution. Unfortunately, it is very difficult to estimate $F_{Bt}$ in the field.

*Comparison with the Caprio model (1998).*

The Caprio model (1998) forms the basis of the model Monsanto uses to assess the durability of Bollgard II. The Caprio model considers a case such as Bollgard, in which there is a single resistance locus and only two types of habitat, Bt crop and refuge. For simplicity, here we will address this model, rather than the multi-patch model presented by Monsanto, although the general comments should apply to both. Also, we focus on the high-dose case corresponding to TBW.

To compare to the Caprio model, we derived a model that explicitly accounts for densities and conforms to assumptions 1-7 above. For population growth in Bt fields, we make the simple assumption that if larvae survive Bt, they have density-independent population growth; in other words, $f_{Bt}(x_{Bt}) = F_{Bt}$ for all densities $x_{Bt}$. For population growth in the refuge fields, we make the simple assumption that the population densities of larvae surviving in the refuge have a fixed value $x_{refuge}$. Thus, density-dependent population growth in the refuge is strong enough to bring populations up to carrying capacity in the refuge every generation. This carrying capacity can be affected by insecticide spraying in the refuge, however, such that if the insecticide leaves only a fraction k of insects alive, the carrying capacity is reduced to $k\,x_{refuge}$. We selected these assumptions about $f_{Bt}$ and $f_R$ only because they are simple; as discussed above, other assumptions give essentially the same results. We will refer to this as the $F_{Bt}$-fixed model, since it assumes a constant value of $F_{Bt}$.

Figure A2-1A compares times to resistance computed from the Caprio model (red dashed line) to those predicted by the $F_{Bt}$-fixed model just described (black line). In the Caprio model,

the time to resistance is much more sensitive to the amount of refuge Q than in the $F_{Bt}$-fixed model.

To understand why these models differ, we constructed a modified version of the Caprio model that explicitly incorporates population densities and satisfies assumptions 1-5 and 7 above. It turns out to be impossible to incorporate density into the Caprio model in a way that satisfies all assumptions 1-7. In the modified Caprio model, the reproductive rate of larvae in Bt fields that survive Bt is 1/(kQ). In other words, to formulate the Caprio model to include densities, the value of $F_{Bt}$ must be inversely proportional to the survival of larvae from spraying in the refuge, k, and the proportion of refuge fields, Q. The fact that this modified Caprio model gives the same predicted rates of resistance evolution is shown in Figure A2-1A by the correspondence between the lines for the modified Caprio model (green line) and the original Caprio model (red line). The dependence of $F_{Bt}$ on kQ in the modified Caprio model is shown in Figure A2-1B.

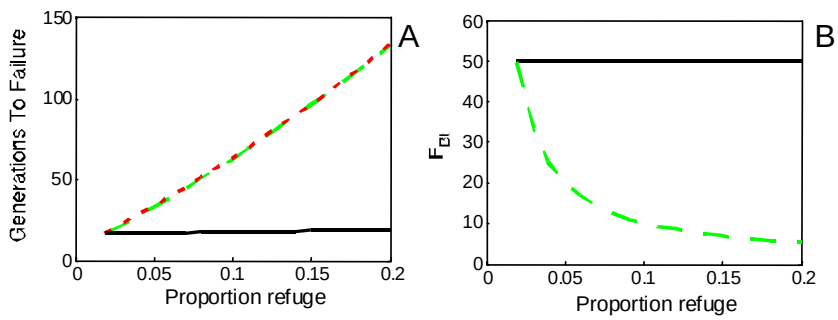


**Fig. A2-1.** (**A**) Generations to resistance (from resistance allele frequency of 0.002 to 0.5) and (**B**) corresponding values of $F_{Bt}$ for resistant insects in Bt crops versus the proportion refuge $Q$. The red dashed line is the Caprio model (1998). The dotted green line is the model with fixed size of the insect population in the refuge, $x_{refuge} = Q$, and $F_{Bt}$ of resistant insects in Bt crops the same as in the refuge ($F_{Bt} = 1/Q$). The solid black line has a constant $F_{Bt}$ of 50. Other parameter values are: $s_{RR} = 1$, $s_{RS} = 0.001$, $s_{SS} = 0.001$, $k$ (survival from insecticide) = 1, and $R$ (proportion of insects leaving natal habitat) = 1.

*The models make different qualitative predictions about factors affecting resistance evolution*

The $F_{Bt}$-fixed model makes different predictions from the Caprio model about the consequences of spraying insecticides in refuges. For the $F_{Bt}$-fixed model, the rate of resistance evolution is not sensitive to insecticide spraying in the refuge (Fig. A2-2). This seemingly unintuitive pattern is a direct mathematical consequences of the theoretical result about the asymptotic rate of resistance evolution described above, in which the rate of resistance evolution does not depend on the reproduction rate of insects in refuges. This contrast between models illustrates how implicit assumptions in models can change qualitative conclusions drawn from the models.

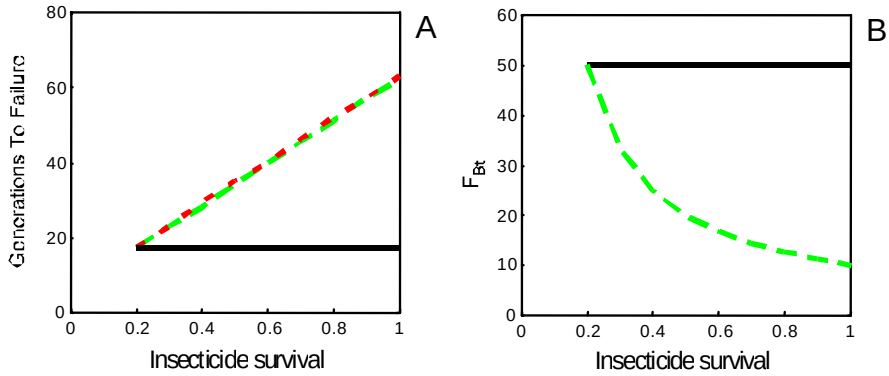


**Fig. A2-2.** (**A**) Generations to resistance (from resistance allele frequency of 0.002 to 0.5) and (**B**) corresponding values of $F_{Bt}$ for resistant insects in Bt crops versus the survival of insects from insecticide in the refuge, $k$. The red dashed line is the Caprio model (1998). The dotted green line is the model with fixed size of the insect population in the refuge, $x_{refuge} = kQ$, and $F_{Bt}$ of resistant insects in Bt crops the same as in the refuge ($F_{Bt} = 1/kQ$). The solid black line has a constant $F_{Bt}$ of 50. Other parameter values are: $Q = 0.1$, $s_{RR} = 1$, $s_{RS} = 0.001$, $s_{SS} = 0.001$, and $R$ (proportion of insects leaving natal habitat) = 1.

Although the lack of effect of insecticide spraying may seem unintuitive, it has a simple explanation (Ives and Andow 2002) using the following reasoning:

(i) The rate of resistance evolution depends on the proportion of the susceptible population that is killed by Bt.

(ii) In the high-dose case when the frequency of a resistance allele is low, essentially the entire population is in the refuge.

(iii) The proportion of the susceptible population killed by Bt equals the proportion of females that leaves the refuge and oviposits in Bt fields.

(iv) Reducing the population <u>size</u> in the refuge does not change the <u>proportion</u> of females leaving the refuge.

(v) Therefore, reducing the size of the population in the refuge through spraying will not change the rate of resistance evolution.

While this argument begins to break down mathematically when the frequency of the resistance allele reaches 0.1, by this time there will be only a very few more generations before the resistance allele reaches 0.5 and control fails.

# Matlab code for the modified Caprio (1998) model

```
% Caprio.m

% written by Anthony R. Ives, 9 June 2006

% produces a model similar to Caprio 1998, but with explicit insect
% densities and a two patch environment (removing the third "patch" of
% dispersing insects in Caprio 1998

clear
clf

% baseline values
R=1;        % proportion of insects leaving natal habitat before mating
m=1;        % proportion of insects leaving mating habitat after mating
Q=.05;      % proportion of refuge
k=1;        % survival from insecticde in refuge
sRR=1;      % survival of RR genotypes in Bt crop
sSS=.001;
sRS=.001;
L=1;        % survival of all genotypes in refuge (excluding insecticide)
F1=50;      % fecundity of females in Bt crops
x0=k;       % population size of insects in refuge

% iterate over three cases:
% 1 - Caprio assumption of population growth
% 2 - fixed fecundity in Bt crops
% 3 - fecundity in Bt crops = Q*k
for flag=1:3
    output=[];
    for z=.02:.01:.2
        Q=z;

        % this allows males and females to have different proportions
        % leaving Bt and refuge fields
        R1m=R;
        R1f=R;

        R2m=R;
        R2f=R;

        m1m=m;
        m1f=m;
        m2m=m;
        m2f=m;

        % survivals
        ORR=[sRR 0;0 k*L];
        ORS=[sRS 0;0 k*L];
        OSS=[sSS 0;0 k*L];

        % initial densities X and frequencies P
        X=k*[0;Q];
        P=.002*[1;1];

        t=1;
        while (P'*X)/sum(X) < 0.5

            t=t+1;

            % set population size in refuge equal to k*Q
            x0=k*Q;

            % pre-mating movement of alleles in males
            z1s=((1-R1m)+(1-Q)*R1m)*X(1);
            z1d=(1-Q)*R2m*X(2);
            z2s=((1-R2m)+Q*R2m)*X(2);
            z2d=Q*R1m*X(1);
            Mm=[z1s/(z1s+z1d) z1d/(z1s+z1d);z2d/(z2s+z2d) z2s/(z2s+z2d)];

            % pre-mating movement of alleles in females
            z1s=((1-R1f)+(1-Q)*R1f)*X(1);
            z1d=(1-Q)*R2f*X(2);
            z2s=((1-R2f)+Q*R2f)*X(2);
            z2d=Q*R1f*X(1);
            Mf=[z1s/(z1s+z1d) z1d/(z1s+z1d);z2d/(z2s+z2d) z2s/(z2s+z2d)];

            % movement of alleles and mating to give genotypic frequences
```

```
            WRR=(Mf*P).*(Mm*P);
            WRS=(1-Mf*P).*(Mm*P)+(Mf*P).*(1-Mm*P);
            WSS=(1-Mf*P).*(1-Mm*P);

            % redistribution of densities during premating dispersal
            d11=((1-R1f)+(1-Q)*R1f);
            d12=(1-Q)*R2f;
            d21=Q*R1f;
            d22=((1-R2f)+Q*R2f);

            D=[d11 d12;d21 d22];
            X=D*X;

            % genotypic densities
            XRR=WRR.*X;
            XRS=WRS.*X;
            XSS=WSS.*X;

            % post-mating movement of females
            d11=((1-m1f)+(1-Q)*m1f);
            d12=(1-Q)*m2f;
            d21=Q*m1f;
            d22=((1-m2f)+Q*m2f);

            % postmating movement and selction
            D=[d11 d12;d21 d22];
            XRR=ORR*D*XRR;
            XRS=ORS*D*XRS;
            XSS=OSS*D*XSS;

            % calculation of new frequencies and densities
            P=(XRR+XRS./2)./(XRR+XRS+XSS);
            X=XRR+XRS+XSS;

            % 1 - Caprio assumption of population growth
            if flag==1
                X=X./sum(X);
            end

            % 2 - fixed fecundity in Bt crops
            if flag==2
                X(1)=F1*X(1);
                X(2)=x0;
            end

            % 3 - fecundity in Bt crops = Q*k
            if flag==3
                F1=1/(Q*k);
                X(1)=F1*X(1);
                X(2)=x0;
            end
        end
        output=[output;z t F1];
    end;

    c='rkg';
    figure(1)
    subplot(2,1,1)
    plot(output(:,1),output(:,2),[c(flag),'-'])
    xlabel('Proportion refuge')
    ylabel('Gens To Failure')
    hold on

    if flag==2 | flag==3
        subplot(2,1,2)
        plot(output(:,1),output(:,3),[c(flag),'-'])
        xlabel('Proportion refuge')
        ylabel('R0')
        axis([0 .2 0 60])
        hold on
    end
end
hold off
```

# APPENDIX 3

*Evolution of Resistance Hotspots*

The hotspot phenomenon can be evaluated only in a model that includes explicit spatial descriptions of the landscape. A spatially explicit 2-loci model (described in more detail in Appendix 4) demonstrates that hotspots and rapid evolution are possible for Bollgard II. The model used by Monsanto is spatially implicit, since insects are assumed to disperse uniformly over the region modeled. Even though CBW and TBW are highly mobile, it is unlikely that TBW are well-mixed over the regions considered during the cotton growing season (see Panel response to Charge 6).

The spatially explicit model is designed to illustrate some key factors that affect the rate of resistance evolution. It is not designed to make detailed predictions or incorporate all of the complexity likely to affect resistance evolution. Therefore, we did not incorporate many elements of realism included in the spatially implicit Monsanto model. In particular, we consider only Bt and refuge fields, and assume that the proportion of Bt and refuge fields in the environment does not change through time. Monsanto assumes that both CBW and TBW only use cotton in half of their 6 annual generations. Rather than having generations with and without selection, here we simply assume that there are 3 selected generations per year. We assume refuge fields are distributed randomly over space; in reality, refuges will likely be clustered, leaving relatively larger areas of contiguous Bt fields where hotspots of resistance are likely to occur. Finally, we assume that the resistance alleles at both resistance loci have initial frequencies of 0.002, and that one of the toxins (Cry 2Ab2) is much more effective than the other (Cry 1Ac), as is the case for CBW. See Appendix 4 for more details.

Two different versions of the model were produced corresponding to the Caprio 1-locus model and the $F_{Bt}$-fixed model described in Appendix 2. The only difference between these models is the assumption about density-dependence. In the Caprio version of the spatial model, the reproductive rate of larvae in Bt fields that survive Bt is 1/(kQ). In the $F_{Bt}$-fixed model, $F_{Bt}$ = 50.

Figure A3-1 shows the spatial distribution of allele frequencies produced in a simulation of the Caprio version of the model when there is 5% refuge and both males and females have a dispersal radius of 6 fields. The simulation shows a “hotspot” of resistance evolution in which the allele frequency of both resistance alleles reach a peak. This type of hotspot occurs, loosely speaking, because there is a limited number of insects dispersing from the refuge into a region of contiguous Bt fields. Although in this model hotspots are generated by limited dispersal, hotspots can be generated in other ways. For example, hotspots will occur even when refuges are within dispersal range of Bt fields if the numbers of susceptible insects from the refuge entering the Bt fields is low. This possibility is apparent from the information provided by Monsanto showing high variation in the number of insects captured in different traps. Therefore, hotspots can occur even when the dispersal distance is much greater than 6 fields, as used in this model.

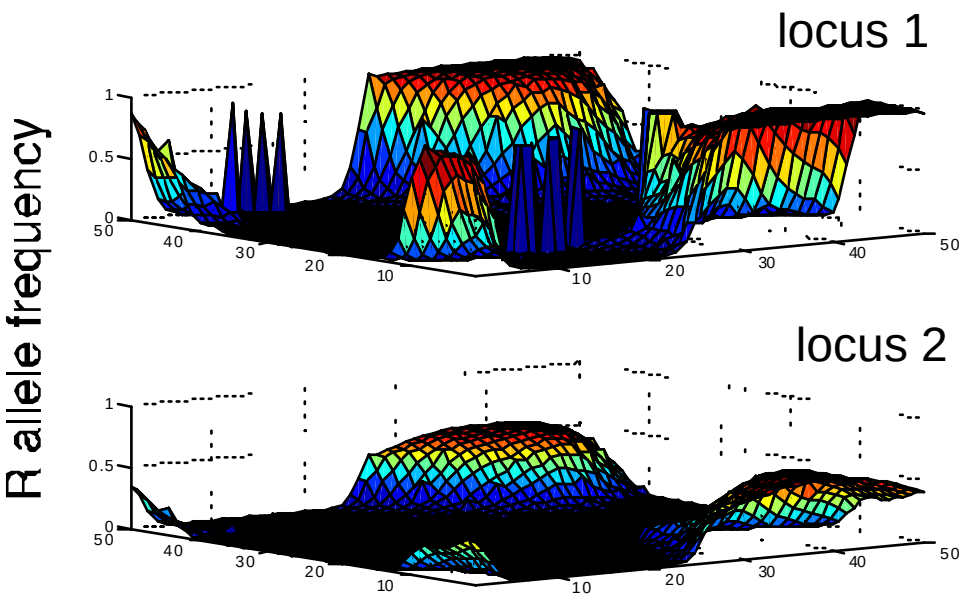


Figure A3-1: Years to crop failure due to resistance evolution in the spatially explicit 2-loci Caprio model (Appendix 4) in which the effective refuge area is 5%, and males and females have a dispersal radius of 6 fields. There is no cost of resistance. In this simulation, resistance failure occurred in 4 years. Other parameter values are: $s_{aa} = 0$, $s_{Aa} = 0.001$, $s_{aa} = 1$, $s_{BB} = 0.1$, $s_{Bb} = 0.2$, and $s_{bb} = 1$.

Table A3-1 gives the number of years required for resistance evolution for several models and assumptions about dispersal. The first model is the spatially explicit version of the model based on Caprio (1998). When there is limited dispersal of both males and females (labeled "limited dispersal") or global dispersal of males and limited dispersal of females (labeled "limited female dispersal"), hotspots occur when the effective refuge size, $R_{eff}$, is 5% but not 10%. This illustrates that the likelihood of hotspots can be very sensitive to the effective refuge size. Note that the hotspots occur even when there is global dispersal of males. While there is considerable information showing male dispersal is high, female dispersal is less well understood (see Charge 1). In the model, limited female dispersal is sufficient to cause hotspots even when males show global dispersal.

The $F_{Bt}$-fixed version of the model in which the maximum reproduction rate of resistant females in Bt fields is $F_{Bt} = 50$ similarly shows hotspots when there is 5% refuge. We considered a further case in which all male and female insects leave their natal fields if they emerge in refuge ($R_{refuge} = 1$), but 20% of males and female remain in the Bt fields if they successfully emerge there ($R_{Bt} = 0.8$). This corresponds to the case in which males and females remain in suitable habitat. This case similarly shows hotspots.

**Table A3-1**. Years to crop failure due to resistance evolution in the spatially explicit 2-loci model (Appendix 4). Values corresponding to evolution in "hotspots" are given in bold. "Limited dispersal" corresponds to a maximum dispersal distance of 6 fields, and "limited female dispersal" corresponds to global male dispersal and a maximum dispersal distance of 6 fields for females. Parameter values common to all simulations are: $s_{aa} = 0$, $s_{Aa} = 0.001$, $s_{aa} = 1$, $s_{BB} = 0.1$, $s_{Bb} = 0.2$, and $s_{bb} = 1$.

| Model | $R_{eff}$ | Infinite dispersal | Limited dispersal | Limited female dispersal |
|---|---|---|---|---|
| Caprio 2-loci | 10% | 155 | 161 | 135 |
| | 5% | 74 | **8.7**† [4, 48] | **18**† [8, 56] |
| $F_{Bt} = 50$ | 10% | 32 | 22.8† [8, 29] | 27.8† [10, 31] |
| $R_{Bt} = R_{refuge} = 1$ | 5% | 31 | **3.1**† [3, 4] | **12.2**† [6, 23] |
| $F_{Bt} = 50$ | 10% | 39 | 22.9† [5, 32] | 32.9† [10, 36] |
| $R_{Bt} = 0.8$ $R_{refuge} = 1$ | 5% | 37 | **3.5**† [3, 5] | **7**† [5, 8] |
| $F_{Bt} = 50$ | 10% | 1366 | > 334 | > 334 |
| $R_{Bt} = 0.8$ $R_{refuge} = 1$ cost of resistance | 5% | 1383 | **4**[*1] [3, > 334] | **14**[*2] [6, > 334] |

† Values are averages of 10 simulations, with the minimum and maximum years to failure given in brackets.

[*1] In this set of simulations, the median number of years to resistance was 4, and 1 of the 10 was > 334 years.

[*2] In this set of simulations, the median number of years to resistance was 14, and 2 of the 10 were > 334 years.

As a final case, we included a cost of resistance in which the survival of larvae homozygous and heterozygous for the resistant allele to the most effective toxin (Cry 2Ab2) have reduced survivals of 0.8 in Bt and refuge fields compared to the survivals of 1 for susceptible larvae in refuge fields. This represents a strong cost of resistance that increases Bt crop durability to over 1300 years when insects are completely mixed (as assumed by Monsanto). However, when there is limited dispersal of females only, the median durability of Bt crop is 14 years. This illustrates that hotspots can overwhelm a cost of resistance, because within the hotspot (unlike the refuge) there is little competition between resistant and susceptible insects. Therefore, the cost of resistance has little effect to slow resistance evolution.

Note that the times to resistance produced by this model are not inconsistent with the results from a detailed, spatially explicit model presented by Caprio (2006) for the Panel. This

model assumes that the effective, unstructured refuge is approximately 10% and there is a cost of resistance. Caprio reports that 25% of simulations without a structured refuge led to resistance in less than 25 years. In Table A3-1 the case with a cost of resistance and $R_{eff} = 0.10$ gives an average time to resistance of 33 years. In general, the Caprio model provided to the Panel (Caprio 2006) seemed to predict shorter times to resistance than the model presented by Monsanto (Gustafson and Head 2005), although the reason for this difference is unclear. The models differ in so many ways, lack of strong concordance might be expected.

We use this modeling exercise only to make three points: (1) hotspots can occur for a two-toxin product (Bollgard II) , as they do for a one-toxin product (Peck et al. 1999, Sisterson et al. 2004), and lead to rapid resistance evolution, (2) hotspots can occur even when males are broadly dispersive when females have limited dispersal, and (3) hotspots may overwhelm the effects of a cost of resistance. However, this model suffers from model uncertainty just like the model used by Monsanto. Rather than a completed model that can give quantitative predictions, instead we view it as only a starting point to develop a greater understanding of resistance evolution to a two-toxin product. Unfortunately, our current level of understanding resistance evolution for two-toxin products is too limited to make quantitative predictions.

The Panel realizes that the EPA would like a model that is rigorously validated and capable of making predictions about the rate of resistance evolution. Unfortunately, for the case of Bollgard II, such a model does not exist. Therefore, the Panel feels that it is best to acknowledge our ignorance rather than hide it. While it might be tempting to use the complexity of predicting the rate of resistance evolution as an excuse to rely on simple models and simple analyses, this runs the risk of ignoring processes that might lead to rapid resistance.

A particular limitation of the 2-loci model presented here is that it is an "infinite population model" in which population densities are modeled rather than individual insects. In effect, infinite population models include fractional individuals, and model the mean frequency of resistance and the mean density of insects. For example, if there are two resistance loci with resistance alleles at frequency 0.001, then the probability of a doubly homozygous resistant individual (assuming completely random mating) is $10^{-12}$. Thus, the population size would have to be roughly $10^{12}$ before there was a reasonable chance of finding a doubly resistant individual. Preliminary simulations show that infinite population models likely overestimate the time to resistance rather than underestimate it relative to models that explicitly following integer individuals and individual alleles. Therefore, hotspots would be more likely in more-realistic simulation models. Nonetheless, this is an issue for further research.

# APPENDIX 4

*A spatially explicit 2-loci model of resistance evolution*

This Appendix derives a spatially explicit version of Monsanto's base model. Monsanto does not provide equations necessary to reconstruct their model, so several specific assumptions about their model had to be made.

Two versions of the spatially explicit model were derived. The first is based on Caprio (1998). We also used a modified version was also derived in which both the population size in the refuge, $x_{refuge}$, and the per capita population growth rate of resistant females in Bt fields, $F_{Bt}$, were fixed. To simplify the presentation, we assume that there are only Bt and refuge fields, rather than distinguishing the multiple types of refuges. Furthermore, we assume that the proportion of refuge in the environment does not change through time. Because only half the generations of either CBW or TBW are likely to be subjected to selection for Bt resistance, we assume that 50% of the generations are unselected. This is approximated by assuming there are 3 generations per year, all of which are selected. We do not present a formal analysis of the model, and it has not been independently proofed. Nonetheless, code is provided at the end of the Appendix.

Below we first describe a spatially implicit model that should be comparable to Monsanto's model (although without incorporating multiple habitat types and variation in habitat types through time). This spatially implicit model could be modified and used to verify Monsanto's calculations. Here, it is used to verify the computer code for the spatially explicit model, since both models give the same output when the spatially explicit model includes "global" dispersal of insects over the entire region.

## 1. *Spatially implicit model*

The spatially implicit model assumes that if males and females disperse from their natal fields, they disperse evenly throughout a region and settle in Bt and refuge fields in proportion to the area of Bt and refuge fields. In Monsanto's model, all males and females are assumed to disperse from their natal field, although here we generalize by including the assumption of Caprio (1998) that a proportion $R$ of insects disperse while $1-R$ remain in their natal fields. After dispersal, mating is random and females oviposit in the fields in which they mate. Because the Monsanto model assumes all insects disperse, post-mating dispersal makes no difference to the model, so for simplicity we have not included it.

Selection occurs on the larvae within fields. Rather than use the formula provided by Monsanto to calculate the survivals of the 9 possible genotypes, here for simplicity we assume that the survival of 2-loci genotypes is equal to the product of survivals for each locus separately. Thus, if the survival of genotype *Aa* to one toxin (e.g., Cry 1Ac) is $s_{Aa}$ and the survival of genotype *Bb* to a second toxin (e.g., Cry 2Ab2) is $s_{Bb}$, then the survival of the genotype *AaBb* to both toxins is $s_{AaBb} = s_{Aa}s_{Bb}$. In the model, genotypes are state variables rather than gene frequencies, because strong selection will generate linkage disequilibrium even when alleles of different loci segregate independently.

*2. Spatially explicit model*

The spatially explicit model includes the same processes modeled within Bt and refuge fields as in the spatially implicit model. These processes, however, are mapped onto space. Space consists of a 50 x 50 grid of cells with "wrap-around" boundaries (i.e., on a torus). Cells are assigned randomly as either Bt or refuge, with the probability that a cell is refuge being *Q*. When insects disperse from cells, they are distributed among neighboring cells. In particular, up to a maximum distance of *n* (selected as a parameter in the model), the dispersing population is distributed by a geometric distribution in all directions from their natal field.

Note that the way in which refuges are dispersed in the spatially explicit model is conservative, in that refuges are spread uniformly throughout space. If refuges were clustered, there would be larger areas of Bt fields without nearby refuges, and hotspots would be more likely.

To include a cost of resistance, we assume that survivals of resistant genotypes are reduced in both Bt and refuge fields. To compute the cost for different genotypes, we assume survivals are multiplicative in the same manner as survivals from Bt.

Matlab code for the spatially implicit model

```
% Caprio2loci.m
% Tony Ives, 16 June 2006

% This is for EPA use only and is copyrighted by Tony Ives

clear

%size of grid
N=50;

% flag2=1 for fixed R0, flag2=2 for Caprio model
flag2=2;

% reproductive rate in Bt fields (under flag2=1)
F1=50;

%dispersal of males (rm) and females (rf) from Bt (1) and refuge (2) fields
R1m=1;
R1f=1;

R2m=1;
R2f=1;

% proportion of refuge
Q=.05;
x0=Q;

% spraying survival in refuge
k=1;

% initial gene frequencies
p1=.002;
p2=.002;

s1=[0 .001 1];
s2=[.1 .2 1];

Z=s1'*s2;
Z=Z(:);
```

```
sAABB=Z(1);
sAaBB=Z(2);
saaBB=Z(3);
sAABb=Z(4);
sAaBb=Z(5);
saaBb=Z(6);
sAAbb=Z(7);
sAabb=Z(8);
saabb=Z(9);

s=[sAABB sAaBB saaBB sAABb sAaBb saaBb sAAbb sAabb saabb];

% calculate V for mating of genotypes
VV(:,:,1)=[1 .5 0;.5 .25 0;0 0 0];
VV(:,:,2)=[0 .5 1;.5 .5 .5;1 .5 0];
VV(:,:,3)=[0 0 0; 0 .25 .5;0 .5 1];
for i2=1:3
    for i1=1:3
        V(:,:,3*(i2-1)+i1)=kron(VV(:,:,i2),VV(:,:,i1));
    end
end

% add cost of resistance
s1=[1 .8 .8];
s2=[1 1 1];

Z=s1'*s2;
k=Z(:);

O1=diag(k.*s);
O2=diag(k);


% initially assume genotypes at H-W and unlinked
Wi=[(1-p1)^2 2*p1*(1-p1) p1^2];
Wj=[(1-p2)^2 2*p2*(1-p2) p2^2];

W=Wi'*Wj;
W=W(:);

W1=W;
W2=W;

X=[10^-4;x0];

P1=p1;
P2=p2;

t=0;
output=[0 P1 P2 0];
while (P1 < 0.5 | P2 < 0.5) && X(1) < x0/2 && t < 2000

    t=t+1;
    % pre-mating movement of males
    z1s=((1-R1m)+(1-Q)*R1m)*X(1);
    z1d=(1-Q)*R2m*X(2);
    z2s=((1-R2m)+Q*R2m)*X(2);
    z2d=Q*R1m*X(1);
    Mm=[z1s/(z1s+z1d) z1d/(z1s+z1d);z2d/(z2s+z2d) z2s/(z2s+z2d)];
    Mm=kron(Mm,eye(9));

    % pre-mating movement of females
    z1s=((1-R1f)+(1-Q)*R1f)*X(1);
    z1d=(1-Q)*R2f*X(2);
    z2s=((1-R2f)+Q*R2f)*X(2);
    z2d=Q*R1f*X(1);
    Mf=[z1s/(z1s+z1d) z1d/(z1s+z1d);z2d/(z2s+z2d) z2s/(z2s+z2d)];
    Mf=kron(Mf,eye(9));

    Wm=Mm*[W1;W2];
    Wf=Mf*[W1;W2];

    % mating
    WW=[];
    for i=1:2
        Wmi=Wm(9*(i-1)+1:9*i);
        Wfi=Wf(9*(i-1)+1:9*i);
        for j=1:9
            WW=[WW;sum(sum((Wfi*Wmi').*V(:,:,j)))];
        end
    end
```

```
    W1=WW(1:9);
    W2=WW(10:18);

    % redistribution of densities during premating dispersal
    d11=((1-R1f)+(1-Q)*R1f);
    d12=(1-Q)*R2f;
    d21=Q*R1f;
    d22=((1-R2f)+Q*R2f);

    D=[d11 d12;d21 d22];
    X=D*X;

    % selection
    Wn1=O1*W1;
    Wn2=O2*W2;

    W1=Wn1./sum(Wn1);
    W2=Wn2./sum(Wn2);

    % density-dependent population growth
    if flag2==1
        BtXincrease=F1*sum(Wn1);
    else
        BtXincrease=(sum(Wn1)/sum(W1))*x0/(X(2)*sum(Wn2)/sum(W2));
    end

    X(1)=BtXincrease*X(1);
    X(2)=x0;

    % compute frequencies
    P1=(X(1)*(sum(W1([3 6 9]))+.5*sum(W1([2 5 8]))) + ...
        X(2)*(sum(W2([3 6 9]))+.5*sum(W2([2 5 8]))))/(X(1)+X(2));
    P2=(X(1)*(sum(W1([7 8 9]))+.5*sum(W1([4 5 6]))) + ...
        X(2)*(sum(W2([7 8 9]))+.5*sum(W2([4 5 6]))))/(X(1)+X(2));

    output=[output;t P1 P2];
end
GensToFailure=t
YearsToFailure=ceil(t/3)

figure(1)
semilogy(output(:,1),output(:,2),'k',output(:,1),output(:,3),'b')
xlabel('Time (generations)')
ylabel('Allele frequencies')
hold on
```

## Matlab code for the spatially explicit model

```
% Caprio2lociSpatial.m
% Tony Ives, 16 June 2006

% Modified from a single-locus model written by Nic Lehmann-Ziebarth and
% Tony Ives

% This is for EPA use only and is copyrighted by Tony Ives

clear

%size of grid
N=50;

% flag2=1 for fixed R0, flag2=2 for Caprio model
flag2=2;

% reproductive rate in Bt fields (under flag2=1)
F1=50;

%dispersal of males (rm) and females (rf) from Bt and refuge fields
rm1=1;
rm2=1;
rf1=1;
rf2=1;
rm=[rm1 rm2];
rf=[rf1 rf2];

% proportion of refuge
Q=.05;
x0=Q;
```

```
% spraying survival in refuge
k=1;

% male dispersal: (1) horizontal/vertical; (2) diamond; (3) global dispersal
disperseflagm=2;

% female dispersal: (1) horizontal/vertical; (2) diamond; (3) global dispersal
disperseflagf=2;

%nearest n neighbor movement
n=6;

% initial gene frequencies
p1=.002;
p2=.002;

s1=[0 .001 1];
s2=[.1 .2 1];
%s2=[1 1 1];

Z=s1'*s2;
Z=Z(:);

sAABB=Z(1);
sAaBB=Z(2);
saaBB=Z(3);
sAABb=Z(4);
sAaBb=Z(5);
saaBb=Z(6);
sAAbb=Z(7);
sAabb=Z(8);
saabb=Z(9);

s=[sAABB sAaBB saaBB sAABb sAaBb saaBb sAAbb sAabb saabb]';

% calculate V for mating of genotypes
VV(:,:,1)=[1 .5 0;.5 .25 0;0 0 0];
VV(:,:,2)=[0 .5 1;.5 .5 .5;1 .5 0];
VV(:,:,3)=[0 0 0; 0 .25 .5;0 .5 1];
for i2=1:3
    for i1=1:3
        V(:,:,3*(i2-1)+i1)=kron(VV(:,:,i2),VV(:,:,i1));
    end
end

% add cost of resistance
%s1=[1 .8 .8];
s1=[1 1 1];
s2=[1 1 1];

Z=s1'*s2;
C=Z(:);

% set up survivals
O1=k.*C.*s;
O2=k.*C;

O=[O1 O2];
AABB=O(1,:);
AaBB=O(2,:);
aaBB=O(3,:);
AABb=O(4,:);
AaBb=O(5,:);
aaBb=O(6,:);
AAbb=O(7,:);
Aabb=O(8,:);
aabb=O(9,:);

% set up dispersal matrix Mm
if disperseflagm==1,
    %xx is movement rate to 4 nearest squares.
    %Distribution over squares is pseudo geometric (scale by 1/2)
    M=zeros(N,N);
    xx=(8*(1-.5^n))^(-1);
    for w=1:n
        xw=xx*.5^(w-1);
        M=M+xw*(diag(ones(N-w,1),w)+diag(ones(w,1), N-w));
    end
    Mm=M+M';
end
```

```
% set up dispersal matrix Mf
if disperseflagf==1,
    %xx is movement rate to 4 nearest squares.
    %Distribution over squares is pseudo geometric (scale by 1/2)
    M=zeros(N,N);
    xx=(8*(1-.5^n))^(-1);
    for w=1:n
        xw=xx*.5^(w-1);
        M=M+xw*(diag(ones(N-w,1),w)+diag(ones(w,1), N-w));
    end
    Mf=M+M';
end

if disperseflagm==2,
    switch n,
        case 2
        load 'M50_n2'
    case 3,
        load 'M50_n3'
    case 4,
        load 'M50_n4'
    case 5
        load 'M50_n5'
    case 6
        load 'M50_n6'
    end
    Mm=M;
end

if disperseflagf==2,
    switch n,
        case 2
        load 'M50_n2'
    case 3,
        load 'M50_n3'
    case 4,
        load 'M50_n4'
    case 5
        load 'M50_n5'
    case 6
        load 'M50_n6'
    end
    Mf=M;
end

%This creates random distribution of Bt sites over the field
Btgrid=1+(rand(N,N)<Q);

O=zeros(N,N,9);
O(:,:,1)=AABB(Btgrid);
O(:,:,2)=AaBB(Btgrid);
O(:,:,3)=aaBB(Btgrid);
O(:,:,4)=AABb(Btgrid);
O(:,:,5)=AaBb(Btgrid);
O(:,:,6)=aaBb(Btgrid);
O(:,:,7)=AAbb(Btgrid);
O(:,:,8)=Aabb(Btgrid);
O(:,:,9)=aabb(Btgrid);

%grid of male and female movement proportions
RM=rm(Btgrid);
RF=rf(Btgrid);

% grid of initial densities
X=10^-4*ones(N,N);
X(Btgrid==2)=x0;

% initially assume genotypes at H-W and unlinked
Wi=[(1-p1)^2 2*p1*(1-p1) p1^2];
Wj=[(1-p2)^2 2*p2*(1-p2) p2^2];

W=Wi'*Wj;
W=W(:);

for i1=1:N
    for i2=1:N
        WW(i1,i2,:)=W;
    end
end
W=WW;
```

```
t=0;
Tmax=10^3;
Plist=[];
while t < Tmax && (p1 < .5 | p2 < .5)
    t=t+1;

    % dispersal of male alleles
    for i=1:9
        Xs=(1-RM).*W(:,:,i).*X;
        Xd=RM.*W(:,:,i).*X;

        if disperseflagm==1,
            %disperse males and alleles to 4 nearest cells
            Xd=(Mm*Xd+Xd*Mm);
        elseif disperseflagm==2,
            %This is diamond dispersal
            Xd=reshape(Mm*Xd(:),N,N);
        else
            %global dispersal
            Xd=mean(mean(Xd))*ones(N,N);
        end
        Xm(:,:,i)=Xs+Xd;
    end
    Wm=Xm./repmat(sum(Xm,3),[1 1 9]);

    % dispersal of female alleles and females
    for i=1:9
        Xs=(1-RF).*W(:,:,i).*X;
        Xd=RF.*W(:,:,i).*X;

        if disperseflagf==1,
            %disperse males and alleles to 4 nearest cells
            Xd=(Mf*Xd+Xd*Mf);
        elseif disperseflagf==2,
            %This is diamond dispersal
            Xd=reshape(Mf*Xd(:),N,N);
        else
            %global dispersal
            Xd=mean(mean(Xd))*ones(N,N);
        end
        Xf(:,:,i)=Xs+Xd;
    end
    Wf=Xf./repmat(sum(Xf,3),[1 1 9]);
    Xf=sum(Xf,3);

    %mating
    for i1=1:N
        for i2=1:N
            for j=1:9
                WWm=reshape(Wm(i1,i2,1:9),9,1);
                WWf=reshape(Wf(i1,i2,1:9),9,1);
                W(i1,i2,j)=sum(sum((WWf*WWm').*V(:,:,j)));
            end
        end
    end

    % pre-selection allele frequencies
    Wo=W;

    %selection
    for i=1:9
        Wn(:,:,i)=W(:,:,i).*O(:,:,i);
    end
    surv=sum(Wn,3)./sum(W,3);
    W=Wn./repmat(sum(Wn,3),[1 1 9]);

    % compute new densities
    X=surv.*Xf;
    if flag2==1
        X(Btgrid==1)=F1*X(Btgrid==1);
    else
        X(Btgrid==1)=(1/(k*Q))*X(Btgrid==1);
    end

    X(Btgrid==2)=x0;

    % compute gene frequencies
    p1=0;
    p2=0;
    for i1=1:N
        for i2=1:N
```

```
            p1=p1+X(i1,i2)*(sum(W(i1,i2,[3 6 9]))+.5*sum(W(i1,i2,[2 5 8])));
            p2=p2+X(i1,i2)*(sum(W(i1,i2,[7 8 9]))+.5*sum(W(i1,i2,[4 5 6])));

            P1(i1,i2)=sum(Wo(i1,i2,[3 6 9]))+.5*sum(Wo(i1,i2,[2 5 8]));
            P2(i1,i2)=sum(Wo(i1,i2,[7 8 9]))+.5*sum(Wo(i1,i2,[4 5 6]));
        end
    end
    p1=p1/sum(X(:));
    p2=p2/sum(X(:));

    Plist=[Plist; t p1 p2 mean(mean(X))];

end

% printed output
GensToFailure=t
YearsToFailure=ceil(t/3)
meanX=mean(X(:))

if 1
    % graph of gene frequencies through time
    figure(1)
    semilogy(Plist(:,[2 3]))
    xlabel('time')
    ylabel('allele frequency')

    % graph 3D picture of gene frequencies
    figure(100)
    subplot(2,1,1)
    surf(P1)
    view(2)
    axis([1 N 1 N 0 1])

    subplot(2,1,2)
    surf(P2)
    view(2)
    axis([1 N 1 N 0 1])
end
```

## Appendix 5

Post meeting comments from one Panel member regarding:
*County-level Variation in Non-cotton Cultivated Hosts of the Tobacco Budworm*

In response to the charge to assess the adequacy of natural refuges by region (Charge 9), one Panel member provided the following additional analyses and comments concerning the adequacy of Monsanto's spatial sampling scheme for extrapolating to areas that were not sampled. Such comments were provided by the Panel member after the meeting and were not considered for review by the Panel, thus they do not reflect a consensus Panel position.

Monsanto's assertion that non-cotton hosts constitute an adequate natural refuge to delay counteradaptation by TBW to Bollgard II cotton in the East region (i.e., Monsanto's "North Carolina" and "Georgia" regions; see Fig. 9-1 under Charge 9, or Fig. 2 in Gustafson and Head 2005) depends in large part, at least for the states north of Georgia, on the data Monsanto provided on host use by TBW from five counties in North Carolina (three counties for two years and two counties for one year). Cropping data from the United States Department of Agriculture, National Agricultural Statistics Service (USDA, NASS 2006) show that these sampled counties are not as representative of the entire South Carolina, North Carolina, and Virginia area as one could desire (Fig. A5-1). In South Carolina and North Carolina, there exist multi-county areas containing tens of thousands to a hundred thousand acres of cotton in which (tobacco + peanut) acreage constitutes < 5% of the (cotton + tobacco + peanut) acreage: $P_{(T+P)} < 0.05$. In the counties sampled, $P_{(T+P)} \cong 0.2$ except for $P_{(T+P)} = 0.10$ for Halifax County in which data were collected for a single year. In Virginia, cotton acreage totaled 93,000 acres in 2005, and there were no counties with large cotton acreages for which $P_{(T+P)} < 0.05$. However, there is a narrow-necked peninsula of two counties on the east side of the Chesapeake Bay with 3,000 acres of cotton and no tobacco or peanut acreage. A peninsula in North Carolina, more broadly attached to the mainland than the one in Virginia, contained 23,000 acres of cotton and no tobacco or peanut acreages in 2005. The restriction of gene flow between a peninsular population and its mainland population could significantly accelerate the rate of development of counteradaptation. Thus, there are county-scale or larger areas in all three of these states that may not have sufficient non-cotton, cultivated hosts to warrant extrapolation from TBW host use data acquired in the counties sampled in North Carolina. Consequently, North Carolina should receive additional sampling, and both South Carolina and Virginia should be sampled directly.

In Georgia in 2005, 1,210,000 acres of cotton were grown primarily in the southeastern two thirds of the state along with 750,000 acres of peanut and 16,000 acres of tobacco. There were no counties with significant cotton acreage for which $P_{(T+P)} < 0.05$. In Florida in 2005, cotton (102,000 acre), peanut (76,000 acre), and tobacco (4,000 acre) were grown in the panhandle area contiguous with agriculturally similar areas in Georgia and Alabama (see below); and $P_{(T+P)} >> 0.05$ for all counties. Thus, significant refuges of non-cotton, cultivated hosts appear to be present in cotton production areas of both Georgia and Florida. However, Florida should be sampled directly to test the expected outcome.

In Alabama in 2005, no tobacco was grown; and peanut (225,000 acre) was grown in 13 counties in the extreme southern and southwestern parts of the state only. A total of 193,000 acres of cotton was produced in this area, but $P_{(T+P)} >> 0.05$ for all counties. In the rest of Alabama, but primarily in the northern half of the state, 357,000 acres of cotton were grown. In addition, a total of 25,300 acres of cotton was grown in three counties on the southern border of Tennessee contiguous with the cotton production area in Alabama. Only 425 acres of tobacco was produced in one of these Tennessee counties. Thus, southern Alabama appears to have a significant refuge of non-cotton, cultivated hosts, but central and northern Alabama is apparently much more similar to Mississippi. However, both of these areas in Alabama should be sampled directly.

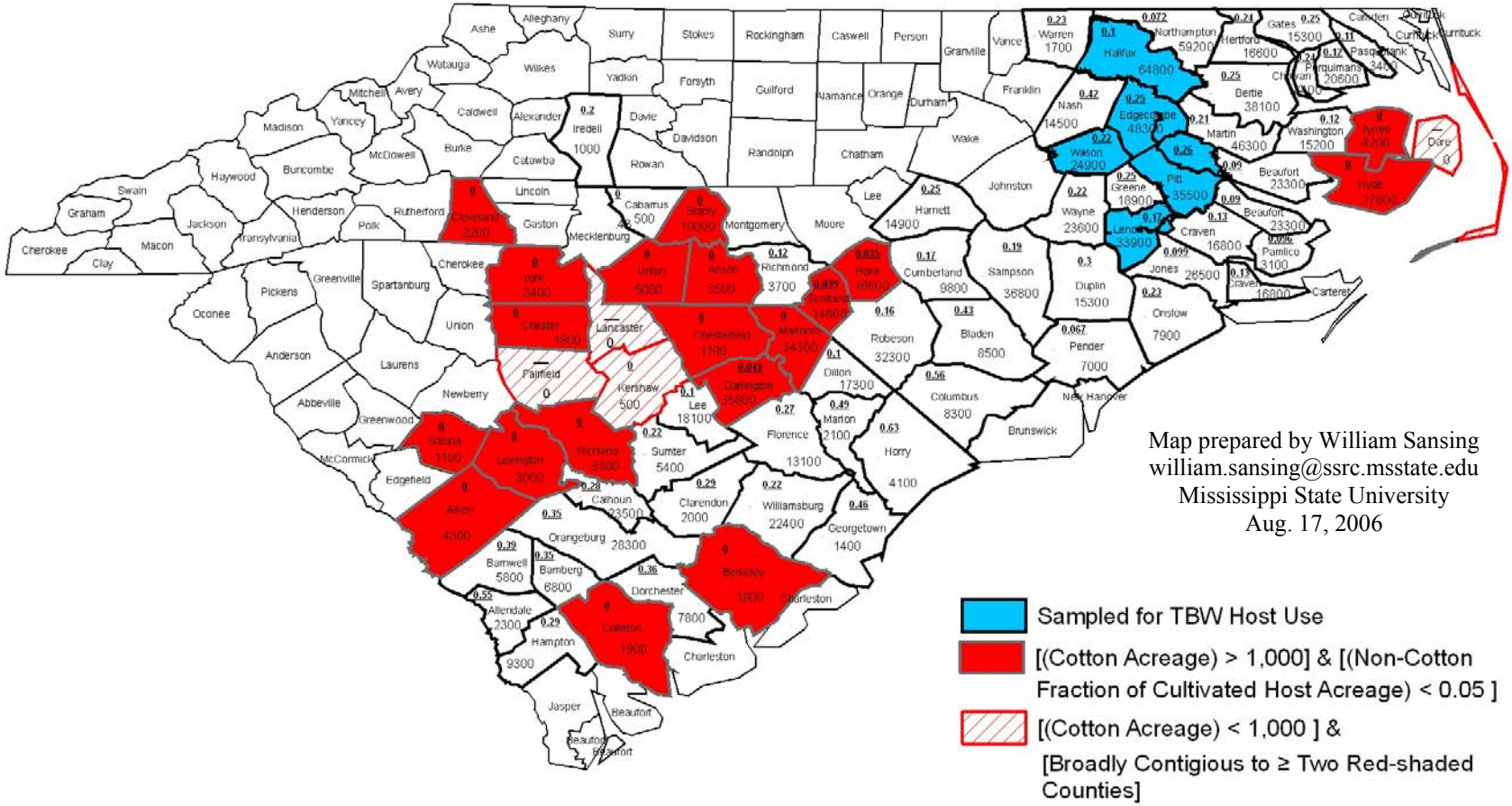


**Fig. A5-1.** County-level variation in proportion cultivated hosts of tobacco budworm (TBW) that are non-cotton with respect to locations of Monsanto's sampling to quantify non-cotton vs. cotton host use by TBW in North Carolina and South Carolina in 2005. Proportion of acreage planted to cotton, tobacco, and peanut that was planted to tobacco or peanut ($P_{(T+P)}$) is written above the county name and the cotton acreage harvested is written below the county name (USDA, NASS 2006). $P_{(T+P)} < 0.05$ in the counties shaded red. Monsanto's sampling for TBW males for host plant analysis was performed in the counties shaded blue.