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

OFFICE OF CHEMICAL SAFETY AND
POLLUTION PREVENTION

March 3, 2011

MEMORANDUM

SUBJECT: Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting Held December 8-9, 2010 to Address Scientific Issues Associated with Insect Resistance Management for SmartStax™ Refuge-in-the-Bag, a Plant-Incorporated Protectant (PIP) Corn Seed Blend

TO: Steven Bradbury, Ph.D.
Director
Office of Pesticide Programs

FROM: Sharlene R. Matten, Ph.D.
Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

THRU: Laura Bailey, Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Frank Sanders
Director
Office of Science Coordination and Policy

Attached, please find the meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, VA on December 8-9, 2010. This report addresses a set of scientific issues associated with "Insect Resistance Management for SmartStax™ Refuge-in-the-Bag, a Plant-Incorporated Protectant (PIP) Corn Seed Blend."

Attachment

cc:
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SAP Minutes No. 2011-02

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

Insect Resistance Management for SmartStax™ Refuge-in-the-Bag, a Plant-Incorporated Protectant (PIP) Corn Seed Blend

December 8-9, 2010
FIFRA Scientific Advisory Panel Meeting
held at
One Potomac Yard
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 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 Environmental Protection Agency (EPA), Office of Pesticide Programs (OPP), and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. FQPA 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 Sharlene R. Matten, Ph.D., SAP Designated Federal Official, via e-mail at matten.sharlene@epa.gov.

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

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SAP Minutes No. 2011-02

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

Insect Resistance Management for SmartStax™ Refuge-in-the-Bag, a Plant-Incorporated Protectant (PIP) Corn Seed Blend

December 8-9, 2010
FIFRA Scientific Advisory Panel Meeting
held at
One Potomac Yard
Arlington, Virginia

Steven G. Heeringa, Ph.D.
FIFRA SAP Chair
FIFRA Scientific Advisory Panel

Date: 03/03/11

Sharlene R. Matten, Ph.D.
Designated Federal Official
FIFRA Scientific Advisory Panel Staff

Date: 03/03/11

Panel Members for the Meeting of the Federal Insecticide, Fungicide and Rodenticide Act Scientific Advisory Panel (FIFRA SAP) to Consider and Review Scientific Issues Related to Insect Resistance Management for SmartStax™ Refuge-in-the-Bag, a Plant-Incorporated Protectant (PIP) Corn Seed Blend

December 8-9, 2010

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INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) has completed its report of the SAP meeting regarding scientific issues associated with **"Insect Resistance Management for SmartStax™ Refuge-in-the-Bag, a Bt Plant-Incorporated Protectant."** Advance notice of the SAP meeting was published in the *Federal Register* on **October 27, 2010**. The review was conducted in an open Panel meeting on **December 8-9, 2010** at One Potomac Yard, Arlington, Virginia. Materials for this meeting are available in the Office of Pesticide Programs (OPP) public docket or via Regulations.gov, Docket No. EPA-HQ-OPP-2010-0772. Steven Heeringa, Ph.D., chaired the meeting. Sharlene Matten, Ph.D., served as the Designated Federal Official. Steven Bradbury, Ph.D., Director, Office of Pesticide Programs (OPP), and Keith A. Matthews, J.D., Director, Biopesticides and Pollution Prevention Division (BPPD) provided opening remarks at the meeting. Presentations of technical background materials were provided by Jeannette Martinez, BPPD and Alan Reynolds, BPPD.

The Agency is currently evaluating the SmartStax™ [1] Refuge-in-the-Bag (RIB) product, a multi-trait plant-incorporated protectant (PIP) corn seed blend consisting of a mixture of 95% *Bacillus thuringiensis* (*Bt*) corn seed and 5% refuge corn seed for insect resistance management (IRM) of *Ostrinia nubilalis* (Hübner) (European corn borer, ECB), *Diatraea grandiosella* (Dyar) (southwestern corn borer, SWCB), and *Diabrotica sp.* (corn rootworm, CRW), the primary target pests. SmartStax RIB was developed jointly by the Monsanto Company and Dow AgroSciences, LLC. In July 2009, Monsanto Company and Dow AgroSciences, LLC obtained registrations for SmartStax with a 5% reduced structured refuge requirement in the U.S. Corn Belt based on the multiple modes of insect control. SmartStax was brought to the market in 2010. In December 2009, Monsanto and DAS applied for additional registrations of SmartStax to allow a 5% seed mixture refuge option ('Refuge in the Bag') in the U.S. Corn Belt.

The focus of this FIFRA SAP was on IRM considerations associated with SmartStax RIB for control of ECB, SWCB,` and corn rootworm. IRM considerations associated with another *Bt* PIP corn seed mixture targeting corn rootworm Optimum® AcreMax™1[2] Corn Rootworm-Protected Corn (Pioneer Hi-Bred) were addressed in the February 2009 FIFRA SAP meeting (http://www.epa.gov/scipoly/sap/meetings/2009/february/232009finalreport.pdf).

During a February 1998 FIFRA SAP meeting (see http://www.epa.gov/scipoly/sap/meetings/1998/february/finalfeb.pdf), the Panel concluded that seed mixtures should not be considered as a viable IRM refuge option for ECB and corn earworm in *Bt* corn. The concern was that ECB larvae can move from plant to plant within corn fields (including from refuge plants to *Bt* plants and vice-versa) which could reduce the effectiveness of the seed mixture at preventing pest resistance.

[1] SmartStax® is a registered trademark of Monsanto Technology LLC. SmartStax (MON 89034 × TC1507 × MON 88017 × DAS-59122-7) is a combined trait corn product with multiple effective modes of insect control for the key above-ground and below-ground corn pests.
[2] Optimum® and AcreMax™ 1 are trademarks of Pioneer Hi-Bred.

Subsequent to the 1998 SAP, new biological data and simulation modeling were developed to support the potential use of a seed mixture IRM strategy in *Bt* corn. The Office of Pesticide Programs considered these data and utilized the Office of Research and Development's (ORD) Population Genetics (POPGEN) model to evaluate the risk of ECB resistance developing in a seed mixture environment. The Agency requested that the FIFRA SAP address scientific issues associated with the SmartStax RIB IRM strategy relative to the effectiveness of block refuges currently required for lepidopteran pests of *Bt* corn.

PUBLIC COMMENTERS

Oral statements were presented by:

1) J. Lindsey Flexner, Ph.D., Pioneer Hi-Bred International, Inc.

2) Graham Head, Ph.D., Monsanto Company

3) Nicholas Storer, Ph.D., Dow AgroSciences, LLC

4) Robert Bowman, past-President of the Iowa Corn Growers Association, National Corn Growers Association

5) Gregory Jaffe, J.D., The Center for Science in the Public Interest

Written statements were provided by:

1) David Onstad, Ph.D., University of Illinois

2) Mike Caprio, Ph.D., Mississippi State University

3) Bruce Hibbard, Ph.D., United States Department of Agriculture, Agricultural Research Service, University of Missouri

4) Lawrent Buschman, Ph.D., Kansas State University

5) James Reed, President, Illinois Corn Growers Association

6) Dean Taylor, President, Iowa Corn Growers Association

7) Gregory Ruehle, CEO, Independent Professional Seed Association

8) Rob Korff, Korff Farms, Inc., Norborne, Missouri

9) Leon Corzine, Corn Grower, Assumption, Illinois

10) Kenneth McCauley, Corn Grower, White Cloud, Kansas

11) Terry Elsbernd, Corn Grower, Decorah, Iowa

12) Randy Schertz, Corn Grower, Eureka Illinois

13) Gary Duffy, President, South Dakota Corn Growers Association

14) Steve Hudson, Corn Grower, Indiana

15) Ron Litterer, Corn Grower, Iowa

16) Graham Head, Ph.D., Monsanto Company and Nicholas Storer, Ph.D., Dow AgroSciences, LLC

17) Laura Higgins, Ph.D., Pioneer Hi-Bred, a DuPont Business

18) David Morgan, President, Syngenta Seeds, Inc.

SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

Overall Panel Summary

The Panel was requested to consider the durability of the 5% SmartStax seed mixture given the available biological, ecological, and genetic information, and IRM modeling. SmartStax corn is a multi-toxin double pyramid in which there are three *Bt* toxins targeting lepidopteran stalk-boring (and ear feeding) pests (Cry1A.105, Cry2Ab2, and Cry1Fa) and two *Bt* toxins targeting corn rootworm (Cry34/35Ab1 and Cry3Bb1). The Panel concluded that a 5% SmartStax seed mixture would have comparable durability to SmartStax planted with a 5% structured refuge for CRW resistance management. However, the Panel stated that resistance management for a pyramid should focus on the pest(s) with the greatest likelihood of resistance in a seed mixture compared to a structured refuge. In this case, the Panel was more concerned about the evolution of resistance by the European corn borer (ECB) and southwestern corn borer (SWCB) than corn rootworm (CRW) because of the difference in selection intensity to a high-dose versus a low-dose of *Bt* toxins expressed in SmartStax corn. As stated in the 1998 SAP report, for high-dose cases when toxicity of the cultivar causes low survival of heterozygous pest individuals, seed mixtures will have lower durability than structured refuges with the same percentage of *Bt* plants. This point was illustrated by the modeling exercise performed by the Panel during the meeting (see Appendix 2). There are also greater uncertainties for seed mixtures than for structured refuges due to the lack of information about larval movement for ECB and SWCB and how larval movement affects the survival of heterozygotes. The Panel identified many uncertainties associated with the Monsanto, Dow and EPA/ORD modeling efforts and stated the models contained assumptions that will lead to overestimates of durability for the 5% SmartStax seed mixture. The Panel also considered CEW resistance associated with corn to be a serious resistance risk to both corn and cotton, but could not quantify the role of selection associated with 5% SmartStax seed mixtures in the Midwest and migration between the Midwest and the South on the rate of evolution for *Bt* resistance in CEW. The overall conclusion of the Panel is that the 5% SmartStax seed mixture will be substantially less durable than SmartStax planted with a 5% structured refuge for resistance management of the lepidopteran pests, ECB and SWCB.

Part A: Biology of European Corn Borer, Southwestern Corn Borer, Corn Earworm, and Corn Rootworm

Charge Question 1

European corn borer (ECB) has both local and long distance dispersal capability. Currently, the proportion and frequency of individuals in a population engaging in dispersal before or after mating is unclear. While it has been established that ECB mate in aggregation sites near cornfields, mark-release-recapture studies in the U.S. have typically had a low recapture success (<1%). Recently it was suggested that 1-day old female ECB may engage in obligate pre-mating dispersal (Dorhout et al. 2008).

Please comment on the uncertainties regarding ECB movement including mating sites, pre-mating dispersal, and the proportion of the population engaging in long-distance dispersal. How might these aspects of ECB movement affect a potential seed blend strategy?

Panel Response Summary

The Panel concluded that a comparison with empirically fitted parameters cannot be made to estimate the effect of seed mixtures on resistance evolution. This is because both known biological variation and uncertainty concerning ECB adult dispersal make it a challenge to construct a reasonable "worst-case" scenario for between-field refuges. Major sources of uncertainty about adult dispersal include: dispersal by susceptible and resistant ECB, sex-specific movement, pre-mating vs. post-mating movement, mating biology and sexual selection, wind and weather events. Known factors generating variation in dispersal include: sex pheromones, humidity, geographic and seasonal variation, diurnal cycle, and age of the adult.

Theoretical considerations indicate that a seed mixture strategy will increase the rate of resistance evolution compared to the present structured refuges. In general, greater male movement will tend to delay and greater female movement will tend to speed up the rate of resistance evolution, and that for ECB and SWCB, intermediate rates of adult dispersal will have the slowest rate of resistance evolution. The effect of a seed mixture would be to increase the rate of adult dispersal between *Bt* and refuge plants, thereby increasing the rate of resistance evolution.

The quantitative increase in evolutionary rate can be evaluated theoretically. The Panel suggested that to compare the rate of resistance evolution between seed mixtures and structured refuges, resistance evolution for the structured refuges should be determined for adult movement rates that minimize the rate of resistance evolution. Additional investigations to reduce uncertainties are also required and research on mating biology and sexual selection should be encouraged.

Charge Question 2

Scientific Advisory Panels (1998 and 2000) discouraged the Agency from the use of Bt seed mixtures to control lepidopteran target pests because substantial larval movement could be expected between Bt and non-Bt plants which could lead to more rapid selection of resistance. BPPD has reviewed new data developed by Dow and Monsanto simulating the effects of SmartStax on various instars of potentially mobile Lepidoptera. These data provide evidence that SmartStax is highly toxic to 1st, 2nd, and 3rd instars. But, there was greater survivability among 4th and 5th instars. While it has been established that ECB disperse as neonates, there is some uncertainty with respect to lepidopteran propensity for dispersal off non-Bt plants as later instars. BPPD notes that simulation models incorporating data on high larval mortality on SmartStax plants have (in some cases) predicted that seed blends may be as durable as structured refuges.

Please comment on ECB larval plant-to-plant movement including uncertainties about late-instar movement and the potential effect on the durability of a seed blend strategy.

Panel Response Summary

The Panel considered the ecological and evolutionary context of ECB larval movement in answering this question. Based on the known behavior and ecology associated with ECB larval movement, the Panel developed several plausible ECB larval movement hypotheses that would be expected to increase the rate of resistance evolution (by increasing the fitness of heterozygotes) via one of the four general larval movement scenarios applicable to many species. The uncertainty about late-instar movement has been overstated by Dow and Monsanto as late instar ECB exhibit considerable movement.

The Panel concluded that there were insufficient data to parameterize these specific ECB hypotheses and evaluate the four scenarios empirically to quantify the durability of the seed mixture strategy. Remaining significant uncertainties include: genotype-specific mortality (especially heterozygote mortality) of moving and sedentary larvae, rate (or percent) of larval moment, and other aspects of fitness of survivors moving from non-*Bt* plant to *Bt* plants and vice-versa.

Because of these uncertainties, the Panel recommended that the ECB larval movement hypotheses and the general larval movement scenarios be evaluated using specifically designed theoretical evolution models. The Panel examined in detail the structure and results of the Monsanto model and the EPA/ORD model, and found that these models did not evaluate or did not clearly evaluate any of the four general larval movement scenarios or any of the detailed ECB hypotheses that could lead to reduced durability of the seed mixture. The durability of a 5% SmartStax seed mixture as projected by both the Monsanto and EPA/ORD models is overstated, and may be overestimated considerably because these plausible ECB larval movement hypotheses have not been considered.

Charge Question 3

It is typically assumed that, since European corn borer (ECB) and southwestern corn borer (SWCB) are similar in many ways, ECB can serve as a surrogate for SWCB to address uncertainties regarding biology and genetics. The applicants' efficacy data, however, suggest that SmartStax is somewhat less toxic to older instars of SWCB. Results of a larval exposure study by Monsanto showed that SWCB survival was higher than ECB on SmartStax. Should some SWCB larvae disperse as older instars, the rate of adaptation to SmartStax could increase in a seed blend deployment. BPPD concluded that simulation models should incorporate such information in their analyses. There is currently a lack of data on the propensity of SWCB larval plant-to plant movement and on how ECB and SWCB differ in this respect, if at all.

Please comment on the assumption that ECB is a suitable biological surrogate for SWCB and BPPD's concerns that a SmartStax seed blend may affect SWCB differently than ECB.

Panel Response Summary

The Panel agreed that the results from the Monsanto model and EPA/ORD model are inadequate to make any scientifically sound conclusions about resistance evolution in SWCB because they do not include density-dependent mortality, which is an important feature in SWCB population ecology. However, on the broader issue of whether ECB is a reasonable surrogate for SWCB, the Panel expressed different views in the degree to which ECB information could be used for SWCB. Some members of the Panel concluded that ECB is a poor surrogate because of the many ways the two species differ biologically and therefore different models would have to be developed for SWCB. Others suggested that at a structural level the same resistance evolution model can be used for the two species, but that the parameter values must be different.

Charge Question 4

Corn earworm (CEW) was not considered in the applicants' and EPA/ORD's analyses for a 5% SmartStax seed blend based on the assumption that the insect does not overwinter in the Corn Belt where the blend has been proposed. BPPD is concerned, however, that there could be areas in the southern portion of the Corn Belt where CEW may be able to successfully overwinter, particularly in less severe winters. Such areas may need to be identified because they could contribute to increased selection for CEW resistance to Bt corn (including the proposed 5% SmartStax seed blend).

Please comment on the assumption that corn earworm does not successfully overwinter in the Corn Belt and poses less of a risk for resistance. If CEW can potentially overwinter in parts of the Corn Belt, should the insect be considered in the analysis of the proposed 5% SmartStax seed blend?

Panel Response Summary

The Panel discussed CEW long-distance migration from the South to the Midwest in the spring, CEW reverse migration from the Midwest to the South in the late summer, and whether these migratory patterns have an effect on the selection for CEW resistance in the Midwestern Corn Belt. There are several studies demonstrating that the major CEW infestations in the Midwest are caused by CEW moths that originate in southern states. There is also evidence for reverse migration of CEW from the Midwest to southern states. The Panel concluded that recent studies demonstrate that moths have evolved sophisticated mechanisms that enable them to move adaptively from one geographic location to another.

The Panel considered CEW resistance associated with corn to be a serious resistance risk to both corn and cotton. Considering all of the evidence, the Panel could not quantify the role of selection associated with 5% SmartStax seed mixtures in the Midwest and migration between the Midwest and the South on the rate of evolution for *Bt* resistance in CEW. Some Panel members suggested that high adoption of seed mixtures in the Corn Belt might have an effect on selection for CEW resistance on *Bt* cotton later in the season. In a worst-case scenario, there might not be any refuge for CEW within Midwestern corn that is planted as a seed mixture. Other crops and wild plants in the Midwest might be good hosts for CEW, but most of these crops are sprayed

with insecticide and would not be good sources for production of susceptible insects. The Panel suggested that a detailed survey of alternate crops and more information regarding *Bt* pollen exposure scenarios would be useful.

Charge Question 5

To assess dose expression for corn rootworm (CRW) Bt toxins, the level of survival (adult emergence) is typically compared between artificially infested Bt and non-Bt corn plots. However, density-dependent mortality in non-Bt plots can potentially confound the comparison by reducing overall survival and adult emergence. (Density-dependent mortality is not expected in Bt plots due to effects of the toxin on young larvae.) To account for this effect, the dose calculation can be adjusted by removing density-dependent mortality from the control plots. This effectively increases the dose mortality estimate by raising the number of larvae present in non-Bt plots relative to the surviving larvae in Bt plots.

For the SmartStax toxins, Dow/Monsanto made a density-dependent adjustment to their dose estimates based on density/survival relationships developed by Onstad et al. (2006). The resulting dose mortality profile was: Cry34/35Ab1 (99.75%), Cry3Bb1 (99.75%), and Cry34/35Ab1/Cry3Bb1 pyramid (99.95%). On the other hand, BPPD has also considered separate work by Hibbard et al. (2010), which suggests that density-dependent mortality occurs at higher egg density levels than those assumed by Dow/Monsanto. In light of this research, BPPD recommended in its 2009 risk assessment of SmartStax that dose should also be evaluated without a density-dependent adjustment. The non-adjusted dose profile for the SmartStax toxin is: Cry34/35Ab1 (94.2%), Cry3Bb1 (97.5%), and Cry34/35Ab1/Cry3Bb1 pyramid (98.2%).

Please comment on dose estimates for the SmartStax toxins (Cry34/35Ab1 and Cry3Bb1) targeting corn rootworm given the different interpretations of density-dependent mortality.

Panel Response Summary

The Panel agreed that a density-dependent mortality adjustment is merited theoretically; but questioned the accuracy and precision of the compensatory adjustment values used in the Dow model. The Panel agreed with BPPD's recommendation in the 2009 risk assessment of SmartStax to evaluate dose without a density-dependent adjustment and use the non-adjusted dose profile for each *Bt* toxin: Cry34/35Ab1 (94.2%), Cry3Bb1 (97.5%), and Cry34/35Ab1/Cry3Bb1 pyramid (98.2%).
Density-dependent mortality estimates were calculated from field studies conducted under optimal field conditions that the Panel considered unrealistic and therefore the density-dependence mortality was overestimated. The Panel concluded there is no statistically credible method to distinguish between 94% and 99% dose mortality because the dose mortality profiles are poorly estimated. Both the unadjusted and the density-dependent adjusted dose mortality profiles have very high variances. The Panel recommended that the CRW models be rerun using the non-adjusted dose profiles (and lower values) and that the re-analysis consider the variance of dose mortality profiles using first order Monte Carlo simulations.

Charge Question 6

Northern and western corn rootworm studies have shown that male emergence in 5% seed blends can be variable and may be up to 60 times lower compared to emergence in non-Bt plots (Data submitted by Monsanto). This information was not included in any of the models used in the SmartStax seed blend analysis. The SAP (2009) concluded that a reduction in the number of males from Bt seed blends could have a negative impact on the effective refuge. BPPD is concerned with the potentially negative effects a reduction in male emergence might have on product durability.

Please comment on the potential effects of lowered male emergence of Northern and Western corn rootworm on the durability of the seed blend and whether this information should be incorporated into the risk assessment.

Panel Response Summary

The Panel expressed concern for the potential effects of lowered male emergence and recommended inclusion of these data in risk assessment. Existing data regarding adult male emergence are limited and highly variable. The Panel recommended further studies to gather additional data on male and female emergence ratios and consideration of the impacts of beetle fitness and emergence timing on mate selection and overall mating.

Part B: Modeling of Resistance Evolution

Charge Question 7

The durability of the proposed 5% SmartStax seed blend strategy was compared to the durability of a 5% structured refuge for lepidopteran and corn rootworm target pests. Monsanto developed a deterministic three locus model for ECB/SWCB and Dow created a stochastic two locus model for CRW. Separate analyses were conducted using EPA/ORD's two locus and three locus deterministic, probabilistic model to estimate the risk of resistance evolution with a 5% seed blend and structured refuge. The applicants and EPA/ORD each made conservative assumptions, though of differing degrees, for parameters determined to be sensitive in the models. For example, more conservative initial resistance allele frequencies and fitness assumptions significantly lowered the time to resistance in EPA/ORD's model for ECB and SWCB. In Monsanto's modeling of ECB and SWCB, a greater degree of dispersal between compliant and non-compliant fields significantly affected the estimated time to resistance.

Please comment on the appropriateness of the assumptions and inputs used for the following parameters in the Monsanto, Dow, and EPA/ORD models:

- *Initial resistance allele frequency for single traits Cry1A.105, Cry2Ab2, Cry1F, Cry34/35Ab1, and Cry3Bb1 for all modeled pests;*
- *Survival/fitness for all modeled pests; and*
- *Dispersal for ECB and SWCB as modeled by Monsanto and EPA/ORD.*

Panel Response Summary

The Panel pointed out the importance of being mindful of the basic differences in model structure among the Monsanto, Dow, and EPA/ORD models because these differences influence whether certain parameters are present in the model and the range of potential durabilities that can be model outcomes. Assumptions about model structure are in many respects more critical than the parameter values themselves. Model structure involves the equations that characterize dynamic processes, embody assumptions about causality, and specify the parameters that can be quantified. The assumptions and data used to estimate parameter values are subsequent to choices about model structure. In relation to the discussion of parameter values specified in the Charge Question, the Panel addressed structural issues related to the parameters as well as the estimation of the parameter values themselves. An additional discussion about model structure is also available in the response to Charge Question 9.

1) **Initial Resistance Allele Frequency for the Single Traits (Cry1A.105, Cry2Ab2, Cry1Fa, Cry34/35Ab1 and Cry3Bb1) for all Modeled Pests (all models).** The Panel concluded that the initial resistance allele frequencies chosen for Cry1A.105, Cry2Ab2, and Cry1Fa were appropriate for both ECB and SWCB as "best guesses" in the face of limited information. Therefore, some are possibly overestimates and hence may underestimate durability. For WCR and NCR, the initial resistance allele frequencies for Cry34/35Ab1 and Cry3Bb1 were probably underestimates of the actual values and probably overestimate durability. For WCR and NCR the Panel suggested that a different model structure may be more appropriate for assessing resistance development because resistance will likely be determined by multiple loci acting with small effects, not a single major locus. In this situation a quantitative genetics model might be a better model structure. The Panel noted that there is likely to be substantial geographical variation in allele frequencies, and therefore initial resistance allele frequency should not be treated as a single value for a species. Because resistance could develop locally from initially high local allele frequencies and then spread geographically, worst-case scenarios must be used that assume high initial allele frequencies.

2) **Cross-Resistance Potential (Monsanto and EPA/ORD).** Cross-resistance is a special case of the more general problem of estimating survival/fitness values, and the Panel focused on the lepidopteran-active toxins because cross-resistance may greatly reduce durability of SmartStax for targeted lepidopterans. In general, there is considerable evidence to support hypotheses of cross-resistance, especially between Cry1A.105 and Cry1Fa in many insect species. The Panel concluded that there is some evidence of partial cross-resistance of Cry1A.105 and Cry2Ab2 with a Cry1Fa resistance allele in ECB. The Monsanto and EPA/ORD models assumed that there would be no cross-resistance and consistently treated Cry1A.105 as a unique protein involving a novel mode of action with no cross-resistance between Cry1A.105 and each of its component toxins, Cry1Fa and Cry1Ac. The assumption of no cross-resistance would overestimate the durability of SmartStax corn. The Panel agreed that the potential for cross-resistance should be considered in any model.

3) **Survival/Fitness for all Modeled Pests.** The Panel concluded that the fitness values used in the ECB, SWCB, WCR, and NCR models were underestimated for the heterozygotes. For ECB and SWCB potential epistasis among resistance loci was insufficiently examined. The Monsanto and EPA/ORD models ignored density-dependent mortality and complex selection associated with corn kernels. In addition, the Monsanto model did not model between-plant movement of larvae in a way that addresses the risks to resistance evolution. The Panel noted that none of the parameters for a quantitative genetics model for WCR or NCR have been estimated. All of these factors will overestimate the durability of SmartStax.

4) **Dispersal for ECB and SWCB as Modeled by Monsanto and EPA/ORD.** The Panel concluded that adult dispersal for ECB and SWCB would likely randomly mix the adults within fields of SmartStax seed mixtures. Nonetheless, this does not rule out the possibility of non-random mating; for example, timing of adult emergence could lead to resistant individuals having a greater chance of mating with each other. Furthermore, there could be non-random oviposition in seed mixtures; for example, damage to non-*Bt* plants could increase relative oviposition rates on *Bt* plants. In these examples, non-random mating and oviposition would speed resistance evolution. Therefore, seed mixtures do not rule out the possibility that non-random mating and non-random oviposition decrease durability.

Charge Question 8

EPA/ORD encountered challenges in the lepidopteran modeling with partitioning non-multiplicative interactions that occurred between more than two resistance genes since the mortality caused by each locus was not independent. With two gene pyramids this non-additivity can be assigned to the single two locus interaction, but in a three gene pyramid there are three possible two locus interactions. In the absence of data, this non-additivity was partitioned equally among the three two locus interactions. As more than two Bt genes are pyramided, this problem will have to be addressed so that resistance evolution in the target pests to these products can be more accurately simulated.

Does the Panel have any recommendations for distributing non-multiplicative interactions in models to evaluate multi-gene pyramided products?

Panel Response Summary

The Panel expressed concern with the way in which survivals to different single toxins are combined to calculate genotype-specific survival on the pyramided plants in the models of resistance evolution. The Panel emphasized that the way in which survival of different genotypes are combined has a large impact on the predictions that all models make about the durability of pyramided crops. It appeared to the Panel that the Monsanto and EPA/ORD models combined survival rates in ways that generated low heterozygote survival, thereby overestimating durability of SmartStax in all simulations. Information on the survival of the genotypes conferring resistance to one or multiple *Bt* toxins for the target pests of SmartStax is not available, causing large uncertainties in the predictions of any model of resistance evolution.

While theory can suggest ways in which survival to single toxins might be combined to generate survival to multiple toxins, empirical information is sparse (Appendix 2: **High and Low Dose Scenarios and Larval Movement in Seed Mixtures**).

When there is larval movement among plants in seed mixtures, it is necessary to know the survival rates of all insect genotypes both before and after movement. Thus, more information is needed than just the survival of different genotypes on *Bt* plants, as emphasized in the previous paragraph. The concern from the previous paragraph must be addressed for the two or more larval stages before the larvae move and after they move (stage-specific survival rates). In addition to generating genotypic survival rates to multiple toxins for each larval stage, the stage-specific survival rates must be combined across stages. These stage-specific survival rates may be combined multiplicatively if these survival events associated with each stage are independent. All of the models the Panel examined combine the stage-specific survival rates multiplicatively. However, while this might be a reasonable assumption in the absence of empirical evidence, other possibilities should be explored, and to the knowledge of the Panel, this topic has not been investigated theoretically.

Non-multiplicative ways in which the resistance of different loci can combine to determine the stage-specific or total immature survival of all genotypes to multiple toxins are forms of epistasis. Specific mechanisms by which epistasis can occur include, but are not limited to: 1) constitutive, low-level expression of Cry-proteases, 2) developmentally restricted expression of low levels of Cry-protease, 3) genes regulating expression of receptor genes, and 4) cross-resistance. These and other forms of epistasis should be investigated as mechanisms that cause non-multiplicative survival rates of the multiple locus genotypes. The consequences of different forms of epistasis on resistance evolution should be evaluated.

Charge Question 9

Based on a review of the submitted simulation modeling, the preliminary conclusions are:

1) *For CRW, a 5% seed mixture and a 5% structured refuge had comparable durability in both the EPA and Dow models;*

2) *For ECB, a 5% seed mixture was less durable than a 5% structured refuge in simulations with EPA's model. However, ECB resistance did not evolve within 158 generations in any of the simulations with the 5% seed mixture, similar to the level of durability predicted by Monsanto's model. There was no difference in durability between the 5% seed mixture and the 5% structured refuge in Monsanto's model. Resistance did not evolve to either refuge option within 100 generations (the extent to which the model was run);*

3) *For SWCB, a 5% seed mixture was less durable (78 generations) than a 5% structured refuge (118 generations) in EPA's model simulations. Conversely, with Monsanto's model there was no difference in the prediction for durability between the 5% seed mixture and the 5% structured refuge. Resistance did not evolve to either refuge option within 100 generations (the limit of the model simulations).*

Please comment on the reliability of the estimates of resistance evolution by each of the three models in light of the biological and parameter uncertainties identified by BPPD.

Panel Response Summary

The Panel considered the durability of the 5% SmartStax seed mixture given the available biological, ecological, and genetic information, and IRM modeling for ECB, SWCB, and CRW. Due to the uncertainties associated with the Monsanto, Dow, and EPA/ORD modeling efforts, the Panel concluded that there was an insufficient scientific basis for supporting the SmartStax RIB 5% seed mixture as an effective IRM strategy for all pests of concern. For ECB and SWCB, the 5% SmartStax seed mixture may lead to more rapid resistance evolution than 5% structured refuges.

SmartStax corn is a multi-toxin double pyramid in which there are three *Bt* toxins targeting lepidopteran stalk-boring (and ear feeding) pests (Cry1A.105, Cry2Ab2, and Cry1Fa) and two *Bt* toxins targeting corn rootworm (Cry34/35Ab1 and Cry3Bb1). The Panel concluded that a 5% SmartStax seed mixture would have comparable durability to SmartStax planted with a 5% structured refuge for CRW resistance management. However, the Panel stated that resistance management for a pyramid should focus on the pest(s) with the greatest likelihood of resistance in a seed mixture compared to a structured refuge. In this case, the Panel was more concerned for the evolution of resistance by ECB and SWCB than CRW because of the difference in selection intensity to a high-dose versus a low-dose of *Bt* toxins expressed in SmartStax corn. As stated in the 1998 EPA SAP report on *Bacillus thuringiensis (Bt)* Plant-Pesticides and Resistance Management (EPA SAP 1998) and in the scientific literature (e.g., Mallet and Porter 1992; Davis and Onstad 2000), for high-dose cases when toxicity of the cultivar causes low survival of heterozygous pest individuals, seed mixtures will have lower durability than structured refuges with the same percentage of *Bt* plants. This point was illustrated by the modeling exercise performed during the meeting (see Appendix 2). There are also greater uncertainties for seed mixtures than for structured refuges due to the lack of information about larval movement for ECB, and SWCB, and how larval movement affects the survival of heterozygotes.

The Panel was also concerned about maintaining susceptible populations if seed mixtures are planted over a wide geographical area. This problem is much greater for 5% SmartStax seed mixtures than for 5% structured refuges due to the mortality caused when larvae move from non-*Bt* to *Bt* plants in seed mixtures. Simply, IRM depends on the persistence of susceptible insects, and if broad adoption of seed mixtures threatens the regional persistence of the pest, then the risk of resistance evolution may increase greatly.

The Panel concluded that the Monsanto, Dow and EPA/ORD models all contain attributes that could lead to overestimates of the durability of the 5% SmartStax seed mixture, especially for SWCB. The Panel noted that modeling resistance durability involves high levels of uncertainty, and interpreting model results must be done in light of this uncertainty. Several areas of uncertainty were highlighted: decision model uncertainty, model completeness, and parameter uncertainty.

The Panel recommended four areas that needed further theoretical investigation to assess and design IRM strategies for SmartStax seed mixtures.

1) Modify the Monsanto, Dow, and EPA/ORD models to remove the attributes that likely lead to overestimates of durability, or develop new models that more accurately assess durability;

2) Investigate the integration of IRM strategies with adaptive IRM management approaches that include well-defined triggers for taking remedial actions and clearly identify the most appropriate remedial actions;

3) Model IRM at the regional scale to account for risks of resistance evolution that may result from long-term, region-wide suppression of pest population densities; and

4) Investigate the incremental introduction of products such as the 5% SmartStax seed mixture, which have high levels of risk uncertainty, especially for lepidopteran pests. This incremental approach would decrease the percent of non-*Bt* in a seed mixture based on coupling monitoring for resistance and population density.

DETAILED RESPONSES TO CHARGE QUESTIONS

Part A: Biology of European Corn Borer, Southwestern Corn Borer, Corn Earworm, and Corn Rootworm

Charge Question 1

European corn borer (ECB) has both local and long distance dispersal capability. Currently, the proportion and frequency of individuals in a population engaging in dispersal before or after mating is unclear. While it has been established that ECB mate in aggregation sites near cornfields, mark-release-recapture studies in the U.S. have typically had a low recapture success (<1%). Recently it was suggested that 1-day old female ECB may engage in obligate pre-mating dispersal (Dorhout et al. 2008).

Please comment on the uncertainties regarding ECB movement including mating sites, pre-mating dispersal, and the proportion of the population engaging in long-distance dispersal. How might these aspects of ECB movement affect a potential seed blend strategy?

Panel Response

The Panel concluded that a comparison with empirically fitted parameters cannot be made to estimate the effect of seed mixtures on resistance evolution because of the challenges involved in filtering through the known causes of variation and sources of uncertainty concerning ECB adult dispersal to construct a reasonable "worst-case" scenario for between-field refuges. Major sources of uncertainty about adult dispersal include: dispersal by susceptible and resistant ECB, sex-specific movement, pre-mating vs. post-mating movement, mating biology and sexual selection, wind and weather events. Known factors generating variation in dispersal include: sex pheromones, humidity, geographic and seasonal variation, diurnal cycle, and age of the adult.

Theoretical considerations indicate that a seed mixture strategy will increase the rate of resistance evolution compared to the present between-field refuges. In general, greater male movement will tend to delay and greater female movement will tend to speed up the rate of resistance evolution, and that for ECB and SWCB, intermediate rates of adult dispersal will have the slowest rate of resistance evolution. The effect of a seed mixture would be to increase the rate of adult dispersal between *Bt* and refuge plants, thereby increasing the rate of resistance evolution.

The quantitative increase in evolutionary rate can be evaluated theoretically. The Panel suggested that to compare the rate of resistance evolution between seed mixtures and structured refuges, resistance evolution for the structured refuges should be determined for adult movement rates that minimize the rate of resistance evolution. Additional investigations to reduce uncertainties are also required and research on mating biology and sexual selection should be encouraged.

Theoretical Expectations

Models of resistance evolution for high dose-refuge events generally find that the rate of evolution is slowest for intermediate levels of adult movement (Comins 1977; Caprio 2001; Ives and Andow 2002; Storer et al. 2003; Sisterson et al. 2004). Evolution is fastest when adults do not move very much from their natal habitat or when they almost always move from their natal habitat, but it is slowest at intermediate rates of leaving.

The reasons for this are not fully understood, but the most compelling explanation is based on the contrasting effect of male and female movement (Ives and Andow 2002; Hu 2008). The Panel discussed how simple patch models illustrate the contrasting effects of female and male movement out of the natal field and its impact on the rate of resistance. When females move more, resistance evolution speeds up. When males move more, resistance evolution is slower. Thus, when both sexes move at intermediate rates, a balance is struck between the speeding up caused by females and the slowing down of resistance evolution caused by males. Female movement speeds up resistance because females produce the offspring that can be selected for resistance. Under the high-dose assumption, most of the adults emerge from refuge habitats. Females that stay in the refuge will have offspring in the refuge, and these offspring cannot be selected for resistance. Females that leave the refuge may return to a refuge, but many will end up in a *Bt* habitat. Offspring produced in the *Bt* habitat will be selected for resistance. Hence the more females move, the greater the proportion of offspring that will be selected for resistance, and the faster the rate of evolution.

Male movement slows down resistance because males disrupt RR homozygote genotypes in *Bt* fields. Under the high dose assumption, most of the (few) adult RR genotypes will occur in the *Bt* fields. As indicated previously, most males will emerge from refuges. Males emerging from refuges will typically be SS genotypes. When these males leave, many will end up in *Bt* fields. These males compete with the (few) RR males emerging from the *Bt* fields for the (few) RR females. If there are sufficient males from the refuge and they have equal or superior intra-sexual selection coefficients, then most offspring produced from RR females will be RS heterozygotes, which are susceptible to *Bt*.

At extremely low rates of adult movement these relationships break down, and resistance evolution becomes very fast. This happens because there is so little movement that there is virtually no dispersal between the refuge and *Bt* habitats. Thus, evolution in the *Bt* habitats occurs as if there was no refuge.

In contrast to patch models, spatially-explicit models include movement distance. In other words, movement distance is ignored in patch models, and these models assume that adults disperse far enough that they can reach any field type in proportion to the area of the field type. Spatially-explicit models produce results somewhat different from patch models when all adults have highly restricted movement distances, such as movement only to the adjacent field (nearest neighbor dispersal). Additional theoretical results with these spatially-explicit models demonstrate that if only 2% of the adults move a long distance (and all others move only to the nearest neighbor dispersal), results are identical to those obtained from the patch models. In other words, if only a small proportion of ECB and SWCB adults move several kilometers, then

movement distance is an insignificant factor in resistance evolution, and can be ignored. This will be addressed in the next section of the response to this question.

One of the key assumptions for the high-dose refuge strategy was that there must be random mating for IRM to be successful. This is not entirely true. A distinction must be made between local mating and global mating. Local mating occurs in a specified area that is a part of the geographic extent of the whole population. Such an area could be a crop field for ECB or SWCB. Global mating occurs over the entire geographic extent of the population, and comprises many local areas (crop fields).

Nearly all of the IRM models assume that there is local random mating and this is a critical assumption. If there is local (non-random) assortative mating, resistance evolution can be quite fast. Local random mating means that in the local environment (crop field), each female mates with any of the locally available males with equal probability, and each male mates with any of the locally available females with equal probability. Obviously mate choice and sexual selection violate local random mating and could generate novel evolutionary dynamics

Global non-random mating is typically associated with slower rates of resistance evolution than global random mating, which is a counter-intuitive result. Global random mating means that each female mates with any male in the entire population with equal probability and each male mates with any female in the entire population with equal probability. This can occur if all adults disperse from their natal sites and settle randomly in fields (including their original natal field) before mating. If some adults do not disperse from their natal sites, there will be global non-random mating. When there is some global non-random mating, there will be some assortative mating among fields, with RR x RR matings more likely in *Bt* fields than in the population at random. This should result in faster resistance evolution. However, because intermediate levels of movement produce the slowest rates of resistance evolution and some global non-random mating must be associated with intermediate levels of movement, the effect on resistance evolution will be determined by the balance between these forces (intermediate dispersal slowing it down and global non-random mating speeding it up). Ives and Andow (2002) showed that for high-dose events, the effect of global non-random mating is much weaker than the effect of intermediate levels of dispersal, and in all cases, the effect of global non-random mating had insignificant effects on the rate of resistance evolution. Thus, some may (wrongly) conclude that any factor (e.g., seed mixes) that increases dispersal to 100% will reduce the rate of resistance evolution because global mating becomes random. In actuality, the opposite is true, and seed mixes are expected to speed up resistance evolution compared to refuges in different fields.

Movement Distances of ECB and SWCB

In general, Panel members agreed that sufficient ECB and SWCB disperse far enough that spatially-explicit models were not necessary for understanding resistance evolution for high dose-refuge systems. This suggests that for these two species, intermediate rates of adult dispersal will have the slowest rate of resistance evolution. If there are sex-specific differences in adult movement, high male dispersal and low female dispersal would delay resistance

evolution the most. Conversely, low male dispersal and high female dispersal would result in the fastest resistance evolution.

The Panel discussed research studies concerning dispersal distances of ECB and SWCB. Research on dispersal of ECB provides somewhat conflicting evidence related to the spatial scale of movement. Chiang (1972) showed that when ECB first invaded Minnesota, it spread across the state at a rate of 100 km/yr, implying a high rate of dispersal of 50 km per generation. Showers (1993) examined the spread of a persistently introduced deleterious genotype, and found that it occurred as far away as 32 km from the release area. Chiang et al. (1965) found that during the spring large numbers of adults migrate from central Iowa to southern Minnesota, a distance of 150 km. These dispersal estimates indicate that a small proportion of adults disperse long distances. This long distance movement may be insufficient to delay resistance evolution on its own, but it is sufficient to imply that patch models are adequate for understanding resistance evolution and explicitly spatial models are not essential.

Mark release recapture (MRR) studies by a number of researchers (Legg 1983; Showers et al. 2001; Bailey et al. 2007; Reardon et al. 2006) have been used as evidence for long-distance dispersal of ECB. The results of these studies indicated that the actual distances marked individuals moved were typically much less than 7 km. Based on many studies concerning low recapture rates of marked individuals, it can be inferred that low recapture rates are common. However, the reported rates of recapture for ECB are not low compared to other insect species. Many factors influence recapture rates. For example, capturing or rearing individuals, handling and marking them, and release procedures may have considerable influence on the subsequent behavior of marked individuals such that their normal behavioral sequence is disrupted and their recapture probability is reduced. Thus, low recapture rates do not indicate that long-range dispersal occurred. MRR results are consistent with long-range dispersal of ECB, but the observations cited in the previous paragraph provide stronger evidence for small amounts of long-range dispersal of ECB.

Compared to ECB, there is considerably less research on movement of SWCB. Where the species co-exist, there are anecdotal observations that SWCB is equal to or less dispersive than ECB (Guse et al. 2002; McCauley et al. 1995). SWCB is also an invasive species, originating in Mexico. It spread eastward at rates between 20-55 km/yr (Fairchild et al. 1965) or 10-27 km/ generation, which is considerably slower than ECB. Additional evidence of long-range dispersal comes from pheromone traps, which are highly attractive to males from considerable distances (Goodenough et al. 1989). Thus, it is likely that there is sufficient long distance dispersal that spatially-explicit models are not essential for understanding resistance evolution in this species.

Rates of Evolution for Within-Field vs. Structured Refuges

Compared to between-field refuges, the effect of within-field refuges is to increase the dispersal rates of adults from both *Bt* and non-*Bt* habitats. This is because the probability of leaving a habitat is inversely related to the size of the habitat. A within-field refuge is essentially a fine scale mixture of very small units of *Bt* and non-*Bt* habitat. Block refuges within fields would require adults to move across a field before they had dispersed from their natal sites (perhaps as much as 1 km). Strip refuges would require adults to move a few rows before they had dispersed

from their natal sites (perhaps as much as 100 meters). Seed mixtures would require adults to move only a few meters before they had dispersed from their natal sites (perhaps less than 10 meters). Female ECB always move more than 10 meters after eclosion and before mating and oviposition, so for seed-mixtures, dispersal rates will be 100%, and greater than for between field refuges.

The main consequence is that seed mixtures will have faster resistance evolution than between field refuges, *ceteris paribus*.

How Much Faster is Resistance Evolution?

The Panel concluded that no direct empirical comparison can be made to estimate the effect of seed mixtures on resistance evolution because of the challenges involved in filtering through the known causes of variation and sources of uncertainty concerning ECB adult dispersal (see lists below) to construct a reasonable "worst-case" scenario for between-field refuges. Lacking this information, the Panel suggested that the relative rate of resistance evolution between seed mixtures and between-field refuges could be estimated by specifying intermediate adult movement rates for between-field refuges that minimize the rate of resistance evolution and then comparing the projected evolutionary rates to those of 100% adult dispersal (seed mixtures).

Although this worst-case scenario is equivalent to a case where *Bt* and non-*Bt* crops are planted in the same fields every year (not rotated), in the absence of additional information, it is an appropriate baseline comparison. First, even when fields are rotated, there are two sequential generations each year without rotation. Thus, the worst-case scenario is at least half realistic. Second, when fields are rotated there may still be spatial autocorrelation with fields near a previous *Bt* field more likely to be *Bt* the following year. This spatial autocorrelation will give lower dispersal between field types. Finally, the appropriate physical scale of the relevant spatial autocorrelation is not known. This will depend quantitatively on the adult dispersal kernels for males and females. These dispersal kernels will be costly to acquire, so investigating a worst-case scenario may alleviate the need for additional expensive experiments.

Factors Generating Variation in Dispersal

1) **Sex Pheromone.** Multiple males can be attracted from hundreds of meters away to single calling females (based on MRR data and dispersal from known sources). Pheromone sources within 80-90 meters of each other probably compete for males.

2) **Humidity.** An ECB adult that finds itself in a habitat with low relative humidity (RH) will leave that habitat in search for a habitat with higher RH. This movement probably occurs during the night or early morning because during the day, it is difficult to find adults in drier habitats (during the day they are in humid, so-called aggregation sites). Adults have been observed to disperse hundreds of meters to locate a suitable moist habitat to stay in during the day.

3) **Geographic and Seasonal Variation.** Known geographical and seasonal differences in adult dispersal have been linked to variation in daytime RH in the habitats in the

landscape. There are probably other factors causing geographic and seasonal variation in dispersal, but these are poorly known. For example, there is a rainfall gradient that results in wetter summers in the eastern part of the North American range of ECB and drier summers in the western part of the range. In addition, the northeastern Corn Belt is wetter than the southwestern Corn Belt. Nebraska, Kansas and South Dakota and parts of Iowa are dry and ECB is typically found during the day only in the so-called aggregation sites throughout the entire growing season. There is some evidence that ECB will move to the irrigated parts of fields in these dry areas. ECB are typically found in daytime aggregation sites during the first generation throughout its geographic range. This is because corn is too short to provide sufficient moisture during the day. It is not clear how far adults will move to find aggregation sites, i.e., do they pass over suitable sites and disperse long distances or do they tend to go the closest site. Dispersal of later generations appears to vary considerably by geographic region. For example, in Pennsylvania, North Carolina, and Maryland adults are typically found in corn fields during the day. This is probably also true for Minnesota. At these locations, the RH inside corn fields during the day can be quite high providing a suitable daytime habitat for ECB.

4) **Diurnal Cycle.** Adults can be observed dispersing from daytime resting sites at dusk. During the early part of the evening, females have been observed drinking water and ovipositing. Ovipositing females lay 1-3 egg masses in a night. Later in the night, often starting around midnight, females will release sex pheromone to call for males. It is not known how frequently an individual will call in her lifetime, and it is not known if the same individual will oviposit and call in the same night. Sometime later and as necessary, adults will seek high humidity habitats to stay during the day. While mating has been observed in the aggregation sites, it is not clear where most of the mating occurs.

5) **Age of Adult.** Adults emerge from pupae either during the early evening (typically before midnight) or the early morning (typically after dawn). About 50% emerge at each of these times, and the sex ratio of emerging adults is similar at both times. Males emerge several days before females. After emergence, adults spend about 2 hours hardening up their cuticle and wings. Females will disperse from their emergence site typically after midnight and do not release pheromone prior to this dispersal (for night emerging moths). Day emerging moths presumably disperse soon after hardening their wings, but this is not clearly known. Dorhout et al. (2008) suggested that it is possible that ECB has an obligate pre-mating long-distance dispersal. Flight mill data tend to over-estimate willingness to fly, flight durations and flight distances; therefore, interpretation of these data should be confirmed using independent means.

Major Sources of Uncertainty about Adult Dispersal

1) **Dispersal by Susceptible and Resistant ECB.** There is no information whether susceptible and resistant ECB emerging from non-*Bt* plants would exhibit similar dispersal, pheromone calling and response, and mating behavior. Until resistant ECB are found, it will not be possible to evaluate this.

2) **Sex-Specific Movement.** Rather little data exists on sex-specific movement of ECB. Although some data may be said to indicate that males are more dispersive than females, a sounder conclusion is that the two sexes are equally dispersive, and perhaps males are more dispersive than females. Because sex-specific dispersal is likely to depend on the environment, such as relative male and female density, and nearby food and oviposition resources, it may take some effort to understand the conditions under which sex-specific dispersal could disrupt resistance management.

3) **Pre-mating vs. Post-Mating Movement.** Relatively little theoretical work has been done on the influence of pre- and post-mating movement, so there are no clear expectations for how these affect resistance evolution. Some recent theoretical investigations suggest that there is little difference in the effect of pre- and post-mating female movement, but other investigations have reported significant effects. The reasons for the different theoretical results are not yet clear. However, little data are available to characterize pre- and post-mating dispersal in ECB. It is clear, however, that both sexes disperse before mating and females disperse after mating. The relative movement rates have not been quantified.

4) **Mating Biology and Sexual Selection.** Although technically not a dispersal factor, the interaction between mate selection, dispersal and survival selection may have significant effects on the evolution of resistance. Sexual selection (including intra-sexual and inter-sexual selection, also known as mate competition and epigamic selection) is known to have significant effects on evolution. In the resistance evolution literature, mating has been incorporated by assumed random or non-random mating. However, a behavioral evolution perspective has been missing. This perspective can generate conditional assortative and disassortative mating that is not possible to incorporate simply through assumptions of non-random mating.

5) **Wind and Dispersal.** Sustained periods of prevailing winds and acute wind events (e.g., passage of cold fronts) might disperse ECB and SWCB long distances. Although the dispersal of ECB in the spring from Iowa to Minnesota probably was assisted by the warm prevailing spring winds (Chiang et al. 1965), the role of winds in long distance dispersal is poorly understood, and its potential influence on resistance evolution remains to be evaluated.

Charge Question 2

Scientific Advisory Panels (1998 and 2000) discouraged the Agency from the use of Bt seed mixtures to control lepidopteran target pests because substantial larval movement could be expected between Bt and non-Bt plants which could lead to more rapid selection of resistance. BPPD has reviewed new data developed by Dow and Monsanto simulating the effects of SmartStax on various instars of potentially mobile Lepidoptera. These data provide evidence that SmartStax is highly toxic to 1st, 2nd, and 3rd instars. But, there was greater survivability among 4th and 5th instars. While it has been established that ECB disperse as neonates, there is some uncertainty with respect to lepidopteran propensity for dispersal off non-Bt plants as later

instars. BPPD notes that simulation models incorporating data on high larval mortality on SmartStax plants have (in some cases) predicted that seed blends may be as durable as structured refuges.

Please comment on ECB larval plant-to-plant movement including uncertainties about late-instar movement and the potential effect on the durability of a seed blend strategy.

Panel Response

The Panel considered the ecological and evolutionary context of ECB larval movement in answering this question. Based on the known behavior and ecology associated with ECB larval movement, the Panel developed several plausible ECB larval movement hypotheses that would be expected to increase the rate of resistance evolution (by increasing the fitness of heterozygotes) via one of the four general larval movement scenarios applicable to many species. The uncertainty about late-instar movement has been overstated by Dow and Monsanto as late instar ECB exhibit considerable movement.

The Panel concluded that there were insufficient data to parameterize these specific ECB hypotheses and evaluate the four scenarios empirically to quantify the durability of the seed mixture strategy. Remaining significant uncertainties include: genotype-specific mortality (especially heterozygote mortality) of moving and sedentary larvae, rate (or percent) of larval moment, and other aspects of fitness of survivors moving from non-*Bt* plant to *Bt* plants and vice versa.

Because of these uncertainties, the Panel recommended that the ECB larval movement hypotheses and the general larval movement scenarios be evaluated using specifically designed theoretical evolution models. The Panel examined in detail the structure and results of the Monsanto model and the EPA/ORD model, and found that these models did not evaluate or did not clearly evaluate any of the four general larval movement scenarios or any of the detailed ECB hypotheses that could lead to reduced durability of the seed mixture. The durability of a 5% SmartStax seed mixture as projected by both the Monsanto and EPA/ORD models is overstated, and may be overestimated considerably because these plausible ECB larval movement hypotheses have not been considered.

General Larval Movement Scenarios Applicable to Many Species

A seed mix may cause faster resistance evolution if larvae move among plants in such a way that RS heterozygotes have higher survival than when they are restricted to feeding only on *Bt* plants. This increases the heterozygosity of resistance so that resistance is less recessive (or more dominant). Four larval movement scenarios are identified that could increase heterozygosity and thereby speed up resistance evolution. These four scenarios are not mutually exclusive, but they could be readily incorporated into resistance evolution models, such as the EPA/ORD model.

1) **The BNI Scenario.** This scenario proceeds as follows: the RS heterozygote hatches on a *Bt* plant, feeds a little bit, but before it dies, it moves to a non-*Bt* plant, where it completes development. Individual probability of survival of an RS heterozygote must be higher

than for an SS susceptible larva undergoing the same scenario. With a higher survival, heterozygosity increases. This is labeled the BNI scenario because a larva moves from a *Bt* plant to non-*Bt* plant with a difference in individual probability of survival.

2) **The NBI Scenario.** This scenario proceeds as follows: the RS heterozygote spends its early life on a non-*Bt* plant, and late in life it moves to a *Bt* plant, where it completes development. Individual probability of survival of an RS heterozygote must be higher than for an SS susceptible larva undergoing the same scenario. With a higher survival probability, heterozygosity increases. This is labeled the NBI scenario because a larva moves from a non-*Bt* plant to a *Bt* plant with a difference in individual probability of survival.

3) **The BNP and 4) NBP Scenarios.** Two additional scenarios focus on average population survival rates under the assumption that there is no difference in individual survival probability between RS and SS larvae. The BNP scenario proceeds as follows: the RS heterozygote larvae have a greater propensity to move from a *Bt* plant to a non-*Bt* plant than the SS susceptible larvae. Thus even though the RS and SS larvae that move have the same individual survival rate, because more RS larvae move, the average survival rate of the RS larvae is higher than the average survival rate of the SS larvae. This is because the average is taken over both the larvae that move and the larvae that do not move. Hence, and heterozygosity increases. This is labeled the BNP scenario because a larva moves from a *Bt* plant to non-*Bt* plant with a difference in average population survival. Analogously, the NBP scenario requires that the probability of moving from a non-*Bt* plant to a *Bt* plant is lower for a late instar RS heterozygote than an SS larva. In this case a greater proportion of SS larvae become exposed to a *Bt* plant, which reduces their average survival compared to the RS heterozygote larvae, and heterozygosity increases. This is labeled the NBP scenario because a larva moves from a non-*Bt* plant to a *Bt* plant with a difference in average population survival.

Other General Larval Movement Scenarios

There are also many scenarios by which larval movement may have limited effect on resistance evolution (Glaum et al. 2011). Movement of SS larvae from Bt plants can reduce the selective pressure on the population, sufficiently to counteract the effects of increased heterozygosity. In addition, genotype specific feeding behavior can equalize the probability of survival of RS and SS larvae. For example even if the RS heterozygote has higher individual probability of survival than an SS larva on a fixed dose of Cry toxin, the RS heterozygote may consume more Cry toxin, resulting in a higher dose that causes the same mortality as the smaller amount consumed by the SS larva.

Larval Movement: Empirical Observations

The Panel made several general points concerning larval movement, and specific points concerning ECB movement that are relevant to the four general modeling scenarios described above.

1) Movement by neonate larvae will occur from both *Bt* and non-*Bt* plants.

2) Movement by late instars will be primarily from non-*Bt* to *Bt* plants. This is because early stages will have already died on or dispersed from the *Bt* plants.

3) For ECB and SWCB, density-dependent dispersal and density-dependent mortality may occur on non-*Bt* plants, but are unlikely to occur on *Bt* plants. The number of eggs in a typical egg mass laid on a single plant is greater than the number of larvae that can mature to adults on a plant. These density-dependent effects may mitigate the effect of the BNI and BNP scenarios if enough larvae move to non-*Bt* plants to trigger these effects, although significant mitigation is probably unlikely.

4) There is considerable larval movement of ECB on a plant after the 3rd instar (White and Andow 2007; Schmidt et al. unpublished data). In general, movement appears to be associated with molting.

5) Plant morphology and larval food resources differ substantially between the first and second generation on corn. The first generation is associated with whorl-stage corn, and larvae can feed on young leaves and young stalks. The second generation is associated with anthesis, silking and ear development. During the second generation, larvae have available many food resources, including young leaf blades (in tillers), mature leaf midribs, collars and sheaths, young and mature stalk, tassel, stamens, pollen, ear buds and developing ears, including, silks, kernels, husks, and cobs.

6) The results presented in the docket suggest survival of susceptible 4th and 5th instars of ECB and SWCB is high when they moved from non-*Bt* to *Bt* plants. The results for ECB (RPN-09-075, Head et al. 2009) may underestimate this effect because control larvae had a low rate of survival (42%). This compresses the range of possible survival of treatment larvae to 0-42%, which increases type 2 statistical error. In addition, the low control survival may be indicative of a weak ECB colony (e.g., pathogen infected). Weakened larvae might be more susceptible to the experimental treatments, resulting in artificially low survival. The SWCB dataset may be more reliable because of the high survival rate of control larvae (86%). Thus, some larval stage after the 1st instar is likely to fit the NBI and NBP scenarios.

7) ECB overwinters as diapausing 5th instar larvae in corn stalks. Pre-diapausal activities are indicated by cessation of feeding, voiding the gut and initiating a wandering stage. After they wander they locate a suitable overwintering site (1st generation ECB locate a suitable pupation site after the wandering stage) and become quiescent. Morphologically, diapausing larvae have large fat bodies and appear plump and whiter especially along the dorsal midline. It is possible that a significant portion of 5th instar larvae found surviving on *Bt* plants at the end of the year are in diapause and not feeding. Although their present and future exposure to *Bt* toxin will be virtually nil, each larva does choose a suitable overwintering site in the corn stalk and passes this stalk material through its gut. Thus, all diapausing larvae found in *Bt* plants must be able to survive consumption of some level of *Bt* toxin at the end of the season. In the spring, overwintered larvae become

active. Most must feed on water, and some proportion will feed on the old corn tissue before pupating. Relative survival of RR, RS and SS diapausing larvae on *Bt* and non-*Bt* plants and tissues is not known. In a noctuid pest of cotton (*Trichoplusia ni*), >50% of susceptible 5th instars survived to pupate and ~88% of these pupae eclosed whether the 5th instars were starved or exposed to Cry1Ac/Cry2Ab cotton tissue (Li et al. 2007). The role of movement of diapausing larvae of resistance evolution is not well understood.

Detailed ECB Movement Hypotheses: First Generation Larvae

The Panel described how the movement of first-generation larvae may speed up the evolution of resistance by fitting one of the four scenarios described above. There are a few circumstances under which known aspects of ECB larval movement would likely result in faster resistance evolution.

Egg masses are typically laid on the undersurface of expanded leaf blades, between the arch and the collar. When eggs hatch, larvae hang out near the egg mass to harden cuticle. Some larvae may cannibalize or eat the unhatched eggs or remaining egg chorion. Some larvae commence feeding, probably on leaf hairs, while others start to walk either up or down the undersurface of the leaf blade (most of the behavioral observations described here and subsequent sections are reported in Lamb 1992). Larvae prefer the hairless undersurface to the richly pubescent upper surface of the leaves because the hairs interfere with their walking. If a larva ends up on the upper surface of a leaf blade, it will tend to migrate to the hairless midrib, where it can quickly move. About half of the larvae move up the blade and the other half move down the blade. Those moving down the leaf blade end up crawling into the leaf whorl, where they may initiate feeding. Most larvae will move around inside the whorl, but it is not known how many will leave the whorl for another plant. It is likely that larvae are relatively sedentary during this period, and the BNI and BNP scenarios are not likely to be initiated at this point in larval development. Typically, larvae feed in the whorl through the 3rd instar. Most later instars move out of the whorl, in some cases because the whorl disappears as the plant develops, and these 4th and 5th instars bore into the young stalk. Larvae may move between plants at this time, but rates of movement are not known. If larvae move between plants at this time, the NBI and NBP scenarios could occur.

The neonates moving up the blade end up at the leaf tip, and there they silk off, and dangle to be dispersed in the wind. A significant fraction of these will land on a lower leaf on the same plant. In humid environments, larvae can walk for more than 12 hours without feeding. More larvae will move up and down rows than across rows (Ross and Ostlie 1990), but when considering the distance moved across rows is farther than the distance moved to an adjacent plant in a row, the probability of moving a given distance is similar within and across rows. These larvae cannot be involved in the BNI or BNP scenarios unless they feed before they move. The amount of feeding by neonates before moving is not known.

Recent studies on ECB larval movement with partial Cry1Ab resistance have documented enhanced dispersal of neonate larvae from *Bt* plants and *Bt* diet (Prasifka et al. 2009a, 2009b; Goldstein et al 2010; Moser et al. unpublished data). Partial resistance provides some tolerance to Cry toxins, but resistant homozygotes cannot complete development on a *Bt* plant. Hence, all

genotypes are phenotypically susceptible on a *Bt* plant. Thus, to avoid confusion with major resistance alleles, which is the focus of the response to this question, the Panel designates the partial resistance genotypes with lower case letters: *rr* is a partial resistant homozygote, *rs* is a partial resistant heterozygote, and *ss* is a susceptible homozygote. Goldstein et al. (2010) found that *ss* neonates left *Bt* plants within 24 h ~1.8 times more frequently than non-*Bt* plants. Prasifka et al. (2009a) observed a higher rate of dispersal of *rr* neonates from Cry1Ab plants than *ss* ones, possibly due to heightened mortality of *ss* compared to *rr* neonates following toxin exposure. On *Bt* diets containing *Bt* corn leaf tissue, *rr* neonates moved shorter durations and distances than *ss* ones, and importantly, heterozygous *rs* neonates moved intermediate durations and distances. In addition, *rr* neonates remained on the *Bt* diet longer than *rs* or *ss* ones (Prasifka et al. 2009b). At low concentrations of Cry1Ab, *rs* neonates remained on *Bt* diet longer than *ss* ones. These results have two implications for resistance evolution in seed mixtures. First, they may indicate that the BNP scenario is unlikely because more *ss* larvae may leave a *Bt* plant than *rs* ones. Second they may indicate that the BNI scenario is possible, because *rs* larvae can feed on *Bt* diets when *ss* ones cannot. Both indications, however, must be considered provisional until major resistance can be evaluated.

Major resistance to Cry1Fa has been recovered from a Cry1Fa-selected laboratory ECB colony at the USDA-ARS in Ames, IA. In unpublished results on larval neonate recovery, resistant (RR) and susceptible neonate larvae (SS) dispersed at enhanced rates (95-100%) from pyramided *Bt* plants; whereas, Cry1Fa (RR) resistant individuals had more natural dispersal rates (around 50% of recovered individuals) from single-toxin Cry1Fa expressing plants (Moser et al. unpublished data). Cry1Fa (RS) heterozygotes dispersed at an intermediate rate (around 67% of recovered individuals) off of single-toxin Cry1Fa expressing plants, whereas the recovered susceptible (SS) larvae dispersed at rates near 100% off of the single-toxin Cry1Fa maize (Moser et al. unpublished data).

Detailed Movement Hypotheses: Second Generation Larvae

The Panel stated that the consequences of movement of second generation larvae are more complex than movement of first-generation larvae because of the multiple food sources the larvae have available. This complexity generates a richer behavioral context and there are many more ways, compared to the first generation, by which larval movement can speed up resistance evolution via one of the four scenarios

Egg masses tend to be laid on the undersurface of the leaf blade within 20cm of the leaf collar on mature leaves at or slightly above the primary ear. Egg masses occur less frequently at other sites, but egg masses can be found on lower leaves, on the leaf sheath, on the upper surface of the leaf blade, on tillers, on ear husks and so on. Newly hatched larvae will walk either up or down the leaf blade. Those moving up the blade will disperse by silking off the leaf tip. It is not known if these larvae eat before dispersing. Those moving down the blade and those settling on new plants will encounter the undersurface of the collar and continue walking down the outside of the leaf sheath. This will lead them to the upper surface of the collar of the leaf below their original leaf.

The fate of the neonate larva depends on what awaits them in this collar. If the leaf is an older leaf on the bottom part of the plant, the collar loosely encircles the stem, and the larva tends to crawl inside the collar and down into the leaf sheath, were it will feed on the inside of the leaf sheath. On a slightly higher leaf, but still below the primary ear, the collar is tighter, but an ear bud may force the sheath to expand, creating a small gap that the larva enters. The larva can be found feeding on the tips of the ear bud leaves, which is the morphological homolog of the leaf blade, or on the leaf sheath. Rarely do they feed directly on the developing ear bud. At an ear leaf, the larva will ignore the collar and leaf sheath and either enter the ear from the ear tip or from the ear shank. Rarely does it bore through the husks on the side of the ear. When it enters the ear tip, it will feed on silks until they become too dry, or on developing kernels. A larva enters the silk channel and eats or makes its way to the ear where it feeds on the developing kernels. A neonate and a 2nd instar can be found feeding on a single kernel, often inside the kernel. Older larvae bore between the kernel rows, feeding on multiple kernels. When the kernels become dry, they are much less suitable food for the neonates so older larvae are found in the cob. On leaves above the ear, the neonate larva will typically find a cache of corn pollen in the collar. If the pollen has been moistened, such as from dew, it supports a community of yeasts and other microbes that make it an enticing stew. Larvae feeding on this mixture are typically larger and more active than those without this food. If the pollen is not moistened, it will be blown out of the leaf collars and will be more evenly distributed on the upper surface of the leaf blade. Here tiny clumps of pollen, as small as 0.5 mm in diameter, will support a remarkably diverse group of fungi, which the larvae will eat. The yeasty pollen caches in the collars also attract a diverse group of insects, including minute pirate bugs, which will consume large numbers of neonate ECB larvae in minutes. If the larva makes it to the pollen cache, it buries itself in the cache where it is safe from predation by pirate bugs.

Third instar larvae may be found at these same sites, but many larvae leave these sites and colonize the midrib of a leaf blade. Although it has not been quantified, there is considerable movement between plants, because midrib damage is common on plants showing no signs of early larval feeding. The midrib appears to be a suitable site only for 3rd instars, because it is rare to find other instars in midribs.

Fourth and fifth instars are typically found in tunnels that they bored into the stalk or cob. Some of these tunnels were initiated from the collars, sheaths and ears where the larvae occurred during their younger stages. Many, however, are initiated from new locations and larvae must have moved to make these tunnels. Unlike bark beetles, ECB larvae often abandon their tunnels and move to new sites where they start a new one.

The BNI and BNP scenarios (described above) can occur only if the younger instars feed on the *Bt* plant before they disperse. Collar, sheath, and ear husks and shanks are toxic and the BNI and BNP scenarios could occur from these locations by older neonates and 2nd instars. These tissues typically express high doses of Cry toxin. The pollen caches and silks may have lower concentrations of Cry toxin, which could discriminate between RS and SS individuals (killing the SS and allowing the RS to survive) and would be one BNI scenario.

The kernel is a complex tissue. The pericarp is maternal tissue, the germ is a combination of maternal and paternal genomes, and the endosperm is two parts maternal and one part paternal.

Part of the endosperm is composed of starch and/or sugar, which contains little protein. A larva feeding on a kernel will encounter the pericarp first, followed by the endosperm and lastly the germ. The effect on the larva will depend on the maternal and paternal plant genotypes. If the maternal plant is a *Bt* plant, then all of the kernel tissues will express Cry toxin, although the concentrations may differ among the tissues depending on whether the paternal plant was a *Bt* plant or not. If the maternal plant was a non-*Bt* plant, then the pericarp will not express Cry toxin. If the paternal plant was a *Bt* plant, the endosperm and germ will express Cry toxin, although the concentrations may be less in the endosperm than the germ. If the paternal plant was a non-*Bt* plant, none of the kernel tissues would express Cry toxin. Thus, when cross pollination between *Bt* and non-*Bt* plants occurs, the ear is a mosaic of expressing and non-expressing tissues. Cross pollination will be common in seed mixtures, and uncommon in between field refuges. This mosaic may facilitate the BNI and NBI scenarios (described above), and it may also reduce the effectiveness of the seed-mix refuge.

The NBI scenario is likely to occur during some stages of larval development. Older larvae move several times, so this is not a barrier to the scenario. The high survival rates reported for 5^{th} instar SS larvae on *Bt* plant tissue compared to the low survival of neonates indicates that survival rates increase as the larva matures. Increased survival as development occurs will also likely take place for RS and RR genotypes. These results indicate that the "killing power" of Cry toxin attenuates with larval development. Given the known toxicity mechanisms, this attenuation is likely to be genotype specific, and there will be some stages where RS survival is higher than SS survival on *Bt* plants.

Unlike the other three scenarios discussed above, there is little evidence about ECB larval movement supporting or refuting the NBP scenario (described above). Perhaps it is unlikely or perhaps there needs to be some experiments conducted to determine if ECB larval movement could fit this scenario.

Uncertainty about Larval Movement and Evaluation of Monsanto and EPA/ORD Models

The Panel examined in detail, the structure, parameterization and results of the Monsanto and EPA/ORD models in relation to the potential effect of larval movement on the evolution of resistance. The Monsanto model did not incorporate structural equations to model larval movement and the approximations used in that model are structured to minimize the effect of larval movement on the rate of resistance evolution. Hence this model has overestimated the durability of seed mix refuges (and overestimated the difference between seed mixes and between-field refuges).

The EPA/ORD model did incorporate structural equations to model larval movement, however, none of the scenarios or hypotheses described above were evaluated. Hence, this model has also overestimated the durability of seed mix refuges (and overestimated the difference between seed mixes and between-field refuges). The larval movement scenarios that were assessed with the EPA/ORD model were substantially more sophisticated, realistic, and easy to understand than the assessment provided by the Monsanto model. The Panel concluded that elaboration of the EPA/ORD model would be superior to generalizing the Monsanto modeling approach for

evaluating the evolutionary consequences of the general larval movement scenarios and specific ECB larval movement hypotheses described above.

There are several sources of uncertainty about ECB larval movement. At the highest level of abstraction, there is uncertainty about model structure. Although there is a considerable amount known about ECB larval movement, investigations have not systematically examined larval movement behavior to have some degree of confidence that all movement hypotheses likely to increase the rate of resistance evolution have been identified. For example, it is not known if neonate larvae feed on leaf hairs shortly after ecolosion and prior to dispersal from the plant they hatched out on. Without this information, it is not possible to know if the immediate movement of neonate larvae affects the rate of resistance evolution. Even less is known about movement of SWCB larvae.

At an intermediate level of abstraction, it is not known if any of the processes and parameter values is correlated with each other or if they are independent. For example, the neurological basis of insect behavior suggests that larvae with a greater propensity to move may also have corresponding increases in adult movement behaviors (i.e., higher activity in larvae translates to increased activity in adults, as seen for *Drosophila foraging* and *shaker* genes; Engel et al. 2000; Pereira and Sokolowski 1993; Ueda and Wu 2006; Ishimoto et al. 2005). If movement is governed by a genetic component (such as the homolog of the *foraging* locus) in ECB, selection for enhanced larval locomotion may give rise to correlated movement responses that speed up resistance evolution either by larval or adult movement.

These kinds of correlational uncertainties are difficult to analyze because of there are so many possibilities (there are $n(n\text{-}1)/2$ correlations that need to be evaluated, where n is the number of parameters). The Panel suggested one way to evaluate correlation uncertainty is to identify the correlations that may increase the rate of resistance evolution the most. One way to do this is to parameterize the model with the extreme values and look for parameter combinations that give rapid resistance evolution. This has an advantage of requiring only 2^n simulations, which is the same order as the number of parameter correlations, and provides more informative results than random draws from a parameter distribution, which will rarely evaluate extreme values. Once a short list of parameter combinations are identified, a smaller number of correlations can be evaluated more thoroughly, and once the key correlations have been identified, then it will be possible to randomly sample parameters from a joint probability distribution and to determine if there is a biological basis for the identified correlations.

Finally, there is considerable parameter uncertainty associated with larval movement. In other words, the quantitative value of the larval movement parameters is poorly known for either the first or second generation ECB. This kind of uncertainty can be evaluated through Monte Carlo simulation, such as drawing on known or suspected probability distributions of the parameters as was done with the EPA model. However, it is critical that the models are structured to evaluate the four general larval movement scenarios, and this has not been done.

Charge Question 3

It is typically assumed that, since European corn borer (ECB) and southwestern corn borer (SWCB) are similar in many ways, ECB can serve as a surrogate for SWCB to address uncertainties regarding biology and genetics. The applicants' efficacy data, however, suggest that SmartStax is somewhat less toxic to older instars of SWCB. Results of a larval exposure study by Monsanto showed that SWCB survival was higher than ECB on SmartStax. Should some SWCB larvae disperse as older instars, the rate of adaptation to SmartStax could increase in a seed blend deployment. BPPD concluded that simulation models should incorporate such information in their analyses. There is currently a lack of data on the propensity of SWCB larval plant-to plant movement and on how ECB and SWCB differ in this respect, if at all.

Please comment on the assumption that ECB is a suitable biological surrogate for SWCB and BPPD's concerns that a SmartStax seed blend may affect SWCB differently than ECB.

Panel Response

The Panel agreed that the results from the Monsanto model and EPA/ORD model are inadequate to make any scientifically sound conclusions about resistance evolution in SWCB because they do not include density-dependent mortality, which is an important feature in SWCB population ecology. However, on the broader issue of whether ECB is a reasonable surrogate for SWCB, the Panel expressed different views in the degree to which ECB information could be used for SWCB. Some members of the Panel concluded that ECB is a poor surrogate because of the many ways the two species differ biologically and therefore different models would have to be developed for SWCB. Others suggested that at a structural level the same resistance evolution model can be used for the two species, but that the parameter values must be different.

ECB and SWCB are phylogenetically and taxonomically related in the Crambidae, morphologically similar, share similar life cycles, corn is their major host, feed on corn stalks in similar ways, and have a similar toxicity spectrum to Cry toxins, among a long list of similarities. In many ways, ECB is a better surrogate for SWCB than its congener, sugarcane borer (*Diatraea saccharalis*), which has a wider functional host range and is considerably less sensitive to many of the Cry toxins that kill both ECB and SWCB. However, there are many differences between ECB and SWCB (Guse et al. 2002; Onstad et al. 2002, see discussion below). Some of these differences have no apparent relation to resistance evolution, but others are extremely important, especially density-dependent mortality. Because the Monsanto and EPA models did not include a density-dependent mortality function, the Panel agreed that the results from the Monsanto and EPA/ORD model were inadequate to make any scientifically sound conclusions about resistance evolution in SWCB.

A functional difference between these species, for example, is that SWCB is more tolerant of low humidity than ECB. This is evident from the geographic distribution of the species: ECB inhabits a moister climatic zone than SWCB. This difference, however, may result in differences in adult movement. As summarized in the answer to charge question 1 above, many aspects of adult ECB movement are driven by their constant search for sufficiently moist daytime habitats. Hence, perhaps adult SWCB are less dispersive than adult ECB. If this were true, the effect of a

seed mix would accelerate resistance even more in SWCB than it would for ECB. Onstad et al. (2002) have suggested that SWCB adult are less dispersive than ECB adults, especially before mating.

Modeling efforts by Ives and Andow (2002) indicated that higher net reproduction fecundity speeds up the rate of resistance evolution for high-dose events. Research suggests that SWCB probably has a higher fecundity and higher larval survival than ECB (Knutson and Gilstrap 1990). Therefore, SWCB may evolve resistance faster than ECB. However, it is not clear how a seed mix refuge might accentuate such a difference.

SWCB larvae are reported to be less sensitive to Cry toxins than ECB larvae (registrant submitted study, RPN-09-075, Head et al. 2009). An important implication is that RS heterozygote survival may exceed 5%, for one or more of the toxins and this neutralizes the advantages of the high-dose strategy. The high-dose strategy may be further undermined with increased interplant movement of SWCB larvae via the BNI and NBI scenarios described in the response to Charge Question 2.

A very significant difference between the species involves the way the larvae use the plant and associated intra-specific larval interactions. ECB larvae can be found in many locations on a corn plant. In contrast, SWCB larvae end up concentrated in tunnels at the base of the stalk, near ground level (Knutson 1987; Knutson and Gilstrap 1990; Chippendale and Sorenson, accessed Dec. 2010). SWCB is cannibalistic, and when the larvae concentrate in this way, typically only one survives. ECB occasionally cannibalizes, but this is incidental and in most cases many larvae can coexist even when they come in close contact with each other. As such, density-dependent mortality is an important consideration for SWCB.

The Panel recommended that the Monsanto and EPA/ORD resistance evolution models include a strong density dependent mortality function in modeling the evolution of SWCB resistance. Ives et al. (2011) demonstrated that models without density-dependent mortality, gene "frequency only" models exhibit qualitatively and quantitatively different evolutionary trajectories than models with even mild density-dependent larval mortality (see also Neuhauser et al. 2003). The Monsanto modeling and EPA/ORD modeling did not include density dependent mortality for SWCB. This means that predictions from these modeling efforts are not reliable in estimating the evolution of SWCB resistance. The Panel indicated that density-dependent selection (soft selection, Wallace (1981)) might be operating in this system in contrast to the traditional concepts of viability selection (hard selection, Wallace (1981)). The larvae that win out in cannibalistic encounters in the presence of *Bt* are likely to be those that have a slight fitness advantage from being more resistant to *Bt* and therefore healthier. Such selection can be very strong even when a similar number of insects successfully develop from a seed mix as in a pure stand of non-*Bt* corn. Examples of such selection have been documented for the sheep blowfly (McKenzie and Whitten 1982, 1984; McKenzie 1996) and the potential for this type of density-dependent advantage has been noted in several studies involving corn earworm larvae (Stinner et al. 1997; Dial and Adler 1990). Periodic, locally high densities of SWCB may drive resistance via density-dependent selection if a higher level of fitness is evident in heterozygotes or resistant homozygotes. Neither the Monsanto nor the EPA model allows for density-dependent selection. This may also be important for CRW resistance evolution.

Charge Question 4

Corn earworm (CEW) was not considered in the applicants' and EPA/ORD's analyses for a 5% SmartStax seed blend based on the assumption that the insect does not overwinter in the Corn Belt where the blend has been proposed. BPPD is concerned, however, that there could be areas in the southern portion of the Corn Belt where CEW may be able to successfully overwinter, particularly in less severe winters. Such areas may need to be identified because they could contribute to increased selection for CEW resistance to Bt corn (including the proposed 5% SmartStax seed blend).

Please comment on the assumption that corn earworm does not successfully overwinter in the Corn Belt and poses less of a risk for resistance. If CEW can potentially overwinter in parts of the Corn Belt, should the insect be considered in the analysis of the proposed 5% SmartStax seed blend?

Panel Response

The Panel discussed CEW long-distance migration from the South to the Midwest in the spring, CEW reverse migration from the Midwest to the South in the late summer, and whether these migratory patterns have an effect on the selection for CEW resistance in the Midwestern Corn Belt. There are several studies demonstrating that the major CEW infestations in the Midwest are caused by CEW moths that originate in Southern states. There is also evidence for reverse migration of CEW from the Midwest to Southern states. The Panel concluded that recent studies demonstrate that moths have evolved sophisticated mechanisms that enable them to move adaptively from one geographic location to another.

The Panel considered CEW resistance associated with corn to be a serious resistance risk to both corn and cotton. Considering all of the evidence, the Panel could not quantify the role of selection associated with 5% SmartStax seed mixtures in the Midwest and migration between the Midwest and the South on the rate of evolution for *Bt* resistance in CEW. Some Panel members suggested that high adoption of seed mixtures in the Corn Belt might have an effect on selection for CEW resistance on *Bt* cotton later in the season. In a worst-case scenario, there might not be any refuge for CEW within Midwestern corn that is planted as a seed mixture. Other crops and wild plants in the Midwest might be good hosts for CEW, but most of these crops are sprayed with insecticide and would not be good sources for production of susceptible insects. The Panel suggested that a detailed survey of alternate crops and more information regarding *Bt* pollen exposure scenarios would be useful.

Corn Earworm Overwintering

The CEW is thought to overwinter as far north as 40° North latitude that approximately corresponds to the area of the last spring freeze being before April 30 (Snow and Copeland 1971; Hardwick 1965). Climatic data reveal that the area of last spring freeze being before April 30 has recently (1971 – 2000) extended far northward (go to http://cdo.ncdc.noaa.gov/cgi-bin/climatenormals/climatenormals.pl?directive=prod_select2&prodtype=CLIM2001&subrnum%20to%20Freeze/Frost%20Data%20from%20the%20U.S.%20Climate%20Normals). Therefore, overwinter survival of CEW is plausible in several states within the Corn Belt, including all of

Kansas, Missouri, Iowa, Illinois, eastern Nebraska, southern regions of South Dakota, Minnesota, Wisconsin, Michigan, and most of Ohio. The geographic extent of the proposed allowable areas for use of a 5% seed mixture also overlaps with at least one area where cotton is currently planted (40,000 acres were planted in Kansas in 2010). The important questions concern the origin of the CEW adults that produce larvae infesting Midwest corn.

Long-Distance Migration from the South to the Midwest

Although some of the CEW larvae infesting Midwest corn may come from a local overwintering population, there are a number of studies that demonstrate that the major infestations in most of the Midwest are caused by CEW moths that are flying up to the Midwest from Southern states. Several studies indicate that CEW moths and larvae appear in areas of the Midwest after periods when the wind is appropriate for enhancing the area moths can traverse in one to a few nights (Pair et al. 1987). Airborne radar tracked a dense cluster of CEW moths from infested corn fields in the Lower Rio Grande Valley of Texas and Mexico for a distance of 400 km in a single night (Wolf et al. 1990). The long-distance flight of CEW moths have been closely correlated with a strong wind feature in the lowest km of the atmosphere (i.e., low-level wind jet) that provides frequent migration opportunities northward from the Southern Plains to the Northern Plains (Westbrook et al. 1998). The meteorological data are reinforced by data indicating the presence of citrus pollen on CEW found in the Midwest (Lingren et al. 1993, 1994). Based on these data, CEW moths must have flown to the Midwest from much more southern areas.

Reverse Migration

If moths simply fly from the South to the Midwest and die there in the winter as pupae then this is an evolutionary dead-end and would not contribute to resistance development (McNeil 1987). The idea that noctuid moths moved north following the availability of lush vegetation has been considered likely (Rabb and Stinner 1978; Kennedy and Storer 2000), but the mechanism triggering this northward movement does not appear to be proximate recognition by the moths of lush crops from long distances. The prevailing perspective in the 1970's through much of the 1980's was that there was no reverse migration back to the South later in the season. However, more data on CEW indicates that they also migrate back south (e.g. Pair et al. 1987; Gould et al. 2002). Pair et al. (1987) found that wind direction is often favorable for southward migrations in the fall. Radar observations have identified abrupt peak concentrations of CEW and other similar-size insects flying southward in the wake of passing cold fronts in the fall in Texas (Beerwinkle et al. 1994). Gould et al. (2002) found that a large fraction of late season CEW moths captured in Louisiana and Texas have C^{13} signatures indicating that they came from larvae that fed on C4 plants even though there are very limited local C4 hosts available for CEW moth production at that time. These results support other findings of reverse migration by the CEW moths. For example, Dowd (2001) (cited in Gould et al. 2002) estimated that there can be 16,000 large CEW larvae per hectare of Midwest field corn in the late summer, and that only 50% of these late season larvae in Wisconsin and Pennsylvania go into diapause (unpubl. data). These two results combine to indicate that there are a large number of potential southward migrant moths in the Midwest late in the season. More recently, southward migration from the Corn Belt associated with the passage of a cold front was simulated using an atmospheric transport model (Westbrook 2008).

Helicoverpa armigera is very closely related to *Helicoverpa zea* (i.e., CEW). The data from China on late summer southward migration of *H. armigera* is more extensive (Feng et al. 2009 and references within) than the studies on *H. zea* late summer migrations in the U.S. The Feng et al. (2009) study is the most comprehensive and demonstrates that in the late summer *H. armigera* moths are found in appropriate air layers for correct movement and survival, and that when air movement is not in the correct direction, the *H. armigera* moths correct for that by their flight orientation as if guided by a compass.

Beyond the data on CEW and *H. armigera*, studies on a number of other noctuids provide strong evidence of northward migration in summer and southward migration in the fall (Showers et al. 1993; Showers1997; McNeil 1987). The question arises as to whether this reverse migration results from a behavioral adaptation or whether some moths in the North simply are flying during a time when the wind is moving south and they get swept up in these winds. Recent, high profile articles demonstrate that moths have behavioral systems that seem to be as sophisticated as those of migratory birds in determining long distance flight paths that are adaptive (Chapman et al. 2008, 2010; Reynolds et al. 2010).

The Panel concluded that recent studies demonstrate that moths have evolved sophisticated mechanisms that enable them to move adaptively from one geographic location to another.

Contributions of these Southward Migrants

The Panel considered the question of whether these southward migratory movements have evolved as adaptive mechanisms and whether they always wind up bringing moths to areas where they can overwinter. For some moths, the answer seems to be "yes" (McNeil 1987; Chapman et al. 2010). However, for the CEW, not enough experimental work has been done to determine the final fitness of moths that fly south. It is not known if they fly to the Southern U.S. or if they fly much further to more tropical areas.

In some years these migrants may not contribute much to next season's population and most of the next season's moths may come from insects that remained in the South all summer. In other years, however, there is evidence that the local populations produce an extra "suicide generation" in areas like North Carolina (Stinner et al. 1978) and would therefore not contribute substantially to the next season's population. In these years it could be that migrants from the North contribute the most genes to the future population.

Selection for CEW Resistance

Considering all of the evidence, the Panel could not make any definitive statement about whether CEW being selected for *Bt* resistance in the Midwest will wind up in the next season's *Bt* cotton in the South and then be back in Midwest corn later in the season should growers plant seed mixtures (*en masse*) with only a small percentage of non-*Bt* corn germplasm. Some Panel members suggested that high adoption of seed mixes in the Corn Belt might have an effect on selection for CEW resistance on *Bt* cotton later in the season. Currently non-*Bt* corn is considered to serve as a refuge for CEW that are being selected by *Bt* cotton. There appear to be plans for intensifying the use of *Bt* corn in the South. If this is done based on the use of seed mixtures, then there might be very little in the way of a refuge for CEW in the South at some times in the year. The Panel considered CEW resistance associated with corn to be a serious

resistance risk to both corn and cotton. The dose of insecticidal toxin in Bt cotton is a major factor determining the level of resistance risk (see Appendix 1**"Empirical Methods for Estimating Dose and Efficacy"** and the Panel's response to Charge Question 8).

Other crops and wild plants in the Midwest might be good hosts for CEW, but most of these crops are sprayed with insecticide and would not be good sources for production of susceptible insects. Some Panel members suggested that a detailed survey of alternate crops is needed. The Panel discussed other situations in which selection for CEW resistance would be increased.

Some Panel members suggested that selection on the CEW for *Bt* resistance would occur whether the refuge is planted as part of seed mixture or is maintained as a structured refuge. SmartStax corn is the result of hybrid crosses involving four different events, MON 89034 (*cry1A.105* and *cry2Ab2* genes) x TC1507 (*cry1Fa* gene) x MON 88017 (*cry3Bb1* gene) x DAS-59122-7 (*cry34/35* genes). As these genes are not on the same construct they will segregate independently during breeding and selection.

Some Panel members suggested that CEW might be selected for resistance faster in a field planted with the SmartStax Refuge-in-the-Bag seed mixture consisting of 5% non-*Bt* seed and 95% SmartStax seed because research by Chilcutt and Tabashnik (2004) indicated that kernels of non-*Bt* plants that are fertilized by *Bt* corn pollen can produce *Bt* toxin. These researchers found that if the percent non-*Bt* corn is ≤10% almost all of the non-*Bt* corn would be fertilized by *Bt* corn pollen. Therefore, the selection on CEW for *Bt* resistance might be even stronger as the SmartStax RIB is a 5% seed mixture. CEW are likely to feed on kernels that are fertilized by *Bt* corn pollen as the small percentage of non-*Bt* corn plants will likely be pollinated by *Bt* corn pollen coming from only a short distance. The Panel indicated that more information regarding *Bt* pollen exposure scenarios would be useful.

Corn kernels are complex tissues genetically and will express *Bt* toxins in a complex manner. The pericarp is a normal maternal diploid, and will express the maternal phenotype. This means that all (or none) of the toxins will be expressed in the pericarp of all of the kernels on a plant in a seed mixture. The germ (embryo and cotyledon) is a diploid combination of a haploid maternal and haploid paternal genome, and the endosperm, which makes up the bulk of the kernel, is a triploid combination of two haploid maternal genomes and one paternal genome. The haploid genomes in the endosperm are identical to the haploid genomes in the germ, and the endosperm is homozygous for the maternal set and hemizygous for the paternal set. Assuming that *cry1Fa* and *cry1A.105/cry2Ab2* are hemizygous and on different chromosomes, a pyramid plant will produce the following germ/endosperm maternal haplotypes: ¼ no *cry* genes, ¼ *cry1Fa* only, ¼ *cry1A.105/cry2Ab2* only, and ¼ with both *cry1Fa* and *cry1A.105/cry2Ab2*. The pollen produced by this plant would have the same haplotypes in the same ratios. The non-*Bt* plants in a seed mixture will produce all non-*cry* haplotypes. Self-pollinated pyramided plants will have kernels with the following phenotypes in the germ and endosperm: 1/16 no Cry toxin, 3/16 Cry1Fa only, 3/16 Cry1A.105/Cry2Ab2 only, and 9/16 with both Cry1Fa and Cry1A.105/Cry2Ab2. Similar frequencies can be worked out for the other crosses in a seed mixture. Assuming random mixing of pollen and a 5% seed mixture, the entire field will have a mixture of kernel phenotypes in the germ and endosperm, with 8.3% no Cry toxin, 19.3% Cry1Fa only, 19.3% Cry1A.105/Cry2Ab2 only and 53.1% both Cry1Fa and Cry1A.105/Cry2Ab2. If *cry1Fa* and *cry1A.105/cry2Ab2* are homozygous and on different

chromosomes, the entire field would have 0.2% no Cry toxin and 99.8% both Cry1Fa and Cry1A.105/Cry2Ab2.

The CEW will be differentially selected for resistance when feeding on SmartStax corn kernels. This is expected to result in stronger selection for CEW resistance. If the *cry* genes are hemizygous, CEW will feed on kernels containing only one or two toxins instead of all three, which will select for single toxin adaptations. If the *cry* genes are homozygous, there will be virtually no refuge for CEW in the seed blend. The Panel noted that future experiments may show that the first instar CEW could avoid the toxic kernels, but this is unlikely for larger larvae. Larger larvae are likely to feed on multiple kernels and may experience an average of all of the kernels in the ear, which would be less than the toxin concentration in the pyramided plant. For hemizygous *cry* genes, the average kernel in a pyramided plant may have ~74%, and the average kernel in a non-Bt plant may have ~47% of the toxin in the intact pyramided plant. These lower concentrations may result in more rapid resistance evolution. These factors would rarely occur in a structured block refuge.

Charge Question 5

To assess dose expression for corn rootworm (CRW) Bt toxins, the level of survival (adult emergence) is typically compared between artificially infested Bt and non-Bt corn plots. However, density-dependent mortality in non-Bt plots can potentially confound the comparison by reducing overall survival and adult emergence. (Density-dependent mortality is not expected in Bt plots due to effects of the toxin on young larvae.) To account for this effect, the dose calculation can be adjusted by removing density-dependent mortality from the control plots. This effectively increases the dose mortality estimate by raising the number of larvae present in non-Bt plots relative to the surviving larvae in Bt plots.

For the SmartStax toxins, Dow/Monsanto made a density-dependent adjustment to their dose estimates based on density/survival relationships developed by Onstad et al. (2006). The resulting dose mortality profile was: Cry34/35Ab1 (99.75%), Cry3Bb1 (99.75%), and Cry34/35Ab1/Cry3Bb1 pyramid (99.95%). On the other hand, BPPD has also considered separate work by Hibbard et al. (2010), which suggests that density-dependent mortality occurs at higher egg density levels than those assumed by Dow/Monsanto. In light of this research, BPPD recommended in its 2009 risk assessment of SmartStax that dose should also be evaluated without a density-dependent adjustment. The non-adjusted dose profile for the SmartStax toxin is: Cry34/35Ab1 (94.2%), Cry3Bb1 (97.5%), and Cry34/35Ab1/Cry3Bb1 pyramid (98.2%).

Please comment on dose estimates for the SmartStax toxins (Cry34/35Ab1 and Cry3Bb1) targeting corn rootworm given the different interpretations of density-dependent mortality.

Panel Response

The Panel agreed that a density-dependent mortality adjustment is merited theoretically; but questioned the accuracy and precision of the compensatory adjustment values used in the Dow model. The Panel agreed with BPPD's recommendation in the 2009 risk assessment of SmartStax to evaluate dose without a density-dependent adjustment and use the non-adjusted

dose profile for each *Bt* toxin: Cry34/35Ab1 (94.2%), Cry3Bb1 (97.5%), and Cry34/35Ab1/Cry3Bb1 pyramid (98.2%).

Density-dependent mortality estimates were calculated from field studies conducted under optimal field conditions that the Panel considered unrealistic and therefore the density-dependence mortality was overestimated. The Panel concluded there is no statistically credible method to distinguish between 94% and 99% dose mortality because the dose mortality profiles are poorly estimated. Both the unadjusted and the density-dependent adjusted dose mortality profiles have very high variances. The Panel recommended that the CRW models be rerun using the non-adjusted dose profiles (and lower values) and that the re-analysis consider the variance of dose mortality profiles using first order Monte Carlo simulations.

The Panel considered three aspects of the density-dependent mortality dose profile in their response to this charge question: 1) larval mortality factors, 2) density-dependent mortality estimates, and 3) statistical significance of the density-dependent mortality estimates.

Factors that Influence Corn Rootworm Survival Rates

The Panel identified a number of biological and ecological factors that cause the dose mortality profile to be highly variable, and therefore the expected dose-mortality in SmartStax corn fields is probably significantly less than the Dow values reported to the Panel. Some of these factors are not fully known or understood, so they cannot be controlled or manipulated. Hybrid characteristics influence root structure, size and regeneration. These characteristics, combined with environmental conditions such as soil type and excessive rains or drought conditions and timing of these events, can have significant impacts on CRW larval mortality regardless of density. Larval survival rates were calculated from fields studies conducted under optimal field conditions, e.g., consistent environmental conditions, uniform distribution of eggs when they are typically found in clumps across the field and no CRW behavioral variants. Given that typical field conditions are not optimal, the Panel concluded that the larval survival rates used to calculate dose mortality are overestimates of what is expected under natural field conditions.

One panel member commented that his research on the performance of single-gene *Bt* CRW corn varieties, YieldGard, Cry 3Bb1 and Herculex, Cry 34/35Ab, with a near isoline (control) showed that efficacy of the *Bt* CRW corn depended on the pest density (Tollefson, unpublished). As the density of larvae attacking the *Bt* CRW corn roots increased, the loss of larvae due to density-dependent mortality on the near isoline increased. This reduced the relative number of adults that emerged from the near isoline and it appeared that the *Bt* CRW corn provided greater protection. In some instances, there were more adults that emerged from the *Bt* CRW corn than from the near isoline, due to density-dependent mortality on the unprotected roots.

The protection provided by a *Bt* CRW corn variety that expresses a moderate dose may be reduced if the pest pressure is high. One panel member commented that this information has been provided by some manufacturers to explain why their *Bt* CRW corn did not provide the level of root protection that the customer expected. The Panel discussed how refuge damage can be used to assess pest density by evaluating root injury where soil insecticides have been used for control. Soil insecticides will not provide complete control of the CRW larvae attacking the corn

roots. The level of control is usually in the 40-60 % range. If there were a structured refuge with an insecticide applied to the soil for CRW protection and there was obvious root injury in the refuge then it is possible to conclude that CRW pressure was high and the *Bt* CRW corn could not protect itself against a high number of larvae. If an insecticide were used on the refuge and there was not a noticeable amount of injury on plants in the refuge, but lodging and root injury was present in *Bt* CRW corn fields then it is possible to conclude that the *Bt* corn PIP was not expressing lethal dosages in that field. This implies that the dose mortality profile is highly variable, and therefore the expected dose-mortality rate in SmartStax corn fields is probably significantly less than the Dow values reported to the Panel. The Panel agreed with BPPD's interpretation of the data and the recommendation that the effective SmartStax dose mortality should be estimated without the inclusion of a density-dependent mortality adjustment. Using the unadjusted dose mortality in the model may provide a more accurate prediction of the durability of the CRW protection provided by SmartStax corn. The Panel identified a number of biological and ecological factors that cause the dose mortality profile to be highly variable, and therefore the expected dose-mortality in SmartStax corn fields is probably significantly less than the Dow values reported to the Panel.

Density Dependent Mortality Estimates

Density dependent mortality does occur in natural populations of CRW. In support of Dow/Monsanto, the Panel suggested that this density dependence may occur at quite low egg densities, as illustrated by the following.

Density-dependent larval survival was modeled using Hassell's (1975) formulation for density dependence for insect populations with discrete generations, x the insect population density at generation t and t+1, $x_{t+1} = \left[x_t\left(1+\alpha x_t\right)^{-\beta}\right]$. The parameters, α and β, were estimated from field experiments on the survival of naturally laid eggs (Gray and Tollefson 1988) or survival of inoculated eggs (Branson and Sutter 1985; Elliot et al. 1989) using nonlinear least squares (SAS 1999, Proc NLIN). There were clear differences in the fitted equations for natural and inoculated eggs (Fig. 5-1, Table 5-1). The field data suggested that α = 10.6 and β = 0.52 were reasonable values for density-dependent egg to adult survival of *Diabrotica virgifera* in non-transgenic corn when insecticides were not applied. These values indicated that density dependence did not intensify until egg densities exceeded about 100,000/ha and that density dependence was moderately strong, allowing the density to return towards a carrying capacity smoothly without overshooting. The Hibbard et al. (2010) conclusion that density-dependence may only occur at very high densities may be due in part to their efforts to estimate per capita mortality. This would normally be the appropriate analysis, but in this case, per capita mortality has high statistical error because it compounds the error in the density estimates. The Hassell method described above is more sensitive for detecting density dependence.

Table 5-1. Estimated density-dependent larval survival parameters for western corn rootworm, based on nonlinear least squares regression. Parameter values were α and β, as developed in Hassell, with statistics from the analysis, $p<<0.05$ for both egg inoculations and field observations. Measurement error is not taken into account with this analysis.

	α	β	F (d.f.)	r^2
Egg inoculations (Branson and Sutter 1985; Elliot et al. 1989)	5.3	0.68	37.09 (2, 2)	0.974
Field observations (Gray and Tollefson 1988)	10.6	0.52	156.81 (2,10)	0.969

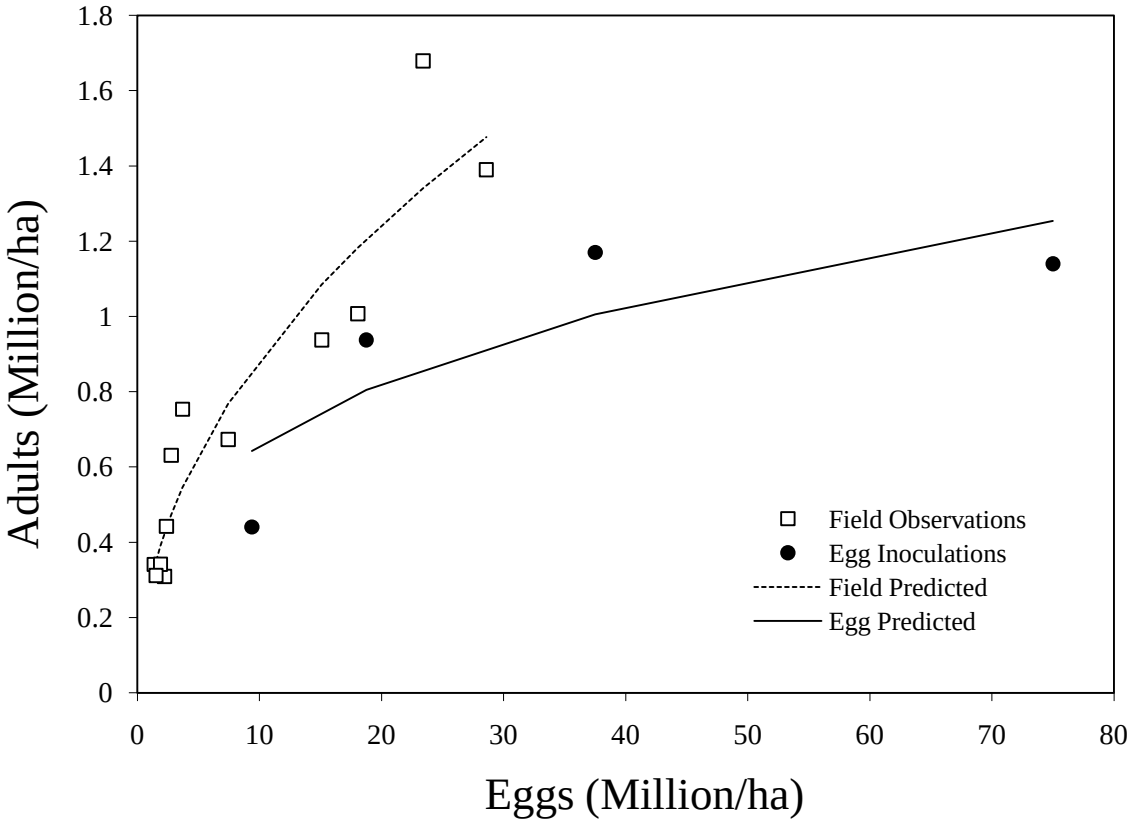

Fig. 5-1. Density-dependent egg to adult survival of western corn rootworm on corn. Egg inoculation data were from Branson and Sutter (1985) and Elliot et al. (1989), showing weighted means from 5 years for the two studies. Field observations were from Gray and Tollefson (1988) and were from 3 years and 4 tillage systems. Lines were fitted by non-linear least squares.

Statistical Significance of Density Dependent Mortality Estimates

In theory, the Panel agreed that parameters should be estimated as accurately as possible, and that adjusting dose mortality with a density-dependent adjustment would be helpful, especially as density dependence is strong and occurs over a wide range of CRW densities. In this case, the

Panel had serious reservations over manipulating field collected mortality estimates to adjust for density-dependent mortality or any other factor that may be considered. There were two reasons. First, the Panel is concerned that the values stated in the charge question may not be accurate because they were collected under ideal conditions. Many factors reduce mortality in typical field conditions, so the typical mortality value may be less than those in the charge question. A density-dependent adjustment may provide a false sense of accuracy. Second, the adjustment may have little statistical meaning. The original estimates of mortality are not precise or repeatable enough to support the adjustment. While parameters should be estimated as accurately as possible, any improvements should provide statistically significant improvements. If the parameter estimates are not very precise, adjusting them gains little statistical improvement. This point is elaborated below.

The Panel concluded there is no statistically credible method to distinguish between 94% and 99% dose mortality because the parameters necessary to make the density-dependent adjustment to dose were poorly estimated. Both the unadjusted and the density-dependent adjusted dose mortality profiles have very high variances. The general formula given in Hibbard et al. (2010, p. 83) as reported in Storer et al. (2006) is

$$D = (1 - (AY(E) / A_0) \times 100 , \qquad \text{[Eq. 1]}$$

where D is dose mortality (in percent), A is adult emergence from the *Bt* CRW-protected variety, A_0 is adult emergence from the non-*Bt* variety, and $Y(E)$ is the predicted survival of viable eggs at the infestation level used.

A claim was made that mortality due to Cry34/35Ab1 toxin dose in the isoline corn estimated at 96.71% was different from the estimate of 99.88% from the toxic dose of transgenic corn targeting rootworms, but there was no discussion as to the statistical significance of this difference. The uncertainty of estimates using this equation could be computed using the variability measured for the individual components by using an approximate estimate of the variance of D given below that is derived using the method of statistical differentials (see for example Elandt-Johnson and Johnson (1980).

$$\frac{V(D)}{(100\%)^2} \approx \left(\frac{Y(E)}{A_0}\right)^2 V(A) + \left(\frac{A}{A_0}\right)^2 V(Y(E)) + \left(\frac{AY(E)}{A_0^2}\right)^2 V(A_0)$$
$$+ 2\left[\left(\frac{AY(E)^2}{A_0^3}\right) Cov(A, A_0) + \left(\frac{AY(E)}{A_0^2}\right) Cov(AY(E)) + \left(\frac{A^2 Y(E)}{A_0^3}\right) Cov(A_0, Y(E))\right] \qquad \text{[Eq. 2]}$$

where $V(A)$, $V(A_0)$, $V(Y(E))$ are estimates of the variance of the equation components and $Cov(A,A_0)$, $Cov(A,Y(E))$ and $Cov(A_0,Y(E))$ are the estimated co-variances for pairs of the equation components. A simpler estimate for the variance of D could be obtained if it is possible to assume that the co-variances are close to zero. Equation 2 could be used in a simple *t*-test to assess the statistical significance of the difference in estimated mortality and/or to establish confidence intervals.

A, A_0, and $Y(E)$ typically have a high variance, so D will have a high variance. This can be illustrated with the data provided by the registrants (Table 5-2). In no case is the estimated SE(D) less than 10%. Two facts are used in this calculation. First $V(x) = SE^2(x)$, where x is either D, A, A_0 or $Y(E)$. Second, $Cov(A,Y(E)) = Cov(A_0,Y(E)) = 0$, because $Y(E)$ is estimated independently of A and A_0. In addition, we assume $Cov(A,A_0) = 0$. In reality, A and A_0 are likely to be positively correlated, and if they are positively correlated, the estimated SE(D) reported in Table 5.2 is underestimated. Finally, it should be noted that the estimated SE(D) for each of the four *Bt* varieties in a year are not independent, because the same A_0 and SE(A_0) (and if Cov(A,A_0) $\neq 0$, then the Cov(A,A_0) are also correlated among the four *Bt* varieties) enter into equation [2] for all four varieties. This non-independence will tend to overestimate or underestimate the SE(D) for the four varieties in a quantitatively similar manner, and it will also reduce the degrees of freedom associated with the estimated SE(D). In conclusion, it is not possible to statistically distinguish a D = 90 from a D = 99 for corn rootworm. Given the uncertainty in the estimation of density-dependent mortality, the Panel decided that the unadjusted (no density-dependent mortality factor) dose should be used in the models.

Table 5-2. Estimation of the standard error on the density-dependent adjusted dose mortality.[1]

		D	SE(D)	n	A or A_0^2	SE(A) or SE(A_0)^2	$Y(E)^3$	SE($Y(E)$)^3
2006								
A	Mon 89034 TC1507 Mon 88017 DAS 59122-7	99.60	23.52	16	6.6	2.5	5	2
A	Mon 88017 DAS 59122-7	99.55	24.13	16	7.4	2.2	5	2
A	DAS 59122-7	98.92	56.93	16	17.6	5.1	5	2
A	Mon 88107	98.57	77.48	16	23.4	7.3	5	2
A_0	Control			16	81.6	15.2		
2007								
A	Mon 89034 TC1507 Mon 88017 DAS 59122-7	99.33	39.72	8	11.9	3.8	5	2
A	Mon 88017 DAS 59122-7	99.61	23.56	8	7	2.3	5	2
A	DAS 59122-7	97.72	139.14	8	40.5	14.2	5	2
A	Mon 88107	98.71	74.88	8	23	6.7	5	2
A_0	Control			8	88.9	26.7		

1. D, A, $Y(E)$ and A0 are as in equation [1], and SE(D) is the square root of V(D) from equation [2]. All values in percent.
2. 2006 data are from Page 166, MRID 474449-11 (Vaughn et al. 2008); Page 27, RPN 07-262 Vaughn et al. (2008), and 2007 data are from Page 168, MRID 474449-11 (Vaughn et al. 2008); Page 29, RPN 07-262 (Vaughn et al. 2008)
3. According to Hibbard et al. (2009), $Y(E)$ varies between 5-25%. SE($Y(E)$) was estimated from Onstad et al. (2006); SE($Y(E)$) is positively correlated with $Y(E)$, so SE(D) is positively correlated with $Y(E)$, and the tabulated SE(D) are lower bounds.

Charge Question 6

Northern and western corn rootworm studies have shown that male emergence in 5% seed blends can be variable and may be up to 60 times lower compared to emergence in non-Bt plots

(Data submitted by Monsanto). This information was not included in any of the models used in the SmartStax seed blend analysis. The SAP (2009) concluded that a reduction in the number of males from Bt seed blends could have a negative impact on the effective refuge. BPPD is concerned with the potentially negative effects a reduction in male emergence might have on product durability.

Please comment on the potential effects of lowered male emergence of Northern and Western corn rootworm on the durability of the seed blend and whether this information should be incorporated into the risk assessment.

Panel Response

The Panel expressed concern for the potential effects of lowered male emergence in SmartStax RIB 5% seed mixture fields. The Panel recommended further studies to gather additional data on male andfemale emergence ratios. The impacts of beetle fitness and emergence timing on mate selection should also be considered in potential mating scenarios as described by Kang and Krupe (2009) since the fitness of female and male adults may influence mate selection. Further, the Panel advised EPA to evaluate male and female emergence ratios in all species, behavioral traits (WCR variant and NCR diapause-resistant) and WCR pesticide resistant populations in field trials with naturally-occurring populations and in field trials that were artificially-infested with corn rootworm.

Adult male emergence counts, beetle weight and emergence timing data provided to the Panel were limited and highly variable. Based on these data, the Panel made the following observations: males emerging from SmartStax RIB 5% seed mixture may be less fit and not as proficient in mating with females as males that emerged in non-seed mix fields. The loss of males and reduced fitness in the 5% seed mixture fields could mean that there would be fewer than expected males present when the receptive females are emerging. The Panel also examined whether adults emerging from 100% SmartStax plots experienced any development delays. Emergence timing data reflects temporal separations in adult emergence in the SmartStax RIB 5% seed mix fields versus non-*Bt* corn fields, although some adults emerging from 100% SmartStax plots did not experience any developmental delays. The later data does not support the hypothesis that larvae that survive on *Bt* plants experience emergence delays resulting in a bimodal distribution from the seed mix plots. Genetic mechanisms may be involved in the survivability of *Bt* feeding larvae, but supporting data are not currently available to determine survival mechanism(s) with little or no apparent loss of fitness. Based on the data provided it is not clear if the temporal separations in adult emergence in the SmartStax RIB 5% structured refuge fields are a result of feeding on *Bt* plants or other factors.

The mixing of emerging adults from refuge plants and SmartStax plants and mate selection may be more important than male/female ratio. A Panel member reported previous studies have shown that CRW move about 17 meters per day (Coats et al. 1986). Adult males typically do not engage in long-range dispersal (Tollefson and Coats, unpublished), but female adults have been shown to travel as far as 25 miles in long distance flights (Coats et al. 1986). In addition, juvenile hormone plays a role in female adult long distance movement behaviors (Coats et al.

1987). Panel members indicated timing of emergence of males and females from Bt and non-Bt is also important to mating of any resistant and susceptible adults.

Part B: Modeling of Resistance Evolution

Charge Question 7

The durability of the proposed 5% SmartStax seed blend strategy was compared to the durability of a 5% structured refuge for lepidopteran and corn rootworm target pests. Monsanto developed a deterministic three locus model for ECB/SWCB and Dow created a stochastic two locus model for CRW. Separate analyses were conducted using EPA/ORD's two locus and three locus deterministic, probabilistic model to estimate the risk of resistance evolution with a 5% seed blend and structured refuge. The applicants and EPA/ORD each made conservative assumptions, though of differing degrees, for parameters determined to be sensitive in the models. For example, more conservative initial resistance allele frequencies and fitness assumptions significantly lowered the time to resistance in EPA/ORD's model for ECB and SWCB. In Monsanto's modeling of ECB and SWCB, a greater degree of dispersal between compliant and non-compliant fields significantly affected the estimated time to resistance.

Please comment on the appropriateness of the assumptions and inputs used for the following parameters in the Monsanto, Dow, and EPA/ORD models:

- *Initial resistance allele frequency for single traits Cry1A.105, Cry2Ab2, Cry1F, Cry34/35Ab1, and Cry3Bb1 for all modeled pests;*
- *Survival/fitness for all modeled pests; and*
- *Dispersal for ECB and SWCB as modeled by Monsanto and EPA/ORD.*

Panel Response

The Panel pointed out the importance of being mindful of the basic differences in model structure among the Monsanto, Dow, and EPA/ORD models because these differences influence whether certain parameters are present in the model and the range of potential durabilities that can be model outcomes. Assumptions about model structure are in many respects more critical than the parameter values themselves. Model structure involves the equations that characterize dynamic processes, embody assumptions about causality, and specify the parameters that could be quantified. The assumptions and data used to estimate parameter values are subsequent to choices about model structure. In relation to the discussion of the parameter values specified in the Charge Question, the Panel addressed structural issues related to the parameters as well as the estimation of the parameter values themselves. An additional discussion about model structure is also available in the response to Charge Question 9.

1) **Initial Resistance Allele Frequency for the Single Traits (Cry1A.105, Cry2Ab2, Cry1Fa, Cry34/35Ab1 and Cry3Bb1) for all Modeled Pests (all models).** The Panel concluded that the initial resistance allele frequencies chosen for Cry1A.105, Cry2Ab2, and Cry1Fa were appropriate for both ECB and SWCB as "best guesses" in the face of

limited information. Therefore, some are possibly overestimates and hence may underestimate durability. For WCR and NCR, the initial resistance allele frequencies for Cry34/35Ab1 and Cry3Bb1 were probably underestimates of the actual values and probably overestimate durability. For WCR and NCR, the Panel suggested that a different model structure may be more appropriate for assessing resistance development because resistance will likely be determined by multiple loci acting with small effects, not a single major locus. In this situation a quantitative genetics model might be a better model structure. The Panel noted that there is likely to be substantial geographical variation in allele frequencies, and therefore initial resistance allele frequency should not be treated as a single value for a species. Because resistance could develop locally from initially high local allele frequencies and then spread geographically, worst-case scenarios must be used that assume high initial allele frequencies.

2) **Cross-Resistance Potential (Monsanto and EPA/ORD Models).** Cross-resistance is a special case of the more general problem of estimating survival/ fitness values, and the Panel focused on the Lepidopteran-active toxins because cross-resistance may greatly reduce durability of SmartStax for targeted lepidopterans. In general, there is considerable evidence to support hypotheses of cross-resistance, especially between Cry1A.105 and Cry1Fa in many insect species. The Panel concluded that there is some evidence of partial cross-resistance of Cry1A.105 and Cry2Ab2 with a Cry1Fa resistance allele in ECB. The Monsanto and EPA/ORD models assumed that there would be no cross-resistance and consistently treated Cry1A.105 as a unique protein involving a novel mode of action with no cross-resistance between Cry1A.105 and each of its component toxins, Cry1Fa and Cry1Ac. The assumption of no cross-resistance would overestimate the durability of SmartStax. The Panel agreed that the potential for cross-resistance should be considered in any model.

3) **Survival/Fitness for all Modeled Pests.** The Panel concluded that the fitness values used in the ECB, SWCB, WCR, and NCR models were underestimated for the heterozygotes. For ECB and SWCB potential epistasis among resistance loci were insufficiently examined. The Monsanto and EPA/ORD models ignored density-dependent mortality and complex selection associated with corn kernels. In addition, the Monsanto model did not model between-plant movement of larvae in a way that addresses the risks to resistance evolution. The Panel noted that none of the parameters for a quantitative genetics model for WCR or NCR have been estimated. All of these factors will overestimate the durability of SmartStax.

4) **Dispersal for ECB and SWCB as Modeled by Monsanto and EPA/ORD.** The Panel concluded that adult dispersal for ECB and SWCB would likely randomly mix the adults within fields of SmartStax seed mixtures. Nonetheless, this does not rule out the possibility of non-random mating; for example, timing of adult emergence could lead to resistant individuals having a greater chance of mating with each other. Furthermore, there could be non-random oviposition in seed mixtures; for example, damage to non-*Bt* plants could increase relative oviposition rates on *Bt* plants. In these examples, non-random mating and oviposition would speed resistance evolution.

Therefore, seed mixtures do not rule out the possibility that non-random mating and non-random oviposition decrease durability.

The Broader Philosophical Context of Modeling

Before addressing the specific issues related to the model parameters, the Panel made philosophical comments about mathematical models. All models are idealizations and abstractions of reality. They pull from the world those processes we already think are important and provide conceptual landscapes to structure our thinking, let dynamic complexity play out, and allow us to make judgments and predictions about what we will find in the world. It is sometimes naively assumed that if we get the parameters right, then what we want to know about the world will just fall out—that parameter values alone are what drives uncertainty about the processes the models are designed to capture. This belief is a fallacy that leads to overconfidence in the output of models.

The Panel pointed out that it is important to be mindful of the idealistic nature of models. That is why the Monsanto, Dow, and EPA/ORD models differ in fundamental ways. There is a growing body of literature from philosophers of science examining how simulation is being used to make scientific inference. These considerations are important in understanding how to interpret the Monsanto, Dow, and EPA/ORD models, and the context for their proper use. To illustrate the philosophical issues surrounding modeling, consider the following questions:

1) Why do the frequency-only models provided by Monsanto, Dow, and EPA differ? The models are ostensibly targeting the same processes.

2) Why are different results obtained? It is more than the choices of parameter values alone. Differences arise from how the models picked out the abstractions and idealizations that they wanted to capture. This is fine, and pluralistic approaches are being touted as the way to study complex systems. William Wimstatt has developed this into the formal conceptual framework he calls 'robustness,' the idea that truth emerges from agreement between independent false models (Wimstatt 2007). Richard Levins made a similar point over four decades ago (Levins 1966). Nonetheless, from a practical point of view of designing IRM strategies, there needs to be a formal way of adjudicating among models when they give different results.

3) Why does each model assume that there is a single locus with alleles coding for resistance to single toxins? A strong case can be made for such model structure when dealing with cultivars that have a high concentration of toxin relative to the pest's susceptibility. This may be true for ECB, but it is clearly not the case for the CRW. For CRW, these models may be less appropriate than quantitative genetic models.

4) How can we assess the models in detail, as opposed to having to simply trust the modelers? The Panel made suggestions to make the models more transparent, and more easily interpreted. First, using peer-reviewed models would have helped provide assurance that the models had been properly evaluated by independent modelers. Second, testing the model using bridge principles is important to assure evaluators that the models were capturing relevant processes in the right way. For example, a model

should be able to be run under the assumptions of standard population-genetics theory to obtain known theoretical results. Testing these results would have been very helpful and provided a bridge to the more complex cases the model was targeting. Peck (2008) has examined the philosophical underpinnings of simulation models and concluded that simulations can be a vital tool for understanding complex ecological questions, but they require more work than other types of models. The Monsanto, Dow and EPA/ORD models were hard to compare because the Panel was not given access to their testing and algorithms.

Initial Resistance Allele Frequency for Single Traits Cry1A.105, Cry2Ab2, Cry1Fa, Cry34/35Ab1, and Cry3Bb1 for all Modeled Pests

ECB and SWCB

The Panel concluded that the estimated initial allele frequency values chosen for Cry1A.105, Cry2Ab2 and Cry1Fa may be overestimates (conservative values) of the actual ECB and SWCB values. However, the data provided to the Agency and in the published literature to estimate initial allele frequencies for each toxin are sparse except for Cry1A toxins. Therefore, the Panel acknowledges that this conclusion is necessarily tentative, and the values used for initial allele frequencies should be regarded by EPA as guesses rather than science-based estimates.

Virtually no data exist to support the estimated values used for the initial frequency for resistance to Cry2Ab2. If we assume that resistance to Cry2Ab2 is similar to that for Cry1Ab in ECB and SWCB, then we would also assume that initial resistance allele frequencies were similar. If we assume that resistance to Cry2Ab2 is rare in ECB and SWCB, then we should also allow that there could be significant cross-resistance between the Cry1A.105 and Cry 2Ab2 toxins.

There are few data available to support the estimated values used for the initial frequency for resistance to Cry1Fa. Some unpublished information indicates that ECB may have resistance alleles to Cry1Fa at frequencies considerably above 0.001. Thus, the values used in the models may be conservative, or they may be near or below the actual resistance frequency (for Cry1Fa). There are no data for SWCB, so we must rely on the assumption that resistance allele frequencies are similar in both species.

The Panel pointed out that there could be substantial geographical variation in initial resistance allele frequencies for all toxins and all pests. This raises the concern that resistance could evolve locally and then spread to other geographical areas. Therefore, for IRM a worst-case scenario (i.e., the highest frequency of resistance alleles) should be adopted, because if this worst-case occurs in one geographical location, it may determine the overall durability of SmartStax (see also Charge Question 9). The Panel cautioned against assuming that there is one value for the frequency of resistance to a given toxin in a given target pest.

WCR and NCR

The Panel stated that the estimated resistance allele frequencies for Cry34/35Ab1 and Cry3Bb1 used in the models were not overestimates of the expected values. This is because the *Bt* toxin

concentrations in the corn cultivars being examined are low relative to susceptibility of WCR and NCR. In this situation resistance will likely be determined by multiple loci acting with small effects, not a single major locus (as assumed), and a quantitative genetics model might be more appropriate for assessing resistance development. For lepidopteran species examined for resistance to high dose events, high frequencies or multiple loci of low-level resistance that could be effective against low-dose events have been located. NCR and WCR have not been examined in this way, but the rapid development of a resistant colony of WCR to mass-selection by Cry3Bb1 on a rather small effective population size implies that resistance is common in this species.

The Panel suggested that a starting point for a quantitative genetics model might be to assume that there are many alleles with small effects that exhibit additive gene action for resistance to either Cry34/35Ab1 or Cry3Bb1. Under these conditions, assumptions about the initial additive genetic variance for each trait, the initial mean value for the trait and any genetic interactions between the genes associated with Cry34/35Ab1 and Cry3Bb1 would have a significant effect on the rate of resistance evolution. In general, the initial allele frequencies for quantitative loci with small effects will be substantially higher than that assumed by the models where there is a resistance allele at a single locus for each toxin, and evolutionary rates may be considerably faster.

Uncertainties Regarding Cross-Resistance Potential between Cry1A.105 and Cry1Fa

Based on the provided materials, the Panel concluded that the overall cross-resistance potential of the chimeric CryA.105 protein remains unclear with regard to the Cry1Fa toxin. The Panel agreed that the potential for Cry1Fa and Cry1A.105 cross-resistance should be considered in any model. Data presented in the review materials indicate there is some level of partial cross-resistance in the major allele for Cry1Fa resistance in ECB to both Cry1A.105 and Cry2Ab2 (see discussion below). In the MON 89034 BRAD, EPA indicated that additional information was needed concerning the cross-resistance potential of Cry1A.105, Cry1Fa and Cry1Ac (US EPA 2008). When SmartStax was registered in 2009, further analysis of the possibility of cross-resistance was required as a term and condition of the registration (US EPA 2009a). The Panel received no subsequent materials regarding this issue and could not discern how Monsanto and Dow were addressing this registration requirement. A response to BPPD requests for additional information was compiled and reviewed for CRW modeling in the July 1, 2009 IRM review document, but did not include new data regarding cross-resistance potential of the Cry1A.105 and Cry1Fa toxins (BPPD 2009b). The Panel agreed that the potential for Cry1Fa, Cry2Ab2 and Cry1A.105 cross-resistance should be considered in any model. The IRM models provided to the Panel assumed that there would be no cross-resistance and consistently treated Cry1A.105 as a unique protein involving a novel mode of action with no cross-resistance between Cry1A.105 and each of its component toxins, Cry1Fa and Cry1Ac. Additional discussion on cross-resistance is found in the Panel's response to Charge Question 8.

Indications from *Plutella xylostella*

There are many species that have evolved resistance to *Bt*, but the most well-studied is. *Plutella xylostella* L. (diamondback moth, DBM), As much as known about DBM resistance, there is

much still unknown about the mechanisms and molecular genetics of insect resistance to the various Cry toxins. DBM was the very first species to evolve resistance to *Bt* in the field, and the species that causes by far the most geographically widespread control failures. DBM is a pest in crucifers which is subject to intensive selection in the field as chemical pesticides and *Bt* sprays are applied frequently. DBM evolved resistance rapidly to chemical pesticides and it was no surprise that DBM would be the first species to evolve resistance to *Bt* in the field. Tabashnik et al. (1997) discovered that a single resistance gene in DBM conferred high levels of resistance to four *Bt* toxins, Cry1Aa, Cry1Ab, Cry1Ac, and Cry1F. Despite considerable effort, the mechanism of DBM resistance to *Bt* still remains unresolved (e.g., Baxter et al. 2008). The Panel considered the possibility that a single resistance gene may confer resistance to multiple *Bt* toxins in other insect species, but has not yet been selected strongly enough to be found.

On the other hand, various studies suggesting that insensitivity to Cry toxins may not evolve via a single genetic mechanism (Baxter et al. 2005; Heckel et al. 2007). Consistent with the findings of Tabashnik et al. (1997), Baxter et al. (2008) reported that a single quantitative trait locus (QTL) confers resistance to Cry1A toxins in the DBM, but that it segregated independently of genome positions that encode the known glycoprotein receptors of *Bt* toxins. Similarly, single nucleotide polymorphism (SNP) markers for *bre5*, aminopeptidase N, or cadherin were shown not to co-segregate with Cry1Ab resistance in the ECB (Coates et al. 2008).

Although the DBM is the most persuasive example, there is also evidence for cross-resistance between Cry1A and Cry1Ftoxins in other species, such as *Heliothis virescens* (tobacco budworm, TBW) (Gould et al. 1995) that may be due to sharing at least one common receptor (Jurat-Fuentes and Adang 2001) and *H. armigera, Spodoptera exigua* (Hernández and Ferré 2005). Hernandez and Ferré (2005) reasoned that their binding data along with previous binding data and observed cases of cross-resistance suggested that this pattern of cross-resistance is widespread among lepidopteran species.

Three Cross-Resistance Studies

During the meeting, the Panel requested that EPA provide them with additional information concerning the assessment of cross-resistance between Cry1A and Cry1Fa toxins.

BPPD IRM Review of the Potential for Cross-Resistance between Cry1Ab and Cry1Fa (US EPA 2010a)

One of these documents was the USEPA-BPPD IRM review of the potential for cross-resistance between Cry1Ab and Cry1Fa toxins based on data provided by Pioneer Hi-Bred International (US EPA 2010a, found in SAP docket for this meeting, EPA-HQ-OPP-2010-0772 and also in the EPA regulatory docket: EPA-HQ-OPP-2010-0183-0028). Pioneer used a number of approaches to investigate Cry1Fa-Cry1Ab cross-resistance including structural comparisons, midgut (receptor) binding assays, pore formation analysis, and tests with Cry1Fa-resistant colonies. Based on these findings, EPA concluded that cross-resistance is unlikely between Cry1Fa and Cry1A.105 in the three species of concern here, the ECB, SWCB, and CEW.

The Panel highlighted several points from the EPA review (US EPA 2010a, quoted below) that raised concerns about cross-resistance.

1) *These data largely show that Cry1Ab and Cry1F elicit toxic responses through separate pathways. Midgut binding studies identified several shared binding sites in each of the targets which could indicate some degree of cross resistance. On the other hand, experiments with Cry1F-resistant ECB showed no survival on Cry1Ab. The potential for low-level cross-resistance between Cry1F and Cry1Ab was addressed by exploring a range of values using a sensitivity analysis with the simulation model. (page 2)*

2) *Results from the Cry1F competition for Cry1Ab binding were similar for ECB, SWCB, and CEW (described in Appendix 1 of MRID# 480056-01). In all cases, the presence of Cry1F did not inhibit Cry1Ab binding with BBMV [brush border membrane vesicles] proteins except at the highest concentrations of competitor. About 20% (ECB and CEW) to 40% (SWCB) competition (reduction in Cry1Ab binding) was observed at the higher concentrations of Cry1F. Conversely, homologous competition (Cry1Ab/Cry1Ab) showed approximately 90% less ^{125}I-Cry1Ab binding at the maximum concentrations. A different pattern of results was observed with the reciprocal Cry1Ab competition for Cry1F (Alexa-Fluor marked) binding. Heterologous competition with Cry1Ab reduced Cry1F binding by approximately 70% (ECB) and 50% (SWCB). (The CEW binding tests were deemed unreliable because of high non-specific binding in the Cry1F/Cry1F homologous competition.) The high level of competition shown in the ECB and SWCB tests suggested that at least some Cry1F receptors are shared with Cry1Ab. (page 5)*

3) *However, for Cry1F-resistant ECB, the presence of Cry1F did not significantly inhibit Cry1Ab binding indicating that the shared binding site was lost (modified to no longer recognize Cry1F). Since homologous competition (Cry1Ab-Cry1Ab) still existed with the resistant colony, it is likely that an unshared Cry1Ab binding site remained viable. (page 5-6)*

4) *Cry1F competition assays for Cry1Ab binding sites showed that two classes of receptors are found in ECB, SWCB, and CEW: one that is recognized by Cry1F and a second that is not recognized by Cry1F. (page 7)*

5) *Separate research conducted by Pereira et al. (2010) found that Cry1F recognizes binding sites of 120, 200, and 250 kDa in ECB and that BBMV binding was not reduced with ECB selected for Cry1F resistance. The authors suggested that the mechanism for Cry1F resistance in ECB is specific and unrelated to Bt toxin resistance mechanisms observed in other insects (e.g., reduced BBMV binding, increased activity of gut proteases). BPPD also notes that competitive binding studies conducted with a separate insect, diamondback moth (Plutella xylostella), showed some competition between Cry1F and Cry1Ab for a common binding site, but also that Cry1Ab binds to separate sites independent of Cry1F (Granero et al. 1996). (page 11)*

Ultimately, the Panel was not as certain as EPA that there is a low likelihood of cross-resistance between Cry1A.105 and Cry1Fa for ECB and SWCB and concluded that predictions of cross-resistance remain uncertain. The Panel cited other studies that also indicated cross-resistance

between these toxins. For example, the DBM does show cross-resistance between Cry1Ac and Cry1Fa (Tabashnik et al. 1997) and showed some competition between Cry1Fa and Cry1Ab for a common binding site (Granero et al. 1996). In fact, there have been several binding studies for different insects, including DBM, ECB and TBW, that showed Cry1Fa and Cry1Ab/Cry1Ac share a common binding receptor as well as unique binding receptors (Denolf et al. 1993; Hua et al. 2001; Jurat-Fuentes and Adang 2001; Hernández and Ferré 2005). Members of the Panel would not exclude the possibility that a mechanism of resistance as found in DBM (i.e., one binding receptor modification conferring resistance to multiple *Bt* toxins) exists in one or more species including ECB, SWCB, and CEW. The Panel remained unconvinced that there was a low likelihood of cross-resistance based on the studies conducted with the Cry1Fa-resistant colony. Finding that Cry1Fa resistance did not confer resistance to Cry1Ab might be due to multiple pathways to resistance (Baxter et al. 2005; Heckel et al. 2007).

MON 89034 BRAD (US EPA 2008)

The Panel considered a second EPA document, the MON 89034 Biopesticides Registration Action Document (BRAD) (US EPA 2008). The BRAD contained a detailed review of the likelihood of cross-resistance between all three toxins (US EPA 2008). The Panel observed that BPPD had concerns about the potential for cross-resistance between Cry1A.105 and Cry1Fa that were not resolved. Monsanto developed a corn event, MON 89034, which produces the Cry1A.105 and Cry2Ab2 insecticidal toxins to protect against corn stalk-boring pests such as ECB, SWCB, and CEW. MON 89034 is one of four events, MON 89034 x TC 1507 x MON 88017 x DAS 59122-7, used to create SmartStax. As detailed in the BRAD (US EPA 2008), Cry1A.105 is a chimeric protein consisting of domains I and II and the C-terminus of Cry1Ac and domain III of Cry1Fa with overall amino acid sequence identity to the Cry1Ac, Cry1Ab and Cry1Fa proteins of 93.6%, 90.0% and 76.7%, respectively. The X-Ray crystallography studies demonstrated that there was high main chain structural similarity between the modeled crystal structures of Cry1Aa, Cry1Ab, Cry1Ac, and Cry1A.105 proteins (US EPA 2008). Such close similarity causes a heightened interest in examining the potential for cross-resistance between Cry1Ac and Cry1Fa and Cry1A.105 toxins. In their review, BPPD concluded that cross-resistance was a real possibility for these toxins. The Panel agreed with this conclusion and highlighted two major points of interest from the BRAD to support this conclusion.

1) **Competition binding experiments indicate a common receptor.** BPPD presented several lines of evidence that indicated that Cry1Fa and Cry1Ab/Cry1Ac share a common binding receptor although each of these proteins has unique binding receptors as well (Denolf et al. 1993; Hua et al. 2001; Jurat-Fuentes and Adang 2001; Hernández and Ferré, 2005). (US EPA 2008, discussion on pgs. 79-80). Specific competition binding experiments using brush border membranes from ECB demonstrated that Cry1F shares a binding site with Cry1Ab/Cry1Ac, though the level of cross-resistance between Cry1F and Cry1Ab/Cry1Ac is not as strong as Cry1Ab vs. Cry1Ac." Hernández and Ferré's conclusion (as cited in the BRAD) was

   …in the case of corn, primary pests susceptible to Cry1Ab and Cry1Fa, such as ECB (and SWCB and CEW), would necessitate the importance of establishing the binding site

model for this species in order to develop an appropriate resistance management strategy" (Pages 76-77).

2) **ECB-resistant colonies.** Cross-resistance studies using ECB resistant colonies indicated that Cry1Ab-resistant ECB were partially resistant to Cry1F although Cry1F-resistant ECB were not cross-resistant to Cry1Ab and only slightly resistant to Cry1Ac. EPA noted that similar trends have also been shown with TBW (see Head and Storer 2008).

   BPPD's conclusions stated in the 2008 BRAD were:

   "In the case of corn, primary pests susceptible to Cry1Ab and Cry1Fa, such as ECB (and SWCB and CEW), would necessitate the importance of establishing the binding site model for this species in order to develop an appropriate resistance management strategy" (Pages 76-77)."

   And

   "Cry1F can be considered partially cross-resistant to Cry1Ab and Cry1Ac. The availability of binding sites may explain the partial cross-resistance: Cry1Ab and Cry1Ac could have more different sites to bind with than Cry1F so that resistance to Cry1F still allows for some binding of Cry1Ab or Cry1Ac." (Page 91).

The Panel concluded that the issue of cross-resistance between Cry1A.105 and Cry1Fa was left unresolved in the BRAD.

SmartStax IRM Plan (MRID 474449-11, Appendix 1)

The Panel considered a third document, the SmartStax IRM plan submitted to BPPD by Monsanto and Dow (MRID 474449-11, entitled "Insect resistance management plan for MON 89034 x TC1507 x MON 88017 x DAS-59122-7 (Head and Storer 2008). Appendix 1 (Schlenz et al. 2008) contained a summary of a study that examined the response of Cry1Fa-resistant (and otherwise susceptible to Cry1A.105 and Cry2Ab) and Cry1Fa-susceptible (a colony susceptible to all three toxins) ECB and fall armyworm (FAW) colonies to Cry1A.105 and Cry2Ab2. Slopes of the concentration-mortality relationship were estimated from data provided in this study and are shown in Table 7-1. The statistical error associated with these slope estimates was not reported. However, the variation in mortality among replicate experiments was small for about 1/3 of these experiments (MRID 474449-11, Appendix 1, Pages 45-47 Schlenz et al. 2008)), so perhaps these estimates are not too variable.

Resistant FAW and ECB had lower slopes than susceptible colonies when exposed to Cry1Fa (Table 7-1). This may be expected, because the resistant colonies should be able to tolerate Cry1Fa better than the susceptible colonies. Unexpectedly, the resistant FAW and ECB colonies also had lower slopes than the susceptible colonies when exposed to either Cry1A.105 or Cry2Ab2 (Table 7-1). Results of this study indicate partial cross-resistance in the Cry1Fa resistance allele in ECB against Cry1A.105, Cry1Fa-resistant ECB had a resistance ratio of 133, and against Cry2Ab2, they had a resistance ratio of 12. In addition, growth inhibition was

significantly reduced on Cry1A.105 and slightly, but not significantly reduced on Cry2Ab2. These data suggest that partial cross-resistance occurs with a Cry1Fa resistance allele in ECB. To evaluate the potential cross-resistance to enable realistic modeling, the concentration-response relationship for the Cry1Fa RS heterozygote on Cry1A.105 and Cry2Ab2 is essential. In addition, it is critical to evaluate survival of these Cry1Fa genotypes on plants expressing Cry1A.105 and Cry2Ab2, together and separately. However, the absence of an estimated statistical error makes any inference about cross-resistance uncertain. The Panel recommended a full statistical analysis of the concentration-mortality data.

Table 7-1. Partial cross-resistance in Cry1F resistance allele in ECB against Cry1A.105 and Cry2Ab2. Data from MRID 474449-11, Appendix 1, (Schlenz et al. 2008, p. 44). Slopes and GI_{50} and LC_{50} were estimated with a probit model.

Insect Strain[1]	Protein Toxin	Growth Inhibition[2] Slope	GI_{50} (95%CI) ng/cm^2	Mortality[2] Slope	LC_{50} (95% CI) ng/cm^2
s-ECB	Cry1A.105	0.00	<1.5	1.10	6.1 (3.9-8.9)
r-ECB	Cry1A.105	0.89	5.9 (2.2-15.8)	0.64	813 (488-1478)
s-ECB	Cry2Ab2	0.99	27.1 (14.6-50.4)	1.56	595 (451-784)
r-ECB	Cry2Ab2	1.09	39.3 (22.3-69.4)	0.62	7206 (3476-20108)

1. s-ECB is a colony susceptible to Cry1Fa, r-ECB is a colony resistant to Cry1Fa.
2. s-ECB and r-ECB are significantly different when the 95% CIs do not overlap.

Survival/Fitness for all Modeled Pests

ECB and SWCB

The fitness values used for the ECB/SWCB models include assumptions about survival that likely lead to overestimates of durability. These models assume low heterozygote survival and independent fitness of each Cry toxin (multiplicative fitness), and ignore density-dependent larval mortality, general knowledge about ECB larval movement, and complex selection in the ear of non-*Bt* plants in seed mixes. In a few cases, non-multiplicative fitness (epistasis) was considered, but this was not done in a realistic way or in a way that would lead to underestimates (conservative estimates) of durability. In addition, the Monsanto model did not model between-plant movement of larvae in a way that addresses the risks associated with that movement.

Theoretical Considerations

Assuming that the initial frequencies of *Bt* resistance alleles are below 0.001, the single most influential parameters in the ECB model are those associated with the fitness values of heterozygous individuals. This can be seen by examining a single-locus model for a single *Bt* toxin. Although SmartStax has multiple stacked toxins, the same principles of resistance evolution derived from single-locus models are still relevant.

The evolutionary dynamics of a single-locus, high-dose system can be approximated by

$$\frac{\Delta p}{p} = (1-Q)LF(p+h)$$

for complete adult dispersal, where *p* is the frequency of the resistance allele, *Q* is the proportion of refuge, *L* is the survival of RR in the *Bt* field (typically set to 1), *F* is the fecundity per capita in the *Bt* field, and *h* is the degree of dominance (Ives and Andow 2002). This is a second-order approximation, and assumes that survival of SS homozygotes on *Bt* plants is very low. The left-hand side of the equation is the rate of change of resistance allele frequency. The proportion of *Bt* is represented by (1-*Q*) and is always between 0 and 1. The reproduction of RR homozygotes in *Bt* fields is represented by *LF*. Both (1-*Q*) and *LF* are always much greater than *p*. Dominance *h* is the main factor that determines the rate of resistance evolution, because except when resistance failure is approached ($p > 0.01$), the magnitude of the right-hand side of the equation is dominated by the value of *h*. Specifically, *h* will be greater than *p* during most of the time of resistance evolution, and therefore dominance will be the most significant factor driving the rate of resistance evolution.

These concepts generalize to the multi-locus case when all genes are high-dose. The term (1-*Q*) will continue to exert its role. *LF* must be generalized to encompass a multi-dimensional fitness differential, and *h* must be generalized to encompass multi-dimensional heterozygote fitness. This generalized *h* will determine the rate of the evolutionary process if it is much larger that the corresponding R allele frequencies.

The Monsanto, Dow, and EPA/ORD analyses did not include detailed explorations of epistasis in the multi-locus fitness interactions that affect dominance *h*. A few cases were modeled with epistasis with the EPA/ORD model. However, the types and magnitudes of epistasis considered were not worst-case scenarios, and therefore they may overestimate durability compared to the predictions that would be made if realistic epistasis were considered (see also Charge Question 8).

Empirical Considerations

The fitness of heterozygotes for ECB and SWCB was assumed to be only 2-5 fold (2x and 5x) that of the susceptible genotypes, in comparison to the assumption in Dow's CRW model that heterozygotes are 25-fold more tolerant of the Cry proteins than susceptible homozygotes. In the absence of RS genotypes, one could look to the only widespread field resistance to *Bt*, in the DBM, where heterozygotes seem to be at least 5X more tolerant than susceptible larvae (Tang et al 1997). If the survival of heterozygous ECB and SWCB were higher than 5-fold the survival of susceptible homozygotes for all toxins, then resistance of ECB and SWCB would evolve at a much higher rate than that predicted by the Monsanto and EPA/ORD models and durability would be overestimated.

In addition, as explained in more depth in the Panel's response to Charge Questions 8 and 9, the fitness values of larvae that move from plant-to-plant are determined from a restricted set of assumptions. The values used in the models do not seem to reflect the empirical data from Davis

and Onstad (2000), and general knowledge about ECB larval movement is not considered (see response to Charge Question 2).

Because some ECB feed within corn ears, they, like CEW larvae (see the response to Charge Question 4) may be feeding on kernels that have zero, one, two, or three toxins, or if kernel tissue from several kernels is mixed during feeding (such as for later instars), some average of these. This could allow survival of heterozygous larvae. The toxicity of these kernels to larvae is not known, nor is the fraction of ECB larvae that feed on kernels. This is an empirical question in need of further experimental data.

Because the SmartStax RIB 5% seed mixture approach under consideration will use the same corn plants as refuges for corn rootworm and lepidopteran pests, it is important to recognize that if there is damage to the refuge plants from rootworm, these plants may grow more slowly than the plants that are protected from rootworm. Similarly, in years when ECB first generation damage to whorl stage corn is high, the damage to refuge plants could make them less robust than the *Bt* plants. By the time of the second generation of ECB moths and CEW moths are flying and choosing host plants, the refuge plants may have been crowded out by the faster growing *Bt* plants and would not be attractive to ovipositing moths. This could significantly decrease the effectiveness of non-Bt plants to serve as a refuge.

WCR and NCR

The fitness values used for WCR and NCR will likely lead to overestimates in durability.

Theoretical Considerations

The EPA/ORD model appears to assume that the default value for *h* should be 0.05, with the minimum at 0.01 and the maximum at 0.2. These assumptions would be theoretically reasonable if the corn plants provided a classically defined high dose for the NCR and WCR, but this is clearly not the case. Tabashnik et al. (2004) among others have pointed out that there is no reason to assume effective recessiveness of any *Bt* resistance allele unless the toxin is at a high dose. Some *Bt* resistance alleles have been found to have a recessive phenotype in concentration/mortality or concentration/growth assays (Gould et al. 1995), while others are additive or dominant (Gould et al. 1992, 1995). Given the dose in the current corn cultivars, the effective recessiveness of a resistance allele in the field is likely to reflect concentration/mortality and concentration/growth assay results. This leads to a default *h* of about 0.5. Fig. 7.1 A-D demonstrates how sensitive outcomes of the model are to changing the value of *h* when using the Monsanto and Dow data for mortality of susceptible larvae on the one and two toxin plants. This figure is based on output of a simple two-locus model from Jongsma et al. (2010) that gives reasonably parallel results to the EPA/ORD model, which in fact likely leads to overestimates of durability (see Charge Question 9).

The Panel noted that the EPA/ORD modelers conducted a sensitivity analysis in which a Beta distribution was used to describe the values of *h*. Although the extremes for *h* used (0.01 and 0.2) could be included in the analysis, using a Beta distribution means that many values of *h* will be close to the mode value for *h* which is 0.05 (see Fig. 7.1A), and therefore the analysis is

weighted towards giving overestimates of durability (see also responses to Charge Questions 2 and 9). From the EPA document **(**US EPA 2009b) the Panel thought that Dow used an *h* value of 0.32 as their worst-case scenario in the model. Given the moderate toxin dose in the corn cultivars for rootworms, an appropriate worst-case scenario would be to use an *h* of 1.0 and as mentioned earlier, a mode of 0.50.

Beyond the issue of *h* for single alleles, is the consideration that when the dose is moderate any allele that confers higher fitness to individuals than that of the population mean will increase in frequency. In the long term, field resistance to *Bt* corn could involve dozens of alleles, each with small effects. Quantitative genetic models are best for dealing with such situations.

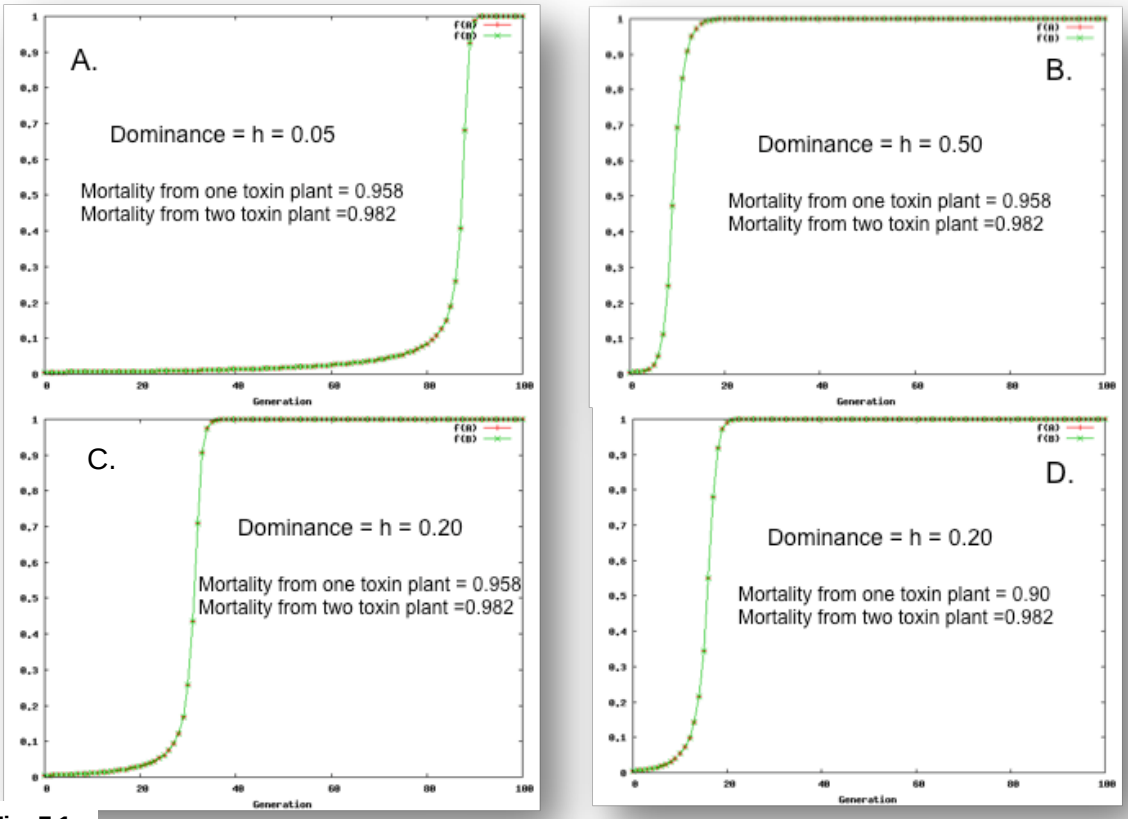

Fig. 7.1 Demonstration of sensitivity to h as well as to the exact degree of redundant killing. In all runs, the initial R frequency is 0.005. Fitness of double heterozygote (RrR'r) is 0.5 that of of RRR'r' individuals.

Empirical Considerations

The Panel examined EPA's SmartStax IRM review (US EPA 2010b) and determined that the Dow model used fitness values for WCR derived from field experiments conducted on test plots of the corn cultivars that were planted under ideal conditions of soil fertility and moisture. Under these conditions, the mortality values of WCR from larvae to adult were very high on each of the single-toxin cultivars and on the dual-toxin cultivar when measured relative to mortality on a non-*Bt* cultivar (Cry3Bb1 = 99.75%, Cry34/35Ab1 = 99.75%, Pyramid = 99.95%). The mortality was slightly lower when there was an assumption of no density-

dependent mortality (Cry3Bb1 = 94.2%, Cry34/35Ab1 = 97.5%, Pyramid = 98.2%). The Panel pointed out that the data were given as mean mortality values without the breakdown of the variance in mortality values. Some locations in some years might have mortality values that were 5-10% lower or higher than the mean. In the response to Charge Question 5, the Panel noted the high variance associated with estimated WCR survival and the potential overestimation of mortality.

The Dow model depended heavily on these data. Based on these mortality values, the model would predict that two toxins inhibit resistance evolution (redundant killing) if there is no cross-resistance to the two toxins. The Panel, however, identified situations in which under sub-optimal field conditions, the mortality due to single toxins would be slightly lower than the values used by Dow in the model and thus resistance would evolve more rapidly, especially if *h* is larger than assumed. The Panel surmised that under stressful conditions, the mortality levels would be low compared to ideal conditions (toxin expression is affected by the plant's health), and when rootworm populations are high or very low; the mortality caused by the toxins would also be affected (see response to Charge Question 5).

The Panel provided a simple example of how important minor variation in these mortality values could be using the Jongsma et al. (2010) model. The question asked was what would happen if under field conditions, instead of causing an average of 95.8% mortality, each of the single toxin plants caused 90.0% mortality. By comparing Fig. 7.1A and B, a small decrease in mortality could cause resistance to evolve in a 40% shorter period of time. The sensitivity analysis run by the EPA/ORD modelers would not pick up this effect because of their focus on parameter uncertainty using a first-order Monte Carlo simulation with a Beta distribution and their choices of minimum and maximum values. This will make the sensitivity analysis of EPA/ORD more likely to overestimate durability. The Panel appreciated that EPA/ORD modelers conducted a sensitivity analysis and suggested that they conduct a more advanced type of uncertainty analysis to differentiate the impacts of each variable in the model on the uncertainty of the overall model predictions (e.g., Xu et al. 2008; Xu and Gertner 2010a, 2010b; see responses to Charge Questions 2 and 9).

Dispersal for ECB and SWCB as Modeled by Monsanto and EPA/ORD

The Panel concluded that the dispersal values used for adult dispersal for ECB and SWCB were probably appropriate. This conclusion is based on the following three factors: (i) the seed mixture will result in fine-scale intermixing of *Bt* and refuge plants on the order of meters, (ii) adults move from their natal plants prior to mating or oviposition and (iii) adult stages move considerably farther than a few meters. Therefore, it is reasonable to assume that adults will disperse far enough and frequently enough that the adult population can be considered randomly mixed at the spatial scale of fields.

As noted in the Panel's response to Charge Question 1, it is not clear what would be conservative assumptions, and it is not clear how processes that are not modeled could affect resistance evolution. For example, because dispersal is extensive, mating is globally random, and the genotypes of offspring are well-mixed on the modeled landscape. However, it is not clear that mating is random, either globally or locally. Sexual selection theory posits that females may be

selective about mates (epigamic selection) and males may compete for mates (intrasexual selection). ECB females do not distinguish between young virgin males and old virgin males, but they tend to mate more frequently with experienced males than virgin males. The mating choices by males and females may have significant implications for resistance evolution because they imply non-random local mating, but these have not been incorporated into resistance evolution models. Such mate-selection models tend to lead to complex evolutionary trajectories and outcomes, in contrast to the population genetics abstractions of assortative and disassortative mating.

A second process that is not modeled is oviposition choice. ECB females will not discriminate between *Bt* and non-*Bt* plants when they are grown in separate fields, but they will discriminate between damaged and undamaged plants when they are grown near each other. In seed mixtures, *Bt* and non-*Bt* plants will be growing side by side. If the non-*Bt* plants are damaged during the first generation, second generation ECB females will discriminate against them, resulting in lower than expected oviposition on non-*Bt* plants (refuge plants), reducing the effectiveness of the seed mixture to delay resistance evolution.

Thus, although the parameter values for adult dispersal are probably appropriate, the model may be missing important processes such as sexual selection and oviposition choice. As a consequence, the parameter values that were used for adult dispersal may have minor influence on the rate of evolution compared to the processes that were totally ignored in the model structure. Hence, the parameter values used in the models may overestimate considerably the durability of the pyramided product, not because the values are wrong, but because the wrong model was used.

Charge Question 8

EPA/ORD encountered challenges in the lepidopteran modeling with partitioning non-multiplicative interactions that occurred between more than two resistance genes since the mortality caused by each locus was not independent. With two gene pyramids this non-additivity can be assigned to the single two locus interaction, but in a three gene pyramid there are three possible two locus interactions. In the absence of data, this non-additivity was partitioned equally among the three two locus interactions. As more than two Bt genes are pyramided, this problem will have to be addressed so that resistance evolution in the target pests to these products can be more accurately simulated.

Does the Panel have any recommendations for distributing non-multiplicative interactions in models to evaluate multi-gene pyramided products?

Panel Response

The Panel expressed concern with the way in which survival to different single toxins is combined to calculate genotype-specific survival on the pyramided plants in the models of resistance evolution. The Panel emphasized that the way in which survival rates of different genotypess are combined has a large impact on the predictions that all models make about the durability of pyramided crops. It appeared to the Panel that the Monsanto and EPA/ORD models

combined survival rates in ways that generated low heterozygote survival; thereby, overestimating durability of SmartStax in all simulations. Information on the survival of the genotypes conferring resistance to one or multiple *Bt* toxins for the target pests of SmartStax is not available, causing large uncertainties in the predictions of any model of resistance evolution. While theory can suggest ways in which survival to single toxins might be combined to generate survival to multiple toxins, empirical information is sparse (Appendix 2: **High- and Low-Dose Scenarios and Larval Movement in Seed Mixtures**).

When there is larval movement among plants in seed mixtures, it is necessary to know the survival rates of all insect genotypes both before and after movement. Thus, more information is needed than just the survival of different genotypes on *Bt* plants, as emphasized in the previous paragraph. The concern from the previous paragraph must be addressed for the two or more larval stages before the larvae move and after they move (stage-specific survival rates). In addition to generating genotypic survival rates to multiple toxins for each larval stage, the stage-specific survival rates must be combined across stages. These stage-specific survival rates may be combined multiplicatively if these survival events associated with each stage are independent. All of the models the Panel examined combine the stage-specific survival rates multiplicatively. However, while this might be a reasonable assumption in the absence of empirical evidence, other possibilities should be explored, and to the knowledge of the Panel, this topic has not been investigated theoretically.

Non-multiplicative ways in which the resistance of different loci can combine to determine the stage-specific or total immature survival of all genotypes to multiple toxins are forms of epistasis. Specific mechanisms by which epistasis can occur include, but are not limited to: 1) constitutive, low-level expression of Cry-proteases, 2) developmentally restricted expression of low levels of Cry-protease, 3) genes regulating expression of receptor genes, and 4) cross-resistance. These and other forms of epistasis should be investigated as mechanisms that cause non-multiplicative survival rates of the multiple locus genotypes. The consequences of different forms of epistasis on resistance evolution should be evaluated.

Below is a discussion of some non-multiplicative ways in which resistance conferred by multiple resistance loci could be combined to determine the survival of heterozygotes. This will be discussed mainly in the context of two-locus interactions, but the results should also be applicable to three-locus interactions.

Multiplicative Interactions among Resistance Loci

Multiplicative interactions among resistance loci refer to the situation in which the survivals of multilocus heterozygotes are equal to the product of the survival of single-locus heterozygotes. To illustrate this, suppose the survival of S_1S_1 and S_1R_1 individuals were known for plants expressing only *Bt* toxin 1, and denoted L_1 and H_1, respectively, with the assumption that R_1R_1 homozygotes have survival 1. Similarly, suppose the survival of S_2S_2 and S_2R_2 individuals were known for plants expressing only *Bt* toxin 2, and denoted L_2 and H_2, respectively. If interactions between locus 1 and 2 were multiplicative, then for plants expressing both toxins the survival of $S_1S_1S_2S_2$ individuals would be L_1L_2, the survival of $S_1R_1S_2S_2$ individuals would be H_1L_2, the survival of $R_1R_1S_2S_2$ individuals would be L_2, etc. This multiplicative manner of combining

Fig. 8-1. Fitness of 2-locus genotypes, showing multiplicative fitness based on the marginal 1-locus fitness values.

		L_1	H_1	K_1
		S_1S_1	S_1R_1	R_1R_1
L_2	S_2S_2	L_1L_2	$\boldsymbol{H_1L_{2*}}$	K_1L_2
H_2	S_2R_2	$\boldsymbol{L_1H_{2*}}$	$\underline{\boldsymbol{H_1H_{2*}}}$	$\boldsymbol{K_1H_{2*}}$
K_2	R_2R_2	L_1K_2	$\boldsymbol{H_1K_{2*}}$	K_1K_2

survival to different toxins assumes that the toxins act independently, in the sense that insects must survive one and then the other of the toxins, with the survival probabilities being independent.

Even when survival rates of heterozygotes to different toxins combine independently (multiplicatively), the impacts of multiple toxins on the rate of resistance evolution do not combine in a simple way. In other words, the rates of resistance to both toxins for insects on single-toxin *Bt* plants do not lead to simple predictions of the rate of resistance evolution to multi-toxin *Bt* plants. When there are pyramid toxins and resistance occurs in the form of diallelic loci, the rate of resistance evolution at all loci is likely determined most strongly by the most-rapidly evolving locus; the most-rapidly evolving locus drags resistance at the other loci along with it (see Appendix 2: **Survival of Heterozygotes**). This result must be interpreted carefully, however. Evolution of multiple resistance loci to pyramid varieties will always be slower, and generally much slower, than the resistance evolution of any one locus separately (i.e., if all other loci are fixed for resistance).

Non-Multiplicative Interactions among Resistance Loci

All non-multiplicative interactions are a form of epistasis. For the two-locus case, there are five survival values involving heterozygotes that could influence the rate of resistance evolution (Fig. 8-1, shown in purple bold lettering with an asterix). For the three-locus case, there are 19 such values. It is likely that changes to the five values in the two-locus case will have differing effects on resistance evolution. Changes to the fitness of the $S_1S_1S_2R_2$ and $S_1R_1S_2S_2$ genotypes (Fig. 8-1, background is shaded in blue) will likely exert the largest effect on resistance evolution, followed by the double heterozygote ($S_1R_1S_2R_2$, Fig. 8-1, underlined). This is because the $S_1S_1S_2R_2$ and $S_1R_1S_2S_2$ genotypes occur significantly more frequently than the others when the resistance alleles are rare, followed by the double heterozygote. This is not fully borne out by the Panel simulations (Appendix 2: **Survival of Heterozygotes**), in that a 5x increase in the survival of the double heterozygote has a similar effect on resistance evolution as a 5x increase in either or both of the survival of the $S_1S_1S_2R_2$ and $S_1R_1S_2S_2$ genotypes. This may depend on how increases in survival are scaled.

One possible scaling is the following. First, designate the genotype-specific survival rates on a single-toxin plant in the margins of the matrix of survival rates on the two-toxin plant (Fig. 8-1). For locus 1 on toxin 1, these are L_1, H_1 and 1, and for locus 2 on toxin 2, these are L_2, H_2, and 1. These will be called single-toxin survival rates. With multiplicative survival rates, the nine 2-locus genotypic survival rates on a two-toxin plant are generated by multiplying the respective single toxin survival rates. These are displayed in the central 3x3 matrix of Fig. 8-1. For each set of single-toxin survival values, H_i can be reformulated as $h_i + (1\text{-}h_i)\, L_i$, where h_i is the

dominance of locus *i* (the relative survival of the heterozygote). The multiplicative fitness of the $S_1S_1S_2R_2$ genotype would be $L_{\tilde{i}}(h_i + (1-h_i)L_i)$, and the double heterozygote would be $(h_{\tilde{i}} + (1-h_{\tilde{i}})L_{\tilde{i}})(h_i + (1-h_i)L_i)$ where *L* is the marginal fitness of one of the SS genotypes, *i* denotes one of the loci and $\tilde{i}$ denotes the other. As an aside, a general rule of thumb is when *h* << 0.05, the event is high dose, and when *h* > 0.1 the event is low dose. Several factors enter into the specific determination of dose, which complicate this rule of thumb, so in the discussion below we use numerical examples which leave little doubt about whether an R allele is to be considered high dose or low dose.

Epistasis occurs when, unlike above, survival is not multiplicative. One way to add epistasis is to multiply *h* by a scaling constant, *e*, in the two-toxin survival values, but not in the single toxin values. When $e \neq 1$, there is epistasis, and when $e > 1$, expression of resistance in the heterozygote is increased in the two locus genotypes relative to the single-locus case. The $S_1S_1S_2R_2$ genotype survival would be $L_{\tilde{i}}(h_i e_i + (1-h_i e_i)L_i)$ and the comparable double homozygote survival would be $(h_{\tilde{i}} + (1-h_{\tilde{i}})L_{\tilde{i}})(h_i e_i + (1-h_i e_i)L_i)$.

Mechanisms Leading to Epistasis

There are numerous mechanisms that lead to epistasis. Although the form and strength of epistasis is ultimately an empirical problem, the degree of epistasis is largely unknown for pyramid *Bt* cultivars. Therefore, considering different potential mechanisms that could drive epistasis may shed light on how epistasis will affect resistance evolution. The Panel focused on one illustrative mechanism, but others are possible (e.g., resistance based on loci regulating cadherin or amino peptidase expression).

1) **Constitutive, Low-Level Expression of Cry-Proteases.** Several species of Lepidoptera are known to have multiple kinds of proteases that are expressed at different times during development. For example, *Sesamia nonagrioides* expresses certain proteases only during later instars (Ortega et al. 1996). These proteases are the likely reason for why this species is able to tolerate and grow on diets containing Cry toxins. It is possible that the higher survivals of later instars of ECB and SWCB are related to the expression of late-larval proteases (designated Cry-proteases) that can partially or fully degrade and detoxify Cry toxins.

   Assume that there is a recessive resistance allele to a Cry1A toxin and another recessive resistance allele to a Cry2A toxin at a different locus. Assume the Cry-protease is not expressed during the 1st and 2nd instars, is expressed at low levels in the 3rd instar, at intermediate levels in the 4th instar, and at high levels in the 5th instar. It would have no effect on survival of the 1st and 2nd instars, increase survival of the 5th instar substantially, and have intermediate effects on 3rd and 4th instars.

   At low and intermediate levels of expression, the Cry-protease might degrade some, but not all Cry toxin, thereby abating some of the effect of the Cry toxin. In doing this, it would have little effect on the survival of $R_1R_1R_2R_2$ homozygotes, which can tolerate even an unabated high dose of Cry toxin, and would have little effect on the survival of

$S_1S_1S_2S_2$ homozygotes, which cannot tolerate slightly abated Cry toxin levels. However, it could cause a significant rise in the survival of heterozygotes. Indeed, survival of the double $S_1R_1S_2R_2$ heterozygote could be improved nearly to that of the $R_1R_1R_2R_2$ homozygote.

A hypothesized effect of low expression of the protease is to increase the single-toxin survival of the heterozygotes. In the example given in Fig. 8-2B, the single-toxin fitness of the heterozygote was increased to 0.2, for which $h \sim 0.2$. This is clearly not high dose, but even so, the survival rate of the single heterozygote on a two-toxin plant ($S_1S_1S_2R_2$ or $S_1R_1S_2S_2$) is still very low (2×10^{-5}). The fitnesses of the single heterozygote and the single resistant homozygotes ($S_1S_1R_2R_2$ or $R_1R_1S_2S_2$) are assumed to be multiplicative, as the SS genotype does not confer any level of resistance. The conditional dominance for allelic variation in resistance at one locus, given that the other locus is fixed as an SS homozygote is $h \sim 0.2$ (Fig. 8-2B).

Similarly, the fitness of the single heterozygote with a resistant homozygote ($S_1R_1R_2R_2$ or $R_1R_1S_2R_2$) is expected to be multiplicative, because the RR genotype is hypothesized to contribute maximally to fitness. The conditional dominance for allelic variation in resistance at one locus, given that the other locus is fixed as an RR homozygote, is also $h \sim 0.2$ (Fig. 8-2B).

Fig. 8-2. Hypothesized epistasis for low to intermediate expression of a late-expressing protease that degrades Cry toxins. A. Multiplicative fitness with the marginal fitness of each locus high dose ($h = 0.029$). B. Hypothetical epistasis for 3rd and 4th instar larvae with low to intermediate expression of the protease, showing non-high dose marginal fitness (shaded, light blue boxes with 0.2 in bold) and epistasis (light red box in the center with value 0.08).

A.

	0.0001 S_1S_1	0.003 S_1R_1	1 R_1R_1
0.0001 S_2S_2	1×10^{-8}	3×10^{-7}	0.0001
0.003 S_2R_2	3×10^{-7}	9×10^{-6}	0.003
1 R_2R_2	0.0001	0.003	1

B.

	0.0001 S_1S_1	**0.2** S_1R_1	1 R_1R_1
0.0001 S_2S_2	1×10^{-8}	2×10^{-5}	0.0001
0.2 S_2R_2	2×10^{-5}	0.08	0.2
R_2R_2	0.0001	0.2	1

The double heterozygote ($S_1R_1S_2R_2$) is expected to show epistasis in the presence of low to intermediate levels of a Cry-protease. This is because the protease may reduce the toxin concentration sufficiently that a single resistance allele allows survival. For example, if the resistance allele results in an alteration of a cadherin-like receptor in the insect midgut, then a heterozygote may have half the population of receptors that can bind Cry toxin. Hence, there are likely to be some concentrations of Cry toxin that are sufficiently low that they do not bind in sufficient quantities or for a sufficient period of time to induce rapid death in the larva. If the protease can produce such concentrations when it is at low to intermediate levels of expression, then the double heterozygote may survive as well as the double resistant homozygote. In the example in Fig. 8-2B, there is a more modest level of epistasis, with $S_1R_1S_2R_2$ survival of 0.08. These considerations result in a conditional dominance with the other locus fixed as an SR heterozygote of $h \sim 0.4$, which is approximately additive gene action. One could assume that $S_1R_1S_2R_2$ survival ranges from 0.04 (no epistasis) to 0.2.

The survival matrix in Fig. 8-2B could be incorporated into a resistance evolution model by assuming that the Cry-protease is expressed constitutively at low levels throughout larval development. Although there is no biological or toxicological evidence either way regarding this assumption for ECB or SWCB, it may be relevant for other species, such as *Helicoverpa* sp.

2) **Developmentally Restricted Expression of Low Levels of Cry-Protease**. Compared to constitutive expression, it is more likely that Cry-proteases are expressed in later instars of ECB and SWCB. Thus, they may affect fitness in a seed mixture via plant-to-plant movement. Glaum et al. (2011) provided a method for calculating the "effective dominance" for resistance for larvae moving in a seed mixture for the single-locus case. It is possible to generalize this method for the multi-locus case. "Effective dominance" could provide an intuitive understanding for how larval movement coupled with stage-specific survival rates affect resistance evolution.

3) **Cross-Resistance**. Cross-resistance is not necessarily modeled using a two-locus model with epistasis. However, it can be done, as indicated in Fig. 8-3. Here survival on a single toxin (the second toxin) is not affected by genetic variation at the second locus, and all survival rates are the same, L_2. The survival rate of the first locus on toxin 1 is the same as in Fig. 8-1. However, survival on a two-toxin plant depends only on the first locus. These are the survival rates for complete cross-resistance.

It is also possible that the second locus influences survival rates on the two-toxin plant even though it has no effect on survival on a single toxin plant (the second toxin). This is illustrated in Fig. 8-4 in which cross-resistance increases the survival of $S_1R_1S_2R_2$ by α and $S_1R_1R_2R_2$ by β. (Note: this and the following examples add survival associated with epistasis. It may be more reasonable to assume a proportional increase in survival related to epistasis, in which case epistasis could be modeled using multipliers).

Partial cross-resistance (see earlier discussion in response to Charge Question 7) has several possible meanings; here it is defined as resistance that provides some increased

survival on all of the toxins, but does not recover full survival on any of the other toxins in the RR homozygote. One possible parameterization of partial cross-resistance is shown in Fig. 8-5A. In this case, the maximum possible survival is L_2. Epistasis could occur for the same reason as detailed for Fig. 8-4. The effect of locus 2, however, should extend to both the S_1R_1 heterozygote and the R_1R_1 homozygote. The effect of the modifier would be to increase the conditional selective differential and in most cases to increase conditional dominance. Finally partial cross-resistance associated with locus 1 could affect survival of heterozygotes at resistance locus 2, which gives major resistance to a different toxin as given in Fig. 8-6. This may be relevant for ECB Cry1Fa resistance.

Fig. 8-3. Complete cross resistance of locus 1. Survival rates associated with locus 2 have no effect on survival on the two toxin plant.

		L_1	H_1	1
		S_1S_1	S_1R_1	R_1R_1
L_2	S_2S_2	L_1	H_1	1
L_2	S_2R_2	L_1	H_1	1
L_2	R_2R_2	L_1	H_1	1

Fig. 8-4. Complete cross resistance of locus 1 with a modifier effect of locus 2. Epistasis is highlighted in in light red boxes, marked in bold with an asterix. .

		L_1	H_1	1
		S_1S_1	S_1R_1	R_1R_1
L_2	S_2S_2	L_1	H_1	1
L_2	S_2R_2	L_1	$\boldsymbol{H_1+\alpha*}$	1
L_2	R_2R_2	L_1	$\boldsymbol{H_1+\beta*}$	1

Constitutive expression of a Cry-protease at sufficiently high levels could cause partial or complete cross-resistance. The Cry-protease could degrade multiple Cry toxins, reducing or eliminating their toxicity to the insect. Similar to other enzymatic mechanisms, constitutive expression of a Cry-protease is likely to be inherited as a dominant allele, or at least having additive gene action. Additive or dominant resistance alleles would lead to rapid resistance to multiple-toxin plants. In the examples provided above, epistasis would further increase the rate of resistance evolution, but it is also possible that epistasis could delay the evolution of additive resistance.

Considerations for the two locus case can be readily generalized to three loci. 1) Increase all three marginal SR survival rates, analogous to the two locus case above (Fig. 8-2), and multiply these through to calculate a new multiplicative survival matrix. This new matrix will be the product of three low-dose marginal survival values. 2) Alter the triple heterozygote survival to have a conditional dominance much greater than any of the marginal dominances. Additive gene action might be one value that could be used. 3) Alter all three double heterozygote survival

rates to have slightly larger conditional dominance than the marginal dominance. This assumes that two different R alleles will improve survival on the third toxin even when the larva has no R allele for the third toxin. A second alternative would be to assume that the conditional dominance of the double heterozygotes is the same as the marginal dominance of the third allele. This assumes that the R alleles do not interact. This second alternative is less conservative than the first, in that evolutionary rates should be slower in the second alternative. However, the first alternative may require an assumption about cross-resistance or some other gene-gene interaction.

Fig. 8-5. Partial cross resistance of locus 1. A. Multiplicative fitness with locus 1 conferring partial cross resistance and locus 2 reducing survival rates. B. Hypothetical epistasis with a locus for partial resistance associated with locus 2, which increases survival of all genotypes with one or more R_1 allele. Epistasis is highlighted in light red and marked in bold with an asterix.

A.

		L_1	H_1	1
		S_1S_1	S_1R_1	R_1R_1
L_2	S_2S_2	L_2L_1	L_2H_1	L_2
L_2	S_2R_2	L_2L_1	L_2H_1	L_2
L_2	R_2R_2	L_2L_1	L_2H_1	L_2

B.

		L_1	H_1	1
		S_1S_1	S_1R_1	R_1R_1
L_2	S_2S_2	L_2L_1	L_2H_1	L_2
L_2	S_2R_2	L_2L_1	**$L_2H_1+\alpha*$**	**$L_2+\alpha*$**
L_2	R_2R_2	L_2L_1	**$L_2H_1+\beta*$**	**$L_2+\beta*$**

Fig. 8-6. Partial cross resistance of locus 1, which provides resistance to toxin 1 and partial cross resistance to toxin 2. Locus 2 provides major gene resistance for toxin 2.

		L_1	H_1	1
		S_1S_1	S_1R_1	R_1R_1
L_2	S_2S_2	L_1L_2	H_1L_2	L_2
H_2	S_2R_2	L_1H_2	$H_1H_2 + \alpha$	$H_2 + \beta$
1	R_2R_2	L_1	H_1	1

Empirical Considerations

Case 1. No major resistance alleles for any of the component toxins are available.

Method 1. In this case, it is possible to estimate L_1, L_2 and their product L_1L_2 (Fig. 8-1). L_1L_2 is efficacy of the pyramided Bt plant, and can be estimated directly by exposing larvae to the *Bt* plant. For high dose events, L_1L_2 is likely to be very small and difficult to estimate accurately. Therefore a standard concentration-mortality experiment can be conducted, incorporating or overlaying diluted plant tissue into or on top of diet. In the dose calculation, the idea was to convert plant tissue concentrations into bioassay equivalents. Here the idea is the opposite, to convert bioassay concentrations into plant equivalents to estimate efficacy on the logit or probit scale. Then the mortality power of the intact plant in bioassay equivalents can be calculated, by extrapolating the concentration-response profile to 100% of the plant concentration. This assumes that the probit or logit model is accurate for extreme mortality values. For example, suppose E, efficacy of the intact plant, is very close to 1.00, but difficult to distinguish from 1.00 statistically. Take a series of dilutions of the plant and measure mortality and estimate the slope as $S = \Delta Logit\mu / \Delta log[C]$, where μ is the measured mortality at plant concentration C. C is expressed as a fraction of the intact plant, which is $C = 1$. In addition, the concentration of each component toxin should be estimated at each dilution. Then estimate a low variance point on the profile, called m, with $[C_m]$ and $Logit\mu_m$. Extrapolate the profile from $[C_m]$ to $[C] = 1$ to estimate a value for $LogitE$. E is the inverse transform of $LogitE$, and this estimated $E = \overline{L_1L_2}$ will always be strictly less than 1.00.

If an $E < 1.00$ can be estimated directly from mortality on the intact pyramided plant, then it is possible to calibrate the dilution assay with the mortality that occurs on the intact plant. It is important when estimating mortality on the intact plant that if inoculations are used that the

relatedness of the larvae is considered, because most insect colonies are started with only a few thousand individuals at best.

Additional bioassays can be conducted with separate, purified toxin components to estimate L_1 and L_2 independently. The concentration-mortality profiles for each component toxin individually should be estimated, using the same methodologies as previously. The concentrations should be similar to the measured concentrations from the plant dilution bioassay. A value of L_1 and L_2 can be estimated from each of the single toxin profiles by interpolating or extrapolating to the equivalent toxin concentration in the intact plant. This will use a statistical method similar to that described for the dilution assay. It should then be possible to test directly the null hypothesis, H_0: $\overline{L}_1\overline{L}_2 = \overline{L_1L_2}$. The null hypothesis is multiplicative fitness (no epistasis) for the $S_1S_1S_2S_2$ genotype, and is one direct test for epistasis.

Method 2. Second, epistasis can be tested indirectly for the single heterozygotes. The slopes of the concentration-response profiles or the entire profile based on all of the data can be used to calculate the expected mortality at toxin concentrations 50x less than that in the intact plant. The bioassay and statistical methodology is the same as for estimating dose, which is described in Appendix 1. The expected mortality at this concentration should be greater than the LC_{50} for high dose toxins, whether evaluated singly or together. According to EPA-SAP (1998), these mortality values may be surrogates for expected heterozygote survival. Designate these mortality rates $\mu_{i,0.02}$, where μ is the mortality rate for profile i when the concentration is 1/50 the full plant concentration (=0.02). When there are two toxins, there are three profiles, and it is possible to calculate $\mu_{1,0.02}$, $\mu_{2,0.02}$, and $\mu_{12,0.02}$, where 12 is for both toxins together. Using the same reasoning as was used in EPA-SAP (1998), $\mu_{1,0.02}$ is a surrogate for H_1, $\mu_{2,0.02}$, is a surrogate for H_2, and$\mu_{12,0.02}$, is a surrogate for the joint fitness, H_1L_2 and L_1H_2. It is then possible to test the following null hypotheses, H_0: $\overline{L}_2\overline{\mu}_{1,0.02} = \overline{\mu}_{12,0.02}$, and H_0: $\overline{L}_1\overline{\mu}_{2,0.02} = \overline{\mu}_{12,0.02}$. These null hypotheses test for multiplicative fitness of H_1L_2 and H_2L_1, respectively (Fig 8-1) forthe single heterozygotes, $S_1R_1S_2S_2$ and $S_1S_1S_2R_2$. It is probably possible to rearrange these null hypotheses so that they are expressed in terms of the original data instead of the derived values.

Case 2. A major resistance allele exists for one of the toxins, but not for any of the others.

Method 1. Assume that the major resistance allele is at locus 1. In this case, the no epistasis hypothesis (multiplicative fitness) can be directly tested using Method 1 described under case 1. The three genotypes, $S_1S_1S_2S_2$, $S_1R_1S_2S_2$, and $R_1R_1S_2S_2$ (Fig. 8-1), can be exposed to dilutions of plant tissue to estimate the joint products L_1L_2, H_1L_2 and K_1L_2. The marginal values, L_1, H_1, K_1 and L_2 can be estimated independently as described above, via four independent bioassays. This allows a direct test of the no epistasis hypothesis for three of the nine genotypes in a two locus problem (Fig. 8-1). Genotype $S_1S_1S_2S_2$ is used to estimate L_1L_2, L_1, and L_2. Genotype $S_1R_1S_2S_2$ is used to estimate H_1L_2, H_1, and L_2. Genotype $R_1R_1S_2S_2$ is used to estimate K_1L_2, K_1, and L_2.

Method 2. Second, indirect means similar to those used in Method 2 described under case 1 can be used to test for epistasis in the genotypes heterozygous for the other locus, the $S_1S_1S_2R_2$, $S_1R_1S_2R_2$, and $R_1R_1S_2R_2$ genotypes (Fig. 8-1). The $S_1S_1S_2S_2$ genotype is used to test for epistasis in $S_1S_1S_2R_2$ as described in case 1. The $S_1R_1S_2S_2$ genotype is used to test for epistasis in $S_1R_1S_2R_2$ similar to the description in case 1. The $R_1R_1S_2S_2$ genotype is used to test for epistasis in $R_1R_1S_2R_2$ similarly.

Method 3. Third, absence of epistasis between resistance loci can be defined as when the resistance mechanism associated with each locus operates independently from the mechanism at the other resistance loci. This can be tested directly when at least one major resistance allele is available at one of the loci. If the mechanisms are independent, then the major resistance allele will confer resistance for one toxin, but will not affect survival on any of the other single toxins. In other words, the $S_1R_1S_2S_2$ and $R_1R_1S_2S_2$ genotypes may improve survival over that of the $S_1S_1S_2S_2$ genotype on toxin 1, but there should be no difference in survival associated with any of the three genotypes on toxin 2.

This can be tested by estimating a concentration-mortality profile for the R_1 allele on toxin 2. If the profiles for the three genotypes, $S_1S_1S_2S_2$, $S_1R_1S_2S_2$ and $R_1R_1S_2S_2$, are not different, the hypothesis of independent resistance mechanisms cannot be rejected and epistasis for toxin 2 is small or absent. If they are different, there is likely to be epistasis. If the $S_1R_1S_2S_2$ and $R_1R_1S_2S_2$ genotypes survive better than the $S_1S_1S_2S_2$ genotype, epistasis will increase survival and speed up resistance evolution. The epistasis will occur in the $S_1R_1S_2S_2$ and $R_1R_1S_2S_2$ genotypes, and may occur in the $S_1R_1S_2R_2$ and $R_1R_1S_2R_2$ genotypes. It may also affect the fitness of the $S_1R_1R_2R_2$ and $R_1R_1R_2R_2$ genotypes if the R_2 resistance is incomplete.

Based on the data summarized in Table A2.4, the major gene for Cry1Fa resistance in ECB shows epistasis for both Cry1A.105 and Cry2Ab2. This epistasis will increase the rate of resistance evolution. Estimation of the three locus epistasis problem is considerably more complex than two locus epistasis because the fitness of each of the 27 three-locus genotypes is determined by the independent action of the three loci plus three kinds of two-locus epistasis and one kind of three-locus epistasis. In some cases, it will be possible to test the null hypothesis of independent three-locus fitness, such as for the direct tests described above (Method 1), but in other cases it may be necessary to evaluate the pairwise null hypothesis that two of the loci have independent fitness (e.g., Methods 2 and 3).

Charge Question 9

Based on a review of the submitted simulation modeling, the preliminary conclusions are:

1) *For CRW, a 5% seed mixture and a 5% structured refuge had comparable durability in both the EPA and Dow models;*

2) *For ECB, a 5% seed mixture was less durable than a 5% structured refuge in simulations with EPA's model. However, ECB resistance did not evolve within 158 generations in any of the simulations with the 5% seed mixture, similar to the level of durability predicted by Monsanto's model. There was no difference in durability between the 5% seed mixture and the 5% structured refuge in Monsanto's model. Resistance did not evolve to either refuge option within 100 generations (the extent to which the model was run);*

3) *For SWCB, a 5% seed mixture was less durable (78 generations) than a 5% structured refuge (118 generations) in EPA's model simulations. Conversely, with Monsanto's model there was no difference in the prediction for durability between the 5% seed*

mixture and the 5% structured refuge. Resistance did not evolve to either refuge option within 100 generations (the limit of the model simulations).

Please comment on the reliability of the estimates of resistance evolution by each of the three models in light of the biological and parameter uncertainties identified by BPPD.

Panel Response

Due to the uncertainties associated with the Monsanto, Dow, and EPA/ORD modeling efforts for ECB, SWCB, and CRW, the Panel concluded that there was an insufficient scientific basis for supporting the SmartStax RIB 5% seed mixture as an effective IRM strategy for all pests of concern. As is well-accepted in the scientific literature (e.g., Mallet and Porter 1992; Davis and Onstad 2000) and concluded in the 1998 SAP Report on *Bacillus thuringiensis (Bt)* Plant-Pesticides and Resistance Management, for high-dose cases when toxicity of the cultivar causes low survival of heterozygous pest individuals, seed mixtures will have lower durability than structured refuges with the same percentage of *Bt* plants. This point was illustrated by the modeling exercise performed during the meeting (see Appendix 2). Also, there are greater uncertainties for seed mixtures than for structured refuges due to the lack of information about larval movement for ECB, and SWCB, and how larval movement affects the survival of heterozygotes.

In particular, the Panel concluded that for ECB and SWCB, the 5% seed mixtures would lead to more rapid resistance evolution than the 5% structured refuges. For CRW, the 5% seed mixture will likely have similar durability to the 5% structured refuges due to the low-dose nature of resistance. Nonetheless, the overall IRM strategy should be based on the target pest with the greatest increase in risk of resistance evolution, which is likely to be SWCB or ECB, rather than CRW.

The Panel concluded that the Monsanto, Dow and EPA/ORD models all contain attributes that lead to overestimates of the durability of the 5% seed mixture, especially for SWCB. Note that the overestimation of the durability of the 5% seed mixture is a separate (though related) issue from the underestimation of the difference between the durability of 5% seed mixtures and 5% structured refuges; indeed, many of these attributes that lead to overestimation of the durability of 5% seed mixtures also lead to overestimate of the durability of 5% structured refuges. The Panel concluded that the estimated generations of durability were not scientifically credible and that these numbers were likely overestimated for ECB, SWCB, and CRW. The degree of overestimation could be considerable, as illustrated by the modeling exercise in Appendix 2.

The Monsanto model overestimated the durability of seed mixtures for several reasons. First, the Monsanto model did not account explicitly for the movement of larvae among plants in seed mixtures and the resulting potential for large increases in the survival of heterozygotes that will greatly speed resistance in ECB and SWCB. Second, they did not report the actual simulated durability with either seed mixtures or structured refuges. The EPA should encourage reporting of mean, 5% risk and 0% risk durability for all simulation models because the relative model results may have meaning even when the estimated number of generations do not. Despite the lack of information provided by Monsanto, the Panel concluded that the relative estimates that

could have been reported by the Monsanto model would underestimate the difference in durability between seed mixtures and structured refuges. Third, the Monsanto model did not consider any fitness values with epistasis. Epistasis can increase survival of single, double and triple heterozygotes that will also greatly speed resistance in ECB and SWCB (see Charge Question 8).

The EPA/ORD model and analyses, although generally superior to those of Monsanto and Dow, also underestimated the difference in durability between seed mixtures and structured refuges, and overestimates the durability of seed mixtures, for both ECB and SWCB. The model explicitly accounts for movement of larvae among plants and explores fitness values with some epistasis. However, it did not explore the realistic larval movement scenarios and hypotheses listed in the Response to Charge Question 2, and did not explore epistasis sufficiently as explained in the Response to Charge Question 8. Specifically, the Panel stated that there were three attributes that likely lead to overestimates in the durability of seed mixtures: 1) the model contains no density-dependent larval survival, 2) the sensitivity analysis could give artificially low estimates of the risk of resistance, and 3) the manner in which survival of heterozygotes is computed could lead to overestimates of durability.

The Panel concluded that the Dow and EPA/ORD models for CRW did not underestimate the difference in durability between a 5% seed mixture and a 5% structured refuge. Independent modeling by the Panel confirmed that there is likely to be no or little difference in the durability of a 5% seed mixture and a 5% structured refuge. Nonetheless, the Dow model did overestimate the durability of seed mixtures (and also structured refuges). The Dow model, as EPA-BPPD noted, uses high-dose values of survival rates of CRW that will overestimate durability. Also, these conclusions come with the caveat that none of the models considered multilocus resistance mechanisms that could operate in the low-dose case of CRW.

The Panel noted that modeling resistance durability involves high levels of uncertainty, and interpreting model results must be done in light of this uncertainty. One source of uncertainty (decision model uncertainty) stems from considering only a limited range of models or comparisons among models. For example, the charge from EPA-BPPD was to compare a 5% seed mixture with a 5% structured refuge, and this constraint on the comparisons limited the information that could be obtained from the models. The Panel also indicated that there was uncertainty about the structure of the models (model completeness). The models may leave out attributes that affect resistance evolution, such as density-dependent larval mortality. A further source of uncertainty that surrounds the predictions of the models is parameter uncertainty. Although a range of parameter values was investigated in the Monsanto and Dow models, and a more thorough sensitivity analysis was done for the EPA/ORD model, the Panel emphasized that there was a high degree of uncertainty for pyramided *Bt* traits and seed mixtures, and recommended the use of sophisticated risk assessment techniques, such as scenario analysis and information gap analysis, to address these uncertainties.

The Panel was not provided sufficient information to estimate how much the durability of 5% SmartStax seed mixture was overestimated or how much the uncertainty mattered. However, the magnitude of this uncertainty is potentially large. To give an illustration of this, one element of the uncertainty about model completeness, the effect of density-dependence, could by itself have

a large effect. If resistance is 20x faster in a seed mixture than a structured refuge with weak density dependence (Appendix 2) and only 7x faster without density-dependence (EPA/ORD model) for Leidoptera, then including density dependence in the models could speed up resistance evolution 2.8x compared to the EPA/ORD model. Multiplying this against the durability predictions reported for the EPA/ORD model, this single source of uncertainty could reduce the projected durability of the 5% SmartStax seed mixture to 57 generations for ECB. Other factors mentioned above could further reduce durability, so the magnitude of uncertainty is clearly large and significant. This example is only for illustrative purposes; the Panel cautions that while the models show that the effects of density dependence can be large, quantifying how large is impossible given the lack of information Monsanto and Dow know about the target pests of SmartStax corn.

If survival of CRW is modeled with low-dose survival rates using the Panel model, the average durability might be 32 generations. This corresponds well with the results reported for the EPA/ORD model, but is substantially less than the results reported for the Dow model. None of these models address uncertainty related to epistasis.

All of these issues related to uncertainty in the model structure and uncertainty about the parameter values are indicated by "-" in Table 9-1. If the durability of the 5% seed mixture is overestimated by a feature in the model, this is indicated by "O". This could occur either because the comparison to a 5% structured refuge is overestimated (ratio of durability of 5% seed mixture/ 5% structured refuge), or because the durability of the 5% structured refuge is overestimated (durability of 5% structured refuge). All of the Lepidoptera models seriously overestimate the ratio of durability of a 5% seed mixture compared to a 5% structured refuge. The models estimated the ratio at 1/7 and 1/20, but both of these are still seriously overestimated. The Lepidoptera models also seriously overestimated the durability of the 5% structured refuge. The Panel warns against basing policy on these durability values, and did not evaluate the overall durability of the Panel model for this reason. However, the Panel understands the temptation to think that durability of 158 generations is a long time, even if it is off by a factor of 2. The Panel model was used to indicate to decision-makers that these long times can be quickly reduced by model and parameter uncertainties. For example, when density-dependent mortality is included (Panel model reported as '+A'), it can reduce durability 2.8-fold compared to a model without density-dependent mortality. As this is only one of the seven ways that durability was overestimated by the Monsanto and EPA/ORD models, at current levels of uncertainty, these durabilities may be considerably overestimated.

Table 9-1. Summary of Panel's evaluation of the evolution models, indicating how the models have overestimated the durability of the 5% seed mix refuge. A = Neither underestimates nor overestimates ratio or durability; U = Underestimates ratio or durability; O = Overestimates ratio or durability; other symbols are defined in footnote to this table.

	Lepidopteran-active toxins (ECB and SWCB)			Coleopteran-active toxins (WCR and NCR)		
	Monsanto model	EPA/ORD model	Panel model	Dow model	EPA/ORD model	Panel model
Ratio of durability of 5% seed mix/ 5% structured refuge	O	O	O	A	A	A
Model Structure						
Larval movement	- O	+	+	N	N	N
Selection in ears	- O	- O	- O	X	X	X
Correlations with larval movement parameters	- ?	- ?	- ?	N	N	N
Parameter Values						
Larval movement hypotheses	- O	- O	- O	N	N	N
Epistasis	- O	+ O	+ (O)	N	N	N
Cross resistance	- O	- O	- O	N	N	N
Adult dispersal	+ (O)	+ (O)	+ (O)	N	N	N
Ratio of durability of 10% seed mix / 5% structured refuge	Not evaluated (decision model uncertainty)					
Durability of 5% structured refuge	O	O	N	O	O	N
Model Structure						
Density dependent mortality	- O	- O	+ A	+ A	- O	+ A
Density-dependent selection	- (O)	- (O)	- (O)	- O	- O	- O
Correct locus number	+ A	+ A	- U	+ O	+ O	+ O
Stochastic	- ?	-/+ ?	- ?	+ ?	-/+ ?	- ?
Non-random oviposition	- ?	- ?	- ?	- ?	- ?	- ?
Local non-random mating	- O	- O	- O	- O	- O	- O

Cost of resistance	- U	- U	- U	- U	- U	- U
Quantitative genetics	X	X	X	- O	- O	- O
Parameter Values						
Initial R allele frequencies	+ U/A	+ U/A	+ U/A	+ O	+ O	+ O
Heterozygote survival	- O	- O	- O	- O	- O	+ A
Epistasis	- O	- O	+ (O)	- O	- O	- O
Cross resistance	- O	- O	- O	- O	- O	- O
Dose mortality	N	N	N	- O	- O	- O
1st order Monte Carlo simulations	- O	+ O	- O	- O	+ O	- O
Temporal variation	- ?	+ ?	- ?	- ?	+ ?	- ?
Spatial variation	- O	+ O	- O	- O	+ O	- O

Symbols are defined as follows: + = structure included in model or appropriate parameter values explored; - = structure missing from model or appropriate parameter values not explored; -/+ = evaluated in some, but not all simulations; N = Not evaluated by Panel; ? = Panel uncertain about direction of effect; () = Panel uncertain that magnitude is significant; X = Not needed in model

The Panel recommended several areas that needed further theoretical investigation to assess and design IRM strategies for seed mixtures:

1) Modify the Monsanto, Dow, and EPA/ORD models to remove the attributes that likely lead to overestimates of durability, or develop new models that more accurately assess durability

2) Investigate the integration of IRM strategies with adaptive IRM management approaches that include well-defined triggers for taking remedial actions and clearly identify the most appropriate remedial actions.

3) Model IRM at the regional scale to account for risks of resistance evolution that may result from long-term, region-wide suppression of pest population densities; and

4) Investigate the incremental introduction of products such as the 5% SmartStax seed mixture, which have high levels of risk uncertainty, especially for lepidopteran pests. This incremental approach would decrease the percent of non-*Bt* in a seed mixture based on coupling monitoring for resistance and population density.

Monsanto, Dow, and EPA/ORD Models Overestimate Durability

Monsanto Model for ECB (SWCB)

The Monsanto model for ECB and SWCB resistance modeled larval movement among plants in seed mixtures implicitly as a decrease in refuge size and appeared to be a frequency-only model (although this was not explicitly stated in their documentation). The Panel indicated that these attributes would overestimate the time to resistance failure of SmartStax for ECB and SWCB. Additional discussion is provided here on the effect of larval movement, while comments on frequency-only models are provided in the discussion of the EPA/ORD model.

The Monsanto model assumes that the effect of larval movement in seed mixtures would increase the mortality of susceptible larvae. For larvae that are initially on non-*Bt* plants, if they move from the plant, they are likely to move to *Bt* plants and thus be killed. As Mallet and Porter (1992) point out, however, the main effect on resistance evolution of larval movement within seed mixtures is to increase the relative survival of heterozygotes. When selection occurs both before and after larval movement between plants, the relative survival of heterozygotes goes up, and this speeds the rate of resistance evolution. This effect on heterozygotes was not included in the Monsanto model. Therefore, this model overestimated the durability of seed mixtures because it excluded the main mode of increased resistance evolution caused by movement of larvae between plants in seed mixtures. See Appendix 2, **Monsanto's Implicit Modeling of Larval Movement**.

Dow Model

The Dow model of CRW resistance used high-dose values that were corrected for density-dependent mortality, in contrast to recommendations by BPPD that dose should not be corrected

for density-dependent mortality (lower dose) (see discussion in response to Charge Question 5). Because of this, Dow's model will likely overestimate the durability of SmartStax with regard to CRW protection.

This notwithstanding, the Panel stated that in low-dose situations, such as for the two *Bt* CRW toxins in SmartStax, a seed mixture is likely similar in durability to a structured refuge (Appendix 2: **High- and Low-Dose Scenarios and Larval Movement in Seed Mixtures**). Therefore, a SmartStax RIB 5% seed mixture will likely have similar durability as a 5% structured refuge (the current refuge requirement) for CRW.

EPA/ORD Model

The EPA/ORD model contained three attributes that will lead to overestimating the durability of the SmartStax RIB 5% seed mixture.

1) The EPA/ORD model is a frequency-only model that contained no density-dependent larval survival for all three target pests (ECB, SWCB, and CRW). As explained below, this will lead to longer times to control failure than comparable models that include density-dependent larval survival.

2) EPA/ORD performed a sensitivity analysis to assess their model, and this sensitivity analysis was performed in such a way that seed mixes will appear to be more durable than they actually are.

3) For ECB and SWCB the model assumes relatively low survival of heterozygotes; as discussed under Charge Question 8, relatively low survival of heterozygotes leads to overestimates of durability.

The Panel addressed 1) and 2) in more detail, as described below.

1) Density-Dependent Larval Mortality. For all models with density-dependent larval mortality, resistance occurs more rapidly than for the corresponding model with no density-dependent mortality, referred to here as "frequency only" model. This is because density-dependent mortality acts primarily on non-*Bt* plants where the density of susceptible larvae can be high. The Panel pointed to the importance of distinguishing between the relationship between density and mortality (sometimes referred to as the strength of density dependence), and the degree of mortality caused by density dependence. Even though in some cases density-dependent mortality may increase only slowly with density, there can still be high density-dependent mortality if densities are sufficiently high. Therefore, the effect of density-dependent mortality on resistance evolution is not determined by the value of a parameter in any model, but instead, depends on both model parameters and the density that larvae achieve in the model.

Density-dependent mortality increases the rate of resistance evolution by decreasing the survival of susceptible insects. This has the effect of increasing the survival of heterozygous insects (partially resistant) relative to susceptible insects. This, in turn,

increases selection on heterozygotes and speeds resistance evolution. In models, the functional form of density-dependent mortality is unimportant provided the insect population is not approaching extinction (i.e., decreasing exponentially). By ignoring density-dependent mortality, the EPA/ORD model will overestimate the durability of SmartStax (see Appendix 2: **Density-Dependent vs. Frequency-Only Models**).

In general, density-dependent models will be appropriate for all pest species. It is not necessary to demonstrate density-dependent mortality in the field in order to use a density-dependent model; instead, density-dependent models should be the starting point for analysis of resistance evolution. This is because there are only a few special cases in which a frequency-only model is appropriate. These include: (a) when the pest population is dropping exponentially to extinction, (b) when density-dependent mortality occurs for the adults (but not for the larvae), and (c) when density-dependent mortality acts equally on all genotypes. The last situation could arise if, for example, a natural enemy attacked pests and did not respond to variation in pest density among *Bt* and non-*Bt* plants within seed mixtures, or between refuge and *Bt* fields when structured refuges are used (Ives et al. 2011). Nonetheless, based on existing evidence density-dependent larval mortality occurs at the scale of plants for CRW, ECB, and SWCB, making density-dependent models necessary.

2) **Sensitivity Analysis.** EPA/ORD conducted a sensitivity analysis by randomly sampling from a distribution of parameter values. The Panel noted that this procedure, while designed to address parameter uncertainty, does not address other forms of uncertainty, such as uncertainty in the structure of the model (e.g., the contrast between frequency-only vs. density-dependent models, or the procedure EPA/ORD used to assign survival rates to genotypes) and uncertainty in the scope of investigation (e.g., whether CEW should be considered in the assessment of IRM for SmartStax).

The Panel suggested that care should be taken in distinguishing sources of variation in parameters when interpreting the parameter sensitivity analyses presented by EPA/ORD. For some parameters (e.g., the probability that larvae move between plants) the greatest source of uncertainty might be lack of information about larval movement. In contrast, for other parameters (e.g., the initial resistance allele frequencies) uncertainty might be due to real variation across the spatial extent of the target pest population. One way to conceptualize this difference is to consider the spatial scale of variation. If all of the variation in the probability that larvae move between plants, for example, is due to lack of information, then this variability will be the same across North America. If a sensitivity analysis based on this variation is conducted and shows, for example, that there is a 0.99 probability that resistance does not occur in 25 years, then rapid resistance evolution is unlikely across entire North America. In contrast, real variation in initial allele frequency might occur at the scale of counties, with some counties having high and some counties having low initial allele frequencies. If there is a 0.99 probability that resistance does not occur in 25 years, and if there are 1000 counties in which the pest occurs, then assuming that populations in different counties are evolutionarily independent, resistance will likely occur in 10 counties within 25 years. Because

resistance to high-dose *Bt* products will likely spread geographically, continent-wide resistance could easily occur within 25 years.

The Panel suggested alternative procedures for risk analysis that should be conducted along with the sensitivity analysis conducted by EPA/ORD. First, the models could be studied in detail to understand what factors mathematically are expected to cause rapid resistance evolution. This might be particularly helpful in comparing predictions from different models or for different pests. Second, EPA/ORD could select parameters from broad, uniform distributions and determine which sets of parameters give rapid resistance. This procedure is conceptually similar to the regression analyses used by EPA/ORD, although instead with a focus on extremes and parameter interactions. This approach would also avoid the difficulties of regression analyses that make linear assumptions about the response of the time to resistance failure on parameter values. Using this process might be helpful in determining interactions among parameters that lead to resistance.

Panel Model

The Panel model also contained several attributes that will lead to overestimating the durability of the SmartStax RIB 5% seed mixture for Lepidoptera pests. These included not evaluating selection in the corn ears, and not evaluating cross resistance and larval movement sufficiently. The Panel model only evaluated two-locus evolution, which will underestimate the durability of both the 5% seed mixture and the 5% structured refuge. However, the ratio between the seed mixture and structured refuge should not be greatly affected by this factor. Consequently, the Panel did not use its model to consider durability in terms of numbers of generations. Instead, the model was used to characterize uncertainty in relative terms, i.e., how many times shorter or longer was durability when the uncertain factor was allowed to vary.

Model Uncertainties

The Panel stated that model uncertainties should be considered when assessing model predictions of the evolution of resistance using different IRM strategies. A general discussion of the types of uncertainties that pervaded all models used by Monsanto, Dow and EPA/ORD and suggestions on how to address these model uncertainties is provided below.

The models used here target what is expected to be the principal process important to the evolution of insect resistances, but they, like all models, represent an abstraction of a much more complex ecological reality. Local processes, scale issues, and spatial substructuring can create conditions that can cause unpredictable events and influence the rate and emergence of resistance). The ecological context of resistance development is often local, scale dependent, and hard to predict (Durrett and Levin 1994). Other authors note that the potential for surprises in the evolution of resistance are found ubiquitously in these complex systems. For example, population substructuring, such as will be found in field-level refuges, is known to convert epitasis into additive genetic variance, which can allow resistance to develop (Takahasi 2007; Jarvis and Cheverud 2009). This is why the Panel stated that it is important to be more cautious about the likelihood of resistance vis-à-vis these models.

The fact that models are abstractions of reality leads to many kinds of uncertainty that should be considered when evaluating risk. Epistemic uncertainty is uncertainty that can be reduced by increased knowledge. There are several kinds of epistemic uncertainty (Regan et al. 2002): ignorance, decision model uncertainty, model uncertainty and uncertainty about causality, interpretive uncertainty, and parameter uncertainty. Each one of these areas is discussed in turn.

1) **Ignorance.** There are many sources of ignorance (Regan et al. 2002), but these cannot be addressed in a single risk assessment, such as being considered in this Panel.

2) **Decision Model Uncertainty.** In the case of examining the durability of 5% seed mixture, this pertains to the IRM management options given to the Panel for consideration. The decision models used by Monsanto, Dow, and EPA/ORD appear to be constrained to a comparison of a 5% block refuge versus a 5% seed mixture refuge. While this is a critical comparison, there is uncertainty that this is the only critical comparison. For example, the Panel could have been asked to compare a 5% block refuge with a 10% seed mixture refuge, or to compare a 5% seed mixture refuge with a 10% seed mixture refuge. Indeed, 20% seed mixture refuges could have been another basis for comparison. Because the modeling results for the 5% seed mixture refuge are likely to be overestimates of durability, the narrow decision model creates decision model uncertainty.

3) **Model Uncertainty and Uncertainty about Causality.** Model uncertainty and uncertainty about causality is a more pervasive source of uncertainty (Regan et al. 2002). The main kinds that are relevant to the present case are associated with model structure and predictions/extrapolations. Some of the main sources of uncertainty about model structure include: completeness of states, relationships among states, and nonlinearities among the relationships (Regan et al. 2002). The model results were not robust to uncertainty about completeness of states. Several of the models did not include state variables for population density and include variables only for allele frequency. These have been referred to as "frequency-only" models. Frequency-only models overestimate durability, sometimes by considerable amounts. The Panel noted that the allele frequency models for CRW may have an inappropriate structure; quantitative genetics models may be less prone to overestimation and would require a different set of state variables. The models were also not robust to uncertainty about the relationships among states. For example, all of the models assumed random oviposition and random mating. Reasonable biological processes presented in the response to Charge Question 7 suggested alternative relationships that would accelerate resistance evolution for ECB and SWCB. Analogous process could be provided for CRW; however, these alternative relationships will be nonlinear. Standard approaches to addressing uncertainty about model structure include qualitative modeling and comparison of alternative models (Regan et al. 2002). Although at least two models were used to evaluate CRW and ECB/SWCB, these models were not distinct enough to be considered alternative models. Thus, the Panel concluded that the uncertainty about model structure was not effectively evaluated.

Several approaches have been used to address uncertainty associated with predictions and extrapolations. These include uncertainty factors, scenario analysis, and other more novel approaches, such as information gap theory (Regan et al. 2005). Uncertainty factors have been widely used in toxicological studies, but they are probably not appropriate for the evolutionary models considered here. Some scenario analysis was done, but the models were not exercised sufficiently to characterize the robustness and conservatism of the predictions. Information-gap theory might be used to address these uncertainties. One of the major sources of uncertainty associated with the extrapolations of the models is the assumed independence of the potential variation in the parameter values. One approach to scenario development is to identify potentially important covariance among the parameter values and specify some biological scenarios that could give rise to this important covariance. This can be done by running the model with systematically varying parameter values that cover the range of possible values of each parameter in combination with variation in all of the other parameters. The output can be screened to identify the worst cases and the parameter values associated with these worst cases can be identified. Inspection of these values will generate hypotheses about important covariance, and the model can be run again to test these covariance hypotheses. Once potentially important covariance is identified, biological scenarios can be generated that show the identified covariance, and these scenarios can be used to guide interpretation of the model output.

4) **Interpretive Uncertainty.** Interpretive uncertainty arises from the necessary subjective judgments in the interpretation of the model output. The major interpretive uncertainty that runs throughout the discussion of the model results is that the model output is believed to be meaningful only in a comparative context, but the model output is often interpreted in an absolute context. Specifically, the credible interpretation of the number of generations to failure is to relate it to some standard for comparison, for example, a 5% block refuge. The results should be presented as the ratio of generations to failure of the 5% mixed seed refuge to the standard for comparison. However, the model results were often interpreted absolutely. For example, if the time to resistance failure was more than 100 generations, then it was considered sufficiently conservative. This results in significant interpretive uncertainty for some of the models because the models were terminated before it was possible to determine the relative rate of evolution for the 5% seed mixture refuge compared to a standard.

5) **Parameter uncertainty.** Parameter uncertainty is associated with measurement error, biased estimation, variation in space and time, and dependencies among parameters. There are many standard approaches for the first three of these, and we have already outlined a robust approach to dependencies among parameters in the broader context of model uncertainty. The models were designed to address some of these sources of parameter uncertainty. Several deficiencies remained. First the stochastic models and the Monte Carlo simulations were not run a sufficient number of times. The number of runs should have been proportional to the x^n, where x is the estimated number of different runs for any one parameter, holding all others constant, and n is the number of parameters. If $x = 3$ and there are 12 parameters, this requires more than 500,000 runs. Most of the models have substantially more than 12 parameters. Second, as noted in our

response to Charge Question 5, measurement errors were not included in the parameterization of dose for CRWs. Third, the Monsanto and Dow models did not consider variation in space and time; while the EPA/ORD model did, more could be done to investigate spatial and temporal variation.

General Modeling Issues for SmartStax IRM

The Panel recommended that emphasis in modeling assessments of stacked cultivars should be placed on durability for the pest that shows the greatest potential rate of resistance evolution. The Panel suspected that this may be SWCB for SmartStax, although acknowledged that this was only a suspicion. Because of the low-dose characteristics shown by CRW to SmartStax toxins, it may have the lowest risk of resistance evolution in SmartStax RIB in comparison to deploying SmartStax with a structured refuge.

The 2009 SAP for Pioneer's Optimum AcreMax 1 seed mixture targeting CRW recommended a 20% seed mixture given that the SAP was comparing this against a 20% structured refuge (the mandated requirement) (EPA SAP 2009). At the same time, the 2009 SAP emphasized that this should not set precedence for future policy decisions regarding seed mixtures due to the unique low-dose characteristics of Optimum AcreMax 1 against CRW. Larval movement for low-dose *Bt* expression is a less-serious threat to IRM than for high-dose *Bt* expression, as illustrated in Appendix 2: **High and Low Dose Scenarios and Larval Movement in Seed Mixtures**. The Panel emphasized that SmartStax RIB did not have the same low-dose characteristics as Optimum AcreMax1 for ECB and SWCB; therefore, the same IRM recommendation is not appropriate.

The Panel recommended several areas that needed further emphasis and theoretical investigation to assess and design IRM strategies for products such as SmartStax RIB:

1) Modify the Monsanto, Dow, and EPA/ORD models to remove the attributes that likely lead to overestimates of durability, or develop new models that more accurately assess durability;

2) Investigate the integration of IRM strategies with adaptive IRM management approaches that include well-defined triggers for taking remedial actions and clearly identify the most appropriate remedial actions;

3) Model IRM at the regional scale to account for risks of resistance evolution that may result from long-term, region-wide suppression of pest population densities; and

4) Investigate the incremental introduction of products such as the 5% SmartStax seed mixture, which have high levels of risk uncertainty, especially for lepidopteran pests. This incremental approach would decrease the percent of non-*Bt* in a seed mixture based on coupling monitoring for resistance and population density.

Each of these recommendations for additional modeling is described in more detail below.

Remove Model Attributes that Overestimate Durability

As described above ("Monsanto, Dow, and EPQ/ORD models overestimate durability"), all three models have attributes that will lead them to overestimate durability of seed mixtures. The Panel recommended that these attributes be changed in the models, and the models should be re-analyzed.

The Panel explained that frequency-only models will overestimate durability relative to density-dependent models. When pest larvae experience density-dependent mortality, the rate of resistance evolution does not change greatly with the percentage of non-*Bt* seed in a seed mixture. However, if adoption of *Bt* crops suppresses pests so effectively that the pest population approaches extinction and density-dependent mortality no longer occurs, a frequency-only model is appropriate. Frequency-only models are much more sensitive to the percentage of non-*Bt* seed in a seed mixture. This is important for IRM because the risk of suppressing pests to very low regional densities, and the consequent potential for very rapid resistance evolution, increases greatly with low percentages of non-*Bt* seed in seed mixtures. Simply, IRM depends on the persistence of susceptible insects, and if broad adoption of seed mixtures threatens the regional persistence of the pest, then the risk of resistance evolution increases greatly.

The Panel emphasized some ways in which the Monsanto, Dow, and EPA/ORD models overestimate the durability of SmartStax seed mixtures. Nonetheless, other attributes of the models will likely lead to underestimates of durability, such as the absence of a cost of resistance. Ideally, we would like to be able to compare those factors that lead to overestimates with those factors that lead to underestimates. The Panel, however, stated that the levels of uncertainty in the models are too high to allow this type of quantitative comparison. The Panel emphasized that the level of uncertainty, and possible overestimation of durability, is likely greatest for SWCB. Furthermore, as noted in the Panel's response to Charge Question 3, ECB is not an adequate surrogate for SWCB. A key difference between SWCB and ECB that will greatly increase the rate of resistance evolution in models is the lesser sensitivity of SWCB larvae to Cry toxins than ECB larvae (registrant study, RPN-09-075, Head et al. 2009). Therefore, RS heterozygote survival may exceed 5%, for one or more of the toxins. This will neutralize the advantages of the high-dose strategy and lead to more-rapid resistance evolution. Given the Panel's concerns about model uncertainty, assessments of IRM with seed mixtures must be extremely cautious, erring on the side of underestimating durability.

The Panel cautioned against using the argument that consistent quantitative (as opposed to qualitative) results among different models provide strong support for predictions about the time to resistance failure. The Monsanto, Dow, EPA/ORD, and Panel models have very different structures, yet they cannot be viewed as giving independent information about the time to resistance (a quantitative result). For example, all are based on the assumption that resistance will occur in the form of major resistance alleles, and that cross-resistance will be minimal. They also share many of the same uncertainties, such as how survival rates of genotypes to different toxins combine (Charge Question 8).

The Panel also cautioned that model predictions concerning the time to resistance are not absolute values. For example, if a model (under a given set of parameters) predicts that the

time to resistance failure is 100 generations, this prediction should not be taken literally as meaning that resistance will not occur within 100 generations. The Panel agreed with the points made by Dr. Nicholas Storer (Dow AgroSciences) in his public comments that models are most valuable in making qualitative comparisons among different strategies, such as between a 5% structured refuge and a 5% seed mixture. This comparison can be made in terms of the relative time to resistance failure of two strategies, but the absolute time to resistance failure should not be used to assess policy decisions.

***Bt* Resistance Monitoring and Remediation Plans**

During the public meeting, EPA provided the Panel (at the Panel's request) with the resistance monitoring and remediation plans for all of the *Bt* PIPs that are part of SmartStax corn. Each registrant was required to develop and implement monitoring and remedial action plan for each PIP. EPA indicated that the current monitoring and remedial action plans for each PIP component of SmartStax corn should be applicable to all PIPs as expressed in SmartStax corn. The Panel noted that there was no unified monitoring and remedial action plan designed specifically for all PIPs as expressed in SmartStax corn. The Panel emphasized the point that monitoring and remediation plans are integral components of IRM strategies. Designing monitoring and remediation plans requires the same scientific information needed to develop IRM strategies. The same high levels of scientific uncertainty discussed throughout this report for the durability of SmartStax seed mixtures are also applicable to the development and implementation of monitoring and remedial action plans.

Based on previous SAP reports for EPA on *Bt* resistance management, the premise for doing monitoring of the frequency and level of resistance in target species is that such monitoring could trigger actions to delay or avoid field resistance from evolving. The current documents provide no valuable guidance beyond requiring a response when confirmed genetically based field resistance causes economic losses in a local area, and the response is only for the local area affected. There is vague language that might imply a possible broader geographic response, but there is no plan for implementation.

The EPA requires that the registrant (e.g., Monsanto or Dow in the case of SmartStax) conduct annual resistance monitoring and implement certain actions should resistance be "suspected" and additional steps should resistance be "confirmed." The Panel indicated that the steps to confirm field resistance are difficult and ill-defined. It could easily take two years to prove that there was "confirmed" resistance even if crops were destroyed by the insects in the same year that resistance was discovered. This is because the remedial action plan stated that "all of the following criteria are met by progeny from the target pest sampled from the area of suspected resistance," before resistance is considered "confirmed." Once "field resistance" is confirmed then the EPA requires that specific remedial action plan must be quickly developed, but the Panel did not see in any of the remedial action plans when these specific remedial action plans needed to be implemented.

The Panel affirmed that the timing of remediation is important. The Panel noted that previous SAPs which addressed monitoring and remediation stated that for most pests, once "field

resistance" is reached the resistance alleles would no longer be locally confined and therefore local action would often be ineffectual.

The Panel indicated that more effective approaches for monitoring single allele resistance (Andow and Alstad 1998; Blanco et al. 2008; Venette et al. 2000; Mahon et al. 2010) and quantitative genetic resistance (Gould 1978; Karowe 1990) have been developed and should be considered. Furthermore, approaches for adaptive response to incipient resistance have been outlined, and these should also be considered (Andow and Ives 2002; Downes et al. 2010).

As currently written, if CRW developed complete genetic resistance to one of the two *Bt* toxins in these cultivars, no action would be taken to change the refuge requirement. This is because it would be impossible to meet the criteria laid out in the documents for establishing "confirmed resistance" under these conditions. Furthermore, if there was complete genetic resistance of CRW to both toxins in the stacked cultivars, it could still take two years or more before testing could meet the criteria in the current document for confirming resistance, and it could take three seasons or more before effective remedial action was taken at even a local level.

As witnessed by the recent disputes over what is genetic resistance to *Bt* in CEW (Tabashnik et al. 2008a, b, c; Moar et al. 2008), the Panel recommended that science-based monitoring and remediation plans be integrated using models to assess monitoring and remediation tactics much more accurately. This will require more precise definitions of field resistance that make it easy to integrate information about field resistance into remediation plans and well-defined triggers that require changes in the IRM plan. The remedial action triggers should be informed by the most current scientific information about IRM and be adaptive to new scientific information. Timing could be critical in responding to resistance, and therefore the steps in a remediation plan should be clearly defined and easily implementable.

Regional-Scale Pest Abundances and Seed Mixtures

Although field trials were performed by Monsanto and Dow, these trials were not on the spatial and temporal scales needed to assess the long-term consequences of 5% seed mixture adoption. Recent work by Hutchison et al. (2010) demonstrated that the wide-spread use of *Bt* plants reduces the region-wide population abundance of ECB; similar region-wide effects are possible for other target pests in high-dose cases of toxicity. Because of the regional spatial scale of this effect, multiple years were required before it was manifested.

The region-wide decrease in pest abundances affects decisions about SmartStax IRM in two ways. First, the benefits of a seed mixture >>5% (e.g., a 20% seed mixture) in reducing pest abundances and crop damage may be as great or greater than the benefits measured by Monsanto and Dow for a 5% seed mixture, because the Monsanto and Dow trials were not performed on sufficient spatial and temporal scales to observe the regional pest suppression demonstrated by Hutchison et al. (2010). In other words, a 20% seed mixture will likely give large benefits to farmers that increase over years as regional pest densities are reduced.
Second, reducing regional pest abundances may introduce risks of resistance evolution. If regional effects are too great, populations could be driven towards extinction, leading to greater

rates of resistance evolution as predicted by frequency-only models. This risk argues against introducing 5% seed mixtures.

Strategies for Increasing the Durability of Seed Mixtures

The Panel considered the possibility that the durability of seed mixtures compared to structured refuges could be counterbalanced by other measures to increase durability. Unfortunately, this approach requires quantitative assessment of the loss of durability due to using a seed mixture that is not possible given the uncertainties in modeling resistance evolution. For example, the loss of durability of seed mixtures depends on the magnitude of movement of larvae among plants and the selection they undergo before and after movement. There is high uncertainty about these key processes (see Appendix 2: **Seed Mixtures vs. Structured Refuges**).
The Panel discussed cautious, incremental approaches to decreasing the percent of non-*Bt* in a seed mixture given the novelty of using seed mixtures for IRM in high-dose situations such as SmartStax for ECB and SWCB. For example, a 20% seed mixture, rather than a 5% seed mixture could be introduced. Modeling could be used to compare different incremental introduction strategies, and use sensitivity analyses, information-gap analyses, for example, could be used to assess the risk of resistance evolution under these different strategies.

The second approach to the introduction of seed mixtures involves the use of adaptive management strategies that integrate modeling and monitoring. Due to model uncertainties, introduction of seed mixtures would be implemented along with monitoring not only for resistance but also for the abundance of target pests. As discussed previously (at the start of "General modeling issues for SmartStax IRM"), monitoring should focus on the target pest that poses the greatest risk of resistance evolution. This incremental approach would decrease the percent of non-*Bt* in a seed mixture based on coupling monitoring for resistance and population density. For example, if a 20% seed mixture was used as the initial IRM strategy then this percentage could be incrementally lowered (perhaps every 5 years) if monitoring had not indicated any signs of resistance or severe population declines. However, if resistance is detected, then remediation should start immediately. Remedial action should also be taken if population densities reach low levels, as this would signal much higher risks of resistance evolution due to the loss of density dependence.

ADDITIONAL DISCUSSION

Identification of Research Needs Concerning Major Corn Pests and Future Use of *Bt* Corn Products

At the end of the meeting, the Panel held an open discussion regarding research needs concerning IRM for the major corn pests and the future use of *Bt* corn products. Each of these research areas were discussed in detail throughout the document. A quick, but perhaps not a complete list of these areas is provided below. These areas are not listed in any priority.

1) Adult and larval movement, especially SWCB
2) Adult dispersal patterns, male and female differences, mating choice (in *Bt* and refuge fields)
3) CRW and density-dependent mortality estimations
4) Survival of heterozygotes
5) Effects of CEW migration on selection for resistance in *Bt* corn and *Bt* cotton
6) Effect of pollination of non-*Bt* corn with *Bt* pollen and selection of CEW resistance in kernels
7) Determine the percentage of ECB feeding in ears and percentage feeding in axils on pollen
8) Re-examine the 1998 SAP definition of high dose with respect to a seed mixture. Selection for resistance to single toxins versus selection for resistance to multiple toxins (combination of single toxins in one plant)
9) Measurement of the effect of redundant mortality in multi-toxin products (pyramids)
10) Uniformity of the seed mixture and uniformity of distribution of non-*Bt* plants in the field
11) Examine other mechanisms of resistance other than detoxification, e.g., behavioral resistance mechanisms
12) Examine cross-resistance potential and its effect on resistance, include cross-resistance (partial cross-resistance) in modeling simulations
13) Interpretation of models: model structure, inputs, outputs – sensitivity and uncertainty analyses

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Appendix 1. Empirical Methods for Estimating Dose and Efficacy

Dose

Dose was estimated using a method suggested by the EPA SAP (1998).

The "dose" of insecticidal toxin in a *Bt* plant is a major factor determining the level of resistance risk. Dose depends on both the concentration of the Cry toxins in the *Bt* plant and the genetic characteristics of the target pest. A "high-dose" is defined as one that kills a high proportion (>95%) of heterozygous resistance genotypes similar to homozygous susceptible genotypes (Tabashnik 1994; Roush 1997; Gould 1998; Andow 2001). For a high dose, resistance is recessive or nearly so. A "low dose" is anything that is not a high dose. All other things being equal, there is a greater resistance risk associated with low dose species than high dose species.

Theory for Dose Calculation

Caprio et al. (2000, p. 808) suggested that a high dose might be defined when the $LC_{50,\,RS}$ for the RS heterozygote is >50 times greater than the $LC_{50,\,SS}$ for the SS susceptible homozygote. The EPA SAP (1998) suggested a similar criterion, using 25 times, instead of the 50 times suggested by Caprio et al. (2000). Because the Caprio et al. (2000) suggestion is based on a literature review, the Panel uses the Caprio et al. (2000) suggestion for calculating dose.

When the R allele is not available, this ratio of LC_{50}'s cannot be evaluated directly. The EPA SAP (1998) suggested several ways to evaluate this when the R allele is not available. The basis for these ideas is that if and only if $LC_{50,RS} > 50\ LC_{50,\,SS}$ for the plant, then the plant toxin concentration, designated $[T_P]$, diluted 50-fold must kill >50% of the SS larvae. In other words, the killing power of the plant toxin concentration is at least 50 times greater than that needed to kill 50% of the SS larvae. Indeed, for any dilution factor x, such that the toxin concentration $[T_P]/x$ kills > 50% of the SS larvae, it can be concluded that $[T_P]$ has at least x times the killing power of the $LC_{50,\,SS}$. Moreover, if $[T_P]/x$ kills y% of the SS larvae, it can be concluded that $[T_P]/x$ is an $LC_{y,\,SS}$. Thus to meet the high dose standard, it must be shown that when $x = 50$, $[T_P]/x$ produces an $LC_{y,\,SS}$ where $y > 50$. If for any $x < 50$, it is demonstrated that $[T_P]/x$ kills <50% of the SS larvae, then $[T_P]/50$ cannot kill > 50% of the SS larvae, and it can be concluded that the plant is low dose.

A test conducted with $x = 1$, provides the killing power, or efficacy, of the plant without dilution. Thus, efficacy measures the killing power when $x = 1$. Specifically, if E is the efficacy, the undiluted plant has an $LC_{E,\,SS}$. Obviously, if $E < 50\%$, the plant cannot be high dose. Using the efficacy data in this way, it is possible to avoid most of the potential problems that were outlined by Caprio et al. (2000, p. 809).

To determine expected dose using EPA SAP (1998) and Caprio et al. (2000), it is necessary to calculate expected efficacy.

Efficacy can be calculated as:

$$E = 1 - (m_t / m_c), \qquad \text{[Equation A1.1]}$$

with a variance of:

$$V(E) = \left(\frac{1}{m_c}\right)^2 V(m_t) + \left(\frac{m_t}{m_c^2}\right)^2 V(m_c), \qquad \text{[Equation A1.2]}$$

where E is efficacy, m_t is the mean of the treatment, m_c is the mean of the control, and V is the variance, assuming $Cov(m_t,m_c)=0$.

The means may be any measure of pest attack under field conditions. In general, various measures of efficacy are of interest because comparison of the various measures provides reinforcing evidence of the accuracy of any one measure. For the estimate of dose, mortality (survival) may be the more appropriate measure, but the mortality estimates must be consistent with other measures of efficacy.

Finally, the slope of the concentration-response relationship is needed. Using the slope and one known point on the concentration-mortality profile for the plant-expressed toxin(s), it is possible to calculate the dilution factor that would produce the $LC_{50,\,SS}$. The dilution factor, x, is the factor by which the undiluted plant matter must be diluted in order to produce an $LC_{50,\,SS}$. It is calculated as:

$$x = 10^{\left[\frac{(\mathrm{Pr}obit(E) - \mathrm{Pr}obit(0.5))}{S}\right]}, \qquad \text{[Equation A1.3]}$$

where E is efficacy, and S is the slope in the concentration-mortality relationship, in this case with probit transformed efficacy data. A straight-forward modification of this equation can be made if a logit model were used instead of a probit model.

Note that by using an independently estimated E, the concentration-mortality profile is scaled to units of dilution on the x-axis. It is known that laboratory bioassays often do not predict field mortality at the same toxin concentration. There are many possible reasons for this that are mostly related to the inability to accurately mimic in the laboratory the exposure of an insect feeding on a plant. Assuming that the laboratory mortality assay models field mortality to some proportionality constant that can be applied to the laboratory toxin concentration, using E in the *Probit* equation implicitly defines the hypothetical proportionality constant. To illustrate this point, let p be the hypothesized proportionality constant. Define $[C_a]$ to be the toxin concentration in the laboratory bioassay that gives a mortality equal to $Probit[E]$. Define $[C_p]$ to be the toxin concentration in the plant that produced efficacy E. Then $p = [C_a]/[C_p]$. The assumed existence of proportionality constant is not unusual or extreme, because it is also assumed in justifying a discriminating dose bioassay for use on field populations.

It might appear that it is important also to assume that the slope of the concentration-mortality bioassay is equal to the slope of the diluted intact plant-mortality profile. This additional assumption is not essential, because it is entailed in the assumption of proportionality constant. To illustrate, suppose there is mortality E on the intact plant ($[C_{p,intact}] = 1$), and mortality M at a half dilution ($[C_{p,half}] = 0.5$). The slope on a *Probit* scale would be equal to $(Probit(E)-Probit(M))/(log\,[C_{p,intact}] - log\,[C_{p,half}]) = (Probit(E)-Probit(M))/(log\,1 - log\,0.5)$. Assume that there is a proportionality constant, and as above, let $[C_a]$ be the toxin concentration in the

laboratory bioassay that gives a mortality equal to *Probit*[*E*]. Then from the definition in the previous paragraph, $[C_a] = p\,[C_p]$, or equivalently, $[C_p] = [C_a]/p$. This means that $[C_{a,1}] = p\,[C_{p,\,intact}]$ and $[C_{a,2}] = p\,[C_{p,half}]$. The slope of the laboratory bioassay is determined from *Probit*(*E*), *Probit*(*M*), $[C_{a,1}]$, and $[C_{a,2}]$, where *Probit*(*E*), *Probit*(*M*), and $[C_{a,1}]$are independently determined and $[C_{a,2}]$is only constrained by *p*, the proportionality constant. The slope of the laboratory bioassay is equal to (*Probit*(*E*)-*Probit*(*M*))/(*log* $[C_{a,1}]$ – *log* $[C_{a,2}]$). Substituting, this is equivalent to (*Probit*(*E*)-*Probit*(*M*))/(*log p* $[C_{p,\,intact}]$ – *log p* $[C_{p,half}]$) which simplifies to (*Probit*(*E*)-*Probit*(*M*))/(*log* $[C_{p,intact}]$ – *log* $[C_{p,half}]$), proving that the assumption of proportionality is sufficient.

A straight-forward modification of this method can be made if a logit model were used to analyze the raw data instead of a probit model. In some ways a logit model might be superior. The dilution factor, *x*, is likely to have a large standard error. To reduce the compounding of error that occurs during the multiplication and division of random variables, it might be better to estimate log *x*. If this is done, the logit transform may enable relatively easier access to the raw data in constructing a MLE for log *x* or for bootstrapping error estimates.

Estimating Pest Attack in the field

Efficacy data were provided by the registrants in MRID 474449-11 (Head and Storer 2008), which are summarized in Table A1-1. The data are from field plots during 2006 and 2007 with either natural pest populations or artificial infestations. There are diverse measures of pest attack that are useful for checking for consistency in measures of control efficacy.

Table A1-1. Measures of levels of pest injury and damage on pyramided and single gene products by the major Lepidopteran pests of corn[1]

	ECB/SWCB[2]	SWCB[2]	CEW[2]	SCB[2]	FAW[2]	FAW PR[2]	
	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)	mean (SE)
Leaf Damage Rating (0-9)							
MON 89034 TC1507 MON 88017 DAS 59122-7	0.2 (0.2)	0.5 (0.3)			0.7 (0.4)	2.3 (0.2)	
TC1507	0.0 (0.0)	1.1 (0.2)			0.8 (0.2)	6.5 (0.3)	
MON 89034	0.2 (0.1)	1.1 (0.4)			0.6 (0.2)		2.1 (0.1)
Control	5.2 (1.8)	8.4 (0.4)			7.0 (0.7)	7.1 (0.2)	7.4 (0.2)
	1 loc, n=4	4 loc, n=16			7 loc, n=28	2 loc, n=8	2 loc, n=8
% Infested Plants							
MON 89034 TC1507 MON 88017 DAS 59122-7			24.2 (7.1)				
TC1507			50.9 (5.7)				
MON 89034 Control			57.4 (5.2)				
			15 loc, n=35-36				
Larvae/ Plant							
MON 89034 TC1507 MON 88017 DAS 59122-7		0.0 (0.0)	0.1 (0.1)	1.1 (0.3)			
TC1507		0.0 (0.0)	0.4 (0.2)	1.9 (0.3)			
MON 89034		0.0 (0.0)					
Control		0.5 (0.1)	0.9 (0.3)	13.1 (1.9)			

		1 loc, n=4	3 loc, n=12	1 loc, n=2	
Cavities/ Plant					
MON 89034 TC1507					
MON 88017 DAS 59122-7	0.6 (0.3)	0.1 (0.1)		0.1 (0.1)	
TC1507	0.4 (0.3)	0.0 (0.0)		0.9 (0.5)	
MON 89034		0.0 (0.0)			
Control	3.9 (1.3)	4.8 (0.4)		18.5 (3.0)	
	7 loc, n=14	2 loc, n=8		1 loc, n=2	
Stalk Damage (cm/plant)					
MON 89034 TC1507					
MON 88017 DAS 59122-7	0.0 (0.0)	0.6 (0.4)			
TC1507	0.0 (0.0)	0.0 (0.0)			
MON 89034	0.0 (0.0)	0.0 (0.0)			
Control	0.6 (0.2)	60.9 (14.7)			
	1 loc, n=4	5 loc, n=20			
Ear Damage Rating (cm/plant)					
MON 89034 TC1507					
MON 88017 DAS 59122-7			0.1 (0.0)		0.6 (0.3)
TC1507			3.8 (0.3)		2.0 (0.4)
MON 89034			0.4 (0.1)		0.8 (0.3)
Control			5.0 (0.5)		3.3 (0.2)
			4 loc, n=15-16		2 loc, n=7-8
Number of Damaged Kernels					
MON 89034 TC1507			6.5 (1.4)		

MON 88017 DAS 59122-7			
TC1507		28.2 (3.4)	
MON 89034		6.1 (0.8)	
Control		29.7 (6.8)	
		2 loc, n=8	
Larvae/Ear			
MON 89034 TC1507			
MON 88017 DAS 59122-7		0.6 (0.3)	
TC1507		2.8 (0.4)	
MON 89034		0.7 (0.5)	
Control		1.4 (0.2)	
		3 loc, n=9-12	

1. Data are from MRID 474449-11. pages 170-180 (Vaughn et al. 2008), and RPN 07-262, pages 31-41 (Vaughn et al. 2008). Data were not collected in the gray cells. "loc" is the number of different geographic locations where the varieties were evaluated. "n" is the number of replicate plots summed across all locations. Data were pooled across sites and years, weighting means and variances by sample size.
2. ECB = European corn borer, SWCB = Southwestern corn borer, CEW = Corn earworm, SCB = Sugarcane borer, FAW = Fall armyworm, mainland USA, FAW PR = Fall army worm in Puerto Rico

Estimating Efficacy from Field Data

Efficacy was estimated using equations A1-1 and A1-2 and the data in Table A1-1. When multiple measures of efficacy were available, they were mutually reinforcing, implying that the data were internally consistent.

The data confirm high efficacy for ECB and SWCB for all three products that were evaluated (Table A2-2). For ECB, efficacy was highest for stalk damage compared to leaf damage rating or cavities per plant. The relatively poor suppression of cavities is probably indicative of the high rates of older larval movement, which is typical for ECB. For SWCB, efficacy was higher for larvae/plant than the injury and damage indexes. For both species, suppression of leaf damage rating was not as high as some of the other injury and damage ratings. This is because the leaf damage rating is very sensitive to small amounts of grazing by neonates, and is not strongly related to yield loss. The percent of infested plants is a good predictor of yield loss in first generation ECB and SWCB, but this parameter was not measured. For these species, the higher reported efficacies are probably more characteristic of field control of larvae than the lower values. We conclude the efficacy of the triple-stack is probably 0.990-1.000 for ECB and SWCB.

Efficacy for SCB is also fairly high, with all values above 0.85; MON 89034 was not evaluated by itself for SCB. The efficacy of the triple-stack was systematically higher than for TC1507, and larval suppression by the triple-stack was 0.916. Cavities per plant were even more strongly suppressed by the triple-stack at 0.995. The efficacy of the triple-stack was probably between 0.916 and 0.995 for SCB.

Efficacy for CEW varied strongly by event. The triple-stack had higher suppression than TC1507 alone, and was systematically higher than MON 89034 alone. For the triple-stack, efficacy was highest for ear damage rating (0.980). Suppression of larvae per plant was 0.889 and suppression of larvae per ear was 0.589 for the triple-stack. An additional trial (In MRID 474449-11, page 420, Huckaba and Storer (2008)) found considerably better suppression of live larvae in the ear, which would raise this value to perhaps 0.690, but without the detailed statistics, it is difficult to combine these values accurately. Larval suppression is probably a better measure of field control.

Efficacy for FAW varied strongly by event and locality. On the USA mainland, TC1507 had lower suppression of ear damage rating than the other events, and MON 89034 was similar to the triple-stack. On the mainland, the triple-stack may have an efficacy of 0.811-0.907. Puerto Rico has a population of Cry1Fa-resistant FAW (Cry1Fa is the active ingredient in TC1507). TC1507 has poor efficacy against the Puerto Rican FAW population; efficacy is similar against the triple-stack and MON 89034. Efficacy is systematically lower against both the triple-stack and MON 89034 in the Puerto Rican population compared to the mainland FAW population. As this might indicate cross-resistance, this should be examined with a controlled experiment.

For estimating dose, the most relevant efficacy measure is neonate to adult survival. None of the reported efficacy measures provide this value, but larval density is the closest measure (Table A1-2). Most of the estimates of efficacy are mutually reinforcing, increasing the

credibility of the data. In no case for the triple stack is $E < 50\%$, so the efficacy data do not by themselves determine if the triple-stack is low or high dose against any of these pests. However, the values allow a rough ranking of species based on the likelihood of being high dose. Of all of the species, ECB and SWCB are most likely to be high-dose, followed by SCB. Of all of the species, CEW and FAW in Puerto Rico are most likely to be low-dose. Mainland FAW is intermediate.

Table A1-2. Measures of efficacy (reduction compared to control) of pyramided and single gene products against the major Lepidopteran pests of corn[1]

	ECB/SWCB	SWCB	CEW	SCB	FAW	FAW PR
	E (SE)	E (SE)	E (SE)	E (SE)	E (SE)	E (SE)
Leaf Damage Rating (0-9)						
MON 89034 TC1507	0.962 (1.000)	0.946 (0.711)			0.907 (0.677)	0.674 (0.070)
MON 88017 DAS 59122-7 TC1507	1.000 (0.000)	0.869 (0.200)			0.884 (0.260)	0.085 (0.053)
MON 89034	0.962 (0.500)	0.869 (0.345)			0.919 (0.351)	0.721 (0.035)
% Infested Plants						
MON 89034 TC1507			0.578 (0.296)			
MON 88017 DAS 59122-7 TC1507			0.113 (0.138)			
MON 89034						
Larvae/ Plant						
MON 89034 TC1507		1.000 (0.000)	0.889 (0.000)	0.916 (0.273)		
MON 88017 DAS 59122-7 TC1507		1.000 (0.000)	0.556 (0.000)	0.855 (0.159)		
MON 89034		1.000 (0.000)				
Cavities/ Plant						
MON 89034 TC1507	0.845 (0.503)	0.990 (1.400)		0.995 (1.000)		
MON 88017 DAS 59122-7 TC1507	0.897 (0.751)	1.000 (0.000)		0.951 (0.556)		
MON 89034		1.000 (0.000)				
Stalk Damage (cm/plant)						

MON 89034 TC1507	1.000	0.990				
MON 88017 DAS 59122-7	(0.000)	(0.639)				
TC1507	1.000	1.000				
	(0.000)	(0.000)				
MON 89034	1.000	1.000				
	(0.000)	(0.000)				
Ear Damage Rating (cm/plant)						
MON 89034 TC1507			0.980		0.811	
MON 88017 DAS 59122-7			(0.002)		(0.413)	
TC1507			0.240		0.393	
			(0.111)		(0.188)	
MON 89034			0.920		0.766	
			(0.250)		(0.398)	
Number of Damaged Kernels						
MON 89034 TC1507			0.781			
MON 88017 DAS 59122-7			(0.221)			
TC1507			0.051			
			(0.249)			
MON 89034			0.795			
			(0.139)			
Larvae/Ear						
MON 89034 TC1507			0.589			
MON 88017 DAS 59122-7			(0.519)			
TC1507			0.000			
			(0.255)			
MON 89034			0.504			
			(0.702)			

1. Data were not collected in the gray cells. ECB = European corn borer, SWCB = Southwestern corn borer, CEW = Corn earworm, SCB = Sugarcane borer, FAW = Fall armyworm, mainland USA, FAW PR = Fall army worm in Puerto Rico

Concentration-Mortality Assay: Slopes and LC_{50}

The registrants provided data that can be used to estimate the slope of the concentration-mortality profile for Cry1Fa, Cry1A.105, or Cry2Ab separately, for some of the species, i.e., only FAW and ECB (MRID 474449-11, Appendix 1, Schlenz et al. (2008)). Slopes of the concentration-mortality profiles for ECB and FAW are shown in Table A1.3. For each species, a colony resistant to Cry1Fa (and otherwise susceptible to Cry1A.105 and Cry2Ab) and a colony susceptible to all three toxins were evaluated. The statistical error associated with these slope estimates was not reported. However, the variation in mortality among replicate experiments was small for about 1/3 of these experiments (Pages 45-47, Schlenz et al. (2008)), so perhaps these estimates are not too variable.

Resistant FAW and ECB had lower slopes than susceptible colonies when exposed to Cry1Fa (Table A1-3). This may be expected, because the resistant colonies should be able to tolerate Cry1Fa better than the susceptible colonies.

Unexpectedly, the resistant FAW and ECB colonies also had lower slopes than the susceptible colonies when exposed to either Cry1A.105 or Cry2Ab (Table A1-3). This suggests that there was some level of epistasis and partial cross-resistance. However, the absence of an estimated statistical error makes any inference about epistasis or cross-resistance uncertain, and the registrant should be encouraged to provide full statistical analysis of the concentration-mortality data.

Table A1-3. Estimated slopes of the concentration-mortality relationships for FAW and ECB colonies against Cry1F, Cry1A.105, and Cry2Ab.[1]

Toxin	Species	Colony[2]	Estimated Slope (Δprobit mortality/ $\Delta Log_{10}[\ ]$)[3]
Cry1Fa	FAW	Susceptible	1.45
		Resistant	0.36
	ECB	Susceptible	1.46
		Resistant	0
Cry1A.105	FAW	Susceptible	1.60
		Resistant	0.63
	ECB	Susceptible	1.10
		Resistant	0.64
Cry2Ab	FAW	Susceptible	0.47
		Resistant	0.46
	ECB	Susceptible	1.56
		Resistant	0.62
Average[4]	FAW	Susceptible	1.17
		Resistant	0.55
	ECB	Susceptible	1.37
		Resistant	0.63

1. Toxins exposed singly in a diet overlay assay. MRID 474449-11 (Schlenz et al. 2008).
2. Susceptible colony is susceptible to all three toxins. Resistant colony is resistant to Cry1Fa, but susceptible to the other toxins.

3. Slopes were taken from Table 1, page 44, MRID 474449-11 (Schlenz et al. 2008). Units are change in probit transformed mortality rate divided by change in $\log_{10}$ toxin concentration.
4. Average does not include Cry1Fa for resistant colonies.

Lethal concentrations, 50% (LC_{50}'s), for the concentration-mortality profiles were reported in Table 1, page 44, MRID 474449-11 (Schlenz et al. 2008). The 95% confidence intervals were also reported, when they could be estimated. As expected, the resistant colonies had a much higher LC_{50} on Cry1Fa than the susceptible colonies. Unexpectedly, the resistant colonies also had a higher LC_{50} for both Cry1A.105 and Cry2Ab (Table A1-4). This suggests some level of epistasis and partial cross-resistance between Cry1Fa and the other two toxins.

Table A1-4. Estimated LC_{50}'s and 95% confidence intervals (in parentheses) associated with the concentration-mortality relationships for FAW and ECB colonies against Cry1F, Cry1A.105, and Cry2Ab.[1]

Toxin	Species	Colony	LC_{50} (ng/cm^2)
Cry1Fa	FAW	Susceptible	377 (279-510)
		Resistant	>10000
	ECB	Susceptible	179 (133-243)
		Resistant	>10000
Cry1A.105	FAW	Susceptible	181.2 (138-240)
		Resistant	>10000
	ECB	Susceptible	6.1 (3.9-8.9)
		Resistant	813* (488-1478)
Cry2Ab	FAW	Susceptible	>10000
		Resistant	>10000
	ECB	Susceptible	595 (451-784)
		Resistant	7206* (3476-20108)

1. See footnotes for Table A1-3.
* LC_{50} for resistant colony is significantly different from the LC_{50} for the susceptible colony.

Estimating Dilution Factors and Determining Dose

As noted above, the registrants provided data that can be used to estimate the slope of the concentration-mortality profile for Cry1Fa, Cry1A.105, or Cry2Ab separately for some of the species (Appendix 1, Schlenz et al. 2008). Although it would have been preferable to characterize concentration-mortality with a mixture of the toxins, the mixture would likely generate a steeper slope and lower estimates of dilution factors. Consequently, use of the average of the separate slopes likely biases the result toward a determination of high-dose. In

addition, it would have preferable to have independent estimates for all of the significant target pests.

Calculated values for x are given in Table A1-5 using equation A1-3. To be a high-dose, $x > 50$. ECB/SWCB has a dilution factor greater than 50 and the triple-stack is generally considered to be high-dose against these pests. The dilution factor for SWCB alone may range from 32 to well above 50. The triple-stack is likely to be high dose for SWCB alone. The dilution factors are lower for SCB, but the triple-stack may be high dose. The dilution factors for CEW and FAW are much lower than 50 and the triple stack are considered to be low dose for these species. Three points should be emphasized about this analysis. First, the logit transform of mortality may provide better parameter estimates than the probit transformation when estimated mortality in the concentration-mortality assay is near 0 or 1. Second, the slopes should be estimated using realistic exposure methods so that the assumption of proportionality of concentrations is met. The diet overlay method may overestimate or underestimate the slope compared to incorporating whole plant tissue. This would, respectively, underestimate or overestimate the dilution factor. Third, the propagation of statistical error in E and S needs to be determined.

In any event, this analysis shows that readily available data can be used to determine dose, and additional standard and/or standardizable experiments can be conducted to obtain improved determinations of dose. Moreover, it shows that dose cannot be inferred from efficacy alone. The slope of the concentration-mortality relationship is also needed. For example, if $E = 0.907$ and $S = 0.55$, the dilution factor would be 254, a high dose even without extremely high efficacy. Indeed, with $S = 0.55$, E can be as low as 0.825 and still be high dose. Alternatively, a very high efficacy, $E = 0.999$, and a steep slope, $S = 2$, would give a dilution factor $x = 35$, which is not high dose. Any slope greater than 1.81 would also give a low dose, despite the extremely high efficacy.

Table A1-5. Estimated dilution, x, of triple-stack plant toxin concentration to reach an LC_{50}[1]

Lepidopteran Target Pest[1]	**Estimated Efficacy (E)**	**Slope**[2] **(Δprobit mortality/ $\Delta Log_{10}[\ ]$)**	**Estimated Dilution Factor (x)**	**Panel Determination of Dose**
ECB/SWCB	1.000	1.37	520[3]	High dose
SWCB	0.980-1.000		32-520[3]	Probably high dose
CEW	0.589-0.889		1.6-11.1	Low dose
SCB	0.916-0.995		15.1-159	Possibly high dose
FAW	0.811-0.907	1.17	5.7-13.5	Low dose
FAW PR	0.674	0.55	6.6	Low dose

1. ECB = European corn borer, SWCB = Southwestern corn borer, CEW = Corn earworm, SCB = Sugarcane borer, FAW = Fall armyworm, mainland USA, FAW PR = Fall army worm in Puerto Rico
2. Average slope for susceptible ECB and FAW or resistant FAW (for FAW PR) exposed to Cry1F, Cry1A.105, or Cry2Ab separately. Slope for ECB was used for SWCB. Slope for FAW was used for CEW and SCB. From Table A2.3.
3. Dilution factor calculated assuming the efficacy was 0.9999 instead of 1.000. If efficacy was only 0.99, the dilution factor would still be at least 50.

Appendix 2: Two-Locus Model of Resistance Evolution

This Appendix provides the Ives et al. model of resistance evolution (Ives et al. 2011) and how it was used by the Panel to address Charge Questions 8 and 9. As a two-locus model, it is most appropriate for investigating the evolution of resistance to SmartStax by CRW. Nonetheless, it is also used here to investigate the high-dose case appropriate for ECB and SWCB. Although there are three toxins targeting ECB and SWCB, and hence a three-locus model of resistance might be called for, the two-locus model can nonetheless be used to investigate the conceptual issues involved in resistance evolution. The factors affecting resistance evolution in the two-loci model are the same as those in a single-locus model (Ives et al. 2011), and there is no reason to suspect that a three-loci model differs.

The description of the model is largely a repeat of what is found in Ives et al. (2011). The base model keeps track of both allele frequencies and insect densities, and is similar to the single-locus model analyzed by Ives and Andow (2002). The base model is described first followed by modifications for seed mixtures and larval movement among plants, for explicit spatial structure and limited adult movement.

Base Model

The base model assumes that adults have high movement rates among fields and therefore are effectively uniformly distributed among refuge and *Bt* fields in proportion to their areal extent; each generation a proportion Q of the adult population occurs in refuge and $1 - Q$ in *Bt* fields. Mating within fields is random, with females producing F offspring. Resistance to each of two *Bt* toxins is governed by diallelic, independently segregating loci, with R_1 and S_1 denoting resistant and susceptible alleles to *Bt* toxin 1, and R_2 and S_2 denoting the resistant and susceptible alleles to *Bt* toxin 2. Thus, there are nine genotypes of offspring whose frequencies within fields are at Hardy-Weinberg equilibrium. The survival of offspring with genotypes R_1R_1, R_1S_1, and S_1S_1 from *Bt* toxin 1 are given by s_{1RR}, s_{1RS}, and s_{1SS}, and similarly s_{2RR}, s_{2RS}, and s_{2SS} give the survival associated with *Bt* toxin 2. Survival on plants containing both of the *Bt* toxins are assumed to be multiplicative, as is expected if toxins have independent modes of action (Raymond et al. 1989). For example, the survival of an $S_1S_1S_2S_2$ individual on *Bt* plants is $s_{1SS} \times s_{2SS}$, and the survival of an $R_1S_1R_2S_2$ individual is $s_{1RS} \times s_{2RS}$. For baseline parameter values, we assume $s_{1RR} = s_{2RR} = 1$, $s_{1RS} = s_{2RS} = 0.0595$, and $s_{1SS} = s_{2SS} = 0.01$; for these values, the dominance of both resistance alleles is $h = 0.05$. Although the model of Ives et al. (2011) can incorporate a cost of resistance, here survival of all genotypes on non-*Bt* plants is 1.

Following any mortality caused by *Bt*, there is density-dependent survival given by $(1 + x)^{-1}$ where x is the density (all genotypes) of surviving larvae within a field. The specific form of this survival function makes little difference for any of our qualitative or quantitative conclusions. Because the model explicitly keeps track of the number of individuals of different genotypes, rather than just genotype frequencies, density-dependent survival changes the rate of resistance evolution. To investigate this effect, the model can be run as a frequency-only model that does not keep track of population densities, only gene frequencies. The frequency-only model is the same as the model including density dependence, except the term $(1 + x)^{-1}$ is removed and

genotype densities are converted to frequencies each generation. The frequency-only model is essentially identical to two-toxin models analyzed previously (Mani 1985; Gould 1986; Roush 1998; Gould 2006).

To run simulations, we assume initial resistance allele frequencies are 0.005 for both resistance genes. Failure of *Bt* crops (i.e., when the insect population is resistant) is assumed to have occurred when both resistance alleles exceed a frequency of 0.5. In some scenarios, in particular when the proportion of refuges (or non-*Bt* plants in seed mixtures) is very small, insect densities can be very low when this criterion for *Bt* failure is reached. Nonetheless, once resistance allele frequencies reach 0.5, the resistant population recovers from low density very rapidly, so using this criterion to assess resistance failure gives similar results to those obtained by using a threshold density of insects.

For the case of seed mixtures, all fields are the same and contain a fraction q of non-*Bt* plants and $(1 - q)$ of *Bt* plants, with females depositing eggs such that larvae initiate on non-*Bt* and *Bt* plants in proportion to their prevalence. We follow Mallet and Porter (1992) in assuming that larvae have two stages (a = young and b = old) and move between plants with probability μ between stages. When they move, they move to non-*Bt* or *Bt* plants with probabilities q and $(1 - q)$. For comparison among cases with different larval movement probabilities μ, we assume that survival on *Bt* plants of susceptible genotypes in different larval stages, s^a and s^b, combine multiplicatively. Thus, the total survival of a susceptible larva to *Bt* toxin 1 that remains on a *Bt* plant is $s_{1SS} = s^a_{1SS} \times s^b_{1SS}$, and the total survival of a heterozygous larva to *Bt* toxin 2 that remains on a *Bt* plant is $s_{2RS} = s^a_{2RS} \times s^b_{2RS}$. Similarly, the total survival of a heterozygous larva to *Bt* toxin 2 that moves from a *Bt* to a non-*Bt* plant is $s_{2RS} = s^a_{2RS} \times w^b_{2RS}$, where w is used to denote the survival on non-*Bt* plants. The survival rates to the two toxins are then combined as before to give the total survival from both toxins; thus, the total survival of a $S_1S_1R_2S_2$ larva that moves from a *Bt* to a non-*Bt* plant is $s^a_{1SS} \times w^b_{1SS} \times s^a_{2RS} \times w^b_{2RS}$. Finally, density-dependent mortality occurs at the scale of individual plants after mortality has been caused by *Bt*; thus, the survival of insects on a plant is given by $(1 + x_p)^{-1}$ where x_p is the density of second-stage larvae per non-*Bt* or *Bt* plant.

The spatially structured model is built on a 50 by 50 grid of same-sized fields, with a proportion Q being refuge fields and a proportion $(1 - Q)$ being *Bt* fields. Assuming that the entire landscape is made up of refuge or *Bt* fields ignores the possibility of fields of other crops or non-crop habitat. Biologically, this is equivalent to assuming that, even though different types of habitats may be available on a real landscape, these habitats are permeable to dispersing adults who move through them as if they were not there. Refuges are distributed randomly on the grid, and crop rotation is included by randomly rearranging refuges on the grid. Cases in which fields are either rotated every three insect generations or never rotated are investigated.

When males disperse from their natal fields, they do so before mating, whereas females disperse from natal fields following mating. The probability of dispersing a linear (Euclidean) distance x from natal fields is proportional to d^x, so dispersal drops off exponentially with distance; the fraction of adults remaining in their natal field is proportional to $d^0 = 1$. At $d = 0.9$, the results from the spatially explicit model are almost identical to the base model that assumes complete spatial mixing of adults. Finally, the grid of fields has "wrap-around" boundaries (i.e., the grid is

on a torus), so that insects dispersing off one side of the grid appear on the opposite side; this assumption makes the dynamics on the 50 by 50 grid better approximate the dynamics expected for a much larger grid while remaining computationally manageable. Comparison of simulations on the 50 by 50 grid to those on a 100 by 100 grid showed no effect of grid size on the conclusions. Within fields, we make the same assumptions as in the base model. There is random mating and no movement of larvae among plants. Following mortality from *Bt* toxins, survival depends on the density within each field according to the equation $(1 + x_{ij})^{-1}$, where x_{ij} is the density of larvae in the *ij*-th field.

Limited Dispersal and Spatial Refuges

Although there are justifiable concerns about low adult dispersal rates causing rapid resistance evolution, generally this requires very low dispersal rates for both sexes (Fig. A2-1a). Also, the risks posed by low dispersal rates depend on the rotation of refuge fields, with lower rotation rates reducing the risk of resistance evolution (Fig. A2-1b). The Panel stated that for the pests targeted by SmartStax, it is unlikely that adult dispersal rates are so low that the risk of resistance evolution are greatly increased, at least under current refuge implementation requirements set by the EPA.

Survival of Heterozygotes

The survival of genotypes, especially of heterozygotes, in comparison to susceptible homozygotes, plays a central role in determining the rate of resistance evolution. Thus, it is necessary to know how the survival rates combine for two or three resistance loci. When larvae move between *Bt* and non-*Bt* plants, it is also necessary to know the survival of all genotypes before and after movement. There is high uncertainty about the survival of all genotypes and how these survival rates are affected by larval movement. The analyses performed by the Panel illustrate the sensitivity of resistance evolution to these uncertainties. Five cases of the relative survival of pre- vs. post-movement larvae were considered.

1) In Case 1, survival rates are equal between pre- and most-movement stages, with survival rates for each stage multiplicatively combining to give the overall survival of larvae.

2) In Case 2, all *Bt* mortality occurs before movement.

3) In Case 3, all *Bt* mortality occurs after movement (Fig. A2-2).

   Comparing these three cases, Case 1 leads to the fastest resistance evolution rate, while Case 2 has slowest resistance evolution rate (Fig. A2-3a). Furthermore, for Case 2 the rate of resistance evolution decreases with increasing larval movement. This happens because all *Bt* mortality occurs before movement, and allowing larvae to move spreads larvae among plants and decreases density-dependent mortality. This decrease in density-dependent mortality favors susceptible larvae that would otherwise be confined to non-*Bt* plants, thereby decreasing the relative survival of heterozygotes and slowing resistance. This explanation can be tested using the frequency-only model (Fig. A2-3b);

when density-dependent mortality is removed, Cases 2 and 3 give the same rates of resistance evolution that are independent of larval movement.

4) In Case 4, most but not all mortality occurs before movement.

5) In Case 5, most of the mortality occurs after movement.

In both of these cases, resistance evolves more rapidly than in Case 1 (Fig. A2-4). Note: Case 4 could be viewed as being intermediate between Cases 1 and 2, while Case 5 could be viewed as being intermediate between Cases 1 and 3. Nonetheless, in both cases the rate of evolution is not intermediate.

To illustrate the sensitivity of resistance evolution to the survival of heterozygotes, consider the case of increasing the survival rates of $S_1S_1R_2S_2$, $S_1R_1S_2R_2$, and $S_1S_1R_2R_2$ individuals by a factor of five (Fig. A2-5a). In each case, the rate of resistance evolution increases, although this increase is least for $S_1S_1R_2R_2$ individuals (Fig. A2-6a). This cannot be solely due to the relative rarity of R_2R_2 homozygotes, because when the initial frequencies of both R_1 and R_2 are the same, the frequencies of $S_1R_1S_2R_2$ and $S_1S_1R_2R_2$ genotypes are the same. When the survival rates of heterozygotes, $S_1S_1R_2S_2$ and $S_1R_1S_2S_2$, and $S_1S_1R_2R_2$ and $R_1R_1S_2S_2$, are increased symmetrically (Fig. A2-5b), the results are similar to those in Fig. A2-6b. This suggests that asymmetrical changes in the survival of heterozygotes can have impacts on resistance evolution almost as large as symmetrical changes.

This last result can be interpreted as meaning that when there are asymmetrical effects on resistance loci, the rate of resistance evolution will be determined by the most rapidly evolving locus. To illustrate this, we considered three cases; (i) $s_{1SS} = s_{2SS} = 0.01$, (ii) $s_{1SS} = 0.01$ and $s_{2SS} = 0.001$, and (iii) $s_{1SS} = s_{2SS} = 0.001$. The rate of evolution for case (ii) is most similar to the rate of evolution to case (i), which gives the most rapid case of resistance (Fig. A2-7).

Monsanto's Implicit Modeling of Larval Movement

In Monsanto's analysis, they model larval movement by decreasing the size of the refuge (proportion of plants that are non-*Bt*) to account for mortality of larvae that move from non-*Bt* plants to *Bt* plants. This implicit method of accounting for the movement of larvae does not include any effects on the selection of heterozygotes. As first demonstrated by Mallet and Porter (1992), this is the main effect by which planting seed mixtures speeds resistance evolution, and therefore the Monsanto model will overestimate the time to resistance failure.

To illustrate this with the SAP model, the model was run under the baseline conditions, and Monsanto's procedure was then used to model the case of reducing the proportion of non-*Bt* plants in the seed mixture in a way that matches the survival of susceptible insects in the SAP model (Fig. A2-8). This demonstrates that the procedure Monsanto used to implicitly model larval movement shows very little effect of larval movement on the time to resistance failure up to the point that the insect population decreases towards extinction.

High and Low Dose Scenarios and Larval Movement in Seed Mixtures

The effect of larval movement to increase the rate of resistance evolution applies mainly to the high-dose case, rather than the low-dose case. This is illustrated in Fig. A2-9 using the low-dose survival rates given in the EPA/ORD model for CRW.

Density-Dependent vs. Frequency-Only Models

The EPA/ORD model included only allele frequencies and not insect densities. This will give higher estimates of the time to resistance failure than a corresponding density-dependent model for the situation in which populations are persistent and density dependence operates at the scale of individual plants (Fig. A2-10).

Seed Mixtures vs. Structured Refuges

In this situation, the rate of resistance evolution between seed mixtures and structured refuges is compared for the case in which insects have long-range adult dispersal and larvae move among plants. For the case of long-range adult dispersal, the case of structured refuges will be the same as the case of seed mixtures when there is no larval movement. Therefore, the decrease in time to resistance failure caused by larval movement represents the lower durability of seed mixtures relative to a structured refuge.

For the baseline parameter values and the density-dependent model, when 0.3 of the larvae move between plants, no seed mixture with the proportion non-*Bt* seeds less than 40% performed as well as a 5% structured refuge (Fig. A2-11 a-c). This is because, when the proportion of non-*Bt* plants is sufficient to maintain the insect population (to the right of the arrows), the time to resistance failure actually decreases with the proportion of non-*Bt* seeds in the seed mixture until the seed mixture contains a majority of non-*Bt* seeds. This unintuitive result is caused by the combined effects of larval movement and density dependence.

When the effect of density-dependent survival is removed by using the frequency-only model, the time to resistance failure increases monotonically with the proportion of non-*Bt* seed in the seed mixture (Fig. A2-11 d-f). In this case, a 5% structured refuge is roughly equivalent to a 20% seed mixture when 0.3 of the larvae move among plants; however, if 0.6 of the larvae move, then a seed mixture of >60% is required for equivalent durability to a 5% structured refuge.

These results show the very large loss of durability caused by a seed mixture under high-dose conditions. For the more-realistic case of the density-dependent model, when there is a modest rate of larval movement (0.3 of the larvae move), there is effectively no seed mixture that provides the durability of a 5% structured refuge. Even under the unrealistically optimistic case of a frequency-only model, a 20% seed mixture is needed.

In conclusion, it is important to remember that comparisons between seed mixtures and structured refuges are only intended to show the very strong effects that larval movement can

have for seed mixtures. The Panel has repeatedly noted that there are sufficient uncertainties in all models that quantitative predictions should be used with extreme caution. That includes the comparisons between seed mixtures and structured refuges.

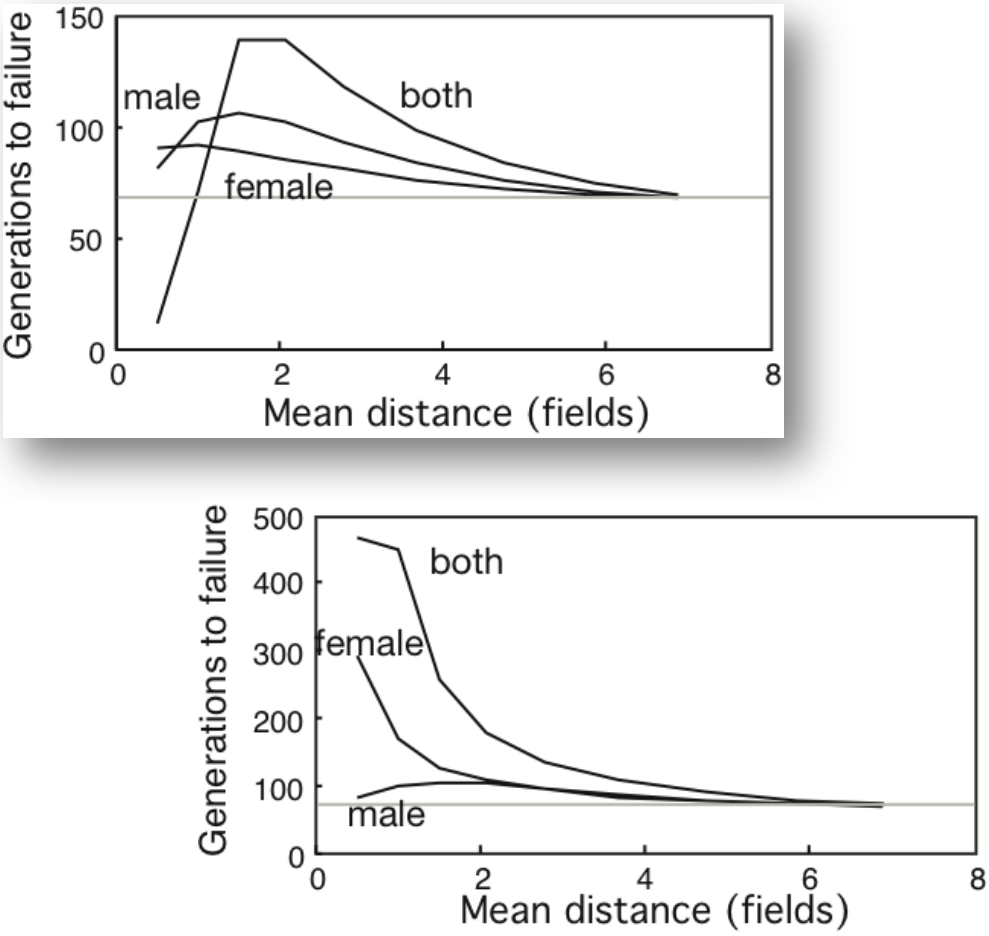

Fig. A2-1. For structured refuges, the effect of adult dispersal distance on the rate of resistance evolution (generations to resistance failure, defined when the frequency of both resistance alleles exceeded 0.5). In the top panel, refuge fields are rotated every three generations, while in the bottom panel refuge fields never rotate. For lines labeled “male”, males have mean dispersal distances given by the x-axis, while females have infinite dispersal distances; the opposite applies for the lines labeled “female”. For lines labeled “both”, the mean dispersal distances of both males and females are given on the x-axis. Gray lines give the case of "infinite" dispersal computed for the spatially implicit model. Data are simulated on a 50x50 grid of fields with “wrap-around” boundaries. Baseline parameter values are: proportion of refuge fields $Q = 0.05$; female fecundity $F = 100$; $s_{1SS} = s_{2SS} = 0.01$; $h_1 = h_2 = 0.05$; and initial allele frequencies 0.005.

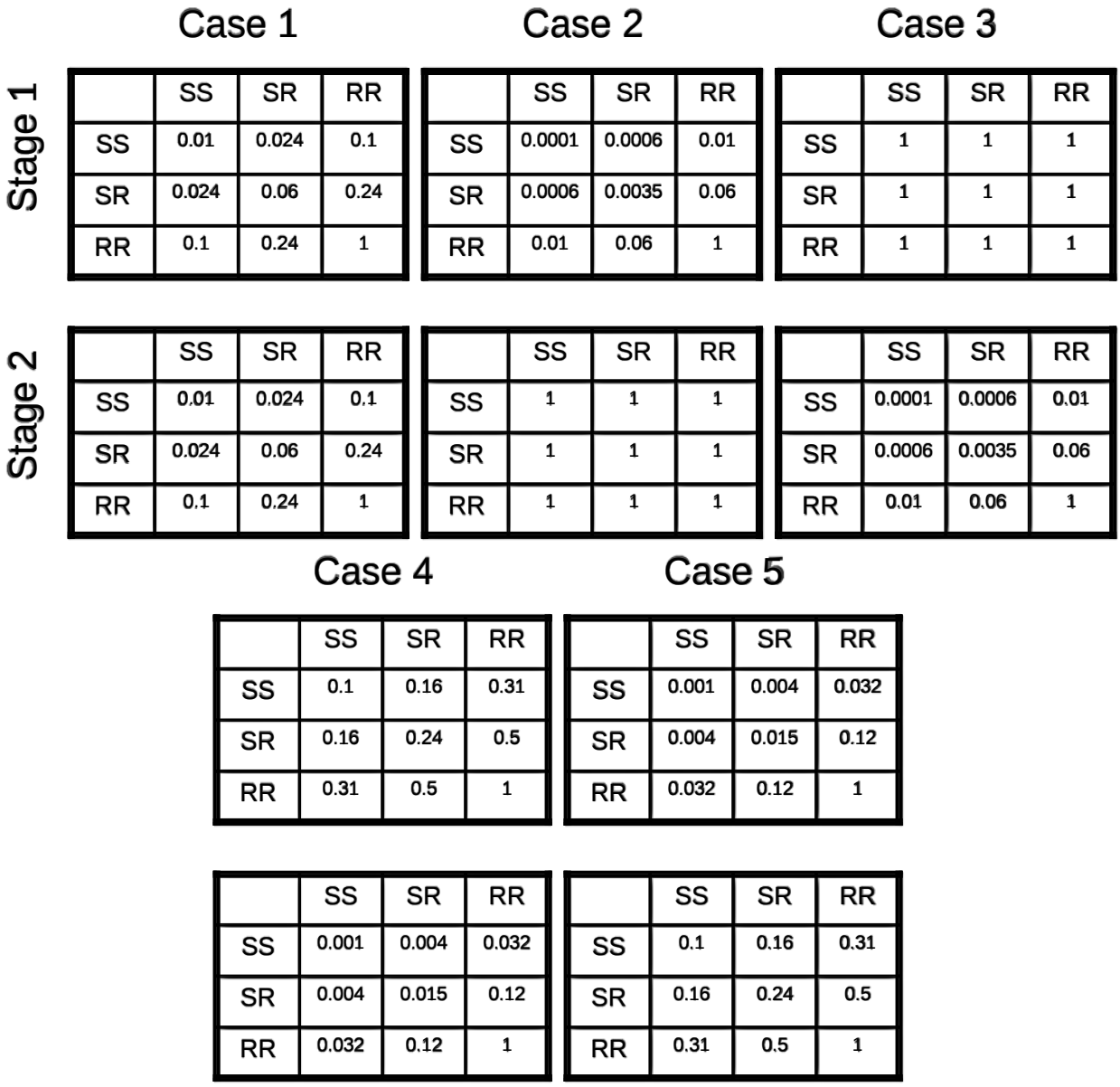

Fig. A2-2. Survival of genotypes before (upper tables) and after (lower tables) for five cases that differently distributed selection on larvae before and after movement between plants. In all cases, the total survival of each genotype on *Bt* plants if there is no larval movement is the same.

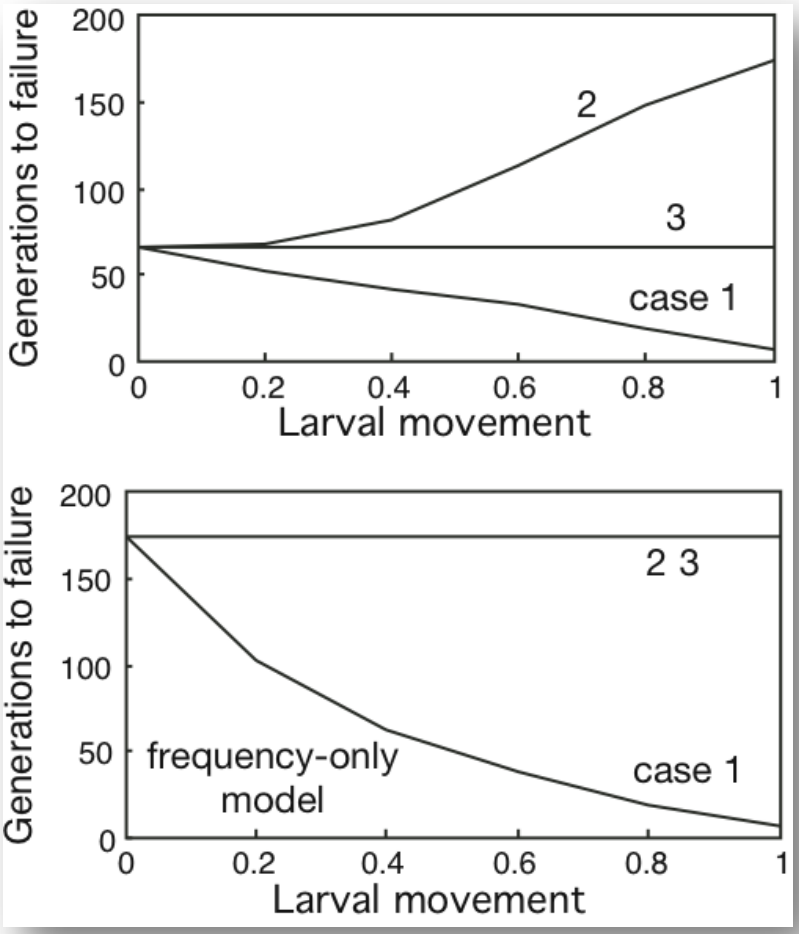

Fig. A2-3. Generations to control failure as a function of the proportion of larvae moving between plants for the density-dependent model (top panel) and frequency-only model (bottom panel). Survival rates for cases 1-3 are given in Fig. A2-2. Baseline parameter values are: proportion of non-*Bt* plants in seed mixture $q = 0.05$; $F = 100$; and initial allele frequencies 0.005.

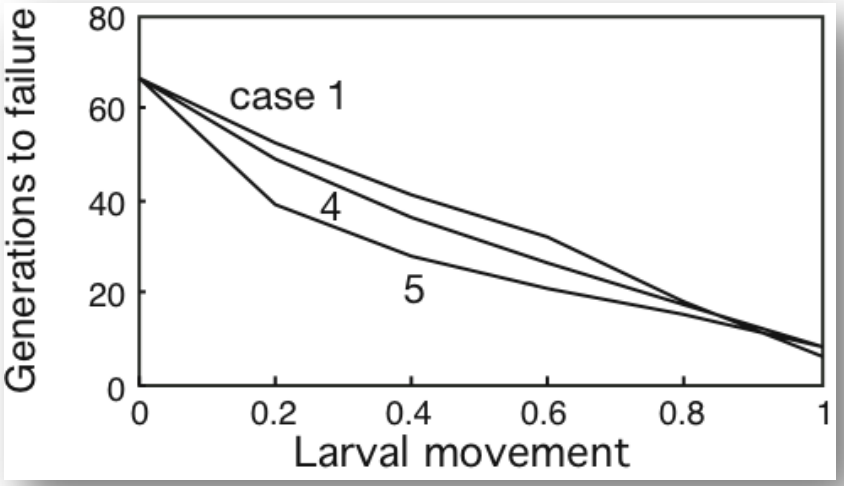

Fig. A2-4. Generations to control failure as a function of the proportion of larvae moving between plants for the density-dependent model. Survival rates for cases 1, 4 and 5 are given in Fig. A2-2. Baseline parameter values are: proportion of non-*Bt* plants in seed mixture $q = 0.05$; $F = 100$; and initial allele frequencies 0.005.

A. Asymmetric Changes

	SS	SR	RR
SS	0.0001	0.0006	0.01
SR	0.0006	0.0035	0.06
RR	0.01	0.06	1

B. Symmetric Changes

	SS	SR	RR
SS	0.0001	0.0006	0.01
SR	0.0006	0.0035	0.06
RR	0.01	0.06	1

Fig. A2-5. Baseline values for total survival of each genotype highlighting those survival rates that were increased by 5-fold in the simulation experiments to investigate the effects of heterozygote survival on the rates of resistance evolution (Fig. A-6). The top table (A) gives the case of asymmetric changes and the bottom table (B) gives the case of symmetric changes. Blue, red, and green shading denote $S_1S_1R_2S_2$, $S_1R_1S_2R_2$, and $S_1S_1R_2R_2$ genotypes (asymmetrical case) and $S_1S_1R_2S_2$ and $S_1R_1S_2S_2$, $S_1R_1S_2R_2$, and $S_1S_1R_2R_2$ and $R_1R_1S_2S_2$ genotypes (symmetric case).

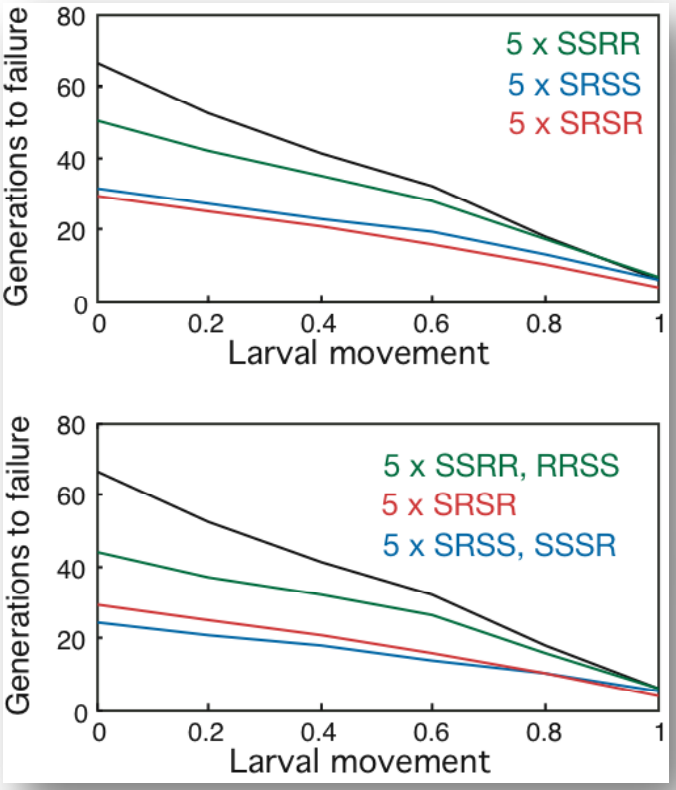

Fig. A2-6. Generations to control failure as a function of larval movement (proportion of larvae moving) for the experiment in which survival of different genotypes are increased by a factor of 5 (see Fig. A2-5). Baseline parameter values are: proportion of non-*Bt* plants in seed mixture q = 0.05; F = 100; $s_{1SS} = s_{2SS} = 0.01$; $h_1 = h_2 = 0.05$; and initial allele frequencies 0.005.

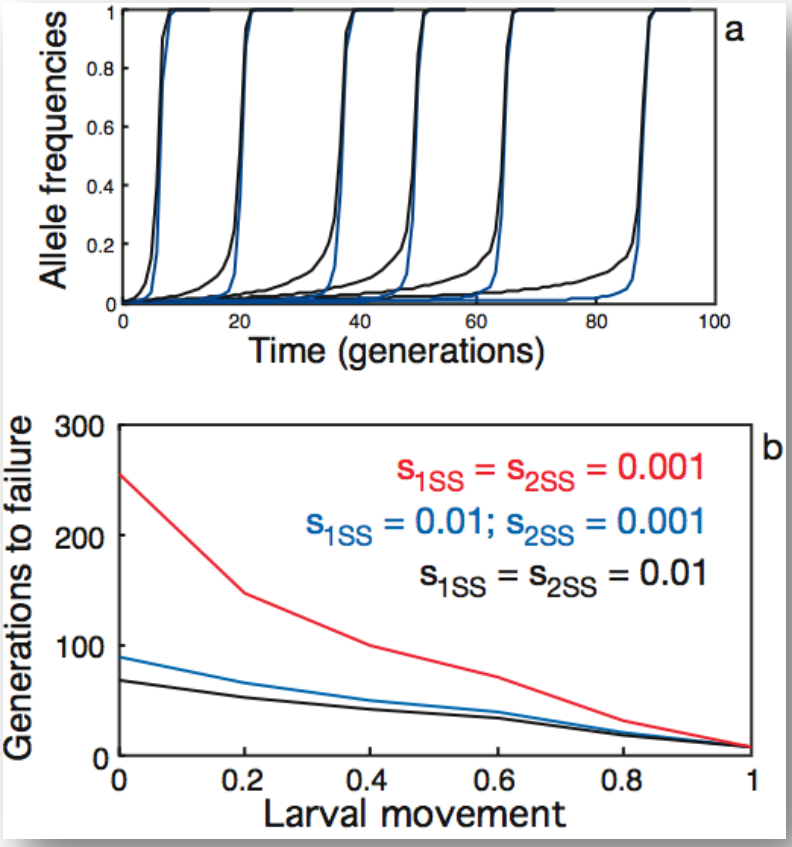

Fig. A2-7. Resistance evolution for two loci under different selection pressures. (a) Example trajectories of allele frequencies when $s_{1SS} = 0.01$ and $s_{2SS} = 0.001$, where the black line corresponds to the frequency of the resistance allele in the fastest-evolving locus, R_1. (b) Generations to resistance failure as a function of larval movement (proportion of larvae moving between plants). Baseline parameter values are: proportion of non-*Bt* plants in seed mixture q = 0.05; F = 100; $h_1 = h_2 = 0.05$; and initial allele frequencies 0.005.

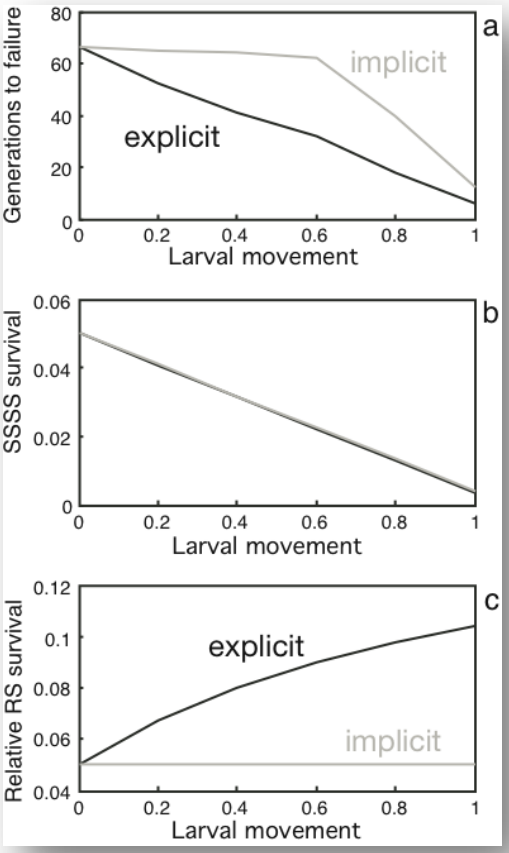

Fig. A2-8. (a) Generations to resistance failure (frequency of both alleles > 0.5), (b) survival of $S_1S_1S_2S_2$ homozygotes, and (c) relative survival of heterozygotes for the cases in which larval movement is explicitly or implicitly modeled. For implicit modeling of larval movement, the survival of $S_1S_1S_2S_2$ susceptibles is decreased (by decreasing the proportion of non-*Bt* plants in the seed mixture, q) (panel b), while there is no change in the relative survival of heterozygotes (panel c). In (a), the break in the slope of the line for implicit larval movement (gray line) corresponds to the point above which the insect population starts to decline towards extinction. Baseline parameter values are: proportion of non-*Bt* plants in seed mixture $q = 0.05$; $F = 100$; $s_{1SS} = s_{2SS} = 0.01$; $h_1 = h_2 = 0.05$; survival is divided equally between pre- and post-larval movement; and initial allele frequencies 0.005.

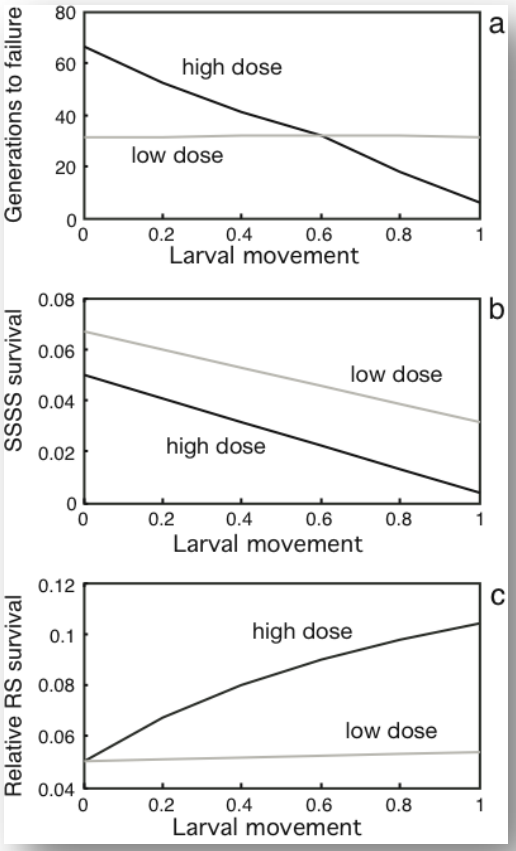

Fig. A2-9. (a) Generations to resistance failure (frequency of both alleles > 0.5), (b) survival of $S_1S_1S_2S_2$ homozygotes, and (c) relative survival of heterozygotes for high- and low-dose cases. In the high-dose case, $s_{1SS} = s_{2SS} = 0.01$ and $h_1 = h_2 = 0.05$, and relative survivals are combined multiplicatively. In the low-dose case, values provided by EPA/ORD for CRW are used: $s_{SSSS} = 0.018$, $s_{SSSR} = 0.019$, $s_{SSRR} = 0.038$, $s_{SRSS} = 0.02$, $s_{SRSR} = 0.0233$, $s_{SRRR} = 0.0861$, $s_{RRSS} = 0.058$, $s_{RRSR} = 0.1051$, and $s_{RRRR} = 1$. Baseline parameter values are: proportion of non-*Bt* plants in seed mixture $q = 0.05$; $F = 100$; and initial allele frequencies 0.005.

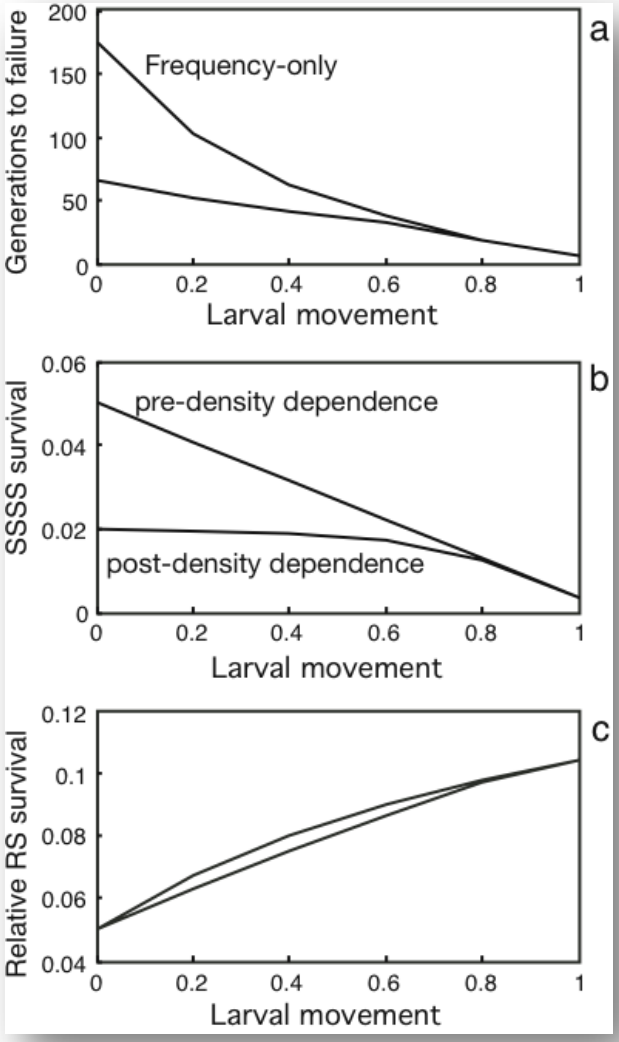

Fig. A2-10. (a) Generations to resistance failure (frequency of both alleles > 0.5), (b) survival of $S_1S_1S_2S_2$ homozygotes both before and after density dependence occurs in the density-dependent model, and (c) relative survival of heterozygotes for the density-dependent and frequency-only models. Baseline parameter values are: proportion of non-*Bt* plants in seed mixture $q = 0.05$; $F = 100$; $s_{1SS} = s_{2SS} = 0.01$; $h_1 = h_2 = 0.05$; survival is divided equally between pre- and post-larval movement; and initial allele frequencies 0.005.

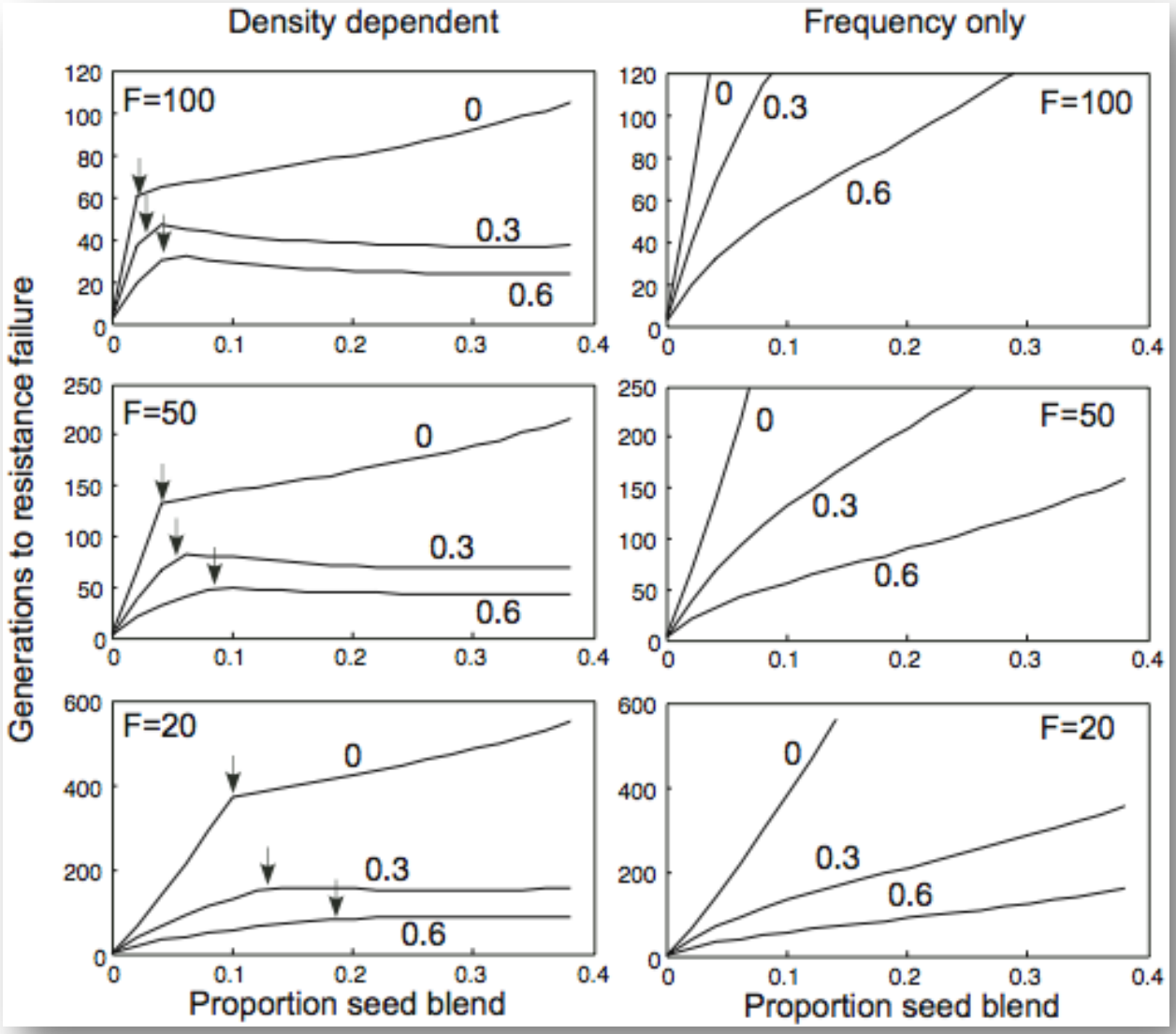

Fig. A2-11. Generations to resistance failure (frequency of both alleles > 0.5) for density-dependent and frequency-only models for F = 100, 50, and 20 (labeled) when larval movement is 0, 0.3, and 0.6. In the density-dependent model, arrows mark the proportion of seed mixtures below which the population decreases towards extinction. Baseline parameter values are: s_{1SS} = s_{2SS} = 0.01; h_1 = h_2 = 0.05; survival is divided equally between pre- and post-larval movement; and initial allele frequencies 0.005.