

**SAP Minutes No. 2014-01**

**A Set of Scientific Issues Being Considered by the  
Environmental Protection Agency Regarding  
Scientific Uncertainties Associated with Corn  
Rootworm Resistance Monitoring for Bt Corn  
Plant Incorporated Protectants (PIPs)**

**December 4-5, 2013  
FIFRA Scientific Advisory Panel Meeting  
Held at the  
Environmental Protection Agency Conference  
Center  
Arlington, VA**

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In preparing these meeting minutes, the Panel carefully considered all information provided and presented by EPA, as well as information presented by public commenters.

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## List of Common Acronyms Used

BMPs	Best Management Practices
BPPD	Biopesticides and Pollution Prevention Division:
CRW	Corn Rootworm
EC50	Effect Concentration /50 percent of the test population
EPA	The United States Environmental Protection Agency
GLP	Good Laboratory Practices
IRM	Integrated Resistance Management
LC50	Lethal Concentration/50 percent of the test population
NCR	Northern Corn Rootworm
NIS	Node Injury Scale
PI	Personal Injury
PIPs	Plant Incorporated Protectants
USDA	The United States Department of Agriculture
WCR	<i>Diabrotica v. virgifera</i> (Western corn rootworm)


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Daniel Schlenk, Ph.D.  
FIFRA SAP Chair  
FIFRA Scientific Advisory Panel  
Date: March 4, 2014



Fred Jenkins, Jr., Ph.D.  
Designated Federal Official  
FIFRA Scientific Advisory Panel  
Date: March 4, 2014



**Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)**  
**Scientific Advisory Panel (SAP)**  
**Scientific Uncertainties Associated with Corn Rootworm Resistance Monitoring for**  
**Bt Corn Plant Incorporated Protectants (PIPs)**  
**December 4-5, 2013**

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## INTRODUCTION

On December 4-5 the FIFRA Scientific Advisory Panel (SAP) met to address scientific issues associated with the “Scientific Uncertainties Associated with Corn Rootworm Resistance Monitoring for Bt corn Plant Incorporated Protectants (PIPs).” As part of the Insect Resistance Management program for Bt corn Plant Incorporated Protectants (PIPs), registrants are required to conduct annual resistance monitoring of the key target insects. Resistance monitoring for corn rootworm (CRW) has been beset by a number of technical challenges. The PIPs registered for control of CRW are considered “non-high dose,” meaning that a proportion of even a susceptible population can be expected to survive exposure to the Bt toxin(s). Opportunities for monitoring investigations are limited because CRW have one generation per year, undergo an obligate diapause period, and can be difficult to maintain in laboratory environments. Testing with artificial diet bioassays (as is done for lepidopteran pests of Bt corn) has yielded highly variable results which have been problematic to interpret. Taken together, these factors have complicated the establishment of a workable (regulatory) definition of “resistance” for CRW (based on bioassay results). The Panel provided recommendations to EPA on how to improve the resistance monitoring program for CRW by addressing the scientific uncertainties associated with this insect. Specifically, the Panel addressed the EPA’s charge to them on questions regarding the following topics: population sampling (random vs. focused), triggers (i.e., field damage to Bt corn) for investigations of potentially resistant populations, bioassay techniques, defining resistance (in the context of bioassay results), and remedial action plans in the event of resistance to contain or limit the spread of resistant populations. Opening remarks at the meeting were provided by Steven Bradbury, Ph.D., Director, Office of Pesticide Programs, and Robert McNally, Director, Biopesticides and Pollution Prevention Division (BPPD), OPP.

US EPA presentations were provided by the following staff:

Alan Reynolds  
Jeannette Martinez

## **Public Commenters**

### **Oral Public comments were provided by (provided in alphabetical order)**

Analiza Alves, Ph.D. on behalf of Pioneer Hi-Bred International  
Tony Burd, Ph.D. on behalf of the Agricultural Biotechnology Stewardship Technical Committee  
Graham Head, PhD on behalf of the Monsanto Company  
Gregory Jaffe on behalf of the Biotechnology Project Center for Science in the Public Interest  
Clinton Pilcher, Ph.D on behalf of Pioneer Hi-Bred International Inc.  
Tony Burd, PhD, Syngenta  
Jim Zimmerman, National Corn Growers Association

### **Written Public Comments were provide by (provided in alphabetical order)**

The Agricultural Biotechnology Stewardship Technical Committee  
Martin R. Barbre on behalf of the National Corn Growers Association  
Tony Burd, Ph.D. and Dennis P. Ward, Ph.D. on behalf of Syngenta Seeds Inc.  
Christian Krupke, Ph.D. Purdue University, West Lafayette, IN  
Chris DiFonzo, PhD. of Michigan State University *et al.*  
Center for Food Safety  
Aaron Gassmann, Iowa State University  
Michael E. Gray, Ph.D. of the University of Illinois, Urbana-Champaign  
Graham Head, Ph.D., Monsanto  
Monsanto Company.  
Clinton D. Pilcher, Ph.D. on behalf of DuPont Pioneer  
Pioneer Hi-Bred International, Inc.  
Center for Science in the Public Interest  
Nicholas Storer, Ph.D. on behalf of Dow Agro Sciences  
John F. Tooker, Ph.D of the Pennsylvania State Extension Pennsylvania State University

## SUMMARY OF PANEL RECOMMENDATIONS

*Charge Question 1a. The Panel is asked to comment on sampling approaches for conducting annual CRW resistance monitoring that support early resistance detection. Please discuss the strengths and limitations of BPPD's proposal for a focused (risk based) sampling approach for the Corn Belt, supplemented with samples from lower risk "fringe" areas for comparison.*

### Panel Summary

The Panel concurs with the Agency's recommendation to expand the sampling area to include other areas with greater than expected damage including Wisconsin, South Dakota, Colorado, and Minnesota (BPPD 2011, 2012). The Panel also concurs with EPA's primary objectives for sampling that include: 1) actively seeking out areas of concern; 2) selecting annual sampling locations based on risk factors; and 3) identifying resistance issues earlier through more proactive monitoring in high risk areas. The Panel recommends that these high risk areas should entail: 1) close proximities to reported field failures; 2) continuous corn production areas; 3) fields that do not rotate the Bt toxin mode of action; and 4) fields that are in non-compliance with refuge requirements. One panel member mentioned that sampling according to the afore mentioned four recommendations should (although being focused) still assure a certain spatial coverage (e.g. systematic grid of survey with focused sampling in each grid). The Panel disagrees that "fringe" areas are more appropriate for the random sampling approach as these areas may not be lower risk depending on production practices (John Tooker Public Comment, Public Docket ID no: EPA-HQ-OPP-2013-0490-0038; Tooker and DiFonzo 2013).

Additional recommendations from the Panel include: 1) a focused approach to track susceptibility of individual populations or discrete geographic locations as this approach will be more likely to identify hot spots and may reduce the likelihood of area-wide resistance (BPPD 2012); 2) a proactive approach to monitoring for greater than expected damage earlier in the season by identifying high risk areas of continuous corn production or areas that lack rotation of Bt toxin modes of action based on sales records; 3) high risk (corn-on-corn, continuous use of the same trait) areas within 4 or 5 zones should be the only target for annual sampling, eliminating random sampling. The "fringe" zones/regions should be considered as one of the above zones and sampled in areas of highest risk; 4) including non-compliance with refuge requirements as a factor for high risk classification since lack of refuge compliance may result in increased resistance in areas that are not currently considered high risk; 5) increasing the amount of acreage surveyed regarding refuge compliance; 6) modifying the regional boundaries used in the Arent Fox surveys to better reflect corn rootworm population variations in species and resistance traits to better reflect field conditions; and 7) that the revision of "regions" boundaries for the Arent Fox survey also be applied to the sampling regions to provide consistency to allow better comparison of data obtained from surveys, sampling and greater than expected damage reports. The current sampling approach is likely to misrepresent potential resistance since the sampling is random and populations are not tracked over time for shifts in susceptibility. This coupled with the associated delays in

susceptibility studies could allow for dispersal of resistant CRW resulting in area-wide resistance prior to identification of a resistance concern.

***Charge Question 1b.*** *How many CRW populations should be collected from within a sampling region to adequately assess susceptibility?*

### **Panel Summary**

The Panel recognizes that when collecting samples from fields with greater than expected damage, the population samples must be adequate to generate enough larvae to conduct all assays (diet and on-plant assays) used to evaluate resistance. The Panel believes the current approach of sampling 12-15 CRW populations mostly from Illinois Iowa and Nebraska seems very low in light of the number of reported cases of suspected resistance and/or greater than expected damage (BPPD 2011). The current approach of a minimum of 2,000 adults collected for each population (EPA 2013) is likely not enough.

The Panel makes the following recommendations: 1) collect a minimum of 4,000 adults per population (This recommendation is based on the presumed resistance allele frequency for CRW); 2) expand areas of annual sampling to include additional “high risk” locations in Wisconsin, South Dakota, Colorado and Minnesota; and 3) annual collections of 10-15 populations by each registrant from the same locations in each of the 4 or 5 zones and bioassays conducted on all four traits presently registered.

***Charge Question 2a. (Corn Rootworm Sampling in Response to Damaged Bt Fields)***  
*The Panel is asked to comment on methods for investigating CRW populations causing unexpected damage to Bt corn. Specifically, please comment on:*

*i. The use of field damage ratings (NIS) as a screen for potentially resistant populations. What sampling triggers should be used for single toxin and pyramided Bt products? Should alternate techniques be considered?*

The Panel agrees that the likelihood of reports describing fields with unexpected damage will be increasing in areas where a single gene Bt product has been continuously used and where fields sown to corn following one or more years of corn has occurred. The Panel believes that a trigger level for further sampling of the CRW population is when a sample of corn roots produces a Node Injury Scale (NIS) rating of 1.0 for single gene expression Bt products and 0.5 for pyramided products. The Panel discussed the logistical problems of requiring more adult sampling and larval bioassays, but concluded it is better to identify resistance in populations in “high risk” areas at this time so that mitigation practices can begin in those areas as soon as possible.

*ii. The use of transect sampling in damaged areas or random sampling throughout the affected field to assess root damage ratings.*

The Panel recommends that transect samples be taken across the reported damaged area to help determine if resistance of the CRW population is the cause and other factors that

may have led to a “hot spot” of damage are ruled out. A transect sampling scheme is more likely to identify the severity of damage in the damaged area and, in high risk areas which frequently lead to adult sampling as a follow up procedure. The Panel believes it is important to reduce the frequency of false negative determinations regarding damage and subsequent resistance in CRW populations in these areas.

*iii. Appropriate sampling locations (i.e., in the vicinity of the damage and/or surrounding areas) for collections of adults if field damage triggers are exceeded.*

The Panel agrees that samples of adult CRW should be obtained from areas as close to the reported and identified damaged field as possible. Samples should be taken as soon as feasible after damage has been determined to be greater than expected. Clearly, because reporting of damage often occurs well after the emergence of adult beetles, samples of adults in the immediate area may not reflect the emergence of individuals responsible for the damage. In addition, beetle density may be quite low in the field and surrounding areas. Sampling for adults more than 1 mile from the damaged area simply reduced the likelihood that the population collected represents the population that was responsible for the damage noted. In cases where sufficient beetles cannot be collected, a follow-up sample is required the following year from the same area the damage was noted. The Panel recommends adult sampling data should include significant detail about the field history (such as information on the field’s rotational crops, Bt expressing hybrids, use of chemical pesticides, etc.) and relation to other neighboring crops and the number of beetles collected, their sex ratio and the number of gravid individuals.

*iv. The deployment of sentinel plots in the vicinity of damaged fields in subsequent seasons to: 1) assess the resistance allele frequency in the area, and/or 2) collect insects if no adults were present at time of the field investigation.*

When sufficient adult CRW cannot be collected from fields with documented damage in the same season, the Panel agrees that beetles should be collected as soon as possible after their emergence the following year in the same location. This collection can be facilitated by the use of sentinel plots of untreated corn (or a surrogate crop like squash or pumpkin). The reality that beetles collected from these plots may not represent those that caused the damage noted the previous year is acknowledged by the Panel. However, this sample method is the best alternative approach for a follow-up beetle collection.

The Panel recognizes that sentinel plots may concentrate resistant individuals that subsequently can spread considerably and increase resistance in the area, but could also serve as a refuge for susceptible beetles that could subsequently dilute the resistance genes in local populations of CRW. A sentinel plot in the same area where damage was documented the previous year planted with a non-Bt similar maturity corn hybrid would serve as the best choice for collecting beetles the following year.

If sentinel plots were planted each year in “high risk” areas to serve as sources for annual collections of adults for subsequent bioassays a rough estimate of the change in allele frequency could be obtained by comparing bioassay results over a number of years.

Currently, our collective knowledge of resistant allele frequency in CRW populations is limited so a more appropriate methodology is unavailable.

*Charge Question 3a (Diagnostic Assays). Please comment on the strengths and limitations of diet bioassay methodologies for early resistance detection with CRW, considering that the currently-registered toxins are less than high dose. What improvements could be made to these bioassays to make them more effective and proactive resistance detection tools?*

### **Panel Summary**

Properly conducted bioassays with artificial insect diet can provide more precise and consistent responses to screen for resistance to Bt, than current on-plant assays. Results from these artificial diet bioassays have been established using standard statistical procedures recognized by the scientific community, including most of the parameters used to evaluate on-plant bioassays. Monitoring efforts conducted with artificial diet bioassays can occupy less space, can yield results in a few days, and be more economical than the current on-plant screening. This is an advantage for most of the research facilities and for IRM mitigation attempts.

Preliminary lethal ( $LC_{50}$ ) and effective concentrations ( $EC_{50}$ ) values have been already established for some of the Bt proteins used to control CRW spp. However, the Panel believes that these methods require further refinement to continue serving as historical records documenting the trends of susceptibility over time. Susceptibility to Bt at a single location can be more accurately followed over time with artificial diet bioassays than with on-plant bioassays because of the more precise nature of the assay and the breadth of data they provide. A single well-maintained stock of purified Bt toxin can last a long time and perform more uniformly than plants where their Bt protein expression can be influenced by environmental and biotic factors and the corn hybrid genetic background.

The Panel recommends keeping the bioassay approach with artificial diet to continue building some of the already observed trends, and share the results among all appropriate researchers, both public and private. Consistency in methodology (e.g.: same type of diet, reference laboratory colony, batch of Bt toxin, proper handling of neonates, etc.), is a necessity for all types of monitoring bioassays. A solid assay can accommodate in a single replicate plate 5 or 6 concentrations of 3 different Bt proteins, providing invaluable information. The Panel also suggests developing a standard diet where neonates can survive on assays beyond 3-4 days with greater than 80% survival.

**Charge Question 3b.** *The Panel is asked to discuss the relative merits and limitations of the two on-plant assays (Gassmann et al. 2011 and Nowatzki et al. 2008). Please discuss the extent to which these assays have different sensitivities to make early corn rootworm resistance determinations? Should other on-plant assay approaches be considered?*

### **Panel Summary**

The Gassmann et al. (2011) approach represents a more realistic situation of field conditions than the Nowatzki et al. (2008) method and the artificial diet bioassays. The Gassmann method may be both easier to follow by others and to confirm results independently, expanding the monitoring effort by the possible inclusion of independent scientists. However, this procedure may benefit from investigating larval behavior and mortality by inspecting the root system over a range of 3 to 17 days after egg hatch to determine if there is a more optimal assay time that can yield more accurate results. Performing an intense/destructive root sample, accounting for the total number of surviving larvae during the recommended period of time and not relying entirely on the larvae collected in alcohol vials after the funnel extraction, may be a great improvement of the method. Confirmation of the transformation event in the Bt hybrid tested and expression of the protein in the roots is necessary to assure that the corn plants are indeed the correct hybrids. Efforts should be taken to obtain corn seed free from pesticide coating. High larval mortality in the soil makes analysis more difficult and problematic, impeding potential Bt resistance to be documented correctly. The use of 10-20 neonates per plant, may present a serious constraint to the methodology.

The Nowatzki et al. (2008) methodology, consisting of “greenhouse plant efficacy,” and “sub-lethal seedling” components, has greatly improved since its publication, as was evident from the public comments during the meeting. This method is easier to standardize because corn seedlings are grown in containers under more consistent conditions inside a growth chamber and not in a greenhouse, also preventing possible insect escapes from the experimental unit. Growth of seedlings may produce an expression of protein more consistent between plants, although not necessarily similar to field conditions. Cross-breeding the collected WCRs with a non-diapausing colony enables testing over a much shorter period of time. The Panel suggests that research efforts are continued to reduce the amount of variability in the assays, to test these methodologies with corn events containing Cry3Bb1 protein, and to develop a more Bt resistant WCRW colony.

Gassmann et al. (2011) and Nowastzki et al. (2008) report that: “...*surviving larvae were collected in vials with alcohol.*” Assuming that a particular population has an unusual high survivorship, the Panel recommends collecting live samples and maintaining part of the sample in artificial diet to continue with studies to confirm resistance by other methods. The surviving colonies would represent a great opportunity to learn more about the biology, behavior and genetics of the CRW. The Panel also recommends evaluating both Gassmann and Nowatzki methodologies with pyramided events, as well as with single-protein events separately. In both methodologies, it is necessary to obtain

information about expression of the Bt toxins in the root system at different time intervals.

**Charge Question 4a.** *The Panel is asked to discuss the merits and shortcomings of the proposed approaches to defining resistance using on-plant assays. What sets of comparisons in the assays are most likely to add value to a weight-of-evidence approach to determining resistance?*

### **Panel Summary**

Gassmann et al. (2011) and Nowatzki et al. (2008) methods measure the response of the western corn rootworm larvae to corn plants that express Bt protein(s). However, limited/declining expression of Bt in the root system is one of the most crucial limitations to these methodologies, and a better understanding of the amount of Bt protein that the larvae are exposed to through time, may facilitate the interpretation of results.

Because the concentration of all the events in the currently commercialized corn hybrids do not meet the definition of “high dose,” the Panel believes that the current scoring criteria of developmental instar proportion of surviving larvae, provides the least variable criteria to discern between putatively resistant and susceptible WCR (*Diabrotica v. virgifera*) larvae.

**Charge question 4b.** *What resistance allele frequency should constitute field resistance for toxins with less than high dose expression? Please discuss the criteria that should be used for these types of toxins (as opposed to high dose toxins) given that a portion of heterozygous insects will survive Bt exposure and drive the evolution of resistance.*

### **Panel Summary**

An effective method to discern Bt resistant CRW from susceptible CRW impeded the establishment of a Bt resistance frequency prior to the release of the Bt corn events. Therefore, the resistance mechanisms in the putatively-resistant CRW colonies have not been thoroughly characterized, making it difficult to establish a resistant allele frequency that should trigger an action by the US E.P.A. The Panel believes that this question does not have a valid scientific answer and opens the possibility for all kinds of speculation.

**Charge question 4c.** *What statistical tests, criteria, and significance levels would be best suited for early resistance detection with the proposed assays? Please discuss how to best avoid false negatives and/or false positives.*

### **Panel Summary**

Probit analysis applied to mortality and larval development instar data obtained in bioassays with insect artificial diets, as well as assessments of the number and developmental instar of surviving larvae at different periods of time for on-plant assays,



seem to be the most adequate methods of analyzing results at the moment. The Panel suggests that bioassays with insect artificial diet should include consistency in terms of methodology, testing materials and reference colonies. Using multiple metrics (e.g. survival, instar development, body size and weight, feeding behavior, root tissue ingestions and exploring gene expression) could be used to reduce the likelihood of false positives and false negatives. Allowing for comparisons between sampled population on Bt and non-Bt corn plus comparisons between sampled populations and susceptible populations (laboratory colonies) will add another indicator.

*Charge question 4d. Please comment on the extent to which incomplete resistance can be identified with on-plant test systems. How should resistance definitions be adjusted to address these scenarios?*

### **Panel Summary**

CRW populations with incomplete resistance may be more fit than the control population on Bt corn, but may not be significantly different. If there are fitness costs for Bt resistance, the resistant population may not survive on non-Bt corn as well as the control colony, or delayed development may occur indicated by reduced body weight/head capsule size of resistant larvae exposed to Bt corn.

Resistance definitions should consider that corn rootworm may have developed more than one response to survive on Bt corn, complicating the use of a single diagnostic tool.

*Charge question 4e. Please discuss the viability of resistance ratios as an option for determining resistant populations, considering the generally low susceptibility of CRW to Bt toxins and the lack of susceptible wildtype populations (i.e., due to widespread adoption of Bt corn). What ratio could be considered as an indicator of resistance for corn rootworm using on-plant assays?*

### **Panel Summary**

Different resistance ratio proposals need to be reconsidered for the current situation of the CRW due to the lack of Bt corn events expressing a “high dose.” Therefore the resistant ratio would need to be ~4x for the CRW for non-high dose corn events. There is limited historical data of the response of this insect that may serve for the calculation of these ratios.

CRW populations obtained from “fringe” areas may serve as “control” populations upon which other populations from “high risk” areas and fields with unexpected damage could be compared. Laboratory populations may be appropriate for a few years, but concern about inbreeding and loss of behavioral traits reflective of field populations may be a limiting factor. The Panel believes that Bt susceptible CRW populations can be obtained in areas where the selection pressure has not been so intense such as in Europe, or from an organic corn grower with a large enough farm area, in the periphery of the US

cornbelt. Also, surrogate species such as *Diabrotica virgifera zea*, the Mexican corn rootworm, can serve as a good indicator for susceptibility, especially if the samples are obtained in areas where Bt corn is not registered (e.g.: Mexico).

*Charge question 5a. Please comment on the strengths and limitations of BPPD's proposal to use resistance allele gradients to define the geographic extent of a resistant population.*

### **Panel Summary**

Mapping allele gradients would be an attractive option if the alleles that confer resistance were well defined, and techniques existed to take many samples from many locations in a timely fashion. Unfortunately, resistant alleles have not been defined. Furthermore, allele presence does not always result in phenotypic expression of resistance. It is feasible that the allele(s) is detected, but not expressed in a manner that confers resistance. A limiting factor is that resistance may be conferred via more than one allele, and the relative importance and interactive contributions of these alleles to resistance is not known. Thus, it is not clear if mapping the gradient of a single allele, a small subset of alleles, or many alleles, would be necessary for inferring maps that reflect the probability of resistant phenotypes. Another limitation is that sample collections could be difficult because abundances of adults could be low or absent at the time collections are required. If when the constraints of defining what allele or alleles confer resistance, and the sampling issue are resolved, the Panel recommends that mapping the presence and frequency of those alleles should be pursued. However, other allele-based studies using neutral genetic markers, discussed as part of the response to question 5(b), could be developed to infer geographic dispersal of populations from locations where resistance is suspected.

Alternatively, it is currently feasible to measure abundance. This can be done *a priori*, in areas of high rates of adoption, and sample resolution guided by other data layers that suggest risk. Information could be obtained within a time frame to influence decisions about deployment of Best Management Practices (BMPs) that are currently deployed in response to suspected resistance. Although BMPs may, or may not, influence the frequency of resistant alleles at a given location, there is an inherent assumption that BMPs can reduce the probability of widespread Bt failure when deployed in response to a problem inquiry field. BMPs help achieve sustainability of Bt crops both at that location and at wider geographic scales, through their influence on population processes. Mapping abundances may help define the spatial scale at which these population processes are being expressed, and tests for spatial dependence in abundance after factoring out other factors (weather, field management, etc.) that contribute to abundance may help define if problem-inquiry fields are arising independently, or if they are more likely to arise close to nearby problem-inquiry fields. The Panel recommends mapping population density gradients as part of Integrated Resistance Management (IRM).

The Panel also recommends clarifying the goal of remedial action, and definitions that enable distinguishing a localized resistant population (a “hot-spot”) from area-wide

resistance. These clarifications are necessary in defining the geographic extents of sampling gradients and in using gradients to define remedial action areas. Remedial action aimed at eliminating a resistant population would entail much longer gradients than those designed to limit and contain allelic frequencies or population abundances below predetermined thresholds. Clarifying these definitions is a necessary component of using gradients to define geographic extents of resistant populations.

*Charge question 5b. What other tools or strategies could be employed to define the remediation zone?*

### **Panel Summary**

The Panel notes seven other strategies to help define remediation zones. The primary suggestion is to adapt from studies that delineate population structure using neutral alleles, such as those that define hybridization zones in northern Italy. Although alleles subject to selection such as those conferring resistance may not follow the same pattern as those detected using neutral markers, they are the most closely related to this question of introgression of alleles among populations. If similar genotypic diversity exists in areas of the US, then similar methods could be developed for areas of adoption. Additional strategies build upon field measures of abundances or bioassays, or landscape and management practices. Thus, a second strategy is to utilize dispersal kernels recently modeled with mark-recapture field experiments or dispersal rates based on historical patterns of abundances during early colonization. A third strategy is to utilize dispersal distances modeled from the introgression of soybean-rotation-resistant variants. A fourth is to spatially structure the sampling in response to damage, and testing offspring to define remedial action zones. The Panel also notes that the bioassay data provided in this review showed temporal trends in survivorship or development rates. In some locations, approximately a county spatial scale, these temporal trends were positive. Thus, a fifth approach is to use spatially-referenced temporal trends in bioassay data to help define spatial locations that could be defined as a remedial action area. A sixth approach is to develop risk maps by categorizing relative risk for a field or land area based on expert opinion, analysis of past Personal Injury (PI) fields, lack-of-rotation, repeated use of the same trait, weather conducive to CRW development, regional corn acreage and corn density in a landscape, and other factors, and use these maps to influence remediation zones. A seventh method is to map the probability of exceeding thresholds using presence/absence categorical data of population abundances or frequencies of specific alleles.

The Panel concurs with the EPA that “A primary goal of resistance monitoring is to detect shifts (in space and time) in the frequency of resistant alleles (i.e., susceptibility changes) before the onset of resistance leads to widespread Bt failure.” However, the goal as stated is not feasible because we are unable to measure allele frequencies or changes in susceptibility with sufficient precision. The Panel believes that the goal should be restated, to include metrics that we have a capacity for measuring with sufficient precision and in a timely fashion. Currently, the only such metric is abundance, and some members of the Panel believe that monitoring abundance should be added,

although not all agreed that monitoring abundance directly related to monitoring resistance. A suggested restatement is: “A primary goal of monitoring is to detect shifts (in abundance, susceptibility, and/or resistant allele frequency) before the onset of resistance leads to widespread Bt failure.”

The Panel notes that the scale of remediation following the establishment of resistance, which ranged from 20 to 50 km radii based on the information available to date, may not be feasible. Thus, achieving resistance management should focus on proactive options that avoid or delay the establishment of resistant alleles in CRW populations.

*Charge question 6a. What remediation approaches could be taken for localized vs. area-wide resistance scenarios?*

### **Panel Summary**

The Panel concurs with the concern stated in the EPA (2013), “...actionable thresholds may not be met (or recognized) until resistance is widespread and effective mitigation (reducing resistant allele frequency) is impractical. In this case, managing resistance through population suppression may be the only alternative.” The Panel also concurs with the statement in the EPA White Paper that “BMPs ... likely need to be implemented ... irrespective of whether resistance testing has been completed (US EPA, 2013).” The context for that statement dealt with responding to a problem inquiry field. The Panel recommends widening the context to deal with factors that increase the risk of becoming areas with the emergence of resistance. The Panel recommends a proactive effort of managing resistance through population suppression achieved through the implementation of an integrated pest management (IPM) plan using best management practices.

The Panel recommends developing BMPs that will address variation in production practices, species behavior and resistance to chemical and cultural practices such as continuous corn, rotation resistance, and production practices. While the Panel anticipates an overlap in BMP recommendations, the Panel believes it is necessary to consider the population and species differences along with differences in production practices and landscapes when developing the BMPs. The Panel recommends the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) Insect Resistance Management Stewardship Subcommittee, the National Corn Growers’ Association and state-based corn associations in cooperation with research and Extension scientists and growers develop an IRM plan and BMPs using an IPM approach that would be implemented prior to suspected resistance (proactive) to reduce the likelihood of heavy selection pressure on corn rootworm by using the same mode of action over an extended period of time as resistance has been shown to develop within 3 to 7 years of use in continuous corn. The Panel recommends inclusion of social scientists into the process to increase the understanding of human behavior with regard to production practices and decision-making. The Panel notes that Extension entomologists are already developing region-specific recommendations, for example, for the eastern corn-growing states (Indiana, Michigan, New York, Pennsylvania, Ohio, Ontario) (Tooker and DiFonzo

2013). The Panel recommends the implementation of the proposed BMPs in advance of suspected resistance.

The Panel believes using the IPM approach as part of BMPs may reduce the need for remediation although the Panel recognizes that not all producers will choose to use the BMPs as part of the production plan unless required. Without the required implementation of BMPs, the Panel concurs with EPA that under the current generic remediation approach too much time may pass before identifying resistant populations. The Panel recommends that remediation plans be in place for all licensed products taking into consideration geographical location of the field, production practice, population resistance, and species' behaviors.

The Panel supports the most conservative remediation plans as outlined by EPA including the recommendation of the use of conventional insecticides to control the adult stage during the present field season and then select an alternative pest control method the following season to reduce the establishment of resistant populations, but only as a last resort to control the spread of resistant populations and not as a population abundance management tool.

*Charge question 6b. Which mitigation measures would be more effective in containing and/or extirpating resistant CRW populations?*

### **Panel Summary**

To mitigate resistant populations, the Panel recommends the use of rotation with a non-host plant would be most desirable, but will not be effective in some geographic locations due to rotation resistant CRW populations that will survive in first year corn by a shift in oviposition by WCR or by extended diapause in the case of Northern Corn Rootworm (NCR). The Panel recommends the use of an alternative Bt toxin as a viable option for rotation resistant populations. The Panel has concerns with the recommendation to use a soil insecticide with the compromised Bt toxin as we believe this will allow resistant corn rootworm to survive by feeding on Bt expressing roots outside of the application band of the soil insecticide and exposing larval populations to both the compromised Bt toxin and the soil insecticide in the band. The Panel believes the use of a soil insecticide with a Bt hybrid should not be done. The Panel only recommends the use of an adult control measure in fields with greater than expected damage during the current season in fields with resistant populations. Dispersal to other fields, although limited, is still a concern as damage may not be noticed until harvest time.

To assist with earlier detection of greater than expected damage, the Panel recommends the use of aerial monitoring using planes or drones in high risk areas especially following major weather events that may contribute to lodged corn. The Panel recommends development of a risk map that identifies: 1) the varying levels of greater than expected damage reports; 2) crop production practices that contribute to resistance including the use of high dose seed treatments (may mask early resistance); and 3) a lack of refuge compliance.

*Charge question 6c. Please comment on the value of theoretical models in designing remedial action strategies for various resistance scenarios.*

### **Panel Summary**

CRW biology models are among the best developed that exist in agricultural entomology. One could reasonably argue that these models have done a good job projecting how varying management and initial biological parameters influence the emergence of resistance. All these models show strong sensitivity to initial resistance allele frequencies, and functional dose (survivorship of heterozygotes on Bt-corn). For some sets of parameters, models have also been predictive. When initial allele frequency is high, years-to-50% resistant allele frequency ranged from 5 to 8 years, which is a good approximation of the time when problem inquiry fields are being found.

We can combine what can be learned from the existing models with empirical information obtained during the years since commercialization to focus both future modeling and remediation strategies. First, the Panel believes that Bt resistant alleles are not rare. Resistance has been selected relatively rapidly in laboratory studies. An independent line of evidence suggesting that resistant alleles are not rare, presented by Dr. Nicholas Miller, a faculty member at the University of Nebraska-Lincoln, is based on the geographic distribution of problem-inquiry fields, and contrasting that to the pattern of rotation-resistant phenotypes. Therefore, the Panel must assume that presence of problems in the field is directly correlated to field-evolved resistance. This assumption may not always be correct, but it is a reasonable first approximation. The problem inquiry fields from Cry3Bb1 plantings show a pattern of sudden appearance in multiple locations with little to no spatial correlation. This is consistent with a process of independent selection of alleles that are not rare, and thus relatively easy to increase in frequency. In contrast, rotation resistance shows a pattern of being selected for in a discrete location, followed by diffusion from that location, and strong spatial dependence in the data (Onstad et al. 1999). Often the published models start with a frequency of 0.001, and contrast scenarios with that as at least an initial baseline. The Panel recommends focusing on scenarios that show higher initial frequencies of r alleles, potentially several orders of magnitude higher.

*Charge question 6d. The current deterministic and stochastic simulation models used for IRM purposes contain many of the following attributes: ecology, population biology, behavior, and genetics of pest, grower behavior (refuge compliance, insecticide spraying, etc), explicit spatial and probability analyses. What other modeling attributes would help improve the analysis of remediation strategies?*

## Panel Summary

Although we have not exhaustively reviewed every CRW simulation model, the Panel recommends the following attributes be included in simulation models:

1. Relatively high r-allele frequencies as initial parameters.
2. Non-random mating.
3. Multiple alleles – polygenic (additive) resistance.
4. Pyramided traits with varying  $h$  (dominance) for each trait. Onstad and Meinke (2010) suggest the presence of single-traits in the landscape reduce the benefit that can be achieved with pyramids.
5. Varying deployments of trait packages in time: deploying each trait sequentially, versus all-at-once in pyramided constructs.
6. Modeling the influence of BMPs on abundances and how that affects rates of resistance across relevant landscapes.

In addition to simulation models, the Panel recommends statistical models describing population genetics and abundance patterns measured in the Corn Belt. The Panel recommends that IRM models are designed to inform decisions about remedial strategies, and proactive IRM efforts. They should include the endpoints of abundance, in addition to the relative frequencies of genotypes, and couple that with efforts to measure abundances in the field. This enables direct monitoring of the variable being modeled, which helps with model validation. The Panel recommends widening the focus of both modeling, and IRM plans, to manage resistance with the use of BMPs.

The Panel summarizes conditions that make it unrealistic to rely upon refuges alone to achieve IRM for WRC given current Bt hybrids (adapted from Krupke, 2012):

1. Lack of a high dose: none of the traits provide a high dose. The survival rate reported in 2002 EPA SAP for MON863 is 17% to 62% survival on MON868.
2. Frequency of r allele: they are not rare
3. Resistance is probably polygenic. There are multiple r alleles
4. Random mating is not occurring.
5. Cross resistance may exist between Cry3Bb1 and mCry3A.
6. Ability to detect incipient locations of resistance and manage them to limit their spread is not working because:
  - a.  $LC_{50}$  not reached until 2nd or 3rd instar (2002 SAP)
  - b. Bioassay problems, discussed in previous charge questions
  - c. Zero foot traffic following planting.
  - d. Lack of spatial continuity in the occurrence of problem fields
  - e. Major disincentives for reporting damage
  - f. Measuring damage is difficult because it occurs underground
  - g. Method for measuring damage (NIS) is only poorly correlated to population abundance

- h. Rescue treatments do not exist
- i. Scouting is imprecise and limited

There is an implicit assumption that we can influence r-allele frequencies and geographic distributions through implementation of BMPs – this forms the base of utilizing BMPs following confirmation of resistance in problem-inquiries. Models should focus on advancing our understanding of this assumption, with current estimates of allele frequencies and assuming polygenic resistant alleles. Questions that should be considered include: 1) What is the relationship between abundances and resistance evolution?; 2) How do BMPs (along with other management practices such as refuges and seed treatments) affect resistance evolution through their influence on abundance? Examples show that this modeling approach is feasible. Addressing how BMPs influence both abundance and r-allele frequency will require inputs defined by landscapes, which vary among regions of the cornbelt. Movement among fields in relevant landscapes will need to be considered. For example, young, recently mated females are most likely to be moving among fields (based on captures from towers placed in fields, and sex-ratios during early colonization). Outputs will probably need to vary across landscapes (as opposed to being expressed as a mean expectation or distribution). This may require models to be developed in a GIS framework.



## DETAILED PANEL DELIBERATIONS AND RESPONSE TO CHARGE

### **CHARGE QUESTION 1**

#### *Annual Corn Rootworm Sampling to Assess Bt Susceptibility*

As a condition of registration, Bt corn registrants are required to conduct annual sampling of CRW populations from locations in the Corn Belt to monitor for changes in susceptibility to Bt toxins. These collections are obtained from random locations within three defined regions covering different western corn rootworm biotypes. The regions also represent areas with high Bt corn adoption and CRW pressure. Region 1 (rotation resistant variety) includes Indiana and eastern Illinois; Region 2 (wild type) consists of western Illinois, Iowa, and Missouri; Region 3 (organophosphate resistant variant) encompasses Nebraska and Kansas. Typically, 12-15 total populations are collected each year, mostly from Iowa, Illinois and Nebraska.

In light of reports documenting field resistance to Cry3Bb1 corn since 2009 (Gassmann et al. 2011, 2012a and 2012b), BPPD concluded that the random sampling approach used for annual monitoring across the three geographic regions of the Corn Belt was not proactive enough for early resistance detection. Instead, BPPD recommended an intensive and focused sampling approach, in which collections should be obtained from areas experiencing Bt crop failures or regions with high risk factors (i.e., causal factors of resistance including continuous use of corn-on-corn with the same Bt trait, non-rotation, or poor compliance with refuge requirements). A purely random and less intense sampling approach could be maintained in areas of the Corn Belt where little or no Bt corn failures have occurred and the selection pressure is lower (i.e. “fringe” areas). These areas could serve as a point of comparison between the higher and lower Bt selection environments, which would supplement the baseline data established in the early years of Bt commercialization.

- a. *The Panel is asked to comment on sampling approaches for conducting annual CRW resistance monitoring that support early resistance detection. Please discuss the strengths and limitations of BPPD’s proposal for a focused (risk based) sampling approach for the Corn Belt, supplemented with samples from lower risk “fringe” areas for comparison.*

The Panel is concerned that there are repeated reports of greater than expected damage in fields using Cry3Bb1 rootworm-protected transgenic corn. Perhaps most troubling to the Panel is that there is a delay in identifying and reporting the failures. This is evident since most of the materials provided to the Panel are discussing the number of crop failures through 2010 (BPPD 2011). While it is possible to determine additional/continued failures of Plant Incorporated Protectants (PIPs) with the Cry3Bb1 toxin from other resources, the documents provided to the Panel by the Agency do not provide an overview of the greater than expected damage for 2012 – 2013. If production practices that have proven to allow CRW to develop in-field resistance have continued to

be deployed, the number of greater than expected damage incidents are likely greater than currently documented. Furthermore, many fields with greater than expected damage may not have been identified/reported. Thus, in-field resistance may be much greater than currently presumed in both continuous corn (Gassmann et al. 2011) and in first year corn in central and east central Illinois (Gray 2013).

In regard to the strengths of the Agency's sampling proposal, the Panel concurs with the Agency's recommendation to expand the sampling area to include other areas with greater than expected damage including Wisconsin, South Dakota, Colorado and Minnesota (BPPD 2011, 2012). Furthermore, the Panel recommends a focused approach to track susceptibility of individual populations or discrete geographic locations as this approach will be more likely to identify hot spots and may reduce the likelihood of area-wide resistance (BPPD 2012). The Panel also concurs with EPA's primary objectives for sampling that include: 1) actively seeking out areas of concern; 2) selecting annual sampling locations based on risk factors; and 3) identifying resistance issues earlier through more proactive monitoring in high risk areas. The Panel recommends that these high risk areas entail 1) close proximities to reported field failures; 2) continuous corn production areas; 3) fields that do not rotate the Bt toxin mode of action; and 4) fields that are in non-compliance with refuge requirements. One panel member mentioned that sampling according to the above recommendation, although being focused, should still assure a certain spatial coverage (e.g. systematic grid of survey with focused sampling in each grid). The Panel does not concur with the public comments specifying that "fringe" areas are more appropriate for the random sampling approach as these areas may not be lower risk depending on production practices (Tooker Public Docket Id-ID: EPA-HQ-OPP-2013-0490-0038; Tooker and DiFonzo 2013).

The Panel recognizes that based on the timeframe of the reports of greater than expected damage, less than adequate samples numbers of CRW may be available due to production practices such as using soil insecticides in conjunction with a Bt trait and conventional insecticides targeting adult populations. The Panel recommends a proactive approach to monitoring for greater than expected damage earlier in the season by identifying high risk areas of continuous corn production or areas that lack rotation of Bt toxin modes of action based on sales records. Despite proactive monitoring, observable damage and potentially resistant populations may be masked by use of insecticide at time of planting in conjunction with the continued use of a Bt hybrid with a seed treatment.

The Panel recommends that high risk (corn-on-corn, continuous use of the same trait) areas within 4 or 5 zones be the only targets for annual sampling. This recommendation would eliminate random sampling. The "fringe" zones/regions should be considered as one of the above zones and sampled in areas of highest risk. Increasingly more "high risk" areas should be noted and examined. For example, in 2010, 27.7% of all corn planted in the U.S. followed corn in 2009 planted to the same acres; 11.3% of all corn acreage planted in 2010 followed corn planted in the same acreage for > 5 years previously (USDA-NASS, 2012). The Panel considered and recommends the concept of sampling some zones with "high risk" areas on a continued basis in the same location for

several years where unexpected damage is likely to recur year after year and thus reduce the logistical costs.

The Panel recommends including non-compliance with refuge requirements as a factor for high-risk classification since lack of refuge compliance may result in increased resistance in areas that are not currently considered high risk. The Panel cites the lack of potential acreage surveyed for refuge non-compliance as described by the Arent Fox report refuge survey data. The survey criteria for participation requires that each grower plant a minimum of 200 acres with at least 50 acres planted to Bt hybrids. The report indicates a total of 920 individuals from the cornbelt surveyed about refuge compliance. Based on the survey criteria the amount of acreage represented could be less than 200,000 of the approximately 80 million acres of corn planted in corn in the heartland (USDA, 2013). The Panel recommends increasing the amount of acreage surveyed regarding refuge compliance. The survey data provided to the Panel indicated:

77% growers surveyed adhere to refuge size requirements

62% growers surveyed adhere to refuge distance requirements

7% growers surveyed are not planting refuge acreage

The Panel recommends modifying the regional boundaries used in the Arent Fox surveys to better reflect corn rootworm population variations in species and resistance traits to better reflect field conditions. Gray et al. (2009) shows an expansive area beyond Illinois and Indiana that have populations of the western corn rootworm rotation resistant variant including eastern Iowa, south central Wisconsin, western Ohio and a few scattered fields in Michigan. This map and other resources should be used to readjust the regional boundaries for all surveys and sampling so they are consistent between one another and better represent corn production practices and CRW populations.

The Panel recommends that the revision of “regions” boundaries also be applied to the sampling regions in order to provide consistency and to allow better comparison of data obtained from surveys, sampling and greater than expected damage reports. For instance since the states within the Arent Fox regions surveyed do not match up with the high-risk area vs. “fringe areas” for sampling, it is difficult to determine true compliance for refuge requirement that can ultimately have an impact on the level of risk in a particular area. The current sampling approach is likely to misrepresent potential resistance since the sampling is random and populations are not tracked over time for shifts in susceptibility. Consequently, the associated delays in susceptibility studies could allow for dispersal of resistant CRW resulting in area-wide resistance prior to identification of a resistance concern.

- b. How many CRW populations should be collected from within a sampling region to adequately assess susceptibility?*

The Panel recognizes that when collecting samples from fields with greater than expected damage, the population samples must be adequate to generate enough larvae to allow for all assays (diet and on-plant assays) used to evaluate resistance. The Panel

believes the current approach of sampling 12-15 CRW populations mostly from Illinois Iowa and Nebraska seems very low especially considering of the number of reported cases of suspected resistance and/or greater than expected damage (BPPD 2011). The current approach of a minimum of 2,000 adults collected for each population (EPA 2013) is likely not enough. Considering the often limited viable offspring from these adult collections, the Panel recommends a minimum of 4,000 adults per population. This recommendation is based on the presumed resistance allele frequency for CRW. If the phenotypic frequency of resistance is one in 1,000 (0.001), then more than 3,000 individuals must be sampled to have a 95% probability of detecting one resistant individual (Roush & Miller 1986). However, the frequency of resistance alleles is unknown, but is expected to be higher than one in 1,000 since the Bt toxin for CRW is less than high dose and the length of product use.

The Agricultural Biotechnology Stewardship Technical Committee (ABSTC) indicates that it attempts to collect multiple samples for each region, but that some collections are not successful as insufficient offspring are produced for testing (EPA 2013). In light of the number of greater than expected damage reports between 2003 and 2010, it seems necessary to increase the number of populations sampled (BPPD 2011) and adopt a proactive monitoring program to detect greater than expected damage earlier in the growing season. The Panel recommends expanding the areas of annual sampling to include additional “high risk” locations in Wisconsin, South Dakota, Colorado and Minnesota.

In addition, the Panel recommends that each registrant collect annually 10-15 populations from the same locations in each of the 4 or 5 zones and bioassays be conducted on all four traits presently registered. Clearly this would require that registrants allow sharing of a standardized protein source and establish a common bioassay technique. Over time this would provide 50-75 assays of populations in each zone that would create a temporal and spatial data set that would provide insight into the evolution of resistance.

## **CHARGE QUESTION 2:**

### *Corn Rootworm Sampling in Response to Damaged Bt Fields*

When unexpected damage in Bt corn occurs, technology providers are obligated to investigate the cause of Bt failure. These investigations, often termed “performance inquiries,” involve a number of procedural steps to assess the causes and circumstances of the corn injury. If CRW are responsible and the level of injury exceeds a root damage trigger based on the Iowa State Nodal Injury Scale (NIS), sampling of local CRW populations is required to test for potential resistance. Currently, the NIS thresholds are 1.0 for single toxin products or 0.5 for pyramided toxins. If insufficient adults were collected during the initial investigation, sampling may have to occur the following season. For determining root damage, the Agency has recommended transect sampling through the injured field sections as opposed to randomly sampling plants from the entire field of concern. The Agency has reservations that random sampling could lead to false negatives by lowering the average root node injury score if actual Bt damage is localized in a portion of the field (due to clustered oviposition). In terms of adult CRW sampling,

The Agency has recommended that collections occur directly in the damaged Bt field(s), specifically in the vicinity of the damage. A concern has been raised, however, that such sampling may be biased relative to surrounding areas and not representative of the overall CRW population.

*a. The Panel is asked to comment on methods for investigating corn rootworm (CRW) populations causing unexpected damage to Bt corn. Specifically, please comment on:*

*i. The use of field damage ratings (Nodal Injury Scale (NIS)) as a screen for potentially resistant populations. What sampling triggers should be used for single toxin and pyramided Bt products? Should alternate techniques be considered?*

The Panel agrees that because registrants are being notified of and required to investigate damaged fields, node-injury scale (NIS) damage ratings will likely be high in the area of damage if CRW feeding is the cause. Therefore, ratings of 1.0 for single toxin products and 0.5 for pyramided products are reasonable. The objective of the Personal Inquiry (PI) from a grower is to establish or rule out the presence of resistance in the local CRW population so setting a relatively low trigger for further testing (diet bioassay/on-plant diagnostic assay) reduces the potential of resistant populations going unreported in fields with greater than expected damage. If mitigation of resistance is the objective of IRM for rootworms, determining that a population is less susceptible to Bt proteins when it is, in fact, not resistant (false positive), represents considerably less risk than not establishing that a population is resistant when it actually is (false negative).

Another potential argument is that population pressure and abundance of CRW in “high risk” areas are normally greater than that in other “fringe” areas of the corn belt due to geographic and edaphic factors other than population resistance so that NIS triggers of 1.5 and 1.0, respectively, would be more reasonable as greater damage is expected with heavy feeding pressure. In addition if plants from the entire field are included in the damage rating process, fewer sites would require the expense and delay of further testing. Both scenarios would likely fail to detect pockets of incipient or resistant CRW populations. A difficult question is, what is the criteria for determining “greater than normal” population activity? It is clear to this Panel that because frequency of damaged fields will likely increase over time in the regions of greatest concern, greater than usual damage events may become more commonplace. If resistance to Bt toxins is the true cause, a more stringent triggering event is increasingly valuable for detecting population resistance on a local and regional scale.

An alternate view by one panelist is that in cases where systematic transect sampling is carried out across the entire field, then NIS thresholds of 1.0 for single toxin products or 0.5 for pyramided toxins might be too high to allow detection of resistance because of the usually aggregated distribution of CRW in a field. Average-across-field NIS thresholds of 0.50 for single toxin products or 0.25 for pyramided toxins might be suggested as it is important to remember yield loss can occur with a root rating of 0.25. If transect sampling is carried out only in damage-hot-spots of a field, then NIS thresholds of 1.0 for single toxin products or 0.5 for pyramided toxins may be too low as rootworms are often spatially aggregated (Toepfer et al. 2007), and may occur in a small

area in such numbers that heavy root damage occurs regardless of control measures. There are many reasons to have hot spots of damage, such as a favorable egg laying micro-climate occurring the previous year. Then, high population and damage spots may not be a result of resistance. It might be even argued that hot-spot sampling would not lead to any quantitatively comparable data. Therefore, a hot-spot NIS threshold of 2 for single toxin products or 1 for pyramided toxins might be suggested. However, another panelist cautions that regardless of favorable egg laying micro-climate considerations, high populations and extensive root injury could be evidence of reduced susceptibility or early resistance and disagrees with the higher NIS rating in hot spots. Conversely, it could be argued that with yield loss at a root rating of 0.25 a NIS rating of 0.5 would be appropriate for both single and pyramid toxins as suggested by a public commenter (Center for Food Safety, Public Comment 2013). This suggestion is supported based on recent data showing a reduced efficacy of Cry3Bb1 with an average root rating of 1.25 in areas with reports of greater than expected damage during the period 2012 – 2013 (Nick Storer, Public Docket Id. no.-EPA-HQ-OPP-2013-0490-0020)

The Panel suggests an alternative approach of recording percent plants lodging in an entire field, or the number of hot spots with lodged plants per field with 2 or 3 such spots serving as a threshold to follow up on resistance investigations. The Panel suggests a more conservative approach to increase detection of early resistance in the field. By sampling plants outside of the damaged area to better represent the overall CRW population, the extent of damage is confused with the severity of damage. Because the objective of the PI visit is to mitigate the problem of resistance development, if necessary, the sampling procedure should be focused on the severity and cause of the damage. By diluting the sample outcome with surrounding area root damage ratings, the objective is blurred and the risk of making a false negative decision regarding potential resistance is increased considerably.

*ii. The use of transect sampling in damaged areas or random sampling throughout the affected field to assess root damage ratings.*

When a PI is undertaken, the objective is to determine the cause of the observed damage. This suggests that a sample within the damaged areas of field would be more valuable to informing the question than samples of the entire field. It would be more appropriate to sample from the damage area and within the field. A transect sample of the visibly damaged plants through the damaged area of the field will lead directly to the cause of damage and provide a reference to the severity (NIS rating) of the damage that has occurred. By randomly including damaged and undamaged plants in the sample, an estimate is obtained of the extent (percent of plants showing signs of damage) of the damage in that locality of the field, but dilutes the estimate of severity of attack. Both pieces of information would seem to be useful in understanding the nature of the damage reported and possibly the failure of the Bt product. The Panel recommends that a transect sample procedure through the damaged areas of the field should be required, which would also separate visibly damaged from undamaged plants and estimate the severity of damage within each of the separated groups. The combination of data from both groups would be an estimate of the extent of damage.

The Panel recommends that a detailed description of the damaged area(s) of the field be recorded and made available in an anonymous version to the Agency's IRM and other appropriate scientists. A suggestion from a public commenter was that a threshold of acceptable damage would be to accept damage of 25% of plants lodged in at least a 1 acre area of a field (Nick Storer; Public Docket Id no. -EPA-HQ-OPP-2013-0490-0020). The Panel feels that this would be a good definition of acceptable damage in areas where cornfields are large and the geography is not diverse, but would not be appropriate for areas where fields are smaller and diversity of terrain and vegetation are present (*eg.* in "fringe" areas). Information included in fields with unexpected damage should include the: GPS location, area of the field and of damaged area(s), history of cropping (crop species and insecticides used) and traits used in the field, refuge compliance and disposition (structured or seed blend), previous PI's for the field, and past frequency of weather events that might influence lodging.

As an example of sampling a hot spot, the Panel describes a hypothetical scenario in which a PI is requested and the company representative determines that the area in question measures approximately 20 x 50m (65 x 165 ft. = 2 acres). The sampling of this area would entail taking a transect through the damaged area looking for damaged plants and selecting 12-15 visibly damaged plants in the transect from one corner to the other of the area and leaving at least 2 m between sampled plants. A transect could take the path of a V or W or be a diagonal line across the damaged area depending on the shape of the area in question. The Panel supports the idea of sequential sampling for plants with damage in the "hot spot" as suggested by a public commenter. The Panel feels that this technique should be investigated further and made available to all registrants to improve their sampling efforts by shortening the decision making time when appropriate. During sampling along a transect, all plants are recorded and rated as damaged or not. If damage exceeds a threshold after 4 or 5 samples, sampling is discontinued and the damaged area is considered to be the result of resistant beetles. Guidelines for continued sampling would be established. Each of the damaged plants is tested for the presence of Bt; those that are not positive for Bt (refuge isolate plants) are discarded from further assessment. The remaining damaged plants are rated for severity of damage and the percent that exceed the established NIS rating (for example 1.0 for single events and 0.5 for pyramided events). If the average rating exceeds the threshold rating and greater than 50% of the plants exceed the rating, further testing for resistant populations should be initiated (sampling beetles for bioassays).

In addition, the proportion of damaged plants in the entire transect sample could be compared with a similar transect in a surrounding area of the field to determine the extent of damage in the area or region, much like the sampling procedures discussed in Question 1. This reasoning would remove the conundrum of which type of sampling to use for determining the need for a subsequent collection of beetles and bioassay. Perhaps a combination of the two sets of transect samples would prove useful for both objectives covered by Questions 1 & 2.

In contrast to the above, if a quantitative damage assessment of reaching a root damage threshold is needed, then a transect sampling over the entire field might be the only

way. Conducting a transect sampling only in damage-hot-spots of a field, would not lead to any quantitatively comparable data as there are many reasons to have hot spots of damage, such as favorable egg laying micro-climate the previous year (Toepfer et al. 2007; Spencer et al. 2009). In case the hot-spot sampling method is chosen, then the average NIS-based damage may not be the key measure. Alternatively, any hot spot of more than 20 m diameter (plant lodging, or root damage of > 1 NIS on more than 50% of plants) should be recorded, and if two or more of such spots occur in a given field, adult sampling is required for subsequent tests. However, one panelist prefers a more conservative approach that would require adult sampling if the NIS level is reached with the sampling criteria established by EPA regardless of the number of hot spots in the field. This panelist recommends that the sampling criteria and NIS should be consistent between registrants and recognizes that single and pyramid traits may have a different NIS. Although based on data suggesting reduced efficacy with Cry3Bb1 the same NIS for both single and pyramid traits in areas of greater than reported damage may be more appropriate than the varied scale.

*iii. Appropriate sampling locations (i.e., in the vicinity of the damage and/or surrounding areas) for collections of adults if field damage triggers are exceeded.*

The Panel feels that it would be most informative if samples of adult beetles for diet bioassays/plant diagnostic assays are obtained from the damaged areas (here damaged and adjacent fields) investigated as a result of a PI. Because of the limited medium and long distance dispersal nature of these insects, a higher proportion of those beetles emerging from the damaged areas will be included in the sample than of those beetles emerging from surrounding areas. If possible, samples of beetles should be obtained from an area no more than 1000 ft. (300m) from the damage and within the same field. Assays using the neonate offspring obtained from rearing these beetles will be the most indicative of potential resistance when data are compared with sources of beetles well away from damaged areas or from standard susceptible laboratory colonies.

It has been suggested that these beetles and their offspring have more fitness than the average beetle in the corn belt because they are surviving in high risk areas where corn-on-corn, non-rotation, single trait Bt, and/or minimal or no refuge fields are common. Fitness (or decreased susceptibility) relative to Bt protein in high-risk areas is the exact issue being confronted. The objective of a resistance management strategy is to demonstrate that resistance to Cry proteins has not occurred and if that cannot be determined then the population is resistant. A critical issue in collection of adult beetles is the timing of collections to obtain the maximum numbers of mated, gravid females that will produce the most number of viable eggs and neonates that best represent the cause of the previously recorded damage where resistance or tolerance is suspected (i.e. after the pre-oviposition period of 12 to 23 days (Spencer et al. 2009) and before the population has considerably dispersed and mixed (Spencer et al. 2007; Carrasco et al. 2010).

The Panel acknowledges that due to delays in reporting damage, the logistics of examining increasing numbers of PIs, and dispersal of CRW, sampling adult populations



in the damaged area the during same season can be difficult. The sooner beetle samples can be taken after assessment of the damaged area, the more likely individuals from that damaged area will be collected. Despite concerns that beetles can migrate out of as well as into the damaged areas, beetles collected close to the damaged areas are most likely to represent the local population that survived on plants in that area. However, if adult sampling can be achieved in the same year to allow timely rearing of offspring for testing, then the entire cornfield could be used in order to obtain sufficient sample numbers. Sampling beetles from the entire field or from areas more than 2-3 km (1-2 miles) away simply (Carrasco et al. 2010) reduces the chance of fairly representing the response in Bt protein assays of those beetles that caused the damage. This is because WCR adult populations perform considerable inter-field movements during their lifespan (Spencer et al. 2009), and nearly equally distribute (mix) across maize fields in an area and lay eggs accordingly.

Wherever adult sampling must be carried out in the following year, samples should be taken from the same field (corn-on-corn) or a neighboring cornfield if sufficient sample numbers are not available from the damaged field. Samples should be taken early in the emergence of adults to maximize the collection of F1 beetles that originated in the previously damaged field. Use of sentinel plots of corn or squash to collect adults that may represent those that caused damage the previous year is problematic, due to supporting the spread of resistance, but would be useful under certain conditions as discussed below.

Sampling can be conducted anywhere in the target field, and not only in damage-hot-spots, because the population will have moved and dispersed considerably by the time damage is assessed (Spencer et al. 2009). Moreover, hot-spots of adults, e.g. in search for food and/or oviposition, later in the adult season may not any more relate to hot-spots of adult emergence due to potential Bt resistance earlier in the adult season (Toepfer et al. 2007).

*iv. The deployment of sentinel plots in the vicinity of damaged fields in subsequent seasons to: 1) assess the resistance allele frequency in the area, and/or 2) collect insects if no adults were present at time of the field investigation.*

The Panel feels that determining the allele frequency for resistance in a population of CRW cannot be achieved at this time and is not easily determined until a more robust set of data is established. These data must be based on a stable bioassay method that includes a standard control population so that considerable data points for each trait can be compared in time and space. These criteria cannot be met at this time.

Because most PI follow-ups are carried out late in the season, in many instances, beetle numbers in the damaged areas or in the field in general may be low or nonexistent and the viability of those beetles collected may be low. Even if a late maturing field is nearby, it should not be used to collect beetles because these late fields act as attractants to beetles throughout the region and would not likely represent the beetles from a neighboring field, let alone those that caused the

damage. It would be imperative that a follow-up sample of beetles be initiated the following year in the same location as that designated as the area of damage. A sentinel plot of non-Bt corn of the same maturity as the rest of the field could be planted much like a varietal demonstration plot. These plots could be sampled for beetles at beginning-to-peak emergence to capture as many beetles as possible emerging from that specific area.

The Panel gives thought to the idea that by planting a sentinel crop the following season, the process would increase the spread of resistant alleles in CRW populations. In areas where local mitigation is prescribed following a year of damage and procedures are completely followed, this may be true. However, at this time, most fields with unexpected damage are being reported from locations where corn-on-corn plantings are common and not likely to diminish significantly. Sentinel plots planted in the same field the season following damage and confirmed to be resistance-based are valuable resources of beetle populations emerging from that localized area.

However, it should be considered that sentinel plots planted with non-Bt corn and without soil insecticide may allow resistant populations to build up in the area while assays are being conducted on beetles collected from fields with greater than expected damage in the area. Conversely, the sentinel plots may provide a population of susceptible beetles that could mate with potentially resistant beetles emerging from Bt corn nearby. However, if female western corn rootworms mate more than once, her eggs are suggested to be mainly fertilized by the sperm of the second or later males (Branson et al. 1977). Since CRW development is delayed when feeding on Bt corn, the second mating has a greater likelihood of a male that developed on Bt corn.

### **CHARGE QUESTION 3:**

#### *Diagnostic Assays*

Insect resistance monitoring for Bt corn toxins has historically been conducted with artificial diet bioassays using dose-response curves to obtain LC<sub>50</sub> or EC<sub>50</sub> measures for laboratory colonies and field populations. BPPD has expressed concerns that diet assays, as presently designed, are inadequate for effectively and proactively detecting resistant populations. Responses to toxin-incorporated diets are often highly variable and a functional “diagnostic” concentration (capable of detecting resistant individuals or populations) has not been developed for any of the registered toxins.

Because of the inherent uncertainties (high variability, less sensitivity) with the present diet bioassay methodology, the Agency has recommended the use of diagnostic on-plant assays as the primary resistance detection tool for corn rootworm. These techniques assess CRW susceptibility directly on Bt corn plants and can measure lethal and sublethal variables. Two such approaches have been developed by Gassmann et al. (2011) and Nowatzki et al. (2008).

- a. *Please comment on the strengths and limitations of diet bioassay methodologies for early resistance detection with CRW, considering that the currently-registered toxins are less than high dose. What improvements could be made to these bioassays to make them more effective and proactive resistance detection tools?*

#### Diet bioassay strengths

The Panel points out that the approach of using bioassays with insect artificial diet and Bt toxins have been conducted for over 40 years (Dulmage et al. 1971). The scientific community is well aware of this approach's basic methodology. Thus, finding ample information in the scientific literature to assess its limitations is possible. Properly conducted bioassays, are those that include an appropriate set of dilutions, good quality insect artificial diet, kept under biologically relevant environmental conditions, and conducted with suitable and uniform insect developmental stages and densities. These bioassays can provide more precise and consistent responses than current on-plant assays to screen for resistance to Bt. Roush and Miller (1986) concluded that diet bioassays are appropriate if resistance allele frequencies are high (0.01), but are not an effective method for detecting early resistance in a population. However, with either an on-plant assay or a diet bioassay the insect population needed to detect a low resistance allele frequency could be substantial.

Results of artificial diet bioassays are routinely examined with established standard statistical procedures recognized by the scientific community. The parameters used in the evaluations of these bioassays can include those that are currently performed with on-plant bioassays (larval weight, head capsule size, etc.), but the opposite may not be true because on-plant bioassays only challenge insects on a putatively constant protein concentration. In terms of logistics, the Panel believes that bioassays conducted with artificial diet can occupy less space and be more economical than the current on-plant screening assay. This provides an advantage for most of the research facilities.

Preliminary lethal ( $LC_{50}$ ) and effective concentrations ( $EC_{50}$ ) values have been established for some of the Bt proteins aiming to control CRW spp., with genetically-engineered corn hybrids (Siegfried et al 2005; BPPD Review, 2013). Although the Panel believes that these methods require refinement, particularly the inclusion of a high concentration to kill/arrest development of all homozygous and heterozygous resistant CRW, it also recognizes that these values already serve as historical records to document trends of susceptibility over time. Trends in susceptibility at a single location may be more precisely followed over time with artificial diet bioassays than with on-plant bioassays because of the more precise nature of the assay and the breadth of data they provide. A single well-maintained stock of purified Bt toxin can last a long time and perform more uniformly than plants where their Bt protein expression can be influenced by environmental, biotic and corn hybrid genetic background.

An example of using current limited data from diet bioassay:

From reports submitted to EPA for the years 2007-2010, inclusive concerning results of bioassays performed on CRW populations collected from locations of unexpected damage following PI's, the following table is presented. The data are for EC<sub>50</sub> values calculated for diet assays conducted using neonate larvae over a four day period.

**Monsanto (Cry 3Bb1) Diet Bioassays 2007-2010 – EC<sub>50</sub> Comparisons**

	2007	2008	2009	2010
# Signif. EC <sub>50</sub> > Lab Strain	3/5	1/8	8/13	5/10
<u>Repeated Bioassay Sites where EC<sub>50</sub> &gt; Lab Strain</u>				
Sherman Co. NE	*26.17	-	*41.33	*83.48
McLean Co. IL	12.68	-	*37.75	48.13
Champaign, IL	-	7.73	*39.88	-
Hamilton Co. IA	-	-	13.90	*119.60
Howard Co. NE	-	-	*40.61	*73.52
Story Co. IA	-	-	*28.88	47.34

\*significant EC<sub>50</sub> (>95% confidence interval) from lab value for that year.

The top line indicates the number of populations which had significant EC<sub>50</sub> (95% confidence interval) values of the total successful bioassays performed that year. The following data report the EC<sub>50</sub> values reported for populations sampled from the same location (County) which were repeated for two or more years. Values with an asterisk indicate significant 95% confidence intervals compared with the lab standard.

Except for 2008, more than half of the populations sampled each year demonstrate a significant difference in growth (final weight) in the bioassays. When bioassays are conducted in the same location for successive years, the trend in EC<sub>50</sub> values increase in all cases. The data presented here, although limited and with certain uncertainties about the diet bioassay technique, are a good example of how diet assays can provide information on the temporal and spatial evolution of resistance in CRW populations collected from “high risk” areas.

The Panel believes that if the registrants’ on-going efforts to produce Bt proteins in high enough concentrations are achieved, many of the current methodological hurdles stated above could be solved. This high-enough protein concentration may serve to establish a diagnostic concentration<sup>1</sup> that could transform the monitoring effort into a faster, reliable and ampler method. One panelist stressed that based on the alarming number of greater than expected damage in the field reports, time is of the essence to identify a diagnostic concentration for the Bt toxins in order for the diet bioassays to have value in identifying resistance. In addition, the development of a diagnostic concentration or tool may not be practical due to selection pressure and commercialization of the Bt toxins.

The Panel agrees with the considerations in other charge questions that consistency in methodology (e.g.: same type of diet, reference laboratory colony, batch of Bt toxin, proper handling of neonates, etc.) is important. This is a valuable consideration that may help explain trends of susceptibility over time. There is also another advantage of this

<sup>1</sup> A diagnostic concentration is a dose capable of killing or arrest development of most (≥99%) of the homozygous and heterozygous resistant insects.

method because many assays can be performed in a small space and entirely within a growth room that provides stable and consistent environmental conditions; a single replicate of 5 or 6 concentrations of 3 different Bt proteins could be performed on a single 96-well plate and repeated sufficiently for a substantial assay. The Panel recommends developing a standard diet where neonates can survive on assays beyond 3-4 days with greater than 80% survival. Composition of current commercial diets is a trade secret and variations of its components may not be proclaimed by the manufacturer. Such minuscule changes can greatly affect the development of insects (Blanco et al. 2009a), and results of bioassays containing Bt proteins (Blanco et al. 2009b). The Panel recommends keeping the bioassays with insect artificial diet to continue building some of the already observed trends, and share the results among all appropriate researchers, both public and private. However, the current criteria used to determine resistance using diet bioassay data is unlikely to identify early resistance that is developing in the field. To support this statement, field collected populations have shown  $LC_{50}$ s exceeding upper limit of 95% confidence, but not been deemed resistant by registrants. In addition, LC measures are highly variable (US EPA, 2013 (Table 2)). The EC data are less variable, but unlikely to detect early resistance due to the lack of sensitivity. In some cases, responses have varied by several orders of magnitude and much is attributed to natural variation and/or laboratory methodology. Meihls (2010) expressed concern about the sensitivity of the LC/EC diet bioassays indicating that resistant colonies cannot always be distinguished from control colonies.

In regard to the limitations of the diet assays, these artificial diet bioassay protocols and toxin sources, ideally, should remain consistent to allow for appropriate interpretation of results with previous assays. The Panel recognizes that science moves forward and creates opportunities for improvements and changes. When these modifications take place, a comparator study should be performed to document the influence that methodological changes have had on the response of insect susceptibility to Bt. A thorough documentation of each step in the protocol and materials utilized (manufacturers, batch numbers, storage conditions, etc.) in the bioassays, are necessary to understand these potential changes. The registrants and other scientists are familiar enough with research conducted under good laboratory practices (GLP) to know the basic information that should be included in the protocols (US EPA.). The Panel is not recommending establishing a GLP for monitoring work. However the Panel is recommending that GLP is considered in order to ensure that these guidelines have thoroughly prepared protocols and documentation. This is very important because the documents provided to the Panel do not contain sufficient information to judge critical variations between methods when changes have occurred. As described above, changes are inevitable; as such, the search for a more concentrated source of protein, and well-documented comparator studies should be requested from registrants to thoroughly understand the impact of changes. The preliminary establishment of Bt susceptibility by Siegfried et al. (2005) and subsequent monitoring effort (US EPA, 2010) performed by a different laboratory, may include substantial methodological changes. Even the most constant methodology performed simultaneously in different laboratories, can yield results with high variation (Blanco et al. 2005). The Panel recommends that these types of comparisons take into consideration the intrinsic and inevitable variation.

In the documents provided by the Agency to the Panel, as well as within the public comments, the concept of “*effective/determination of allele frequency*” has been mentioned. An assessment of the effective CRW diversity/number of alleles in field samples/tested populations, although not a perfect method at the moment, can still be measured after several years of commercialization of the Bt corn hybrids. One approximation, and the simplest, is counting the number of the gravid females in a field sample, but preferably the number of those that produced subsequent generations in the laboratory. If the field sample size recommended is at least 2,000 corn rootworm adults, an assessment of some (~50) of the ~1,000 field-collected females capable of producing progeny can give an accurate indication of the total alleles tested in a sample. If some of their progeny are Bt-tolerant, a partial and imperfect allele frequency can still be determined. The reproductive biology of WCR allows for accurate paternity determinations, because the sex ratio in the genus is ~1:1, and a great proportion of females mate only once (Spencer et al. 2009). Therefore, by dissecting a sub sample of females and counting the number of spermatophores (which is not a difficult task) an indication of the total number of alleles in the sample can be estimated. Another but more elaborate and expensive method is to establish the monitoring by screening single gravid females or isofamilies. The F<sub>1</sub> progeny of a female, putatively the alleles of only two individuals, are challenged on artificial diet bioassays or on-plant assays. Each female produces sufficient larvae (Spencer et al. 2009) to perform this type of work. The response (susceptible or resistant) of the F<sub>1</sub> provides the necessary data. The basis for this work can be found on Andow and Alstad (1998), a methodological modification (Gould et al. 1997), and its applicability (Blanco et al. 2008), and cost (Blanco et al. 2009c), have been already demonstrated for Lepidoptera.

Another issue with the bioassays and the statistical methods employed is that it is commonly assumed that a higher proportion of resistant alleles in a bioassay do not necessarily produce a higher LC<sub>50</sub>/EC<sub>50</sub> response. In most of the cases it is true, but there is also a possibility that a proportion of resistant larvae in a bioassay can produce variable results. For example, the ~25% of resistant WCR larvae detected in some of the monitoring reports provided to the Panel, can produce different outcomes. If these larvae happen to be tested in low enough concentrations allowing for ~25% survivorship, LC<sub>50</sub> values can vary up to 18% using the same data of tolerant larvae scored in different sub lethal concentrations. With this example it is obvious that a bioassay should: include: 1) at least one concentration capable of producing 100% mortality/growth inhibition, a true diagnostic concentration; 2) a minimum of four replications to reduce variability; and 3) a discrepancy of ~18% in the response in the current method which should not be attributed necessarily to a higher resistant allele frequency.

The Panel recognizes the seminal efforts of Dr. Blair Siegfried on the development of the methodology with insect artificial diet (Siegfried et al. 2005), as well as the on-going plans by the registrants to improve it. Understanding that Bt proteins are expensive and laborious to produce, the Panel suggests that the feeding behavior of the WCR larvae on the top of the overlaid toxin bioassay, and an assessment of the toxin ingested, may be necessary to understand some of the variation in the results. To partially answer this

question, a comparative study between diet incorporated and overlaid techniques will add to our understanding on why the latter method has had many shortcomings. It is important to note that both techniques may yield LC<sub>50</sub>/EC<sub>50</sub> results entirely different (Siegfried et al. 2007), but the intrinsic variation within a technique is what needs to be understood. Proteins in this technique are applied to diet surface of wells on test plates that have been filled with diet to avoid denaturation of protein in diet; only the surface of the cell has the protein applied, so consumption of protein may only be at initial feeding of neonate, while subsequent feeding may be on diet below the surface, free of protein. Thus, calculation of the protein in the diet ( $\mu\text{g}/\text{cm}^2$  of diet) may be inconsistent or incorrect and exposure via consumption is also variable and inconsistent. The use of dyes in diet to look for consumption by larvae, or the use of a diagnostic tool to detect Bt protein in the larval gut, may serve as aide. The Panel recognizes that there is no “standard” methodology to conduct this work at the moment. The report of Siegfried et al. (2005) provides more information for others to reproduce these findings. Mortality of larvae on diet wells can become excessive or bacterial contamination may appear beyond 4 days at 24<sup>0</sup>C, so most tests are terminated at that time. There might be some inconsistency of results (3 d in the current monitoring work versus  $\leq 6$  days in Siegfried et al 2005), because larvae have not all fed to the same extent or that some have died at initial contact with a high concentration of protein at the surface of the diet which does not correctly represent the concentration intended.

The Panel also recommends evaluating a representative number of bioassays daily for a time lapse of 3 to 7 days. Studies have shown neonate larvae can survive 3-8 days without feeding (Branson 1989) and Oloumi-Sadeghi and Levine (1989). It is imperative to find out if a different evaluation time, (greater than 3 days-after-initiation) may yield better results. The Panel recommends that the laboratory currently conducting these bioassays adjust to some of the evaluation parameters found in Siegfried et al. (2005) and provide comparisons.

The Panel recommends keeping the monitoring efforts utilizing bioassays with insect artificial diet as another tool to detect shifts in susceptibility. This evaluation and the on-plant efforts can complement each other under the current testing limitations. The Panel recognizes that access to the currently tested proteins to perform bioassays by independent researchers has been restricted. It has been a serious barrier for independent laboratories to contribute to the improvement of these techniques if they do not have access to these toxins. The Panel recommends that the toxins used in the diet bioassays be made available to academic researchers to allow comparative data from independent sources.

Perhaps the greatest limitations of the diet bioassay are the following. Firstly, it may not accurately reflect the larva’s ability to survive on the Bt corn in the field. Secondly; Roush and Miller (1986) concluded diet bioassays are appropriate if resistance allele frequencies are high, but are not an effective method for detecting early resistance in a population. Thirdly, Bt toxin for corn rootworm is a non-high dose. Lastly, the use of the diet bioassays to identify resistance is unlikely as a functional diagnostic

concentration that is capable of detecting resistant populations or individuals has not been developed.

- b. The Panel is asked to discuss the relative merits and limitations of the two on-plant assays Gassmann et al. 2011 and Nowatzki et al. 2008). Please discuss the extent to which these assays have different sensitivities to make early corn rootworm resistance determinations? Should other on-plant assay approaches be considered?*

In regard to the strengths of the on-plant (Gassmann et al. 2011), the Panel recognizes that the Gassmann et al. (2011) approach is more realistic of field conditions than the artificial diet bioassays. Although the published paper on this method has some procedural omissions, once it is understood in its entirety it may be an easy method to follow and to confirm results independently from those provided by the registrants. The adoption of this method may expand the monitoring area by the inclusion of independent scientists.

It is the understanding of the Panel that this method has been performed with different corn events, demonstrating some of its value as it may be useful in comparing durability of the registered toxins both as single toxins traits and pyramid traits. To date, this is the only assay that has confirmed resistance in the field based on its evaluation criteria. In addition, it most closely replicates field conditions.

Concerning the limitations of the Gassmann on-plant assay, the Panel believes that some of these limitations also apply to the on-plant and sub-lethal seedling assays described in Nowatzki et al. (2008). A paper published by Clark et al. (2006) suggests that WCR survival on Bt corn is a result of larval feeding behavior and describes that this insect may be able to discern subtle differences in toxin levels in root tissue, thus allowing them to feed selectively on low expressing root tissues. Furthermore, Spencer et al. (2009) also found that “(WCR) larvae on the resistant maize moved continuously, sampling root hairs or root tissue but not actively feeding. These behaviors were dramatically different from those feeding on isoline maize.” These two pieces of evidence make it imperative to address the following points in both of the current on-plant protocols: 1) conduct on-plant bioassays for a range of 3 to 17 days after egg hatching to find out if there is a better (shorter) time that can yield more accurate results with this method and 2) perform an intense/destructive representative sample of the total number of surviving larvae in the root system and soil during those sample dates, and not rely entirely on the larvae collected in alcohol vials after the funnel extraction. The Panel believes that both methods, destructive and extraction funnels can be compared simultaneously. If the behavior of the larvae is to avoid higher Bt concentrations in roots, these discerning insects may have a delayed development resulting in smaller larvae. These discerning larvae may or may not be Bt resistant, but due to its possible smaller size after 17 days of not actively feeding, they can be categorized as susceptible because of their smaller size. Some of the parameters to evaluate “resistance” in the current methods (artificial diet and on-plant assays) involve the proportion of larvae that have developed into different instars. The Panel suggests looking carefully at the results presented by DuPont/Pioneer in the written and public comments of (DuPont Pioneer, Public Docket Id. no.-EPA-HQ-



OPP-2013-0490-0022 to use them as an indication that shorter exposure time of larvae to roots may yield more accurate results. In addition, the Panel recommends investigating the use of the agar-based growing medium (Clark et al. 2006) that would allow larval root feeding to be observed to better understand behavioral variations and consequences.

The Panel has reviewed the publicly-available information and finds that Bt protein expression in corn changes with time (USDA-APHIS 2001, & 2003; BPPD, 2011), and Bt expression in cotton plants behaves in the same manner, having a ~5x concentration difference when these plants are grown in different environments (Greenplate 1999). Plant growth conditions in greenhouses and growth chambers may also vary from location to location and throughout the year, and from year to year. It is crucial that research be initiated and published about the quantification of Bt proteins over time in different parts of the corn plant, especially in the root system. Such evaluations would be more valuable if they are performed using the transformation events already incorporated into commercial varieties. This may help to elucidate another source of great variation in hybrid performance and insect survivorship. The limited data available of these Bt concentrations in the root system of corn plants come from the transformation event into a variety that generally, allows for effective breeding, not necessarily from a commercially-available variety. Because this transformed event will give rise to other varieties by crossing it with elite lines adapted to specific geographic conditions, this may increase the possibilities that the expression of Bt may be dependent on genotype and environmental interactions. Furthermore, some of these elite commercial varieties may already have integrated some WCR antibiosis traits (Levine and Oloumi-Sadeghi 1991), while others may not, making comparisons even more important. There is very limited data on the expression of these toxins throughout the development of the plants indicating a decline of Bt expression in roots up to ~30% in a period of ~22 days (USDA-APHIS, 2001 & 2003; BPPD, 2011). This lapse of time was similar to the one employed in the Gassmann et al. (2011) and Nowatzki et al. (2008) studies. This may present a double challenge for these particular on-plant bioassays, a sub-optimal Bt expression to effectively control WCRs and its diminishing concentration during the V2-V6 plant developmental stage, during the most actively-feeding period by the pest. These challenges would also be experienced in the field. The main problem with the Bt corn rootworm technology is that it is non-high dose. As a result of the sub-lethal level of toxin, the WCR are developing methods to survive exposure, thus allowing populations to build and exert enough feeding pressure on the corn roots to result in greater than expected field damage.

The Panel also identified the following other areas of improvement of this methodology including:

- 1) The confirmation of the transformation event in the Bt hybrid and the expression of the protein in the roots is necessary to assure that the tested corn plants are indeed the correct hybrids. “Off type” seed is a possibility in any seed batch and the quantification of this proportion is another critical issue on which the Panel recommends obtaining detailed information.

2) This methodology would greatly improve if the corn seeds obtained are free from pesticide applications. The Panel is aware of the efforts made by Gassmann et al. (2011) removing the pesticide from the seeds as they come in a commercial seed bag, but no quantification of the potentially toxic residue was performed after the removal procedure.

3) High larval mortality in the soil makes analysis more difficult and problematic, impeding potential Bt resistance to be documented correctly. Loss of larvae that may have escaped from pots that are not sealed properly can cause greater variation in survivorship and results. The Panel is not aware if this was considered/observed in the Gassmann et al. (2011) and for the greenhouse plant efficacy (Nowatzki et al. 2008) research.

4) An evaluation of the whole soil/root system at various points in time is necessary to understand this potential pitfall.

5) The use of “between 10-20 neonates” per plant, may present a serious constraint in the methodology. The influence that doubling the amount of WCR on root growth and protein expression may have is not known. Is it “logical” to assume that twice the amount of larvae in a bioassay may double the quantity of roots consumed, therefore causing a lower developmental time due to lack of food? Also, what is the influence of twice the pest population on air exchange, larval competition, etc.?

These are only a few questions that the Panel has identified due to the inconsistent insect pressure of this method. Furthermore, the use of larval extraction with Berlese funnels, employed in both of the on-plant bioassays, leaves some uncertainty. It is not known which treatment had 10 or 20 neonates, or an amount in between, in order to calculate survivorship. Again, a thorough inspection of the soil/roots at the end of experiment, to see if any larvae remain, could help to adjust the results. Also, how can “natural” mortality be assessed with this method?

Gassmann et al. (2011) and Nowastzki et al. (2008) mention that: “...surviving larvae were collected in vials with alcohol.” Assuming that a particular population has an unusual high survivorship, the Panel recommends collecting live samples/maintaining part of the sample in artificial diet to continue with the studies and to confirm resistance by other methods, if that particular population demonstrates high tolerance to corn hybrids. The surviving colonies would represent a great opportunity to learn more about the biology, behavior, and genetics of these interesting insects.

Curzi et al. (2012) demonstrated WCR had developed an ability to detoxify plant compounds to allow soybean herbivory by altering gene expression resulting in high digestive enzyme activity. The Panel recommends investigations to see if similar responses to Bt corn could be developed to identify resistant populations. The Panel suggests that registrants provide detailed information regarding additional testing if suspected Bt resistance is identified.

Space and logistics is another consideration that the Panel believes may be of concern, especially if the Gassmann method is compared with the sub-lethal seedling assay proposed by Nowatzki et al. (2008). Greenhouse space to grow seedlings in pots, shelf space in growth chamber/rooms to hold the pots, space for

extraction funnels, and maintaining consistent growing conditions, may be difficult to obtain and/or duplicate with other researchers following this methodology in other locations, year after year. Maintaining consistency of the tested varieties with this method may be also crucial to build a historical trend of susceptibility shifts.

Regarding the strengths of the on-plant assay, the Panel recognizes the great current improvements to the methodology of Nowatzki et al. (2008) that were submitted as public comments.

The Panel agrees that the Nowatzki et al. (2008) sub-lethal seedling method may be easier to standardize because the seedlings are grown in containers under dark and more consistent conditions in a growth chamber. Growth of these seedlings may produce an expression of protein more consistent between plants, although not necessarily similar to field conditions, and because the assay is conducted in closed plastic boxes, it makes for a reduced opportunity for larvae to escape, while maintaining better environmental conditions for the insects. The methods described in Nowatzki et al. (2008) included the verification of the correct transformation event or its isoline, and the Bt expression in the root was verified. The Panel recommends testing Gassmann and Nowatzki methods with pyramided events, as well as with single-protein events separately. A great difference with Nowatzki's method is the crossing of the WCRs with a non-diapausing colony, which enables the monitoring testing over a much shorter period of time. Also, as a proof of concept, the inclusion of different proportions of susceptible and "resistant" WCR larvae to challenge the assays, is something that the Panel recommends continuing with this and Gassmann's protocols.

The Panel recognizes that some of the concerns regarding the possibility of erroneously classifying larvae as potentially resistant due to "insensitivity" of the assay, have been corrected either by adjusting the number of eggs or by reducing the amount of time of the assay (DuPont Pioneer, Public docket Id. no.-ID: EPA-HQ-OPP-2013-0490-0022). Some of the criticisms to the Nowatzki et al. (2008) method expressed by the Panel were answered during the public comment period and in the documentation provided by DuPont Pioneer (DuPont Pioneer, Public docket Id. no.-EPA-HQ-OPP-2013-0490-0022).

In reference to the On-plant assay limitations, screening by this method involved two different assays with live plants: a "greenhouse plant efficacy" assay, and a "sub-lethal seedling" assay (Nowatzki et al. 2008). Below the Panel elucidates on points that were not previously addressed .

The greenhouse plant efficacy assay closely resembles the Gassmann methodology. The Panel believes that some of the uncertainties expressed above (escaping larvae, insects not collected with extraction funnels, killing potentially-resistant larvae in alcohol vials, etc.), are also applicable here.

The Panel suggests that research efforts continue to reduce the amount of variability in the assays and expand testing to include corn events containing Cry3Bb1 and Cry3A1

proteins. In addition, efforts should continue to develop a WCR colony that is considered Bt resistant based on the criteria currently used when field populations from greater than expected damage areas are tested or preferably redefine resistance to better reflect field populations that are feeding on Bt roots, inflicting extensive damage using NIS ratings and surviving. An example of lack of consistency in the current criteria for resistance is the York resistant population that had a mean instar and body area measures significantly lower on Bt than non-Bt in 2010 and in 2011 a mean instar not significantly different between Bt and non-Bt, but lower body area on Bt vs. non-Bt. During the public comment period, Pioneer indicated that this population is resistant to Cry34/35, but this population would not be considered resistant based on the criteria Pioneer is proposing. There needs to be consistency in what is termed resistance and that criteria should be the most conservative option. One panel member recommended comparing the results from the Gassmann and Nowatzki assays using the current registered toxins to evaluate sensitivity and consistency of data outputs.

#### **CHARGE QUESTION 4:**

##### *Defining Resistance*

The nature of resistance for corn rootworm and Bt toxins is not completely understood, though it is likely to affect how bioassay results are interpreted for resistance determinations. A series of laboratory and greenhouse selection experiments with non-high dose Bt toxins have shown that CRW resistance is unlikely to follow a single gene, recessive model as for other target pests of Bt crops with high dose expression (e.g., tobacco budworm, Gould et al. 1995). Instead, research has suggested that CRW resistance may be non-recessive (Meihls et al. 2008) or could involve multiple genes conferring varying levels of tolerance (Lefko et al. 2008). On-plant assays essentially function as a diagnostic screen that will identify populations with genetic resistance (the ability of offspring to survive on Bt plants), but may not clearly identify cases of incomplete resistance or detect other forms of resistance such as avoidance of the toxin by selective feeding on roots with differential toxin expression.

As discussed in the Agency's white paper on CRW resistance monitoring, BPPD and industry have proposed definitions for resistance based on the use of on-plant assays (Gassmann et al. 2011 and Nowatzki et al. 2008). These definitions include different levels of comparisons to make a "weight-of-evidence" assessment -- i.e., 1) the response of a field population on Bt vs. non-Bt corn plants, or 2) the responses of a field population vs. a susceptible laboratory colony on Bt corn plants. To make a resistance determination, EPA believes that two separate measures demonstrating statistically significant reduced susceptibility to the Bt toxin would be needed (e.g., survival, growth).

- a. *The Panel is asked to discuss the merits and shortcomings of the proposed approaches to defining resistance using on-plant assays. What sets of comparisons in the assays are most likely to add value to a weight-of-evidence approach to determining resistance?*

The Panel has already identified several merits and shortcomings of both on-plant assays in Charge Question 3. Both methods demonstrate the actual response of the western corn rootworm larvae to corn plants that express Bt protein(s). Limited/declining expression of Bt in the root system, addressed by the Panel in charge question 3, is one of the most crucial limitations to these methodologies. Currently, this situation of low and declining Bt expression in roots may be difficult to overcome using the approved Bt transformation events, but a better understanding of the amount of Bt protein that the larvae are exposed to through time, and how that interacts with larval feeding behavior, may facilitate the interpretation of results. However, the Panel recognizes that the limited and/or declining expression of Bt in the root system is the environment that larvae are exposed to in the field. From the public comment presentation of Dr. Analiza Alves (DuPont Pioneer, Public Docket Id. no.-EPA-HQ-OPP-2013-0490-0025), the Panel has learned that: 1) optimized insect density in the root system and 2) categorizing the developmental instar of surviving larvae at different periods of time, would alleviate some of the previous variation and provide a more solid statistical method to differentiate between putatively resistant and susceptible larvae.

Because the concentration of all the events in the currently commercialized corn hybrids do not meet the definition of “high dose,” the Panel believes that the current scoring criteria of developmental instar proportion of surviving larvae, provides the least variable criteria to discern between putatively resistant and susceptible CRW larvae. However, we must recognize that there may be some fitness costs associated with resistant populations that may delay development and result in earlier instars than control populations. Populations surviving from on-plant assays should be collected alive and progeny from the survivors should be tested using on-plant assays to determine if the offspring survive thus demonstrating heritability of resistance.

- b. *What resistance allele frequency should constitute field resistance for toxins with less than high dose expression? Please discuss the criteria that should be used for these types of toxins (as opposed to high dose toxins) given that a portion of heterozygous insects will survive Bt exposure and drive the evolution of resistance.*

The Panel is aware that: 1) because there is not a current effective method to discern Bt resistant from susceptible CRW; 2) this impeded the establishment of a Bt resistance frequency prior to the release of the corn events already commercialized, but more importantly; 3) resistance mechanisms have not been thoroughly characterized, allowing for speculations on the nature of its tolerance (multi-gene, incomplete resistance) (Gassmann et al. 2011). These factors make it difficult to establish a resistant allele frequency that should trigger an action by the US EPA. These factors also do not allow for comparisons with previously suggested rare resistant allele frequencies considered for Bt plants and Lepidoptera (e.g.: Roush and Miller 1986). The Panel believes that this question does not have a valid scientific answer and opens the possibility of all kinds of speculation.

External laboratory selection studies have demonstrated that selection for CRW resistance to BT proteins can evolve fairly rapidly (within 3 to 11 generations) therefore,

the Panel assumes that BT resistance alleles are not rare in the field (Meihls et al. 2008, Oswald et al. 2011, Meihls et al. 2012, Lefko et al. 2008, Meihls et al. 2011). Modeling simulations illustrate that if initial resistant allele frequency is high then resistance is predicted to evolve in 5 to 8 years even in the presence of a refuge (Pan et al. 2011). The simulations suggest that the resistance allele frequency in CRW populations was already high when the BT corn hybrids were introduced.

Because resistance has been detected fairly rapidly (3 to 11 generations), the Panel assumes that Bt-resistant alleles are not rare (Meihls et al. 2008, Oswald et al. 2011, Meihls et al. 2012, Lefko et al. 2008, Meihls et al. 2011). Pan et al. 2011 demonstrates only a small influence of refuges when allele frequency is high with time to 50% resistant allele frequency ranging from 5 to 8 years, which suggests that resistant allele frequency in the CRW populations was already high when the Bt corn hybrids were introduced.

In Charge Question 3 part 1, the Panel discussed some of the “imperfect” methods that can give us an approximation to the current presence of resistant alleles in field populations (e.g.: assessing the number of females that contributed with alleles to the tested generation, establishment of isoline testing).

- c. *What statistical tests, criteria, and significance levels would be best suited for early resistance detection with the proposed assays? Please discuss how to best avoid false negatives and/or false positives.*

Probit analysis in bioassays with insect artificial diets (Robertson et al. 2007), and assessments of surviving larvae at different periods of time by developmental instar (DuPont Pioneer; Public docket Id.no.-EPA-HQ-OPP-2013-0490-0022) for on-plant assays, seem to be the most adequate method at this time. The Panel suggests that bioassays with insect artificial diet should include all the considerations expressed in Charge Question 3 part 1, and that the method of proportion of different developmental instars be tested also for Cry3Bb1 events, and the assay developers continue the efforts of refining the methodology.

The Panel also suggests that to further confirm resistance consecutive additional testing should be done when: 1) results of the single, on-plant bioassays are inconclusive due to unclear statistical analysis of a limited sample size; and 2) other factors have the possibility of contributing to greater than expected damage, including CRW populations that might be a result of reduced susceptibility. However, the Panel suggests that it is important to recognize that higher CRW populations in certain fields could be an indication of resistance and greater survivability of the population.

Using multiple metrics (survival, instar development, body size and weight, feeding behavior, root tissue ingestions and exploring gene expression) may be used to reduce the likelihood of false positives and false negatives could be reduced. Allowing for comparisons between sampled population on Bt and non-Bt corn plus comparisons between sampled populations and susceptible populations (laboratory colonies) will add another indicator. During the public comment period (04Dec13) the Panel learned that

some of these parameters may reduce variability and produce solid data. The Panel addressed some of the parameters in Charge Question 3.

One panelist emphasizes that perhaps a more conservative approach for both on-plant assays should be adopted initially until the importance of the possible metrics are better understood – using the agar medium may allow for behavior to serve as a metric.

- d. *Please comment on the extent to which incomplete resistance can be identified with on-plant test systems. How should resistance definitions be adjusted to address these scenarios?*

Early resistance may be more easily determined by a diet bioassay than an on-plant bioassay. The  $LC_{50}$  value and possibly the  $EC_{50}$  values by themselves could provide an estimate of resistance in WCR populations, if an acceptable value for heritable resistance is established. Over a period of time, a successive series of on-plant assays of the same population of WCR can infer resistance by noting increasing values for both  $LC_{50}$  and  $EC_{50}$  that exceed a ratio value compared with a standard population to which all registrants agree.

Populations with incomplete resistance may be more fit than the control population on Bt corn, but may not be significantly different. In addition, if there are fitness costs for Bt resistance, the resistant population may not survive on non-Bt corn as well as the control colony. The fitness costs for resistant populations could be reflected in the developmental stage, body weight/head capsule size, but may still be an indicator of incomplete or early resistance.

Progeny of isofamilies, a possibility indicated in the EPA's white paper (US EPA. 2013), can be tested in a medium such as that proposed in Clark et al. (2006) that may allow non-destructive evaluations at different intervals. Knowledge of the proportion of larvae that die or stop developing may provide an indication of the proportion of incomplete resistance and this non-destructive evaluation can greatly contribute to the establishment of a (late) resistance frequency.

Resistance definitions could be adjusted by considering that corn rootworm may have developed more than one response to Bt corn allowing them to survive, such as physiological and behavioral resistance thus complicating the development of a diagnostic tool that confirms resistance on both diet bioassays and on-plant assays. Use of field data may provide the earliest and most reliable indication of resistance or reduced susceptibility. As an example, performance of Bt corn rootworm toxins data from 2007 through 2011 from locations without greater than expected damage shows a Cry3Bb1 average root rating using the Iowa State University node injury scale 0-3 (ISU NIS) of approximately 0.125 (Nick Storer, Public Docket Id no.-EPA-HQ-OPP-2013-0490-0020) Data from 2012 through 2013 from locations without reports of greater than expected damage indicates approximately a 50% increase in average root injury (NIS 0.25) in fields planted with Cry3Bb1. Locations with greater than expected damage for the period 2012 - 2013 reflect an average root rating of 1.25, five times higher than a root rating

where producers can expect a yield loss (NIS of 0.25). During the public comment session of the SAP meeting, population resistance was defined to “occurs when a large portion of a pest population is field-resistant and causes the Bt corn to fail to confer economic control of the population (Nick Storer, Public Docket Id. no.-EPA-HQ-OPP-2013-0490-0020”. The data from 2012 through 2013 in locations with greater than expected damage meet the economic threshold of 0.25 NIS (Hodgson and Gassmann 2013) and the 2012 through 2013 data from locations with greater than expected damage greatly exceed the economic threshold. These data may reflect population resistance. In the simplest terms resistance can be defined as survivability of a population and the heritability of the survivability trait.

- e. *Please discuss the 1) viability of resistance ratios as an option for determining resistant populations, considering the generally low susceptibility of CRW to Bt toxins and 2) the lack of susceptible wildtype populations (i.e., due to widespread adoption of Bt corn). 3) What ratio could be considered as an indicator of resistance for corn rootworm using on-plant assays?*
1. Gassmann et al. (2011) proposed a resistance ratio (proportion of corrected survival of field population/proportion of corrected survival of control population) as a determination of resistance. Due to the nature of current Bt corn events not expressing a “high dose,” the resistance ratio would need to be lower than the ratio of >10x proposed by Tabashnik (1994). A suggested ratio of ~4x for the CRW for non-high dose corn events may be reasonable at this point. The limited historical data that the BPPD IRM Team has received, may serve for the calculation of these ratios, taking into consideration that methodological changes (different batches of Bt toxins with different concentration, discrepancy between the methods of Siegfried et al. (2005) and Custom BioProducts, etc.) may have altered the overall response of field collected and laboratory colonies.
  2. A “fringe” population at this time may serve as a control population upon which other populations from “high risk” areas and fields with unexpected damage could be compared. These populations may not be sustainable over time as greater than expected damage has been reported in Michigan and New York suggesting reduced susceptibility in “fringe” areas as well, but populations from other “fringe” areas without reports of greater than expected damage may serve as a standard for survival, growth measures. Viability of F<sub>1</sub> generations could be established for all current traits to be used in later comparisons. Laboratory populations may be appropriate for a few years but concern about inbreeding and loss of behavioral traits reflective of field populations may be a limiting factor. Diet bioassays may provide realistic responses over time, but in on-plant assays, behavioral responses may make results suspect. Bt-susceptible *Diabrotica* populations can be obtained in areas where the selection pressure has not been so intense such as in Europe, or from an organic corn grower with a large enough farm area, in the periphery of the US cornbelt. Also, surrogate species such as *Diabrotica virgifera zea*, the Mexican corn rootworm, can serve as a good indicator for susceptibility, especially if the samples are obtained in areas where Bt corn is not registered (e.g.:Mexico).



3. A panelist recommends that a ratio of ~4x for the CRW for non-high dose corn events may be reasonable at this point.

### **CHARGE QUESTION 5:**

#### *Determining Remedial Action Areas*

In the event of CRW resistance, successful remediation will likely depend on the ability to geographically define a remedial action zone in a fashion that adequately encompasses the resistant population(s). To accomplish this objective, a robust scientific approach is needed to assess the extent of resistance. As discussed in the Agency's white paper, this step may be complicated by the fact that corn growers use insecticides to suppress corn rootworm population densities (prophylactically or in response to field damage). These management strategies can reduce population sizes and mask the presence of resistant populations the following year, though resistant corn rootworm populations may be present beyond the visual boundary of failed Bt corn *fields*. One possible scientific approach to delineate a remedial action zone could be to use a resistance allele frequency gradient starting in the center of the area of concern and working outward beyond the known affected field(s). Once a resistance frequency gradient has been determined, a resistance threshold value could aid in demarcating the spread of resistance.

- a. *Please comment on the strengths and limitations of BPPD's proposal to use resistance allele gradients to define the geographic extent of a resistant population.*

Unfortunately, constraints preclude measuring allele frequency from currently being a realistic option. These constraints include the following.

First, the alleles conferring resistance have not been defined. If such a breakthrough occurred in which specific allele(s) were defined, it would be useful to map the frequency (or presence/absence) of allele(s) as gradients from problem-inquiry fields. Mapping allele gradients would be an attractive option if a technique existed to take many samples from many locations, to achieve high sampling resolution. Initial locations could be defined as fields with problem inquiries, and samples taken in concentric rings surrounding that location. Maps could be made if samples only were able to categorize the presence/absence of the resistant allele(s). The sampling would (1) confirm the presence of resistant allele(s) at the location of the fields of problem inquiries, and (2) define the locations where these alleles are no longer present. Mapped representations of an allele frequency would have direct utility for efforts to manage gene flow and introgression. However, there are important implicit biological assumptions in the mapped realization of allele frequency for purposes of managing population abundance of resistant phenotypes. A key assumption is that the presence of the allele(s) results in expression of the resistance. It is feasible, especially for cases where multiple alleles are involved and their expression is influenced by environmental factors, that the allele(s) is detected but not expressed in a manner that confers resistance.

Secondly, it is likely that resistance of CRW to the currently deployed transgenes is conferred via more than one allele. The relative importance of each allele that contributes to resistance, and the interaction among those alleles, is not known. It is also feasible that there are gene-by-environment interactions, resulting in poor expression of resistance under field conditions even if resistant alleles are present. The phenotypic expression of resistance may not be a simple function of the presence of an allele(s). Thus, it is not currently clear if mapping the gradient of a single allele, a small subset of alleles, or many alleles, would be necessary for inferring maps that reflect the probability of resistant phenotypes.

Thirdly, sample collections could be difficult because abundances of adults could be low, or absent at the time collections are required. Currently, collections are not always made in the year that problem-inquiry fields are defined. It also may be difficult to obtain sufficient precision in measuring changes in susceptibility along spatial gradients in a timely fashion for proactive resistance management if many hot spots of resistance occur, or if area wide resistance occurs, due to the workload of adult sampling and running molecular assays. If resistant allele frequencies are estimated from bioassays, it is unlikely that data could be gathered within the time frame needed to implement effective remediation due to the need to collect adults, obtain eggs, complete diapause, and run bioassays.

When the constraints of defining what allele or alleles confer resistance, and the cost of determining the presence of those allele(s) in a population is reasonable, then the Panel recommends that efforts to map the presence and frequency of those genes be pursued. Efforts should also include modeling the spatial patterns of those alleles, which can be used to optimize interpolation algorithms. A spatially defined sampling approach may be applied starting from the hot spot fields (problem inquiry fields), in 0, 5, 20, km steps. This may not work if area wide resistance is detected or if multiple hot spots were detected due to the work load problem. In cases where resistant populations were found at the 20 km step, then more distant sampling is required. Then interpolated maps may be created, and remedial action zones visually defined. Assuming that the detection of resistant alleles is directly related to phenotypic expression of resistance, mapping resistance allele frequency would also help to define action zones to guide mitigation efforts. However, mapping allele frequencies cannot proceed until the constraint of defining what gene or genes confer resistance is determined.

Alternatively, it is currently feasible to measure abundance. This can be done a priori, in areas of adoption, in the year of adoption, and sample resolution guided by other data layers that suggest risk (continuous corn, continuous use of similar traits, historically higher abundances, locations of problem inquiry fields, etc.). Information may be obtained within a time frame to influence decisions about deployment of Best Management Practices (BMPs). There is an inherent assumption that BMPs can reduce the probability of widespread Bt failure when BMPs are deployed in response to a problem inquiry field. Although BMPs may, or may not, influence the frequency of resistant alleles at a given location, BMPs help achieve sustainability of Bt-crops both at

that location and at wider geographic scales, through their influence on population processes. Therefore, we suggest mapping population density gradients as part of IRM.

Although Bt resistance allele(s) have not been defined, other allele-based studies could be used to define remedial action zones (e.g. population hybridization studies).

For the time being, the Panel advises learning from these other allele gradient studies, such as those defining hybridization zones between different CRW populations of northern Italy. They show that hybrid populations with characteristic neutral markers of genetic diversity (microsatellites) spread about 13-21 km per year in intense maize growing landscapes without geographic barriers (Bermond et al. 2012). This is due to short and medium distance dispersal, but not likely due to long distance (stratified) dispersal. The Panel recognizes that alleles subject to selection, such as alleles conferring resistance, may not follow the exact same pattern as those detected through neutral genetic diversity studies, but for the time being these studies are the most closely related to this question.

Studies using these population genetics methods with neutral genetic markers should be conducted in areas at high risk of resistance (continuous corn, unmanaged populations, etc.), and areas where problem-inquiry fields have occurred, to determine if similar spread rates exist in these landscapes and populations. This is, however, only possible if the supposed resistant populations are genetically different (sufficient genetic diversity in the neutral markers) among surrounding populations.

For determining the size of remedial action zones, the Panel indicates that first it is important to clarify whether the remedial action zone is aimed at elimination of resistant alleles (remedy) and/or resistance suppression and containment of resistant alleles. The latter appears more practical. The determination of the size of remedial action zones also requires first defining the potential control measures and the expected efficacy of each measure on population suppression.

The Panel also believes that definitions that distinguish a “hot-spot” versus a “containment zone of area wide resistance” need to be developed. This is an issue of defining the spatial scale of resistant populations. Pan et al. (2011) described several approaches for clarifying “hot spot” versus “containment zone of area wide resistance.”

One option is to define hot spots based on fixed distances from problem-inquiry fields, based on dispersal kernels and/or introgression of microsatellites (see 5b, below).

The spatial scale of resistance can also be partially addressed through tests for spatial continuity of the data. Currently, resistance or problem-inquiry fields are being discussed as “hot-spots”, and it is implied that they are independent. Spatial independence can be tested, and used to help distinguish independent hot-spots versus larger-scale processes. Mapping the location of problem-inquiry fields, and the location of fields where similar traits are deployed but are not resulting in problems, results in a categorical map (locations of 0 and 1's), from which tests of spatial autocorrelation can be applied. Moran's I, or other regression-based approaches to look for spatial dependence in the

numbers as a function of intervening distance, may be used. Population genetics data may have other statistical tools for defining spatial dependence in the data.

Mapping abundances may also help define the spatial scale at which population processes are being expressed. Although abundances are influenced by many factors other than allele frequencies, currently population abundance is the data that has the potential to be collected in much higher spatial and temporal resolution. It may be feasible to devise a statistical approach (such as a multivariate approach) to test for spatial dependence in abundance after factoring out other suspected factors (weather, field management, etc.) that contribute to abundance.

It seems reasonable to look for introgression of resistant alleles among CRW populations in fields placed along a gradient as a function of distance from point-sources that would be first defined as problem-inquiry fields. However, if spatial patterns among problem-inquiry fields are best described as random with respect to distance and direction among fields, then trying to find spatial gradients among fields will be difficult.

*b. What other tools or strategies could be employed to define the remediation zone?*

The Panel proposes the following seven strategies to define remedial action zones:

1. Adapt from population hybridization studies.

CRW populations with characteristic neutral genetic markers (microsatellites) are suggested to spread about 13 to 21 km per year. This is based on a molecular genetics study modeling the spatial spread of allele frequencies of CRW hybrid populations, and two differentiated parental populations in northern Italy (Bermond et al. 2012, 2013). The spatial process measured in this study is due to effects of short and medium distance dispersal but not likely due to long-distance (stratified) dispersal. Using this study, an approximate 20 km radius can be suggested as a remedial action zone, but the Panel is uncertain if this would be feasible. When assigning an approximate 20 km range as the remedial action zone, a significant reduction of the resistance alleles may be achieved, but not with 100 % certainty. This is because (i) a small proportion of CRW disperses about 60 to 100 km per year (Baufeld and Enzian 2001) or up to 200 km/year (Gray et al. 2009); and (ii) efficacy of available alternative control measures far below 100%, except for crop rotation schemes. Furthermore, the dispersal kernel of adult CRW (see 2, below), suggests that a remedial action zone using a radii of 50 km, and deployment of highly effective actions, would be needed to achieve remedy (defined as elimination of resistant alleles) (Carrasco et al. 2010). The Panel is uncertain if this would be feasible.

2. Adapt from dispersal kernels, used to define eradication and containment zones of newly introduced CRW in Europe.

For remedial action zones to eradicate Bt resistance, the experiences from European CRW eradication efforts might be useful. They divide remedial action zones (called eradications zones) into a 1 km “focus” zone around a hot spot (with highly efficient

resistance management measures applied in that area) and additional 5 km “safety” zone (with less strict resistance management measures applied in that safety zone) (EC Decision 2003/766/EC (Anon, 2003). However, according to the dispersal kernel of adult CRW, the focus zone should be extended to 5 km and the safety zone up to 50 km (Carrasco et al. 2010) if the intent is elimination. If this approach is to be adapted for the USA, then the suggested remedial action zone for trying to eliminate resistant populations would be 5 + 50 km per year, which is not feasible. If the detection of resistant populations requires 2 years, then the zone should be even doubled (10 + 100 km). Again, this is unlikely to be feasible because of the scale, and the lack of 100% effective control measures.

If suppression of resistance rather than elimination is the aim, then a containment action zone may be created if area wide resistance is detected, and/or mitigation zones defined around hot-spots of resistance. For containment zones to mitigate area wide resistance, we may also learn from European experiences, where a 40 km containment zone is defined around specific CRW populations (10 km inside population, 30 km outside). Control measures are targeted at the 10 km inside populations, to suppress their populations so much, that spread of the population becomes much less likely (integration of many different control measures). Measures targeted on the outside zones (i.e. more crop rotation) are aimed to reduce the chance of population spread. According to the microsatellite frequency spread studies of Bermond et al. (2012, 2013), 13 to 21 km around populations would be sufficient (instead of 30 km). But according to the dispersal kernel studies of Carrasco et al. 2010, the outside population zone should be extended to 50 km (at 99% safety level, versus 5 km at 50% safety). This translates to suggested containment zones of 20 km (based on studies using microsatellites: Bermond et al. 2013) or 30 km (based on EU Legislation) to 50 km (based on dispersal kernel studies: Carrasco et al. 2010) outside plus 10 km inside a population. The Panel questions whether this is feasible in the USA landscape.

As for mitigation zones around hot-spot areas of resistance, the Panel suggests also applying the 13 to 21 km suggestions by Bermond et al. 2013 (based on studies using microsatellites) or the 5 km – focus zone suggested by Carrasco et al. (2010) considering that up to nearly 10% of a population would breach this zone (based on the dispersal kernels).

A recent review (Gray et al. 2009), and models that may be compared against measured abundances (Onstad et al. 1999, 2003a,b) suggested rates of expansion of WCR ranged from 20 to 200 km/year in North America, and was influenced by direction, prevailing winds, storm fronts, landscape and local management practices. However, such long distances, influenced by stratified dispersal, probably cannot be taken into account when defining remediation zones.

From these studies, we can suggest a containment zone of 20+ 10 km. But this assumes spread is emanating from a single source location, or a single front, driven by dispersal from that source or front. In contrast, the pattern of problem-inquiry fields appears to suggest multiple, independent initiation sites. Efforts to contain introgression of resistant

alleles with a containment zone would be impaired by each appearance of new, independent sites harboring resistant alleles. We believe resistant alleles are not rare, making containment unlikely to be successful for long periods of time in the absence of IRM practices that significantly limit the appearance of new, independent sites harboring resistance.

### 3. Adapt from rotation tolerant population spread studies.

Lessons from the spread of rotation tolerance may be applied to defining containment zones around area wide Bt resistance regions, thus under large scale situations. Onstad et al. (1999) modeled spread of rotation resistance at 10-30 km per year. As for remedial mitigation actions zones at a smaller hot spot scale, lessons from the spread of rotation tolerance may not be applied, as spatial correlations are not clear.

### 4. Adult CRW sampling and Bt susceptibility assessment of offspring in a series of distances from hot spots or problem-inquiry fields.

It is conceptually feasible to use the results from the sampling and bioassays in response to damage, and testing of offspring, to define remedial action zones. Spatial sampling may be applied starting from the hot spot fields (problem inquiry fields), in 0, 5, 20, km steps. If resistant populations were found at the 20 km step, then more distant samplings would be required. This may not work if area wide resistance is detected or if multiple hot spots were detected due to the work load problem. Furthermore, this would require that samples be collected and analyzed in a timely fashion. These data could be used to create interpolated maps, and remedial action zones visually defined.

### 5. Temporal trend in bioassay information and spatial location.

The Panel notes that bioassay data showed temporal trends in LC50s and EC50s. In some locations, at approximately county spatial scale, these temporal trends were positive. Thus, these temporal trends in bioassay data help define a spatial area (a county or group of counties) that could be defined as a remedial action area.

### 6. Develop risk maps.

Risk maps could be developed, and risk expressed as spatial gradients (Baufeld and Enzian 2001). Risk maps could be developed by categorizing relative risk for a field or land area based on expert opinion, analysis of past PI fields, lack-of-rotation, repeated use of same trait, weather conducive to WCR development, regional corn acreage and corn density in a landscape, and other factors. Such risk maps would be useful for determining size of remediation zones and for guiding IRM practices and education efforts aimed at influencing human behavior in ways to minimize resistant establishment or spread. The risk could be buffered out to distances informed from the eradication/containment zones in Europe, the population genetics studies from Europe, the dispersal kernel studies from Europe, or the rotation-resistance studies from the Midwest.

7. Investigate mapping the probability of occurrence of exceeding thresholds, borrowing from statistical tools developed in the geostatistical literature.

Defining the geographic extent of a population as a gradient requires measuring the population (density, frequency of alleles, or presence/absence) with sufficient precision at discrete, known points, so that changes over space can be detected. It is unclear if techniques currently exist to achieve that precision for alleles, but it does exist for measuring abundances.

Statistically estimating density, or presence/absence, of a population at unsampled points involves interpolation. Interpolation can be based on the statistical relationship of points as a function of intervening distance (such as kriging) or assumed spatial relationships (such as inverse-distance interpolation). Sample placement can be optimized from understanding the statistical relationship of samples as a function of their distance and direction. Any map that shows interpolated estimates should be recognized as representing statistical or graphical models.

Ideally, defining the geographic extent of a resistant population would lend itself to interpolated mapping of resistant allele frequencies measured (with precision) at point sources. This approach would probably require many sample sites. When positive values are rare (which they will need to be, if used for resistance monitoring for achieving resistance management), it is important to define where the negative locations are (where resistance is absent). Without the negatives defined, interpolations will often tend to “bleed” from positive locations over wide areas, which may be areas of false positives.

Advances in the ability to map probability of occurrences (positives) in the presence of large numbers of zeros (negatives) could be developed for this purpose. Recta et al. (2012) provide an example for insect count data that have many zeros. Statistical approaches to mapping have also been developed that utilize other data layers (known priors) for estimating values at unsampled locations. These approaches (such as “indicator kriging”) are utilized in the geosciences for defining placement of wells, and mapping probabilities of obtaining positives (defined as economic extraction of a resource). Similar examples may exist for interpolating values for allele frequencies, although the Panel is not currently aware of those examples.

In conclusion, the Panel concurs with the EPA (EPA White Paper 2013) that “A primary goal of resistance monitoring is to detect shifts (in space and time) in the frequency of resistant alleles (i.e., susceptibility changes) before the onset of resistance leads to widespread Bt failure.” However, the goal as stated is not feasible because we are unable to measure allele frequencies or changes in susceptibility with sufficient precision and in sufficient time. The goal should be restated, to include metrics that we have a capacity for measuring with sufficient precision and in a timely fashion.

Currently, the only such metric is abundance, and some members of the Panel felt that monitoring abundance should be added to the definition, although not all members agreed because monitoring abundance may not always be directly related to monitoring resistance. A suggested restatement is: “A primary goal of monitoring is to detect shifts (in abundance, susceptibility, and/or resistant allele frequency) before the onset of resistance leads to widespread Bt failure.”

The Panel cannot come to a final suggestion on the size and structure of remediation zones to mitigate resistance, but suggests taking the above explained studies into account when defining such zones. The Panel did not judge the feasibility of the scale of remediation following establishment of resistance, which ranged from 20 to 50 km radii based on the information available to date. Thus, achieving resistance management should focus on proactive options that avoid or delay establishment of resistant alleles in CRW populations.

### **CHARGE QUESTION 6**

#### *Containment/Mitigation of Resistance*

The terms and conditions for all Bt corn registrations for CRW include a generic remedial action plan, which is to be superseded by a specific remedial action plan (submitted to the Agency within 30-90 days) if resistance is confirmed. BPPD is concerned that the current generic remedial action plans may not be sufficiently proactive to halt resistance from spreading before a specific plan can be developed. Unless the resistant population is extirpated by management actions described in the generic plan, the implementation of targeted and specific measures (i.e., to reduce the spread of or eliminate resistant populations) could be hindered.

As discussed in the Agency’s white paper, BPPD has recommended a more proactive approach of remediation for addressing CRW resistance. Some of the considerations include:

- Creation of species and toxin specific remedial action plans before resistance evolves such that the strategies can be deployed quickly if resistance is confirmed;
- Strategies to identify and address localized (i.e., “hot spots”) vs. widespread (large, continuous geographic areas) resistance (scenarios that could be evaluated with theoretical models);
- Additional research on CRW dispersal (i.e., proportion of long distance and pre-ovipositional movement) to evaluate containment strategies;
- The use of crop rotation and alternate PIP modes of action (other than the compromised toxin) as preferred CRW control strategies for resistance mitigation (as opposed to soil insecticide use with continued planting of the compromised Bt toxin);



- Simulation modeling to help design and evaluate remediation strategies. Simulations should explore effects of dispersal (male dispersal and pre-ovipositional female dispersal) on the success of remediation and provide insight into which remedial actions are the most effective at containing or eliminating resistant populations.

The Panel is asked to comment on the merits and shortcomings of the current remedial action approach and the proposed improvements as well as alternative approaches the Panel may wish to provide. Specifically, please discuss the following issues:

- a. What remediation approaches could be taken for localized vs. area-wide resistance scenarios?*

The Panel concurs with the concern stated in the white paper (pg. 22), that "...actionable thresholds may not be met (or recognized) until resistance is widespread and effective mitigation (reducing resistant allele frequency) is impractical. In this case, managing resistance through population suppression may be the only alternative." The Panel also concurs with the statement (US EPA, 2013 (Page 26)) that "BMPs ... likely need to be implemented ... irrespective of whether resistance testing has been completed." The context of this statement was addressing the situation of responding to a problem inquiry field. The Panel recommends widening the context to deal with factors that increase higher risk of becoming areas with the emergence of resistance. The Panel recommends a proactive effort of managing resistance through population suppression through the implementation of an integrated pest management plan using BMP.

The Agency BPPD IRM team separates population suppression from remediation (EPA White Paper 2013). In this case, the separation does not hold strongly through space and time. The r-alleles are not rare. BMPs applied both prior to, and following, problem-inquiry sites and years, both only affect r-alleles through population processes. In a high-risk setting, rotating to another crop, or different traits, will help achieve IRM whether they are conducted before or after a problem-field inquiry.

Given the prioritization of BMPs to manage abundances as the primary means of influencing resistance, we now turn to consideration of proactive approaches for achieving IRM, including rapid remediation and mitigation. The Panel assumes r-alleles are not rare, that the geographic location of problem-inquiry fields will show low rates of spatial dependence and arise (and will continue to arise) primarily independently through local selection, and secondarily through gene-flow achieved through dispersal. The Panel focuses on efforts to achieve proactive IRM in a situation where r-alleles are not rare and events do not express a high dose. The Panel recognizes the value of including the appropriate use of transgenic cultivars in an IRM plan due to their ability to influence population densities and pesticide-use patterns, and their labor-saving effects.

The Panel has great concern about the viability of all Bt products for corn rootworm, particularly in a landscape of continuous corn. Growers must be directly involved in the design of BMPs that will be relevant to their landscape and farming practice. Thus, there needs to be a process created to involve growers, specifically those who decide upon

cultivar choice. BMP practices can be encouraged through requirements placed on the labels. These might include requirements for rotations (among traits, pyramids, sequential versus concurrent deployments of single or pyramided cultivars depending on initial *r* allele frequencies (Onstad and Meinke 2010), and/or crops) depending on the production practices. The Panel recommends developing BMPs that will address variation in production practices, species behavior and resistance to chemical and cultural practices such as continuous corn, rotation resistance and production practices.

While the Panel anticipates an overlap in the BMPs recommendations, the Panel believes it is necessary to consider the population and species differences along in conjunction with differences in production practices and landscapes when developing the BMPs. The Panel recommends the Agricultural Biotechnology Stewardship Technical Committee (ABSTC), Insect Resistance Management Stewardship Subcommittee, the National Corn Growers' Association and state-based corn associations in cooperation with research and extension scientists and growers develop an IRM plan and BMPs using a proactive integrated pest management approach that would be implemented prior to suspected resistance (proactive). This proactive approach would reduce the likelihood of heavy selection pressure on corn rootworm by using the same mode of action over an extended period of time as resistance has been shown to develop within 3 to 7 years of use in continuous corn. The development of the IRM and IPM plans should be a process that is reviewed annually and revised as needed. Such organizations may also be able to influence the location and sequence of planting single versus pyramided traits, and use patterns of soil insecticides, all of which influence rates of resistance (Onstad and Meinke 2010). Involving the USDA Risk Management Agency may help facilitate options. The Panel recommends inclusion of social scientists in to the process to increase the understanding of human behavior with regard to production practices and decision making. The Panel notes that Extension entomologists are already developing region-specific recommendations, (i.e. the eastern corn-growing states (Indiana, Michigan, New York, Pennsylvania, Ohio, Ontario) (Tooker and DiFonzo 2013).

It was suggested that the selection pressure exerted by continuous corn production, in areas that do not have rotation resistant corn rootworm populations, could be reduced by a rotation plan that allows producers to plant corn 4 out of 5 years by rotating corn with soybean, then a non-Bt hybrid, non-Bt hybrid with a soil insecticide, a pyramided Bt product and followed by a pyramided Bt product or similar rotation schemes. By implementing a rotation plan that alternates frequency of rotation over time, management tools and modes of action, selection pressure and abundances are greatly reduced. Reduced abundances also reduces the rate of resistance, through source-sink dynamics interacting with movement rates and landscapes, as has been shown by Caprio (2001) and Storer (2003). The combination of reduced abundances and reduced selection pressure may delay resistance to the PIPs toxins. The Panel recommends the implementation of the proposed BMPs in advance of suspected resistance.

The Panel believes using the IPM approach as part of BMPs may reduce the need for remediation although the Panel recognizes that not all producers will choose to use the BMPs as part of the production plan unless required. Without the required

implementation of BMPs, the Panel concurs with EPA that under the current generic remediation approach too much time may pass before identifying resistant populations. The Panel recommends that remediation plans for all registered products take into consideration geographical location of the field, production practice, population resistance and species' behaviors. These plans may include elements outlined in the BMPs with an emphasis on crop rotation although areas with rotation resistant corn rootworm will require a multi-year rotation plan that uses the planting of non-Bt with rotation of soil insecticides rather than crop rotation to overcome oviposition and extended diapause populations. With the "hotspot" resistance scenarios, producers who experience higher than expected damage in their Bt field(s) in cooperation with industry representatives can control the implementation of a remediation plan. However, area-wide resistance will be more difficult to implement and may prove ineffective as the boundaries of the resistant populations may not be known and as with refuge, full compliance of the remediation plan may not occur. Area-wide remediation may require removal of the trait for which resistance exists over a wide geographic area. If the removal of a trait due to resistance occurs, the use of any pyramid traits that include the resistant trait needs to be addressed as it would be functioning as a single trait product.

IPM practices include the judicious use of tools within the IPM toolbox as needed, not all at once. The use of a Bt hybrid with a seed treatment and a soil insecticide does not fit in the IPM tool box. The use of soil insecticides with a non-Bt hybrid reduces selection pressure as band of soil insecticide protects roots to prevent yield loss, but provide root tissue outside of the soil insecticide band that are fed upon by CRW larvae. If larvae emerge from the soil insecticide band, random mating with CRW adults emerging from the non-treated area between corn row due to the close proximity reducing the likelihood of resistant individuals from mating with one another. When soil insecticides are used with a Bt hybrid, any CRW adults emerging from the soil insecticide band have been exposed to both the soil insecticide and the Bt toxin. CRW adults emerging from the area between cornrows have been exposed to the Bt toxin so mating between these two populations that have both survived exposure to the Bt toxin plus individuals who have been survived exposure to the soil insecticide. BMPs do not include all management options, rather BMPs are practices that provide adequate management of a pest and at the same time prevent the development of resistance thus preserving the effectiveness of the management tool. The use of a soil insecticide with a Bt hybrid is not a BMP. The issue of using a Bt hybrid with a soil insecticide may be driven by the belief that only Bt hybrids have the elite traits that are desirable.

The Panel supports the most conservative remediation plans as outlined by EPA including the recommendation of the use of conventional insecticides to control the adult stage during the present field season and then select an alternative pest control method the following season to reduce the establishment of resistant populations, but only as a last resort to control the spread of resistant populations and not as a population abundance management tool. Producers should not use conventional insecticides to reduce adult populations to reduce density on an annual basis as this may lead to resistance thus eliminating the usefulness of the conventional insecticide as part of the remediation plan.

It should be noted that the use of SmartStax with a 5% refuge will be less durable and could compromise the second toxin in areas where resistance to Cry3Bb1 is occurring.

*b. Which mitigation measures would be more effective in containing and/or extirpating resistant CRW populations?*

To mitigate resistant populations, the Panel recommends the use of rotation with a non-host plant although this is desirable it will not be effective in some geographic locations due to rotation resistant populations that will survive in first year corn by a shift in oviposition by western corn rootworm or by extended diapause in the case of northern corn rootworm. The Panel recommends the use of an alternative Bt toxin as a viable option for rotation resistant populations, but the Panel has concerns with the recommendation to use a soil insecticide with the compromised Bt toxin as we believe this will allow resistant corn rootworm to survive by feeding on Bt expressing roots outside of the application band of the soil insecticide and exposing larval populations to both the compromised Bt toxin and the soil insecticide. The Panel believes the use of a soil insecticide with a Bt hybrid should not be done. The Panel recommends the use of adult control in fields with greater than expected damage during the current season in fields with resistant populations, but dispersal to other fields, although limited, is still a concern as damage may not be noticed until harvest time. The distribution of potentially resistant populations that arise from multiple locations has a greater likelihood of being transported to new locations via extreme weather events (Center for Food Safety 2013).

To assist with earlier detection of greater than expected damage the Panel recommends the use of aerial monitoring using planes or drones in high risk areas especially following major weather events that may contribute to lodged corn. The Panel recommends development of a risk map that identifies the varying levels of greater than expected damage reports, crop production practices that contribute to resistance, including the use of high dose seed treatments (may mask early resistance) and lack of refuge compliance.

*c. Please comment on the value of theoretical models in designing remedial action strategies for various resistance scenarios.*

WCR biology models are among the best developed that exist in agricultural entomology. One could reasonably argue that these models have done a good job projecting how varying management and initial biological parameters influence the emergence of resistance. All these models show strong sensitivity to initial resistance allele frequencies, and functional dose (survivorship of heterozygotes on Bt-corn). Models can: 1) describe the geographic spread of rotation-resistant phenotypes (Onstad et al. 1999), 2) show that pyramided cultivars slow resistance but that this delay is reduced when single traits are in the landscape (Onstad and Meinke 2010), 3) show the value of fixed as opposed to random refuge sites in several scenarios (Storer 2003, Pan et al. 2011), and 4) show the affect of population dynamics and movement on rate of resistance development (Storer 2003, Caprio 2001.).

For some sets of parameters, models are predictive. For example, the Pan et al. (2011) model, which focuses on contrasting refuge designs, shows only a small influence of refuges when initial allele frequency is high (0.01) (Table 4). Furthermore, when initial allele frequency is high, years-to-50% resistant allele frequency ranged from 5 to 8 years, which is a good approximation of the time when problem inquiry fields are being found.

As described in detail below the Panel suggest combining what is learned from the existing models with empirical information obtained during the years since commercialization to focus both future modeling and remediation strategies. We describe these next.

First, the Panel believes that Bt-resistant alleles are not rare. Often the published models start with a frequency of 0.001, and contrast scenarios with that as an initial baseline. The Panel recommends focusing on scenarios that show higher initial frequencies of r alleles, potentially several orders of magnitude higher. Dr. Nicholas. Miller, Assistant Professor/Research Entomologist at the University of Nebraska-Lincoln summarized arguments for focusing on scenarios with a relatively high initial r frequency at the Entomology Society of America (ESA) 2013 meeting in Austin, TX, and his argument is paraphrased here. The first line of evidence is that selection experiments from five studies (Meihls et al. 2008, Oswald et al. 2011, Meihls et al. 2012, Lefko et al 2008, Meihls et al. 2011) have been able to detect resistance fairly rapidly (3 to 11 generations) from multiple populations across the corn belt (including the areas of Colorado, Illinois, Kansas, Minnesota, Missouri, Nebraska, Ohio, South Dakota, and Wisconsin). The published support based on selection in the laboratory is stronger for Cry3Bb1 than Cry34/35 and mCry3A. Onstad and Meinke (2010) use observed lab selection data fit to simple population genetic models to suggest an initial r allele frequency of  $0.05 \geq r \geq 0.1$  (using Lefko et al 2008) or 0.2 (using Meihls et al. 2008).

A second independent line of evidence presented by Dr. Miller is based on the geographic distribution of problem-inquiry fields, and contrasting that to the pattern of rotation-resistant phenotypes. In both cases, we must assume that the presence of problems in the field is directly correlated to field-evolved resistance. This assumption is not always correct, but it is a reasonable first approximation. The problem inquiry fields from Cry3Bb1 plantings show a pattern of sudden appearance in multiple locations with little to no spatial correlation. This is consistent with a process of independent selection of alleles that are not rare, and thus relatively easy to increase in frequency. In contrast, rotation resistance shows a pattern of being selected for a discrete location, followed by diffusion from that location, and strong spatial dependence in the data (Onstad et al. 1999).

The ability to select for resistance rapidly, from many populations, and the geographic pattern of problem fields strongly support the argument that resistant alleles are not rare. This conclusion is stronger for Cry3Bb1 than Cry34/35 and mCry3A. Models show the ability to delay resistance with refuges is poor when resistant alleles are not rare. Examples can be seen in biologically rich models (Pan *et. al.* 2011), and by simple models based on population genetics presented by Miller at the ESA in Austin,

TX. As the initial r allele frequency is increased, including rates that appear realistic, the ability of refuges to influence increase in r allele frequency is dramatically dampened. With the reduction of refuge size and refuge non-compliance the influence of refuge on resistance lessened further (Center for Food Safety 2013).

One important conclusion regarding scientific uncertainty, quoted from N. Miller, is that “we ... do not know enough about WRC genetics.” We lack a sufficient understanding of which genes contribute to resistance, how they interact, what their current frequency is in field populations, and factors that influence the dynamics of their presence in populations.

The Panel suggests modeling attributes to add or focus on, given our current understanding of the emergence of resistant population. However, the Panel believes that the lack of components are not the most critical element for improving the analysis of remediation strategies or for defining proactive IRM programs.. Instead, the Panel advises that the primary requirements for achieving both proactive IRM and remediation entails an identification of where to focus the initial parameters and endpoints.

- d. *The current deterministic and stochastic simulation models used for IRM purposes contain many of the following attributes: ecology, population biology, behavior, and genetics of pest, grower behavior (refuge compliance, insecticide spraying, etc), explicit spatial and probability analyses. What other modeling attributes would help improve the analysis of remediation strategies?*

Although we have not exhaustively reviewed every WRC simulation model, the Panel recommends the following attributes be consistently included in these models:

1. Relatively high r-allele frequencies as initial parameters. Often the models include this among the scenarios being compared. The Panel suggest focusing on those scenarios, which would improve the ability to see effects under those conditions.
2. Non-random mating. Storer 2003 and Pan et al. 2011 include varying development rates and mating timing tied to when the different genotypes emerge, which results in non-random mating. However, a caged study using N<sup>15</sup> markers reported at the ESA meetings by Krupe et al. suggest higher rates of non-random mating, perhaps not entirely tied to development rates.
3. Multiple alleles (polygenic (additive) resistance). In regards to quantitative genetic variation although the 2002 SAP (EPA 2002) noted that “For low/moderate dose plants, any gene that confers even slight resistance is expected to be favored by natural selection...”, most models assume a single allele conferring resistance..
4. Pyramided traits with varying h (dominance) for each trait. Onstad and Meinke (2010) suggest the presence of single-traits in the landscape reduce the benefit that can be achieved with pyramids.
5. Varying deployments of trait packages in time: deploying each trait sequentially, versus all-at-once in pyramided constructs.

6. Modeling on the influence of BMPs on abundances and how that affects rates of resistance across relevant landscapes. Models and theory (Caprio 2001, Storer 2003) support the idea that population abundances influence the rate of resistance through source-sink dynamics, reflecting complex interactions among population processes (mating, oviposition, and movement), and spatial patterns of Bt corn, nonBt corn, and non-host plants in the landscape. Also, when BMPs are deployed in response to a problem inquiry field, there is an inherent assumption that BMPs can reduce the probability of widespread Bt failure. Although BMPs may, or may not, influence the proportion of resistant alleles at a given location, BMPs help achieve sustainability of Bt-crops at that location and at wider geographic scales, through their influence on population abundances and processes such as the number of individuals participating in dispersal. It should be noted that use of a Bt trait hybrid with a high dose seed treatment and soil insecticide should not be considered a best management practice or considered part of an integrated pest management program, but should be included in models to determine the potential for driving resistance to the Bt toxin and the insecticides. Modeling in a GIS framework using current landscapes would be useful for IRM planning and grower education.

In addition to simulation models, the Panel recommends statistical models describing population genetics and abundance patterns measured in the corn belt. Recent examples of these modeling approaches (Miller et al. 2005; Ciosi et al 2011; also see discussion for charge question 5) helped elucidate origins and spread rates of populations in Europe. The review in Gray et al. (2009) notes that the Miller et al. (2005) analysis used Bayesian approaches that integrate historical and genetic data. These approaches should be applied to areas associated with problem inquiry fields.

Current IRM models often focus on estimating rates of change in genotype frequencies, or time to achieve an arbitrary proportion of the population in a given genotype category, but these measures cannot currently be compared to field data of allele frequencies for validation. In contrast, population abundances can be used for model validation (as in Storer 2003), and keeping populations low contributes to resistance management by reducing the number of resistant individuals that disperse to neighboring areas. The Panel recommends that IRM models include modeling abundance, in addition to the relative frequencies of genotypes, and couple that with efforts to measure abundances in the field for validation. By validating abundances, future models may focus more on parameter values that allows them to become more predictive, at least for abundances, and hopefully also for phenotypes.

The Panel recommends widening the focus of both modeling, and IRM plans, to manage resistance by managing abundances through the use of appropriate BMPs. This enables direct monitoring of the variable being modeled, and model validation. Although earlier reviews of IRM plans for CRW (e.g., US EPA SAP 2002 and 2009) recognized the lack of a high dose and recommended more stringent IRM plans (Center for Food Safety 2013), the current IRM plans rely heavily upon strategies which were

designed to be useful for high dose. This strategy has theoretical merit and has been efficacious for conditions of both high dose and very low frequencies of r alleles. Neither condition is met for the Bt traits currently used for CRW management. The Panel summarizes conditions that make it unrealistic to rely upon refuges alone to achieve IRM for CRW given current Bt hybrids.

- a. Lack of a high dose: none of the traits provide a high dose. The survival rate reported in 2002 EPA SAP for MON863 is 17% to 62% survival on MON868.
- b. Frequency of r allele: They are not rare
- c. Resistance is probably polygenic. There are multiple r alleles
- d. Random mating is not occurring. Lack of random mating was clear in two reports at the Entomological Society of America (ESA) 2013 meeting in Austin, TX. These include N<sup>15</sup> data presented by Steven Smith, a graduate student at Purdue University, and Sarah Hughson, a graduate student at the University of Illinois using different methods. These reports show a higher probability of phenotypes that had developed on Bt plants to mate with each other.
- e. Cross resistance may exist between Cry3Bb1 and mCry3A
- f. The ability to detect incipient locations of resistance and manage them to limit their spread is difficult, and logistical reasons were summarized by Dr. Christian Krupke, Associate Professor, Purdue University, in a symposium at the Entomological Society of America (ESA) 2013 meeting in Austin, TX, and are paraphrased here:
  - i. LC<sub>50</sub> not reached until 2nd or 3rd instar (2002 SAP)
  - ii. Bioassay problems, discussed in previous charge questions
  - iii. Zero foot traffic following planting. Plants with a NIS of 0.5 or 1.0, which are part of the current definition of resistance, may not be well detected. Under some environmental conditions, plants with this level of node-injury may not show lodging or other signs that would trigger reporting greater than expected damage to seed dealers.
  - iv. Lack of spatial continuity in the occurrence of problem fields
  - v. Disincentives for reporting damage may be perceived by growers. These aspects of human behavior are not well studied. They include reluctance to get involved with regulatory agencies, needing to switch among seed dealers, concern about being out of compliance with refuge requirements, or other factors.
  - vi. Measuring damage is difficult because it occurs underground.
  - vii. Method for measuring damage (NIS) is only poorly correlated to population abundance
  - viii. Rescue treatments do not exist
  - ix. Scouting is imprecise and limited

Using refuges to manage the frequency of r alleles is an indirect method. The management action (placement, size of refuges) acts on r-allele frequency indirectly, through its influence on many population processes, such as those that affect population growth, dispersal, and mating, not through direct and selective action on r-alleles. The



Panel recommends that proactive IRM for CRW with current PIPs must embrace additional indirect methods appropriate for low dose traits, not-rare r-alleles, and polygenic r-alleles. The Panel also recommends an approach that can be evaluated under field conditions. This will be an approach that focuses on managing abundances (densities), to keep abundances of resistant phenotype sufficiently low.

Currently, problem-inquiry fields trigger Best Management Plans (BMP). These BMPs strongly influence population dynamics. To achieve IRM for CRW with current PIPs, the sequence of events must be reversed. IRM plans must proactively develop and deploy BMPs, and secondarily rely on detection and remediation of fields found to experience greater than expected damage.

The influence of BMPs upon population dynamics are modeled in several papers (Storer 2003, Crowder and Onstad 2005, Crowder et al. 2005, Onstad and Meinke 2010, Pan et al. 2011). The Panel agrees that not all management practices are BMPs, but there are BMPs that keep WCR abundances sufficiently low (they include crop rotation, rotation among traits, deployment of pyramids perhaps prior to their deployment individually, and others). There is an implicit assumption that we can influence r-allele frequencies and their spread through implementation of BMPs – this forms the rationale of utilizing BMPs following confirmation of resistance in problem-inquiries. Models should focus on advancing our understanding of this assumption, with current estimates of allele frequencies and assuming polygenic resistant alleles. Questions that should be considered include BMPs affects on resistance evolution through their interaction with population dynamics. Examples show that this modeling approach is feasible. For example, Pan et al. 2013 explore how use-patterns of seed treatments and soil insecticides affect population dynamics and r-allele frequency. Storer 2003 contrasts the addition of crop rotation to soil insecticides, in the years prior to Bt-corn deployment, on rates of CRW resistance (Fig. 7B and 7C), and suggest that “population density can have a significant effect on the rate of adaptation”.

Addressing how BMPs influence both abundance and r-allele frequency will require inputs defined by landscapes, which vary among regions of the cornbelt. For example, Crowder and Onstad (2005) and Crowder et al. (2005) modeled the interaction of rotation and transgenic cultivars on CRW dynamics. Movement among fields in relevant landscapes will need to be considered. For example, young, recently mated females are most likely to be moving among fields (based on captures above from towers placed in fields, and sex-ratios during early colonization). Outputs will probably need to vary across landscapes (as opposed to being expressed as a mean expectation or distribution). This may require models to be developed in a GIS framework.

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