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

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT:	BPPD review of data and support materials submitted by Monsanto to amend the insect resistance management requirements for Bollgard II Bt cotton. EPA Reg. No. 524-522. DP Barcode: 327258 Decision: 363974 MRID#: 467172-01, -02, -03.
TO:	Leonard Cole, Regulatory Action Leader Microbial Pesticides Branch Biopesticides and Pollution Prevention Division (7511C)
FROM:	Alan Reynolds, Entomologist Microbial Pesticides Branch Biopesticides and Pollution Prevention Division (7511C) and Sharlene Matten, Ph.D., Biologist Microbial Pesticides Branch Biopesticides and Pollution Prevention Division (7511C)

Action Requested

BPPD has been asked to review data and support materials submitted by Monsanto to justify a request to amend the Insect Resistance Management (IRM) requirements for Bollgard II (Cry1Ac and Cry2Ab2) Bt cotton (EPA Reg No. 524-522). Specifically, Monsanto is requesting to remove the requirement for structured, non-Bt cotton refuges in the southeast U.S. cotton belt (East Coast to Texas). The submitted materials include a description of tobacco budworm (TBW) biology as it pertains to IRM, results of a 2 year sampling program designed to determine the plant host origin of TBW throughout the southeast, an assessment of TBW natural refuge (i.e. production from alternative host plants and crops), a calculation of "effective" refuge (i.e. the proportion of TBW not exposed to Bt proteins), revised simulation models based on the data collected, and an overall assessment of Bollgard II IRM for TBW and cotton bollworm (CBW) in the southeast U.S. cotton-growing region. Information obtained from two follow-up letter from Monsanto to the Agency (dated April 3 and April 7, 2006) is also included in this review. Bollgard (Cry1Ac) Bt cotton (524-478) is not addressed in a separate amendment, though is discussed in the context of the Bollgard II amendment request and will be considered in this review.

Conclusions

1) BPPD agrees with Monsanto that the TBW sampling, gossypol analyses, and effective refuge determinations clearly demonstrate that a significant portion of the TBW population is derived from non-cotton hosts. However, questions remain on the larger issue of whether these alternate hosts can serve as unstructured refuge for TBW for Bollgard II Bt cotton. Within the Cotton Belt, the gossypol data differed greatly by state/region, although resistance modeling scenarios with TBW showed that Bollgard II was predicted to retain durability for at least 30 years for all regions. On the other hand, there are a number of unresolved issues with the data, modeling, and interpretation of the results (detailed below). Should these issues be resolved, BPPD could support the use of natural refuge alone for Bollgard II in the southeastern U.S. cotton region.

2) The strongest case for the use of natural refuge for TBW resistance management can be made in North Carolina and Georgia. In this region, the proportion of non-cotton-origin TBW was consistently high (>90%) throughout the cotton growing season. The trends were similar for both 2004 and 2005. These states are also known to contain substantial acreage of alternate crops (peanut, tobacco, and soybean) that are known to be preferred TBW hosts. Resistance modeling incorporated with the estimations of effective refuge using worst case counties predicted that Bollgard II retained its durability for at least 30 years.

3) In other regions (i.e. the Mississippi Delta, eastern Texas) the use of natural refuge alone for Bollgard II cotton is less certain. In these areas, the data were variable and difficult to interpret. The specific proportions of cotton-origin TBW varied by state and county, though non-cotton-origin TBW usually were significant (at least 20% of the population) except in a few cases. In some counties, the proportion of non-cotton-origin moths was below 20% while in other counties the percentage of non-cotton moths was high (over 90%) during the same time periods. The general trends were high proportions of non-cotton TBW early in the season, followed by a decline after June. These results seem to indicate that cotton is a much more important host for TBW in these states then in the east coast states and that fewer alternate hosts may be available during the cotton season. However, like North Carolina/Georgia, resistance modeling for TBW and CBW with the estimations of effective refuge using worst case counties showed that Bollgard II was predicted to retain durability for at least 30 years (though one scenario in Texas with CBW showed slightly less than 30

years durability).

4) No statistical correlation comparisons were made between the two growing seasons in which samples were taken (2004 and 2005). Because of this, comparisons of the trends between growing seasons can be conducted only on a qualitative basis.

5) BPPD identified a number of questions regarding the TBW sampling methodology. First, the use of pheromone traps restricted the collections to male TBW (females are not attracted to the pheromone). It is unclear whether inferences about female behavior (i.e. host utilization) can be made from data obtained solely from males. Second, BPPD also questions whether the two year sampling period (2004 - 2005) is adequate for an experiment of this magnitude. Though relatively consistent, cropping and landscape patterns can change over time -- a factor that may not be reflected in a study of limited duration. Given the inherent variability in natural and agronomic ecosystems, a two year study may present only a “snapshot” of the host availability and productivity for TBW that may not be representative of future conditions.

6) It was noted that for a number of counties, trapping locations, and trapping periods there were low trap captures of TBW. For many traps, few (or no) moths were collected. Low numbers of TBW have also hampered recent Bt cotton resistance monitoring efforts (see BPPD 2005), possibly due to a suppressive effect from the widespread adoption of transgenic Bt cotton. Data from collection sites with few captures (i.e. <10) were included in the effective refuge calculations, but not in the modeling (where worst case scenarios were used). It is unclear if the low trap captures in some areas may have affected the reliability of the results. Also, low numbers of TBW could present a concern if insufficient susceptible insects were available to mitigate a resistant population emerging locally from a Bt cotton field.

7) Overall, the CBW and TBW modeling predicts that Bollgard II cotton should have more than 25 years of durability in all regions using natural refuge as the only source of susceptible insects. It should be noted that the “number of years” is an imprecise designation of relative value, not an absolute value. On a relative basis, Bollgard II cotton has much higher durability than Bollgard cotton. Monsanto’s modeling does not account for any prior selection for Cry1Ac resistance. Uncertainties in the modeling parameters and assumptions will impact the modeling output. The extent to which the modeling output will be impacted by these uncertainties is unknown. The presence of larger amounts of Bollgard II cotton in the marketplace is predicted to increase the durability of both Bollgard II and WideStrike cotton products that are present in the landscape. On the other hand, larger amounts of Bollgard cotton in the marketplace are predicted to decrease the durability of Bollgard II and WideStrike cotton.

8) Bollgard II (Cry1Ac and Cry2Ab2) currently exists in a mosaic with single gene Bollgard (Cry1Ac), with Bollgard accounting for the vast majority of Bt cotton acreage. Modeling work has shown that in such a scenario, the pyramided product can be at risk for resistance if

there is significant acreage of a single gene variety. The single gene variety may in a sense act as a "stepping stone" for resistance to the pyramided product: if resistance develops to the Bollgard then Bollgard II would effectively become a single gene product (Cry2Ab2). Monsanto's natural refuge proposal contained no details on how this mosaic might be managed. There was no discussion as to whether Monsanto will "phase out" Bollgard to facilitate the adoption of Bollgard II. Rather, it was suggested in the submission that if there is no requirement to plant structured refuge a strong incentive will exist for growers to switch to Bollgard II. However, considering the large acreage of Bollgard currently in the marketplace, it is highly likely that a significant percentage of cotton acreage will remain planted with Bollgard varieties.

9) Field resistance to Cry1Ac places additional selection pressure on the Cry2Ab2 component of Bollgard II cotton. Encouraging the adoption of Bollgard II will increase the overall durability of all three Bt cotton products. From an insect management point of view, removal of Bollgard cotton from the marketplace should be encouraged as quickly as possible.

10) Monsanto's proposal to use natural refuge for Bollgard II represents a switch from structured refuge (i.e. refuge planted and managed by growers) to an unstructured (i.e. unmanaged) refuge. In developing IRM strategies, there have been three major considerations for evaluating structured refuge that could also be applied to natural (unstructured refuge): i) production of a sufficient number of susceptible insects relative to any resistant survivors of the Bt crop, ii) proximity of the refuge to the transgenic crop to facilitate random mating between susceptible (from the refuge) and resistant (from the Bt crop) insects, and iii) developmental synchrony of the refuge with the transgenic crop to promote random mating. For the most part, Monsanto's sampling and gossypol assays have addressed these three criteria. Proximity and synchrony were addressed by the sampling strategy (traps were placed next to cotton fields and monitored throughout the growing season). Production of susceptible insects was not evaluated from a numerical perspective *per se*; rather the proportion of moths originating on cotton/non-cotton sources was assessed. However, BPPD noted (as described in #6 above) that low numbers of TBW trap captures were reported for many areas. Low number of TBW could be a concern if a resistant population were to emerge locally from a Bt cotton field without a sufficient number of susceptible TBW available to mitigate the threat.

11) BPPD generally agrees with Monsanto that a natural refuge strategy (allowing planting of 100% Bollgard II) will result in economic and environmental benefits. Bt cotton refuges are currently planted as non-Bt (conventional) cotton on either 5 or 20% of growers' total cotton acreage and may be treated with pesticides under the 20% option. Benefits to growers will result largely from yield increases and reduced insecticide use on cotton acreage previously planted as refuge. However, these benefits will likely be compromised should the lepidopteran target pests develop resistance to the Bollgard II toxins.

12) If the natural refuge proposal is ultimately accepted for Bollgard II, it is recommended that Monsanto continue to conduct resistance monitoring for the Cry2Ab2 toxin with both TBW

and CBW. Also, considering that replacement of structured refuge with natural refuge may increase the Bollgard II acreage (and selection pressure for resistance), it may be justified to increase the monitoring efforts for Cry2Ab2 (i.e. increased sampling sites and collections).

Background

[NOTE: This review will focus primarily on submitted data for TBW and modeling for TBW and CBW. Previously reviewed data for CBW are summarized below in this section.]

Bollgard Bt cotton (EPA Reg. No. 524-478), expressing the Cry1Ac toxin, was registered for commercial use beginning with the 1996 growing season. The three major target pests of Bollgard are, tobacco budworm (TBW), cotton bollworm (CBW), and pink bollworm (PBW). Bollgard demonstrates high toxicity against TBW and PBW (considered to be a "high dose") and has moderate, but still effective, activity against CBW (a non-"high dose").

Since Bollgard (and other Bt crops) express insecticidal toxins at high levels throughout the growing season, there is likely to be high selection pressure for the development of resistance among the target pests. Therefore, as part of the terms and conditions of the Bollgard registration, EPA imposed insect resistance management (IRM) requirements including the use of structured, non-Bt cotton refuges. These refuges will theoretically produce susceptible insects not exposed to the Bt toxin that should be available to mate with rare resistant insects emerging from the Bt fields, thus diluting the resistance genes in the pest population. In 1995, the initial refuge strategy for Bt cotton included the use of either a 4% untreated refuge or a 20% insecticide-treatable refuge. Other IRM requirements included resistance monitoring, remedial action plans (in the event of resistance), grower education initiatives, and compliance reporting. In 2001, the Agency reassessed the existing Bt crop registrations and revised the refuge requirements for Bt cotton. Based on the reassessment, the following three refuge options were established for Bollgard cotton (TBW and CBW resistance management):

1) A 5% external, untreated structured refuge (must be within ½ mile of Bollgard fields, preferably within 1/4 mile or adjacent);
2) A 20% external, treatable structured refuge (must be within 1 mile of Bollgard fields, preferably within ½ mile);
3) A 5% embedded refuge (must be planted as a continuous block within the Bollgard field and must be must be at least 150 feet wide, but preferably 300 feet wide).

"Structured" refers to the management of the refuge, in which the non-Bt cotton acreage is planted using similar hybrids and agronomic practices as the Bt cotton portion. Similar management of the refuge and Bt crop is important to ensure that susceptible moths from the refuge emerge at the same time and in close proximity to any potentially resistant moths from the Bt field, thus increasing the probability of random mating.

Shortly after the 2001 reassessment, registration was approved for Bollgard II Bt cotton (524-

522). Bollgard II is a "pyramided" event, expressing both the Cry1Ac and Cry2Ab2 Bt toxins. A pyramided transgenic crop can offer advantages for IRM in that multiple toxins are expressed simultaneously. This can decrease the likelihood of pest resistance development, since an insect resistant to one toxin will still be susceptible to the other (provided there is no cross resistance potential between the toxins). Like Bollgard, Bollgard II is targeted primarily at TBW, CBW, and PBW, though with the combination of lepidopteran-toxic proteins, Bollgard II is considered to be a "high dose" for all three pests. Since, Bollgard II exceeds the dose and toxin profile for Bollgard, the same refuge options detailed above were also applied as terms and conditions of the Bollgard II registration.

CBW Alternate Host Analysis

As an additional term and condition of the Bollgard II registration, Monsanto was required to investigate whether alternate (non-cotton) hosts can serve as refuge for CBW. There were concerns that the 5% unsprayed refuge option might not be sufficient for CBW, considering that the pest is typically less susceptible to Bt toxins. It is well known that CBW is highly polyphagous and utilizes a wide range of plant hosts. However, it was unknown whether these hosts might serve as an "unstructured" refuge for large scale planting of Bollgard and Bollgard II cotton. In response to the term and condition, Monsanto submitted an interim and final report on non-cotton CBW refuge to the Agency that have been reviewed by BPPD (see BPPD 2004a).

To assess the suitability of non-cotton hosts, Monsanto conducted a series of experiments during 2002 and 2003 focusing primarily on four alternate CBW hosts: peanut, sorghum, field corn and soybeans. The experiments consisted of a CBW sampling program on the various host crops, a "C_3/C_4" host determination analysis on adult CBW, an analysis of cropping patterns, and satellite mapping of alternate crop hosts. From these data, Monsanto was able to determine CBW production in various crop hosts at different points in time during the growing season.

CBW adults were collected in pheromone traps positioned around the various host crop fields in Arkansas, Louisiana, Mississippi, Georgia and North Carolina. Larvae were also sampled to determine which crop hosts supported larval CBW populations at any given time. The "C_3/C_4" analysis was based on the ratio of ^{13}C to ^{12}C found in the wings of sampled CBW, which can be used to determine which host plants were utilized by the insect during development. The ratios differ for "C_3" and "C_4" plants: C_3 plants include cotton, peanuts, clover, and soybeans; while C_4 hosts include corn and sorghum. Wings from sampled moths were sampled using the "C_3/C_4" assays and compared to known standards. To supplement the sampling and host origin experiments, satellite mapping of the regions (1 or 10 miles) around the traps was also conducted.

Monsanto's analysis showed that CBW larvae were typically found on corn early in the growing season (early June) and on other crops starting several weeks later. CBW presence on sorghum and cotton was detected mid-June/early July, while on sorghum and peanuts populations were observed in mid-July/August. Overall, CBW populations from the alternate crop hosts overlapped occurrence of all CBW generations on cotton and that larval population numbers were comparable between the crop types. From the CBW adult trap captures, similar numbers of adult CBW were observed at all the different crop interfaces in a given state. The comparable numbers captured at each crop interface suggest that the size of the adult CBW populations captured in the traps is not a function of the local host crops. This is because the timing and the number of adults captured is greater than what would be predicted based on the adult emergence numbers from these host crop fields. Monsanto concluded that the CBW adults must be moving broadly across the landscape in search of alternative host crops, corn in the early season and other crop hosts later in the season.

The C_3/C_4 assays confirm the literature on CBW host use and the qualitative predictions made based on the larval populations. Early in the season, the CBW moths collected come from a mixture of C_3 and C_4 plants (20-80% from C_4 hosts). By late June, the CBW moths collected come nearly all from C_4 sources, and remain constant until early to mid August at which point the percentage of CBW moths coming from C_4 hosts declines to around 20-50% by late August. The percentage of moths from C_4 hosts then rose again in the final month of the season to a maximum of 50-80%. These data demonstrate that C_4 alternative hosts (primarily corn and sorghum) are making a significant contribution to the CBW adult population throughout the season. The C_3/C_4 data could not be well correlated with predictions based on the spatial data obtained from satellite imagery. This was likely because the scale of CBW adult movement exceeds the 10 mile range used and that CBW may be migrating from Mexico/south Texas and/or originating from weedy hosts.

Based on the CBW studies, it was concluded that both C_3 and C_4 alternative hosts serve as an unstructured refuge that exceeds the currently required 5% external, unsprayed structured non-Bt cotton refuge option (the smallest refuge choice available). CBW moths are produced on alternative hosts in sufficient numbers throughout the cotton growing season to mate with any putative resistant CBW moths emerging in Bollgard or Bollgard II cotton fields and dilute resistance. For a full discussion of these studies and the BPPD's scientific conclusions, refer to the full review (BPPD 2004a).

Summary of Monsanto's Submission

The data and support materials submitted by Monsanto in their amendment request for Bollgard II are contained in three volumes:

1) "Production of Tobacco Budworm from Alternate Host Plants and the Role of These Host Plants as Natural Refuge for Bollgard II Cotton" (MRID# 467172-01)
2) "Modeling the Impact of Natural Refuge on the Evolution of Tobacco Budworm and Cotton Bollworm Resistance to Bollgard II Cotton" (MRID# 467172-02)
3) "Scientific and Economic Justification for Not Requiring Structured Cotton Refuges for Bollgard II Cotton in the U.S. Cotton Belt from Texas to the East Coast" (MRID# 467172-03)

Overall, the TBW studies and analyses follow the general framework that was utilized for CBW alternate host assessment (summarized in the background section). The substance of the TBW submission can be broken down into six categories: 1) pest biology of TBW as it relates to IRM and refuges; 2) sampling and experiments conducted by Monsanto to determine the origin (host plants) of TBW; 3) a spatial analysis of cropping patterns; 4) a calculation of TBW effective refuge (cotton and alternate hosts) and the use of resistance models incorporating the effective refuge data; 5) the proposed revision to the Bollgard II IRM plan and a summarization of the scientific and economic rationales for the changes. Each separate category will be reviewed independently in this document.

In response to comments from BPPD, Monsanto submitted supplemental information in two letters to Dennis Szuhay (branch chief, Microbial Pesticides Branch, BPPD) dated April 3, April 7, and May 10, 2006. Relevant information from these letters is also included in this review.

I. Pest Biology of TBW

Monsanto's submission included a literature review of TBW and CBW biology compiled by Dr. John H. Benedict (Texas A&M University). The white paper covers life history, utilization of host plants, dispersal/migration, and genetic diversity of TBW and CBW biology (references are summarized in the paper). It is noted that BPPD previously reviewed the biology of the major cotton pests in the 2001 Bt crops reassessment (see EPA 2001); therefore, only the topics directly relevant to IRM, Bt cotton refuge, and the utilization of alternate host plants will be discussed in this review.

Regarding TBW and Bt cotton refuge, Monsanto has identified two primary biological topics of importance: 1) the range of host plants utilized by TBW, and 2) adult movement (i.e. dispersal and migration). Both are important variables in terms of determining whether alternate host plants (non-cotton) can provide adequate refuge for Bollgard II cotton. Assessing the TBW host range can provide an indication of whether non-cotton hosts will be able to produce sufficient numbers of Bt-susceptible adults to dilute any resistance genes arising from Bollgard II cotton fields. Movement is critical for proximity issues (i.e. the likelihood that Bt-susceptible TBW originating from non-cotton sources will be available and proximate to mate with potentially resistant individuals emerging from Bt cotton fields). Both topics were addressed in detail by Dr. Benedict in Monsanto's submission and are summarized below.

a) Host Plant Range of TBW

It is well known from the literature that TBW is a polyphagous insect, utilizing a wide range of crop hosts, vegetables and weeds. Its preferred host has been tobacco, though as an opportunistic pest it readily infests other host plants. The extent to which TBW utilizes plant hosts depends on a number of factors, including agricultural/cropping practices (i.e. landscape mosaics), land use patterns, natural plant fauna, climate, and seasonal variability (e.g. weather, changing cropping patterns, etc.). In addition, as a polyvoltine insect (4-6 generations per growing season), TBW will utilize a succession of crop and plant hosts within each growing season.

The sequence of host utilization by TBW is dependent on geography and available plants. Early season TBW (first generation, March to June) are often found on weeds or sometimes on the seedlings of cultivated crops. The specific hosts vary by state; for example, clover serves as a effective TBW host in the Mississippi Delta region, while alfalfa may also play a role in South Carolina and Georgia. Second and third generation TBW (occurring from June through August) frequently move to cultivated crop hosts. Wild and cultivated tobacco is the preferred host for TBW (common in the Carolinas), though in the Delta region cotton is thought to be a primary host in the absence of tobacco. Other crops (including soybean, peanut, and tomato) or weeds (velvetleaf, deergrass, and others) also provide suitable hosts. The overwintering generations (fourth or later, occurring after August) also utilize a range of available hosts including tobacco, cotton, soybeans, peanuts, alfalfa, and some weeds. Table 1 below is a general summarization (though not complete) of known patterns of host utilization by region through the growing season.

Table 1. TBW Host Range Succession by Selected Regions (Created from information in the white paper "Biology and Dispersal of the Bollworm and Tobacco Budworm in North America" by Dr. John Benedict; contained in MRID# 467172-01)

	1st generation (March - early June)	2nd/3rd generation (June - August)	4th + generation (August - October)
Carolinas	Wild hosts: deergrass, beggarweed, morning glory, prickly sida, bicolor lespedeza, toadflax, Desmodium spp., Ipomoea spp.	Crops: tobacco, cotton, peanut, soybean Wild hosts: deergrass	Crops: regrowth cotton, peanut, soybean, tobacco Wild hosts: deergrass, beggarweed, morning glory, prickly sida, others
Mississippi Delta	Wild hosts: clover, velvetleaf, vetch, geranium	Crops: cotton, soybean, some vegetables	Crops: cotton, soybean Wild hosts: beggarweed, burcucumber, empress tree, spider flower, prickly sida, Royal Paulownia, others
Texas	Wild hosts: paintbrush, bluebonnet, vetch, wild tobacco Crops: alfalfa	Crops: cotton, tomato	Crops: alfalfa, tomato Wild hosts: Amantillo, hillside verbena, wild tobacco

It is important to note that data on TBW host utilization throughout the growing season are limited, often lacking definitive quantitative analysis. Much of the host range information contained in Dr. Benedict's report was based on dated or anecdotal observations. There is likely to be high variation to the hosts and use patterns listed above and many additional wild hosts may exist. More thorough analyses are needed to fully assess the extent to which TBW uses alternate and wild hosts for development.

There is also significant uncertainty regarding the suitability of the various plant host types to produce significant numbers of TBW adults. Though TBW infests a wide range of plants, some hosts are likely to be better than others in terms of adult production since TBW population densities are known to vary between production areas and host plant species. Clearly both tobacco and cotton are productive hosts (and preferred by TBW), considering the management needs for the pest on these crops. However, for cotton and other cultivated crops, insecticide use may play a major role in reducing TBW production. In terms of other plant hosts, such as non-cultivated plants and weeds, there is far less information on their contributions to the overall TBW population.

Some comparative analyses have been preformed in some states regarding TBW adult production (references summarized in the Benedict white paper). In North Carolina, for example, tobacco was shown to be by far the dominant producer of TBW larvae per acre, with cotton, peanut and soybean contributing far fewer insects (insecticide use is thought to curtail production in non-tobacco crops). On the other hand, in South Carolina (with similar cropping patterns), cotton produced more TBW per acre than tobacco and other cultivated crops. A study of wild host plants in Georgia and Florida found high production of TBW on Japanese honeysuckle, clover and wild geranium. Other studies in Mississippi found that geranium is an important early season producer of TBW (before the second generation moves to cotton) and produces more larvae per acre than clover. Royal Paulownia and garbanzo beans have also been identified as potentially major producers of heliothine larvae in Mississippi. In Texas, cotton is thought to be the greatest TBW producer, though some may are also produced by tomato, wild tobacco, and other weedy hosts.

There are also some indications that widespread adoption of Bt cotton has had an impact on TBW numbers across planting landscapes. One of the studies noted in the white paper demonstrated that in one Mississippi county, overall abundance of TBW dropped (from 2.2 males/acre to 0.77 males/acre) over the seven year period after Bollgard cotton was first commercialized.

b) TBW Movement and Dispersal

TBW, like other heliothines, is capable of significant movement during adulthood. Moth movement has been characterized in three different ways: 1) short range movement (most frequent movement, usually 300 - 3000 feet, typically within or near plant canopy for mating, oviposition, feeding, or sheltering); 2) long range movement (less frequent, 1 to 10 miles, occurring above the plant canopy for purposes of finding host plants, movements between emergence and egg laying sites); 3) migratory movement (least frequent movement, wind-based, 10 to 300 miles, flights occurring to a mile above the plant canopy). Movement can result from a number of factors, including: 1) loss of habitat (due to crop and host plant cycles as the growing season progresses); 2) population density (increases in density that might challenge the limits of existing resources); 3) environmental effects impacting TBW survival or reproduction.

As with TBW host plant utilization, there is still significant uncertainty regarding movement. For example, as Dr. Benedict points out, the proportion of TBW engaging in short range, long range, or migratory movement is unknown. However, some studies on the three types of movement (mainly using mark and recapture techniques) have been conducted and are described in the white paper.

The mark-recapture experiments certainly demonstrate the abilities of TBW as strong fliers, capable of flights spanning many miles. Such flights can range over 20 miles, last over 5 hours in duration, and occur at altitudes of up to 3,000 feet at wind-aided speeds of 30 mph or more. The typical TBW may not travel such distances, though, as one study found average lifetime movement to be 6 to 12 miles. In fact, there is some speculation that migratory movements of TBW may occur less frequently than with CBW. Overall, TBW movement is greatest early in the season, as they move through the landscape from overwintering sites to available plant hosts. There is also likely a significant early season influx of TBW from southern sites in Mexico to the United States based on weather and air movement patterns. Later in the season (towards fall), TBW have been observed to migrate southwards towards Texas and Mexico, again based on favorable weather conditions.

Long distance TBW movement is a critical component of gene flow between populations. TBW have fairly genetically homozygous populations from Mexico through the U.S., largely due to migratory movements and interbreeding. Understanding gene flow is important for IRM, particularly how genes for Bt susceptibility and resistance move through populations. For example, with TBW pyrethroid resistance, a seasonal pattern has been observed. Early in the season, resistance is low (a period of high gene flow), while during the cotton season (period of low gene flow), resistance increased.

BPPD Review (Pest Biology)

Dr. Benedict's white paper provides an excellent summary of the current knowledge base for TBW biology. However, the paper also identifies major gaps in the understanding of how TBW utilizes available resources and moves through its environment. As Dr. Benedict points out, much of the information is anecdotal or was derived from a limited geographical region over a short time period (i.e. a "snapshot" of local TBW populations).

Despite the range of information on TBW host plants and potential production of moths, there has been no assessment of these hosts might function specifically as refuge (i.e. a source of Bt susceptible moths) for Bt cotton. The goal of a refuge in IRM is to provide a source of susceptible insects to be available to mate with any resistant individuals emerging from Bt fields, thus diluting the frequency of resistance genes in the population. There are three major considerations for a refuge to function properly: 1) susceptible insect production (i.e. the numbers of moths produced by the non-Bt refuge relative to resistant survivors in the Bt field) – a 500 (susceptible) to 1 (resistant) ratio has been used as a target production goal for a non-Bt refuge (as recommended by the 1998 SAP); 2) proximity (i.e. the distance between the refuge or source of susceptible insects and the Bt field) – susceptible insects must be close enough (or mobile enough) to emerging resistant insects to have a reasonable probability of finding and mating with them; 3) synchrony (i.e. emergence "windows" of resistant insects from Bt fields and susceptible insects from refuge sources) – there must be sufficient overlap between susceptible and resistant emergence to ensure a high probability of random mating. A structured refuge (i.e. a non-Bt portion of the crop) is specifically designed to address these three criteria to minimize the likelihood of resistance development. If an unstructured refuge (e.g. alternate and/or natural plant hosts) function in a similar manner, then these three topics should also be considered.

In terms of the current knowledge of TBW (as provided by the white paper), it appears that alternate plant hosts may provide sufficient production (criteria #1 above) to serve as a source of Bt cotton refuge. This follows from the highly polyphagous nature of TBW and wide range of host plants utilized at different points in the growing season. However, additional information is needed to confirm this hypothesis. For example, in some regions (e.g. the Mississippi Delta) TBW are "funneled" through one preferred host (cotton) with seemingly little use of alternate hosts. As for the other refuge criteria (proximity and synchrony), far less is known for the potential natural refuge. Information on TBW movement is limited, particularly non-migratory movement between hosts that could quantify the availability of Bt-susceptible TBW near Bt cotton fields. Also, the timing of TBW development on non-cotton hosts compared with cotton hosts will have to be addressed to ensure that there is sufficient overlap.

Monsanto has also considered the data gaps for TBW and has designed experiments to address these questions. The knowledge gained from these experiments and other information on TBW pest biology will be discussed and integrated into the subsequent sections of this review that describe the analysis of alternate hosts as potential refuge.

It should also be noted that one of the more interesting studies noted by Dr. Benedict was the work from Monroe county, Mississippi by Dr. John Schneider. This study, conducted over seven years, observed landscape production of male TBW before and after the introduction of Bollgard cotton. After Bollgard was introduced, TBW production dropped from 2.2 per acre to 0.77 per acre (Schneider 2003). Also, in recent resistance monitoring reports for Bt cotton, smaller TBW populations have been sampled, possibly due to a suppressive effect of Bt cotton (see BPPD 2005). Such a suppressive effect due to large acreage of Bollgard cotton could conceivably reduce the overall numbers of susceptible TBW available (as part of a natural refuge strategy) and should be addressed as part of proposed IRM revisions.

II. Experiments to Assess TBW Alternate Hosts and Natural Refuge

In consideration of the limited TBW biological information available, Monsanto designed a large-scale experiment to investigate the potential of alternate host plants to severe as refuge for Bt cotton. The two year experiment, conducted in 2004 and 2005, consisted of two major components: 1) a TBW sampling program in cotton growing regions with large cotton acreage, and 2) bioassays based on detection of gossypol in sampled TBW to determine the plant host origin (i.e. cotton vs. non-cotton). Gossypol is a natural toxin found specifically in cotton that is bound and metabolized in cotton-feeding moths. Data obtained from this experiment were then used to calculate "effective refuge" from alternate hosts and subsequently incorporated into IRM models. The results were also compared with known cropping patterns near sampling sites using spatial analyses. The use and interpretation of the experimental data will be described later in this review.

a) TBW Sampling

Monsanto's TBW sampling program focused on seven cotton growing states: Arkansas, Georgia, Louisiana, Mississippi, North Carolina, Tennessee, and eastern Texas (2005 only). All of these states are characterized by large cotton acreage, with at least 480,000 acres of cotton in each state. Texas (5,710,800 acres), Georgia (1,256,050), Mississippi (1,026,500), and Arkansas (900,200) are the four largest cotton producing states in the country (2004 acreage figures, obtained from Monsanto's report). These 7 states are also well within the known geographical range of TBW.

For each state, three to eighteen counties were selected each season for TBW sampling. The counties were chosen based on density of cotton acreage, with each county having 15-24% of land use devoted to cotton planting. Bt cotton acreage was also high in the sampled counties, with an average adoption of more than 70% Bollgard or Bollgard II (based on 2004 sales data). Sampling sites in each state are detailed in the following table.

Table 2. TBW Sampling by State for Gossypol Analysis (reprinted from Table 4 in Monsanto's submission, MRID# 467172-01).

State	Sampling locations (by county)	
	2004	2005
Arkansas	3 (Drew, Little River, Mississippi)	3 (Craighead, Drew, Mississippi)
Georgia	5 (Decatur, Dooly, Mitchell, Seminole, Terrell)	6 (Appling, Decatur, Dooly, Mitchell, Terrell, Tift)
Louisiana	5 (Bossier, Catahoula, Franklin, Rapides, Tensas)	5 (Bossier, Catahoula, Franklin, Rapides, Tensas) note – TBW collected in only 3 of the counties
Mississippi	18 (Bolivar, Carroll, Chickasaw, Clay, Coahoma, Grenada, Humphreys, Itawamba, Lee, Leflore, Lowndes, Madison, Monroe, Noxubee, Prentiss, Tunica, Washington, Yazoo)	16 (Bolivar, Carroll, Chickasaw, Clay, Coahoma, Humphreys, Itawamba, Lee, Leflore, Lowndes, Monroe, Noxubee, Prentiss, Tunica, Washington, Yazoo)
North Carolina	3 (Lenoir, Pitt, Wilson)	5 (Edgecombe, Halifax, Lenoir, Pitt, Wilson)
Tennessee	10 (Carroll, Crockett, Dyer, Fayette, Gibson, Haywood, Lake, Lauderdale, Madison, Tipton) note – too few TBW were collected to be analyzed during 2004	10 (Carroll, Crockett, Dyer, Fayette, Gibson, Haywood, Lake, Lauderdale, Madison, Tipton)
Texas	None	4 (Austin, Burleson, Fort Bend, San Patricio)

The sampling within each state was conducted by pheromone traps, which capture adult TBW. The traps were usually placed next to cotton fields and were monitored weekly throughout the cotton growing season (April through October, depending on the state). Within each state, 4 to 42 traps were deployed across the sampled counties (generally, states with more sampled counties had a greater number of traps). A total of 73 traps were used in 2004 (traps in Tennessee were not included due to low numbers collected) and 157 in 2005. From these traps, 17,282 moths were collected in 2004, of which 3,385 were used in the gossypol analysis. In 2005, a total of 17,204 adults were sampled including 5,243 that were used in the analysis. Trap collections were high (exceeding 50 per trap) in Georgia and North Carolina in 2004 and in all states except Tennessee in 2005. Lower collection numbers were observed in Arkansas, Louisiana, and Mississippi in 2004 and Tennessee during 2005.

BPPD Review (TBW Sampling)

Monsanto's sampling strategy has addressed the major areas of TBW habitat as it relates to cotton production. Some cotton-growing states in the southeast, were not sampled, including Alabama, South Carolina and Florida. However, these states have either little TBW pest pressure (Alabama) or relatively little cotton acreage (South Carolina and Florida). Generally, the states with large cotton acreage (e.g. Mississippi) received greater numbers of sampling sites (counties) and traps. On the other hand, some states with large cotton acreage (e.g. Georgia) were given fewer traps (<10) over the two year sampling period. In addition, Texas was only sampled during the 2005 growing season. Monsanto targeted major cotton-growing counties for sampling within each state, though the submission did not provide a specific breakdown of the sampling locations within each county (a total number of traps per state was provided instead). From a reading of the statistical analyses of the data it appears that most counties had one trapping location, although some of the sampled counties (e.g. Drew, Arkansas) had up to 24 traps. It is unclear if the number of traps used per county was correlated to the density of cotton acreage within those counties.

Traps were placed in proximity to cotton fields, though the report did not specify whether these were Bollgard fields, conventional (non-Bt) cotton fields, refuge fields, or a combination of the three. If traps were placed exclusively next to Bollgard fields, it is possible that trap captures (and the proportion of gossypol positive moths) could have been lower due to the suppressive effects of high dose Bt cotton. However, since refuges are required to be planted within ½ mile of Bt fields, it is likely that susceptible TBW produced by the refuges would have been included in the sampling. This could also have created a "worst case" scenario of a landscape with high adoption of Bt cotton in which the only trapped gossypol positive TBW originated from planted refuges. On the other hand, if trap deployment was near conventional cotton fields only (with little Bt cotton nearby), the sampling could have produced larger numbers of gossypol positive TBW that might not have occurred with larger Bollgard acreage.

The submitted report provided only a cursory description of the trapping techniques, although some supplemental information on the TBW trapping was provided by Monsanto in response to questions from BPPD (letter to Dennis Szuhay, dated April 3, 2006). All TBW were collected as adults using traps baited with a sex pheromone. The lure used in the traps is a commercial product, "Hercon Luretrap Tobacco Budworm," that is highly attractive and specific to TBW males (no females were sampled or tested for gossypol). The range of attraction of the pheromone lures used with the traps was not discussed (i.e. the ability of the attractant to pull moths in from some distance away), though pheromone trapping is typically used to assess pest populations on a local scale. Also, given the proclivity of TBW moths to move in the environment (see Pest Biology section), it may be reasonable to assume a high degree of mobility in and around cotton fields (a valuable resource for TBW). The area mapping/spatial analysis project (described later in the review) also provided some information on the host plant fauna occurring around the trapping locations.

BPPD also notes that the sampling project encompassed only two growing seasons (2004 and 2005). It is uncertain whether this is a sufficient sampling period to be able to fully assess and quantify the productivity of alternate plant hosts over the large regions of TBW habitat included in the survey. As Dr. Benedict notes in the provided white paper (referenced from Kennedy and Storer 2000), "annual cropping systems are rapidly changing mosaics of plant species, plant phenologies and physical environments through the year – with no two years being identical." Given the natural variability in the natural and agronomic ecosystems, a two year study may present only a "snapshot" of the host availability and productivity for TBW that may not be representative of future conditions. However, it is noted that a similar sampling approach previously taken for CBW (described in the Background section) was also conducted over a two year period and found to be acceptable.

b) Gossypol Bioassays and Analysis (Methodology)

To determine the origin (i.e. host plant) of TBW adults captured in pheromone traps, Monsanto utilized a bioassay to detect the presence of gossypol. Gossypol is a naturally occurring toxin that is found exclusively in cotton plants that functions as a plant protectant with insecticidal activity. Therefore, a bioassay designed to detect gossypol in captured TBW should distinguish adults that arose on cotton from those that originated on other plant hosts. Once ingested by cotton-feeding TBW, gossypol is metabolized and bound. The bound form can then be extracted and detected using a High Performance Liquid Chromatography (HPLC) technique.

Monsanto employed a sequential sampling scheme for testing the collected moths based on the numbers obtained in each trap. For trapping locations in which large numbers of moths were collected, subsets of moths (sets of 10 per trap sampling date) were analyzed by HPLC. For smaller samples, up to 100% of the collected moths were tested. If the gossypol testing results from individual trap locations determined that TBW from non-cotton sources were less than 10%, additional moths were tested.

The gossypol bioassay system was first tested with laboratory-reared TBW that were raised on host plants of known origin. These hosts included cotton, tobacco, soybean, pea, and velvetleaf. Assays were run to determine the level of accuracy with false positives and negatives. The calibration runs detected 100% of moths that originated on cotton (i.e. positive for gossypol), while of the TBW from non-cotton hosts, 81% were found to be negative for gossypol. These results indicate that there may be some degree of false positives, which could inflate the numbers of sampled moths estimated to have originated from cotton.

The gossypol HPLC bioassays were conducted in 96-well plates using a number of positive and negative controls. Positive controls consisted of a calibration standard (known gossypol concentrations) and TBW known to have developed on cotton while negative controls included blank wells (with buffer solution and gossypol-negative extractions) and TBW from non-cotton hosts. Criteria were established for rejecting test runs based on abnormal response ranges for the control standards. Gossypol was extracted by forming a Schiff's base with aniline to create a dianilino-gossypol complex which can be detected by HPLC.

While the gossypol analysis can determine whether TBW developed on cotton, it is unable to differentiate between non-cotton hosts. Therefore, Monsanto developed a separate analytical method to ascertain whether non-cotton TBW developed on tobacco, a highly preferred host in some parts of the insect's range. This test was designed for use with moths collected in North Carolina, a state with high tobacco acreage and large TBW numbers. The assays are based upon the detection of cotinine (a nicotine derivative) using gas chromatography-mass spectrometry (GC-MS) techniques. Moths that test positive for cotinine can be assumed to have developed on tobacco plants based on uptake of nicotine during feeding. As with the gossypol tests, the procedures were evaluated for false positives and negatives, and none were observed. The cotinine assays were conducted using the same general procedures as the gossypol tests (i.e. the same type of control groups) with a subset of moths trapped at locations in North Carolina during the 2004 growing season. Moths subjected to cotinine testing could also be subsequently tested for gossypol (the two processes did not interfere with each other).

BPPD Review (Gossypol Bioassay Methodology)

Monsanto has provided an adequate description of the techniques used for the gossypol and cotinine analyses, though several details were omitted. For example, details of the gossypol extraction were not provided (e.g. how the moths were handled and shipped, where the testing was conducted, and how the extractions were accomplished). Monsanto also did not supply any information on the number of test runs that were rejected due to abnormal performances of the control groups. Such information could give an indication of the precision of the testing techniques, but is not critical for the overall analysis. It is also noted that a more complete description of the gossypol methodology is contained in a draft journal article (Orth et al. *in draft*) that was included in a supplemental submission (Monsanto letter to EPA dated April 3, 2006).

Since the pheromone traps used to sample TBW adults were attractive to males only, no females were included in the gossypol bioassays. Therefore, it was not possible to determine if response trends vary by sex. Since host utilization is a function of oviposition (i.e. the location the female selects for egg laying), there would not likely be variation between sexes in terms of the gossypol results had females been included. On the other hand, as described in Dr. Benedict's white paper, female longevity, fecundity, and reproductive fitness can be influenced by the quality of the host plant. In addition, patterns of movement may be different for females, particularly during and after mating. Prior to mating, females find suitable host plants and release sex pheromone to which the males respond. After mating, females are known to retain spermatophores throughout their lives and may potentially move significant distances for egg laying. The trapping system employed by Monsanto essentially mimics the male component of TBW reproductive behavior, while not fully accounting for the behavior of females. The effect could be a bias in the trap captures – a scenario in which males are present in the trapping locations at greater numbers than females. This could be an important consideration, since the traps are designed to provide a "snapshot" of the TBW population in and around cotton fields. If there is a difference between the numbers of males and females, it is conceivable that the level of random mating between any resistant survivors of Bt cotton and susceptible moths from alternate host plant refuges could be affected. BPPD also notes that the June, 2004 Scientific Advisory Panel (SAP) that assessed IRM considerations for Bt cotton expressed concern regarding the use of pheromone traps that sampled only male CBW (SAP 2004). The SAP stated that the "traps do not provide a meaningful measurement of adult productivity." Based on these concerns, it is recommended that Monsanto address any potential bias in the results that may arise from sampling only male TBW.

The cotinine analysis is a useful tool that should further help characterize the host plant origins of TBW in areas where tobacco and cotton (both are preferred hosts) are grown in proximity. It is noted that nicotine is also produced at lower levels in other nightshade plants that may serve as TBW hosts including tomatoes and green peppers. However, considering the lack of false positives and negatives observed in the preliminary assays, it is unlikely that those plants biased the results.

c) Gossypol Bioassays and Analysis (Results)

The results from the 2004 and 2005 gossypol TBW host origin testing were analyzed and reported independently, although the data patterns were generally consistent in both years. Within each growing season, Monsanto presented the data at the county level (i.e. incorporating all trap locations within a county) as well as by state (pooled from all sampled counties). A further break down of the county level data by trapping location was provided in the statistical analyses (see description later in this section).

Overall, cotton and non-cotton host utilization in TBW was observed to follow seasonal trends in the states tested during the two year experimental period. Early in the growing season (April to June), TBW were found to develop almost exclusively on non-cotton hosts (i.e. close to 100% of tested TBW were negative for gossypol). These results were not unexpected, considering the early season wild host usage that has been previously observed with TBW (see the Pest Biology section). As the growing seasons progressed (July and August), the proportion of TBW developing on cotton (i.e. those testing positive for gossypol) increased before generally leveling off later in the season (September). The exact number of TBW developing on cotton was variable, depending on the collection region. In the eastern states (Georgia and North Carolina), production on non-cotton hosts was much higher than in Delta states (Mississippi, Louisiana, Arkansas) (see the summary by state in table 3 below).

Table 3. Gossypol Bioassay Results by Year and State (Compiled from Figures 2A - 2E and 3A - 3E in Monsanto's submission, MRID# 467172-01).

State	Year	# TBW tested (total)	Percentage of TBW originating on non-cotton hosts [1,2]				
			April/May	June[3]	July	August	Sept/Oct
Georgia	2004	896	---	(n<10)[2]	81.3 - 100	78.3 - 100	100
	2005	544	98.0 - 100	100	100	100	---
North Carolina	2004	684	---	---	98.2 - 100	91.6 - 97.2	72.9 - 88.5
	2005	1392	84.0 - 88.9	(n<10)[2]	100	96.2 - 100	84.6 - 96.0
Arkansas	2004	399	---	95.9 - 98.0	50.0 - 100	20.0 - 92.3	86.0
	2005	1216	25.0 - 93.1	99.3	41.4 - 76.4	25.7 - 44.8	22.2 - 25.0
Louisiana	2004	417	---	100	69.2 - 85.7	7.0 - 54.8	46.7 - 63.4
	2005	245	(n<10)[2]	100	38.5 - 90.0	24.1 - 51.0	35.0 - 56.0
Mississippi	2004	989	---	---	18.8 - 90.9	36.4 - 93.5	22.2 - 70.9
	2005	1076	100	94.2 - 100	54.0 - 100	20.5 - 100	15.4 - 80.0
Tennessee	2004	0	---	---	---	---	---
	2005	82	(n<10)[2]	(n<10)[2]	(n<10)[2]	50.0 - 81.3	---
Texas	2004	NT	---	---	---	---	---
	2005	688	93.8 - 100	18.2 - 95.0	14.3 - 61.5	17.6 - 59.7	24.0 - 63.3

[1] Results are given as a range of the surveyed counties for each monthly trapping period.
[2] Counties with sample sizes of less than ten were not included in the ranges (no confidence intervals were created for samples of less than ten).
[3] Data from 2004 are for May/June trapping period.

The complete host origin data for each state and year surveyed (pooled by state or for individual counties) are found in figures 1, 2, and 3 from Monsanto's submission (MRID# 467172-01). Copies of these figures are included in appendix 1 attached to end of this review.

As can be seen from the ranges included in table 3 above and in the data summarized in Monsanto's figures, there was frequent variability in the results by state, county, year, and month (county level variability is reflected in the ranges for each collection month). As previously discussed, the proportion of TBW originating on non-cotton hosts was consistently high in the eastern states of Georgia and North Carolina. Non-cotton TBW typically accounted for greater than 90% of those sampled throughout the growing season. These states typically have large tobacco (a highly preferred TBW host), peanut, and soybean acreage which may help account for the large non-cotton contribution. In the other surveyed states, the non-cotton contribution was much more variable, particularly in the mid to late season when cotton may be available as a preferential host. For the Mississippi Delta (Arkansas, Louisiana, and Mississippi), the proportion of non-cotton TBW is sampled counties was frequently below 50% after June, though was almost always at least 20%. In Tennessee the testing was confounded by low trap captures which limited the number of moths that could be tested. In the one month (August, 2005) in which significant numbers from Tennessee could be tested, there was high variability among the sampled counties. Like Tennessee, TBW populations from Texas (tested in 2005 only) also displayed wide and variable ranges of non-cotton-origin moths, though the overall non-cotton contribution was lower than in most of the other tested states. Monsanto noted that the variability in Tennessee, Texas, and the Delta states may be due to the lack of alternate host crops preferred by TBW for development. In Texas, the results may have also been affected by differing cotton phenology and TBW population dynamics.

The trends between seasons (2004 and 2005) were generally similar, although there were some notable differences (figure 1 tracks the state level data for each season). In both years, the results from Georgia and North Carolina were the most consistent, with average non-cotton contribution of 90% or more throughout the growing seasons. Likewise, in the other states, early season (April/May) non-cotton TBW proportions were consistently high in both years (> 85%), with one exception in Arkansas during 2005. Monsanto suggested that there may have been some TBW in Arkansas that developed on cotton the previously year and then emerged locally from diapause. Towards the middle of the season (July), the percentage of non-cotton TBW noticeably dropped in both years. This drop continued into August, before leveling off in September and October, though during 2004, the non-cotton percentages actually increased substantially in two Delta states (Arkansas and Louisiana). This dramatic late season increase was not observed during 2005, however, as the non-cotton percentages remained relatively flat after August.

In addition to the gossypol testing, Monsanto conducted cotinine testing on 118 TBW collected from three counties in North Carolina (2004 season only) to distinguish individual originating on tobacco. The moths, which were first tested for gossypol, were trapped during July and August in the midst of the North Carolina cotton season. Of the assayed moths, less than 5% had tested positive for gossypol (development on cotton), none of which also tested positive for cotinine. A total of 30% tested positive for cotinine, indicating larval development on tobacco. The remainder (>65%) are likely to have developed on plants other than tobacco or cotton, indicating that alternate hosts play a large role in the production of TBW. Monsanto suggests that this may also be the case in other states without significant tobacco acreage.

d) Gossypol Bioassays and Analysis (Statistical Analyses)

For the statistical analyses of the gossypol data, Monsanto analyzed the data from 2004 and 2005 independently. Within each year, the percentage of cotton-origin TBW was tabulated for each trap by collection date (within each month). As could be expected in such large scale testing, there was variability in the numbers of TBW captured in individual traps. Some traps collected hundreds of moths (of which a subset was tested) while others captured a single moth (traps with no captures were not included).

To analyze the variation in the trapping and gossypol data, Monsanto utilized the Fisher's Exact Test (FET). This type of analysis is typically done to determine statistical significance with categorical data and small sample sizes, as is the case with much of the gossypol sampling data.

The FET analyses were used for two purposes: 1) to determine whether the gossypol data could be pooled by month (i.e. combining all trap dates within the month), and 2) whether the data could be pooled by county (i.e. combining all of the trap data by month within a county). For the pooling by date determination, Monsanto employed a "trap by month" categorization for the data that included all of the collection dates within a month for individual traps. The Fisher's Exact Test could not be run on trap by month samples in which there was only one collection date (degrees of freedom would equal zero) or the percent of cotton-origin moths was zero (marginal total would equal zero). The county pooling determination involved "county by month" figures, which included all of the traps in the county pooled together for the month. As with the date pooling, county by month samples with only one trap or 0% cotton-origin moths could not be analyzed with a FET.

2004 Analysis

For the 2004 data, there were a total of 102 trap by month data sets that could be analyzed by FET. An additional 96 trap by month sets could not be analyzed by FET, due to having only one collection date or 0% cotton-origin TBW. Of the 102 sets that were tested with the FET, five were found to have a p-value of less than the 0.05 level of significance, indicating that date was a significant factor in the gossypol results. For the other 97 sets, the gossypol results did not significantly vary by date. It is noted however, that there were eight other trap by month sets with p-values between 0.05 and 0.10 (i.e. just below the level of significance). Among the 96 sets that were not analyzed by FET, there were 33 that had more than one week of collection data and 0% cotton-origin TBW (the remainder had only one collection date). Since these sets had no variance (all tested TBW were negative for gossypol) over multiple sampling dates, it can be assumed that trap date was not significant for these cases. The 33 sets not tested by FEW were grouped with the ones tested by FET to produce a total of 135 trap by month sets in which there were multiple trap dates. Therefore, in 130 of these 135 sets, the gossypol results were not shown to be significantly different by trap date. Based on the FET outcomes, Monsanto pooled the gossypol data for each trap by month set, including the five cases in which trap date was found to be a significant factor.

The pooled data from individual traps was then analyzed by FET at the county level. For 2004, there were 38 county/month combinations in which data were collected from multiple traps and could be analyzed by FET. An additional 49 counties were not tested with FET because only one trap in the county yielded data or all traps had 0% cotton-origin moths. The FET analyses showed that three of the 38 tested counties had p-values below 0.05, showing significant differences between individual traps in those counties. For the other 35 counties, the gossypol results did not significantly differ between individual traps within each month, though three of these counties had p-values between 0.05 and 0.08 (close to the level of significance). Based on the FET analyses, Monsanto pooled the individual trap data by county for each month in the experiment (including the three counties with p-values below 0.05).

> *2005 Analysis*
>
> The 2005 analysis was carried out in much the same manner as for the 2004 data. In 2005, there were a total of 170 trap by month data sets that could be analyzed by FET, with an additional 304 trap by month sets could not be analyzed by FET, due to having only one collection date or 0% cotton-origin TBW. Of the 170 cases analyzed by FET, there were nine data sets in which date was a significant factor (i.e. p-value <0.05). An additional 10 other trap by month sets with p-values between 0.05 and 0.10 (i.e. just below the level of significance). Of the remaining 305 data sets, those with more than one week of data and 0% cotton-origin TBW (107 total) were combined with the 170 that were analyzed by FET. This created a total of 277 data sets, of which 268 did not significantly vary by date. Based on these results, the data were pooled for each trap within each month (including the nine cases with significant variance).
>
> At the county level, there were 57 county/month combinations in which data were collected from multiple traps within the county that could be analyzed by FET. The other 116 sampled counties could not be tested by FET because only one trap in the county yielded data or all traps had 0% cotton-origin moths. For seven of the 57 counties tested, there was a significant difference in gossypol results between individual traps (p-value <0.05). Of the counties that were not analyzed by FET, 44 county/month sets had multiple traps all of which trapped no cotton-origin moths. These 44 sets were included with the 57 tested by FET, for a total of 101 data combinations. Since only seven of the 101 sets differed significantly, Monsanto pooled the data from individual traps for each county and month.

Based on the pooled data, Monsanto calculated upper and lower 95% confidence intervals for each county and sampling month. Confidence intervals were not calculated for counties in which less than 10 moths were tested for gossypol. The gossypol results and confidence intervals for each county and month were plotted on figures 2 (2004) and 3 (2005) in Monsanto's report (copies of the figures are attached to the end of this review).

An additional analysis was conducted with data pooled at the state level to determine any significant differences between states during the sampling months. In 2004, comparisons were made in July, August, and September (the other months had too few data points for analysis) using FET. The results revealed that most of the state/month comparisons were significantly different, though several "regional" state comparisons did not differ significantly at the 0.05 level. For example, Louisiana and Mississippi were not significantly different in both July and September. For 2005, FET analysis was not run because of the data structure, although 95% confidence intervals were calculated for each state during each sampling month.

BPPD Review (Gossypol Bioassay Results and Analyses)

BPPD agrees with Monsanto's overall conclusion that the gossypol bioassay results from the two year sampling period clearly show that there is a significant contribution of non-cotton hosts to the overall TBW population. The results followed a seasonal pattern in both years that can be explained by the availability of cultivated and non-cultivated host crops in each region tested. There was variability, however, between different regions (states) in terms of the proportion of TBW originating from non-cotton sources.

In North Carolina and Georgia, the proportion of non-cotton-origin TBW was consistently the highest among all of the tested states. The percent non-cotton TBW for these two states was typically greater than 90% (often close to 100%) throughout all of the testing months in both 2004 and 2005. These results seemingly indicate that cotton plays a relatively minor role in the TBW population as a whole, with alternate plant hosts responsible for the production of the vast majority of TBW. Both states contain significant acreage of non-cotton cultivated crops (including soybean, tobacco, and peanuts) that are known to serve as effective TBW hosts. Tobacco, a highly preferred host, may account for an appreciable percentage of the non-cotton-origin TBW, especially in North Carolina. The cotinine analysis, conducted by Monsanto to test for a nicotine derivative, found that 30% of the tested individuals (collected from North Carolina) were likely to have developed on tobacco. In addition to non-cotton crops, it is likely that wild hosts (weeds) also play a large role in supporting TBW populations in these states.

For the other tested states, particularly those in the Delta region (Mississippi, Louisiana, and Arkansas), the gossypol results were more variable, though a general seasonal pattern could be discerned. Like North Carolina and Georgia, non-cotton-origin TBW were prevalent early in the season (i.e. April - June). However, once the cotton growing season was underway, the proportion of the sampled population originating on cotton increased, in some cases dramatically. The specific amount of cotton-origin TBW varied by state and county, though non-cotton-origin individuals usually were a significant factor (at least 20% of the population) except in a few cases. Some counties, such as Madison, MS (18.8% non-cotton-origin TBW in July, 2004), Franklin, LA (7.0% in August, 2004), and Lee, MS (15.4% in Sept./Oct., 2005) had particularly low proportions of non-cotton-origin moths. Alternatively, in other Delta counties the percentage of non-cotton moths was high (over 90%) during the same time periods. Regardless of the variability, these trends clearly show that cotton plays a much bigger role as a TBW host in the Delta states, probably due to fewer available alternate cultivated crops or wild hosts during the cotton season.

In Texas and Tennessee, the results were also variable. Data obtained in Tennessee were confounded by low trapping numbers and gossypol testing was conducted solely in 2005. From the 2005 collections, only collections in August, 2005 produced significant numbers of TBW for testing, which showed a fairly wide range of cotton and non-cotton-origin moths. Likewise in Texas, sampling was only conducted in 2005. The gossypol results during the cotton growing season (July - October) revealed that the majority of sampled TBW originated from cotton. In three counties, the cotton contribution exceeded 80% of the total. As with the Delta states, it is likely that cotton is an important host for TBW for much of the season.

In relating the TBW sampling and gossypol testing to potential natural sources of (unstructured) refuge for Bt cotton, it is important to consider the three major aspects of structured refuge.

These are 1) production of sufficient numbers of susceptible insects to dilute any potential resistance genes, 2) proximity of the refuge to the Bt field to ensure random mating between susceptible and resistant insects, and 3) synchrony of the refuge with the Bt fields to ensure overlapping emergence between susceptible moths and any resistant survivors of the Bt crop. For the most part, Monsanto's experiments have addressed these three criteria.

In terms of susceptible production, the goal of structured refuge for Bt crops has been a ratio of 500 susceptible insects for every resistant insect that could emerge from the Bt field (see EPA 2001). With a high ratio of susceptible to resistant insects, any surviving resistance alleles can be effectively diluted through random mating to mitigate the potential development of a widespread resistant population. Monsanto's TBW sampling/gossypol experiments did not look at susceptible insect production from a numerical perspective per se; rather the proportion of moths originating on cotton/non-cotton sources was assessed (all trapped moths can be assumed to be Bt susceptible). The experiments clearly showed that a portion of the TBW develops on cotton, with another portion developing on non-cotton crops and wild hosts. However, the numbers collected at individual traps were highly variable, with some traps collecting few or no moths and others trapping over one hundred. In some states (e.g. Tennessee) moth captures were erratic and frequently at numbers too low for testing. Low numbers of TBW have been observed elsewhere as well: recent Bt cotton resistance monitoring efforts have been hampered by low collections of TBW, possibly due to a suppressive effect from widespread adoption of Bt cotton (see Blanco 2005 and BPPD 2005). It is noted that the sampling was conducted under the current IRM strategy for Bt cotton that mandates a non-Bt cotton structured refuge that should (theoretically) have supplied a source of susceptible moths. On the other hand, should the IRM strategy for Bollgard II cotton be revised to replace structured refuge (i.e. non-Bt cotton) with natural refuge, the acreage of Bollgard II cotton may significantly increase while overall non-Bt cotton acreage may decrease. Given this scenario, it is conceivable that the portion of TBW developing on cotton (as determined from Monsanto's experiments) could be reduced, leaving mainly the non-cotton-origin moths. It is not clear whether reducing the cotton-origin portion of the susceptible TBW population would have a measurable effect on the ability to dilute emerging resistance genes (the modeling reviewed in section IV will more fully discuss resistance development scenarios using Monsanto's data).

Both refuge proximity and developmental synchrony criteria were directly addressed by Monsanto's experimental design. For proximity, the TBW traps were placed adjacent to cotton fields (both Bt and non-Bt cotton). Though the traps were baited with a pheromone attractant that could attract moths from surrounding areas, the sampling likely presented an accurate portrayal of the TBW population around the cotton field trap sites. Developmental synchrony was also examined by collecting samples throughout the growing season. Data were assessed for each month of the cotton season and a statistical analysis showed that in the majority of cases the cotton/non-cotton proportions did not significantly differ within the individual sampling months. At variable proportions, the results showed that TBW developing on non-cotton hosts overlapped TBW from cotton during the major part of the cotton growing season (July- September) (see figure 1 attached to this review). These results were not surprising: though TBW may develop on different plant hosts at slightly different rates, there are four to six generations per year that likely create a natural overlap (discussion in Benedict 2004). Based on these considerations and Monsanto's data, it can be assumed that TBW from non-cotton hosts will develop in the same time frames as TBW developing on cotton hosts.

For the statistical analyses, BPPD agrees with Monsanto's criteria for pooling data for across dates for individual traps and across traps for individual counties. The statistical tests (Fisher's Exact Test) showed that in the vast majority of cases, the gossypol results did not significantly differ by trap date for single traps or by trap within counties. Given the lack of significant effects of date and individual trap, Monsanto pooled all of the data, including the few cases that were found to be significantly different. It is unclear whether the pooling of the significantly different cases had any bias on the overall data, though given the few examples, it is unlikely that there would be much of an effect.

III. Spatial Analysis of Cropping Patterns in Cotton Growing States

To support the gossypol testing data, Monsanto conducted an analysis of the alternate host distribution around the trapping sites. This spatial analysis was then compared with the observed proportions of TBW originating from cotton and non-cotton hosts for various counties in the sampling project. In addition, Monsanto analyzed USDA/NASS cropping data for a ten year period in the sampled counties to further assess the presence of alternate non-cotton hosts.

The cropping patterns around trap locations were determined by utilizing aerial photographs that encompassed a one mile radius around the traps. Each trap site was photographed at least once during the two year experimental period. From the photographs, various crop and plant types were identified by ground-truthing and spatial analysis software. Of the known TBW crop hosts, cotton (non-Bollgard), soybean, peanut, and tobacco were included in the compilation of cover crops (wild hosts and weeds were not included). Within individual counties, the crop pattern data was averaged together from multiple traps when necessary. The cropping patterns tabulated from these photographs and the non-cotton TBW contributions (from the trapping/gossypol data) were then tested by regression analysis to check for correlations between the plant hosts observed around traps and the cotton/non-cotton-origins of the trapped TBW.

The results of the spatial analysis/land cover estimates as well as the NASS data (2004 season) from a subset of the sampled counties were summarized in Table 9 and 10 of Monsanto's submission (MRID# 467172-01). Overall, non-Bollgard cotton accounted for a sizable portion of the crop acreage in the one-mile trap radius, averaging 9.6% of the cropped area across all counties. In eight of the 20 counties included in the results summary, cotton had the greatest acreage (relative to peanuts, soybean, and tobacco) within the one-mile area. However, the specific cropping patterns varied by county and region. The percentage of cotton was generally higher in Arkansas and Texas, though individual counties in Georgia, Louisiana, and North Carolina were shown to have large cotton acreage (>10% cotton). In Georgia, peanut acreage was abundant relative to the other cataloged crops, while in North Carolina, soybean was planted on the largest portion of the trapping area. Tobacco acreage was recorded only in North Carolina at low levels (<5% of the total acreage in all counties reported).

The 2004 NASS data, compiled at the county level, generally mirrored the crop pattern trends obtained from the one mile trap radius photography. Soybean acreage was significant at the county level, with seven of 20 counties having greater than 10% of the cropped area devoted to soybean production. Non-Bt cotton was grown in all of the counties (except two), though typically at lower levels than soybean. Tobacco was found only in North Carolina at low acreage (< 5% of the crop area) and peanuts were farmed in both Georgia and North Carolina.

As part of the analysis, Monsanto paired the gossypol results (i.e. the proportion on non-cotton-origin TBW) with the crop pattern trends for individual counties. In general, counties with higher percentages of cotton (i.e. >10%) within the one mile radius (e.g. counties in Arkansas and Texas) had higher proportions of cotton-origin TBW. On the other hand, counties with larger plantings of soybean and peanuts (e.g. counties in Georgia and North Carolina) had greater proportions of non-cotton-origin TBW. To test for any statistical correlations between the gossypol results and the observed crop patterns, Monsanto conducted a regression analysis for both the one mile trap radius and county level data (see Table 10 in Monsanto's submission, MRID# 467172-01). The analysis was conducted with the gossypol data from the months of July, August, and September (the major portion of the cotton growing season). At the trap level (i.e. one mile radius), there was no significant correlation between crop type and the non-cotton contribution to TBW, except for tobacco acreage in August. However, at the county level there was a positive correlation between the non-cotton contribution to TBW and tobacco and peanut acreage in all three months tested. This correlation is not surprising, since in both Georgia and North Carolina (where the tobacco and peanut acreage is located), non-cotton-origin TBW accounted for greater than 90% of the trapped population. Cotton acreage could not be positively correlated with non-cotton TBW contribution in any of the tested months, either at the trap or county level.

In addition to the crop pattern analysis described above, Monsanto also compiled USDA/NASS data for the seven sampled states over a ten year period (1995-2004). These data (presented in Figure 4 in Monsanto's submission, MRID# 467172-01) included acreage figures for cotton, soybean, peanut, and tobacco. Despite some year-to-year fluctuations, the acreage for each crop generally remained stable relative to the other crops in the state. For example, although cotton acreage dipped in Louisiana during 2002, soybean acreage also fell that season. However, in other states, some shifts in cropping patterns appear to be visible. In North Carolina, tobacco acreage dropped over the ten year period, while soybean acreage appears to have increased.

Overall, Monsanto concluded that the spatial analysis data could not fully account for the variability in non-cotton-origin TBW observed throughout the gossypol testing (i.e. the captures of cotton-origin TBW in non-cotton growing areas and vice versa). It was suggested that high TBW mobility may explain some of the results; migratory TBW may have moved great distances during the seasons to be trapped at locations far from their point of origin. However, Monsanto indicated that stable cropping patterns (as evidenced by the ten year NASS data) should provide a steady source of non-cotton refuge in the future.

BPPD Review (Spatial Analysis of Cropping Patterns)

The spatial and cropping pattern analysis by Monsanto provides some support for the trends observed in the gossypol data, though the overall conclusions that can be derived from these data are somewhat limited. For example, crop patterns observed in Georgia and North Carolina (large peanut and tobacco acreage) seem to support the large percentage of non-cotton TBW trapped during the experiments. In Texas, relatively large amounts of (non-Bt) cotton may help explain the low proportion of non-cotton TBW observed in the state. On the other hand, despite significant cotton acreage in some states (Georgia and North Carolina), few cotton-origin TBW were captured.

Monsanto cataloged four crop types as part of the spatial analysis: non-Bt cotton, soybean, peanut, and tobacco. All four are known to be favored plant hosts for TBW and are cultivated on a large number of acres in the pest's geographical range. Bt cotton (i.e. Bollgard or Bollgard II varieties) was not included, presumably because TBW are not expected to survive the high dose of Bt toxin(s) expressed by the plants. However, by excluding Bt cotton acreage, the overall amount (and importance) of cotton in the landscape is reduced. Wild hosts (i.e. weeds) were also not included, likely due to the extreme difficultly in tabulating such acreage (NASS data do not address wild hosts). Given the probable high importance of wild hosts for TBW populations, the lack of statistical correlations seen in the cropping data may be explained by the lack of wild hosts in the analysis. Other cultivated crops such as vegetables were not included, possibly due to relatively small acreage.

Another point to consider is that all four of the crop types compiled by Monsanto are typically managed agronomically. This management could include the use of insecticide treatments (targeted at TBW or other pests) that could reduce TBW populations. Since the gossypol experiments did not distinguish the type of non-cotton host, it is not possible to ascertain how many trapped TBW originated from these managed crop acres relative to wild hosts. To better understand the potential of alternate hosts to serve as Bt cotton refuge, it would be of interest to know the relative importance of each host type (cultivated non-cotton crops and wild/weedy hosts) for the TBW population

Though the submitted report indicated that all traps were photographed aerially at least once during the two year test period, only a subset of the sampled counties was included with the data. Monsanto described this set as a "representative" sample, but no criteria for picking the subset were provided. For Mississippi, a state with high cotton acreage, data were provided for only one county. It is unclear how the inclusion of results from all counties would affect the overall analysis.

The ten year NASS data included in the report show that cropping patterns have been relatively stable over the decade preceding the gossypol experiment. However, it is unclear whether these past trends can serve as a predictor for future years. One crop in particular, tobacco, presents an interesting case. The acreage of tobacco, a highly preferred TBW host, in North Carolina was shown to decline during the ten year period captured in the NASS data. Though still planted on a sizable area of the state, it is unknown how tobacco will be farmed in future years. It is conceivable that tobacco acreage could continue to decline due to social and marketing reasons. If that scenario proves to be the case, it will be unclear how TBW is affected. In addition, the NASS data do not compile wild and weedy TBW hosts. These types of plant hosts, which are also important TBW hosts, can be affected by non-agronomic factors (e.g. development, land use, etc.), which could also impact the makeup of TBW populations. As previously discussed, it is impossible to determine from the submitted data the proportions each host type (non-cotton crops or wild plants) contributes to the overall TBW population.

<u>IV. Calculation of Effective Refuge and Resistance Modeling for Bt Cotton</u>

[Note: All figures and tables referenced are in Appendix 2.]

The effective refuge calculations and modeling work are contained in MRID# 467172-02 (hereafter referred to as Gustafson & Head 2005). Monsanto's computer model used for this study is executed in Microsoft® Excel 2000 (version 9.9.4402 SR-1), and is a modification of Caprio's deterministic, two-compartment, random-mating, random-oviposition model used previously (Caprio 1998a). The modification involved removing the assumption of constant effective refuge size in response to the June 2004's Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel's (SAP) recommendations (SAP 2004). Instead, a regionally-specific annual cycle of effective refuge size was assumed, according to data collected in alternative host studies of CBW (Head & Voth 2004) and TBW (Head & Gustafson 2005). These data were combined with Bt corn planting estimates on either the regional-scale for CBW, or county-scale for TBW, to construct effective refuge sizes for each of what were conservatively assumed to be six annual generations for each pest.

The derivations of effective refuge size for both CBW and TBW are provided below. Monsanto has corrected their calculation of effective refuge size presented in Gustafson & Head (2004) based on the June 2004 SAP's recommendations (SAP 2004). The following equations explicitly account for the lower production of CBW and TBW in Bt cotton where survival of these insects is reduced. All figures and tables regarding effective refuge and modeling can be found in Appendix 2 as well as in Gustafson & Head (2005).

a) CBW effective and natural refuge calculations

The following description of the CBW effective and natural refuge calculations is taken directly from Gustafson & Head (2005).

> "As shown in Figure 1, for the purpose of estimating effective refuge, both compartments of the original two-compartment model were subdivided into different crop types. The *B.t.* compartment was subdivided into the two *B.t.* crops grown commercially in the southern U.S. that express the closely related Cry1Ac and Cry1Ab proteins, *B.t.* cotton

and *B.t.* corn respectively, and the refuge compartment was subdivided into conventional (non-*B.t.*) corn and other C4 hosts, conventional (non-*B.t.*) cotton, and alternative C3 host crops (other than cotton). The effective refuge is defined as the proportion of the overall insect population not exposed to the relevant *B.t.* protein or proteins (see Figure 1). The relative number of adult moths produced by each of these five sub-compartments is given by the following equation:

$$M_{ij} = A_{ij}\, E_{ij}\, LB_{ij}\, LS_{ij} \qquad [1]$$

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

The fractional land areas occupied by the various crop types were calculated as regional averages from a combination of 2004 data from Monsanto, Doane Agricultural Services, and USDA NASS (2005) (see **Table 2**). The three regions are defined as shown in Figure 2. The regions include all contiguous counties in which *B.t.* cotton was planted in 2003-2005, and any adjacent counties in which one of the following CBW host crops was planted in 2004: corn, cotton, peanuts or soybeans.

The regional values for the proportion of *B.t.* corn were taken from the most recent two years of market research data (Doane Agricultural Services 2004). The Mississippi regional value of 15% *B.t.* corn combines the totals for Arkansas, Louisiana, and Mississippi. For Georgia, the state totals for Alabama and Florida were included, giving a regional value of 3% *B.t.* corn. The North Carolina value included South Carolina and Virginia for a total of 10% *B.t.* corn. The eastern Texas value is based on the state-wide average of 12%. Combining values from the additional states for the regional averages did not significantly change the values for each region, but it increases the level of confidence in the estimated regional values and is consistent with the widespread nature of CBW movement indicated by field study results (Head et al. *in draft*; Jackson et al. 2005b).

As shown in **Table 2**, available market research data (Doane Agricultural Services 2004) show that differing proportions, T, of *B.t.* and non-*B.t.* cotton were treated with pyrethroids. When treatment occurred, most cotton, regardless of whether it contained *B.t.*, was sprayed twice. For the purpose of estimating effective refuge, it is necessary to estimate the total proportion of adults emerging from these two cotton compartments. This estimation was made using the following equation, which assumes there are three generations of CBW on cotton, with the last two generations getting sprayed and a proportion, *K*, of larvae killed by each spray:

$$\boldsymbol{LS_{i1} = [T_{i1}\ ((1/3) + (2(1\text{-}K_{i1})/3))] + (1 - T_{i1})} \qquad \textbf{[2]}$$

The average mortality of larval CBW resulting from pyrethroid sprays on non-*B.t.* and Bollgard cotton has been reported as 64.6% and 82.8%, respectively (Greenplate 2004). Thus, *LS* for each region and cotton type can be obtained using equation [2] after inserting the value for the percentage of the crop that is treated in the appropriate region. For the purposes of estimating the size of the effective refuge, the fractional survival of CBW in *B.t.* cotton and *B.t.* corn were set at 15% and 50%, respectively (Kurtz et al. 2004).

The final terms in equation [1] for which estimates are required are related to the CBW larval productivity of the various crop types. Caprio et al. (2004) showed that there is no statistical difference in the number of CBW eggs laid in *B.t.* cotton and non-*B.t.* cotton, thus E_{B1} and E_{R1} may be assumed equal to 1. Similarly, we assume that the number of eggs laid in *B.t.* corn and non-*B.t.* corn also are identical, i.e. $E_{B2} = E_{R2}$. Thus, there are only two unknown parameters remaining: the relative number of CBW eggs laid in corn and the relative number laid in non-cotton C3 crops.

The relative larval productivity of other C3 crops was directly measured in a recent set of field studies (Jackson et al. 2005b). The relative larval productivity of corn (a C4 crop) also was determined in a recent study using $^{13}C/^{12}C$ ratios in captured CBW moths (Gould et al. 2002; Head et al. *in draft*). These C4 host data are summarized in Table 3. A mathematical expression can be derived relating this observed C4/C3 distribution of moths to the relative productivity of the C4 crop, conservatively assumed to be only corn (sorghum is ignored). The fraction of observed moths coming from C4 crops is:

$$\boldsymbol{f}_{\mathbf{C4}} = \frac{M_{B2} + M_{R2}}{M_{B1} + M_{B2} + M_{R1} + M_{R2} + M_{R3}} \qquad \textbf{[3]}$$

Inserting the definition for *M* from equation [1] into [3] and rearranging for the only unknown parameter, E_{B2}, (assumed equal to E_{R2}) gives the following equation:

$$\boldsymbol{E_{B2} = E_{R2}} = \frac{f_{C4}\left(M_{B1} + M_{R1} + M_{R3}\right)}{(1 - f_{C4})\left[\left(A_{B2}LB_{B2}\right) + A_{R2}\right]} \qquad \textbf{[4]}$$

The final remaining unknown in the calculation of *M* is the relative CBW larval productivity of non-cotton C3 crops. Side-by-side strip trials were carried out by Jackson et al. (2005) for several non-cotton C3 crops in five states: Arkansas, Georgia, Louisiana, Mississippi, and North Carolina. The data for each state are summarized in Table 4. These productivity numbers use only the data covering the period when CBW larvae were observed in cotton. A weighted overall average value for E_{R3} for each of the three regions is also shown in **Table 4**. In the case of Georgia and North Carolina, the weighting was based on the acreage of peanuts and soybeans in these two regions, as shown in **Table 2**. In the case of the Mississippi region, the weighting was based on the conservative assumption that any soybeans of maturity group 6 have the same productivity as that of maturity group 5. The calculation also assumed that the proportion of maturity group 5 (and higher) soybeans in the three states was as follows: Arkansas (50%), Louisiana (40%) and Mississippi (20%) (Doane Agricultural Services 2004).

For the eastern Texas region, which was not studied by Jackson et al. (2005b), the relative productivity of non-cotton C3 crops conservatively was assumed to have the lower values found in the Mississippi region.

The effective refuge, R_{eff}, is defined as the proportion of adult moths that would have been produced in the refuge compartment in the absence of any *B.t.*-induced larval mortality:

$$\boldsymbol{R_{eff}} = \frac{M_{R1}+M_{R2}+M_{R3}}{M_{R1}+M_{R2}+M_{R3}+M_{B1}+M_{B2}} \qquad \textbf{[5]}$$

In the calculation of adult moths from the two *B.t.* compartments in equation [5], LB_{B1} and LB_{B2} are both set to 1 to ensure that this calculation is made prior to selection by *B.t.* crops. The assumed and fitted values used in the calculations are shown in **Table 5**. The resulting effective refuge sizes for each of the six annual generations of CBW are shown in **Table 6**. The full version of equation [5] was required for estimating effective refuge only when CBW populations were actively feeding in cotton, which corresponded to generations 3-5 (Head et al. *in draft*; Jackson et al. 2005b). According to these same data, essentially all moths were coming from C4 hosts (conservatively assumed to be corn) during generation 2. The C3 host contribution to adult moths in generations 1 and 6 was assumed to be coming entirely from non-cotton crops in all three regions based on published literature (Benedict 2004). The simpler version of equation [5] for all of these “non-cotton” generations is therefore (with the C3 contribution, M_{R3}, vanishing in generation 2):

$$R_{eff}^{NC} = \frac{M_{R2}+M_{R3}}{M_{R2}+M_{R3}+M_{B2}} \qquad \textbf{[6]}$$

The natural refuge component of the total effective refuge is found by eliminating the structured refuge from [5] as follows:

$$R_{nat}^{CBW} = \frac{M_{R2}+M_{R3}}{M_{R2}+M_{R3}+M_{B1}+M_{B2}} \qquad \textbf{[7]}$$

> For those periods when cotton is not grown, the natural refuge is equivalent to the effective refuge and is still defined by [7].
>
> The modeling of CBW resistance development against Bollgard cotton is described in detail by Gustafson et al. (2005)."

b) TBW effective and natural refuge calculations

The following description of the TBW effective and natural refuge calculations is taken directly from Gustafson & Head (2005).

> "The pooled, county-level estimates of the percent cotton-reared TBW moths were combined with county-level landcover information to estimate the current effective refuge and the natural refuge for each county by month (Head & Gustafson, 2005). For the purposes of this calculation, the following assumptions were made:
>
> - None of the observed TBW moths came from cotton fields containing the *c*ry1Ac gene (in 2004, this corresponded to the Bollgard and Bollgard II brands alone and combined with Roundup-Ready®), therefore all observed cotton-reared moths came from non-*B.t.* cotton;
> - The county is the appropriate scale to relate observed proportions of cotton-reared TBW moths to the frequency of other landcover types; and
> - The non-cotton areas produce TBW moths with a relative productivity, E_{NC}, that varies by month.
>
> Under these assumptions, the relative TBW productivity of non-cotton areas can be related to other known or measured factors:
>
> $$\boldsymbol{E_{NC}} = \frac{(A_{NBTC} / P_{NBTC}) - A_{NBTC}}{A_{NC}} \qquad \textbf{[8]}$$
>
> where E_{NC} is the relative TBW productivity of non-cotton areas within a county for a specific month; A_{NBTC} and A_{NC} are the relative areas of non-*B.t.* cotton and non-cotton landcover types, respectively; and P_{NBTC} is the observed proportion of cotton-reared TBW moths for the month of interest (the subscript is a reminder that all of these moths are from non-*B.t.* cotton).
>
> The current effective refuge for TBW then is defined as the proportion of TBW moths actually produced in the effective refuge compartment (prior to selection by *B.t.* cotton):

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$$R_{eff}^{TBW} = \frac{A_{NBTC} + (A_{NC}E_{NC})}{A_{BTC} + A_{NBTC} + (A_{NC}E_{NC})} \qquad \textbf{[9]}$$

in which the only new term on the right hand side of the equation is A_{BTC}, the relative area of cotton in the county containing the *cry1Ac* gene.

The potential natural refuge for TBW is given by a similar equation, except that it is now conservatively assumed that all current non-*B.t.* cotton acres would contain *cry1Ac*, thereby removing the first term from the numerator of [9]:

$$R_{nat}^{TBW} = \frac{A_{NC}E_{NC}}{A_{BTC} + A_{NBTC} + (A_{NC}E_{NC})} \qquad \textbf{[10]}$$

The effective and natural refuge values that result from the use of equations [8]-[10] are summarized in **Tables 7 and 8**. For certain counties (indicated by a subscript in **Tables 7 and 8**), the non-*B.t.* cotton estimates were erroneously low (<5% of all cotton) due to some combination of inaccuracies in either the sales figures or the total cotton acres or both. These non-*B.t.* cotton values were adjusted to 5% of all cotton for the purpose of estimating current effective refuge. For cases where no cotton-reared moths were observed, a lower bound on the productivity of non-cotton hosts and the resulting refuge sizes was estimated by using the upper 95% confidence limit for the percentage of cotton-derived moths (based on the number of moths collected). However, as a practical matter, this uncertainty is of little concern because it corresponds to cases in which the natural refuge is relatively large.

Table 9 contains the values used for TBW modeling, which were the averages of all available 2004 and 2005 values for the selected counties where the number of moths collected during that month was at least 10. Confidence intervals for any values associated with fewer than 10 moths were deemed to be too unreliable for the modeling work."

c) Description of the Three-Gene Model with Cross-Resistance

The three-gene model for insect resistance evolution used in this study is based on a conceptual model similar to that proposed by Dow AgroSciences (DAS) for its product, WideStrike® cotton, and was reviewed by a recent U.S. EPA Scientific Advisory Panel (SAP) (SAP 2004). However, the SAP questioned some of the mathematical details of the DAS model and Monsanto has made some changes to address the SAP's concerns. The following description of the Monsanto model is taken directly from Gustafson & Head (2005).

“As shown in schematic form in **Figure 3**, the three-gene model is based on the following assumptions concerning the mechanism of activity of the three commercial *B.t.* cotton products (Bollgard, Bollgard II, and WideStrike cotton):

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

We assume the mortality due to each of these toxins to be given by a standard logistic dose response curve with a slope factor of 1:

$$S_{rk} = \frac{1}{(1+(T_{rk}/LD50_{rk}))} \qquad \textbf{[11]}$$

where S_{rk} is the survival probability for a pest that has ingested toxin k, which is bound to receptor r; T_{rk} is the concentration of toxin-receptor complex; and LD_{50k} is the toxin-receptor dose that kills half the pest population. The concentration of the toxin-receptor complex is directly proportional to the expression level, C_k, of the toxin, but also may be impacted by any competitive binding. In the case of Cry1Ac, 60% is bound to receptor A and 40% to receptor B. In the particular case of WideStrike cotton, there is competition between Cry1F and Cry1Ac for receptor A. This competition is modeled through the following equation:

$$T_{rk} = f_{rk}C_k\left(\frac{1-(f_{rz}C_z)}{(f_{rk}C_k)+(f_{rz}C_z)}\right) \qquad \textbf{[12]}$$

where f_{rk} is the affinity (ranging between 0 and 1) of toxin k for receptor r; and the subscript, z, refers to a toxin that competes with toxin k for receptor r. In this model, the concentration of the toxin-receptor complex is simply the expression level for a toxin, such as Cry2Ab2, for which there is no competitive binding involved.

The combined toxic effect of the toxins present in any particular crop product is then determined as the product of all S_{rk} that are relevant to that crop:

$$S_p = \prod_{k=1,m}^{r=1,n} \frac{1}{(1+(T_{rk}/LD50_{rk}))} \qquad \textbf{[13]}$$

For example, in the case of Bollgard II cotton, there are three factors in [13]: two for Cry1Ac and one for Cry2Ab2. The results of applying equations [11]-[13] to the three

> current cotton products are shown in **Table 10** for CBW and **Table 11** for TBW. Most of the expression levels and intrinsic toxicities shown in this table are taken directly from published literature reports (Greenplate et al. 2003; Perlak et al. 2001). The exceptions are the expression levels for WideStrike cotton, which were not published in these reports. These were set to give predicted overall survival probabilities consistent with the levels reported by DAS for WideStrike cotton and its two corresponding one-gene precursor products (SAP 2004).
>
> As shown in **Tables 10 and 11**, a conservative assumption was made with respect to the relative intrinsic toxicities of the two toxic complexes that are formed by the binding of Cry1Ac; the complex formed by binding to Receptor A (the one shared with Cry1F) was assumed to have the same *LD*50 as that observed for Cry1F alone. In sensitivity analyses not included here, we have found that a model which assumes equivalent toxicity for the two bound forms of Cry1Ac results in extremely long durability (>>30 years) for all three *B.t.* cotton products against CBW and TBW, regardless of market share assumptions. This is because such a model requires the co-occurrence of two rare alleles for a genotype that is functionally resistant to Cry1Ac to exist.
>
> The survival probabilities shown in **Tables 10 and 11** are for the fully susceptible, homozygotic genotype, *AABBCC*. To model resistance, we assume that each receptor has an alternate, fully resistant form whose structure is determined by a single, diallelic gene that assorts independently from the other two receptors. We further assume that expression in a heterozygote is additive such that a heterozygote has 50% susceptible and 50% resistant receptors present in its gut. The concentration of the toxin-receptor complex in such a heterozygote is then half the level given by [12]. This reduced concentration is entered into [11] to determine the quantitative impact on the probability of survival due to that particular toxin-receptor combination.
>
> The net result of applying this methodology to the three current cotton products gives the CBW and TBW survival probabilities shown in **Table 12**. These values differ somewhat from the values recently reported by DAS for the same three products (SAP 2004), but they are more consistent with the relative activities of these three products in the field: Bollgard II > WideStrike > Bollgard (Jackson et al. 2005a)."

d) Other modeling assumptions

Monsanto has made several other modeling assumptions. These are provided below.

1. *No fitness costs.* The modeling reported in this study made the conservative assumption that there is no fitness costs associated with the resistant form of any of the three receptors. This assumption is made as a worse case scenario even though there is some empirical evidence that Cry1Ac resistance in common bollworm (a close relative of CBW), *Helicoverpa armigera* (Hubner), and the pink bollworm, *Pectinophora*

gossypiella (Saunders), has a substantial associated fitness cost. In *H. armigera*, Cry1Ac-resistant individuals had significantly slower larval development on non-Bt cotton and lower adult overwintering survival than susceptible individuals (Bird & Akhurst 2004). In *P. gossypiella*, Cry1Ac-resistant individuals were found to have lower overwintering survival (Carrière et al. 2001b) and less tolerance of the cotton defensive compound gossypol (Carrière et al., 2005; Carrière et al., 2004) than susceptible individuals, resulting in fitness being decreased by over 50% (Carrière et al. 2001b; Carrière et al. 2001b).

2. *Resistance is complete.* Resistance to each of the three Cry proteins was assumed to be complete. This is a conservative assumption.

3. *Initial frequency of resistant allele.* The initial frequency of the resistant allele for each of the three receptors, in both CBW and TBW, was set equal to 0.002, based on data reported by Gould et al. (1997).

4. *Proportion of Bt cotton is varied using different refuge scenarios.* The proportion of Bt cotton planted to the three available Bt products was varied in several ways, as shown in **Table 13**. As listed in this table, there were two assumptions concerning refuge, designated “current” and “natural”. These refuge scenarios were defined as follows:

Current Refuge Scenario: The effective refuge (consisting of conventional cotton plus non-cotton hosts) was maintained at current levels, while the relative marketsares of the three available Bt products were allowed to vary. The amounts of non-Bt and Bt cotton remained at current levels.

Natural Refuge Scenario: The refuge was assumed to comprise the natural refuge plus a 5% structured refuge requirement for Bollgard and WideStrike cotton. Bollgard II cotton was assumed to have no structured refuge. The total amount of Bt cotton was higher than currently present in the landscape, but total cotton acres remained at current levels.

5. *Four regions for CBW.* For CBW, the model was run for each of the four regions shown in Figure 2.

6. *Worst-case county for TBW.* For TBW, the model was run for the “worst-case” county in each state (indicated by shading in Tables 7 and 8).

7. *Number of generations of CBW and TBW per year.* For both CBW and TBW, it was conservatively assumed there were six generations of the pest each year.

8. *Resistance definition.* The resistance model was run in a simple, deterministic manner, one region at a time. The number of years until the frequency of each resistance allele exceeded 0.5 was recorded, up to a maximum of 30 years. The final efficacy of each

product was recorded. If Bollgard cotton was in the scenario (all but scenario 4), this product was (conservatively) left in the landscape even after its efficacy had been lost.

e) Monsanto's Modeling Results and Discussion

All results (tables and figures) are found in Appendix 2 and taken from Gustafson & Head (2005).

Cotton bollworm

Monsanto has examined the durability of each of the three Bt cotton products that are available commercially. The Bt protein, Cry1Ac, is common to all three products. The presence of each of these products in the marketplace selects for potential resistance to Bollgard cotton, expressing only the Cry1Ac protein, and also selects for resistance to the other two products through the common selection for Cry1Ac resistance. The products vary greatly in the rate at which they select for resistance to Cry1Ac because of the presence of additional insecticidal proteins in Bollgard II, Cry2Ab2, and in WideStrike, Cry1F. Monsanto examined the durability of each of the three Bt cotton products individually (**Figure 4**) and together (**Table 14**) in the marketplace.

The rate of selection for resistance or intrinsic durability is demonstrated by looking at the durability of each of the products individually in the marketplace, as if they were occupying the entire marketplace and with no structured refuge in place (i.e., all natural refuge) (**see Figure 4**). Based on the modeling results shown in **Figure 4**, Monsanto concludes that resistance in CBW is predicted to take more than 30 years to evolve to Bollgard II cotton in three of the four modeling regions, and more than 22 years to evolve in eastern Texas reflecting the protective impact of two unique insecticidal modes of action for Cry1Ac and Cry2Ab2. If all cotton is grown as Bollgard cotton, CBW resistance is predicted to evolve in less than ten years to Bollgard cotton in Mississippi and eastern Texas, and takes only 12 years to evolve in Georgia, reflecting the presence of only the single insecticidal mode of action. The predictions for WideStrike cotton fall between those for Bollgard and Bollgard II cotton because of the shared receptor for Cry1Ac and Cry1F; CBW resistance is predicted to evolve in 12 years in eastern Texas and approximately 20 years in Mississippi.

Monsanto also modeled the impact of all three products in the marketplace on the selection for Bt resistance (i.e., Cry1Ac, Cry1F, Cry2Ab2) using the full three-gene model. This modeling effort is far more complex than that of modeling the selection for resistance of each of these products individually. The results for CBW with the full model are summarized in **Table 14**. Each of the scenarios included in the resistance modeling are shown in **Table 13.**

There were major differences in the predicted durability of Bollgard expressing only the Cry1Ac and WideStrike cotton product expressing the Cry1Ac and Cry1F proteins and Bollgard II cotton which expresses both the Cry1Ac and Cry2Ab2 proteins due to regional and marketshare-related factors. Bollgard II cotton retained its efficacy against CBW for more than 30 years (the maximum run length of the model) in all, but one of the 56 cases modeled (25.5 years when R =

0.5 and 8% efficacy remaining after 30 years) in Scenario 2-N (**Table 14**) in east Texas). The remaining efficacy after 30 years ranged from 96% to 99% in all scenarios except for Scenario 2-N. Monsanto states that the greater durability of Bollgard II is due to the higher mortality of individuals heterozygous for Cry1Ac resistance in the presence of Cry2Ab2.

The presence of greater amounts Bollgard II cotton (Scenario 2, **Table 14**) in the marketplace also increased the durability of both Bollgard and WideStrike cotton. Conversely, the presence of substantial amounts of Bollgard cotton (Scenario 1, Table 14) in the marketplace tended to decrease the longevity of Bollgard II and WideStrike cotton. These results suggest that the overall durability of Bt cotton will be enhanced by encouraging increased adoption of Bollgard II cotton.

For the region centered on North Carolina, resistance always took at least 30 years to evolve for all three products, even for all of the natural refuge scenarios. The relatively slow rate of resistance evolution in this region reflects the high percentage of natural refuge (and therefore also effective refuge) present throughout the season (Abney et al. 2004). The predicted time to resistance was shorter for the regions centered on Mississippi and E. Texas, where the percentage of effective refuge was smaller than in the regions centered on North Carolina and Georgia during the third, fourth, and fifth CBW generations (**Table 6**), particularly for Bollgard and WideStrike cotton.

Monsanto indicates that based on the conservative assumptions of the model, a natural refuge is sufficient to delay the evolution of CBW resistance to Bollgard II cotton for more than 25 years under all of the scenarios run in the model. The modeling predictions for CBW resistance evolution should be regarded as highly conservative, both because of the nature of the resistance assumed (including complete resistance and no fitness costs for resistance to Cry1Ac, Cry1F, and Cry2Ab2) and the values used for alternative host contribution to CBW populations (Table 6). The estimated contributions of non-cotton alternative hosts used in the model were derived from Head et al. (*in draft*) and Jackson et al. (2005b). Those studies focused only on a subset of CBW hosts.

Monsanto performed additional sampling of CBW populations in Arkansas and east Texas in July through September of 2005, followed by analysis of these moths for gossypol using methods described in Head & Gustafson (2005). These data were not included in the current modeling simulations. In these subsequent studies, the non-cotton contribution to CBW populations in these regions was many times higher than predicted by the earlier studies presented in Head et al. (*in draft*) and Jackson et al. (2005b). For Arkansas, only 3.2% of 993 moths analyzed came from cotton (tested positive for gossypol), while 4.7% of 409 moths collected in east Texas were derived from cotton. The effective and natural refuge sizes assumed in the modeling were highly conservative because >40% of the moths from Arkansas and >70% of the moths from Texas were assumed to be from cotton. Overall, these results suggest that there are greater effective and natural refuge sizes for CBW moths in the E. Texas region and Arkansas (Mississippi Region) than was assumed in the modeling.

Tobacco budworm

Monsanto examined the durability of each of the three Bt cotton products individually and together in the marketplace. The intrinsic durability of all three Bt cotton products is much greater for TBW than CBW because of the "high-dose" of Cry1Ac for TBW expressed in all three products (see EPA 2001; SAP 2004). Based on the modeling simulations, all three products are predicted to retain their efficacy for more than 30 years even if all cotton in a region is planted to that product and no structured refuge is required. The only exceptions occur for Bollgard cotton; for Mississippi and Tennessee. In these cases, Bollgard cotton is predicted to lose some efficacy within the 30 years modeled **[Figure 5].**

With the full three-gene model, the durability of Bollgard and WideStrike cotton is increased in cases where Bollgard II cotton also is present. In all cases, the three products were predicted to retain their efficacy against TBW for more than 30 years in all cases modeled. This is a direct result of the extremely high efficacy of Cry1Ac against this pest, and the fact that Cry1Ac is present in all three Bt cotton products. In the state with the lowest nature refuge for TBW (Mississippi, **Table 13**), resistance to Cry1Ac and Cry1F evolved after 21 years in Scenario 1-N if the structured refuge requirements for Bollgard and WideStrike cotton also were removed **[Table 15].**

BPPD Review (Effective Refuge Calculations and Modeling Efforts for CBW and TBW)

[Note: All figures and tables referenced are in Appendix 2.]

CBW

In 2004, BPPD did an assessment of Monsanto's analysis of the impact of effective refuge size and typical insecticide use practices on predicting of years to resistance of CBW (and TBW) to Bollgard cotton (BPPD 2004b). In this analysis, BPPD agreed with Monsanto's conclusion that the model output is very sensitive to effective refuge size and use of insecticide sprays on Bollgard cotton for CBW control. With this understanding, the 5% external, unsprayed structured refuge option was considered adequately protective to delay TBW and CBW resistance if effective refuge size and typical use practices (i.e. pyrethroid oversprays of Bollgard fields) were included as parameters in the model (BPPD 2004b; SAP 2004). BPPD concluded that the Gustafson et al. (2004) model was limited because it did not consider the spatial and temporal dynamics of CBW alternate hosts by generation. BPPD also disagreed with Monsanto's calculation of estimated effective refuge size. Estimated effective refuge was calculated as the sum of the total acres of conventional cotton, soybean, and other alternate hosts (assumed to be 10%) per county. Rather the estimation of effective refuge size must be a weighted average on each host for each generation. The June 2004 SAP agreed with the Agency's analysis regarding the calculation of the effective refuge size, the spatial limitations of the Gustafson et al. (2004) model and the need to incorporate more explicitly the alternate host data (SAP 2004). The SAP also noted that "the estimation of the total refuge proportion also requires an estimate of the emergence from transgenic crops (e.g. the proportion of refuge is relative to the total number of adults, including those emerging from transgenics)." They further indicated that the estimate of total adult emergence should be corrected for losses due to selection for resistance.

In Monsanto's current modeling study, alternative hosts are more explicitly examined in the model, i.e., five compartments: Bt cotton, Bt corn, non-Bt cotton, non-Bt corn, and non-cotton C3 crops vs. the original two-compartment model (Gustafson & Head 2004) This is shown in **Figure 1**. Spatiality is now included in the model; there are four cotton production regions that differ in the amount of alternate hosts: Mississippi, Georgia, North Carolina, E. Texas. The modeling analysis provides a regional snapshot of the population dynamics of CBW and thus is a limited picture of what might be happening in the landscape (although these snapshots may represent the worst case scenario). Making an extrapolation over many years based on just a few snapshots in time, given the variability of the data, is uncertain.

The effective refuge size is defined as the proportion of the overall insect population not exposed to the relevant Bt protein or proteins. Natural refuge is considered to be the proportion of adults on non-Bt corn and non-cotton C_3 crops. The relative number of adult moths produced in each of the five sub-compartments per unit area is given by Equation 1 above. In this equation, corrections are made for losses due to selection for Bt and pyrethroids for the appropriate sub-compartments. CBW larval productivity is calculated on the various crop types using a combination of direct field data for non-cotton C3 crops (Jackson et al. 2005b), C_{13}/C_{12} isotope analysis data for the productivity on corn (Gould et al. 2002; Head et al. *in draft*), egg production on Bt and non-Bt cotton (Caprio et al. 2004 indicate no difference), and egg production on Bt cotton and Bt corn is assumed to be equal. The fractional land areas occupied by the various crop types were calculated as regional averages from a combination of 2004 data from Monsanto, Doane Agricultural Services, and USDA/NASS. The average mortality of larval CBW resulting from pyrethroid sprays on non-Bt and Bollgard cotton has been reported as 64.6% and 82.8%, respectively (Greenplate 2004). It was recommended by both BPPD (BPPD 2004b) and the SAP (SAP 2004) that pyrethroid mortality be incorporated into the model. The fractional survival of CBW in Bt cotton and Bt corn were set at 15% and 50%, respectively (Kurtz et al. 2004).

In Gustafson & Head (2005), Monsanto addressed BPPD's and the SAP's previous concerns regarding the calculation of the effective refuge size (weighted per host per generation), inclusion of alternate hosts (five sub-compartments vs. two-compartments), increased spatiality (four regions), impact of multiple Bt cotton products (three gene model and scenarios varying the level of adoption of each product) and larval mortality caused by pyrethroid sprays and *B .t.* (no corrections were made in the original model). This means that Monsanto's current CBW modeling analysis (Gustafson & Head 2005) is more robust than the one previously reviewed by the Agency (BPPD 2004b) and the SAP (2004).

There are limitations to the carbon isotope analysis. There is uncertainty as to the relative non-cotton C_3 crop contribution vs. the C_4 crop contribution. Non-cotton C_3 crop cannot be further subdivided because of the limitations of carbon C_{13}/C_{12} isotope analysis data. This was previously noted in the BPPD review (BPPD 2004a) and by the SAP (2004) of the CBW alternate host data (Head & Voth 2004).

There is agreement, however, that the C_4 crop contribution (primarily corn) is quite high throughout most of the cotton growing season and this contribution can be much greater than that of cotton. There is large variability in the number of moth captures in the pheromone traps due to the impact of immigration and reverse migration, among other factors (Gould et al. 2002). There are also limitations in extrapolating data based on just male moth captures to be representative of the entire population (see previous discussion in Section II of this review and in SAP 2004).

The June 2004 SAP concluded that "without more definitive data quantifying temporal and spatial production of susceptible CBW moths from each of the C_3 and C_4 hosts, and confirmed mating behavior of subsequent adults, the current refuge requirement(s) should continue." It should be noted that this comment was made in the context of the question the Agency posed to the SAP on the relative contribution of the C_3 and C_4 hosts as unstructured (natural) refuge. The Agency had to make a decision as to whether to continue the 5% external, unsprayed refuge requirement for Bollgard cotton based on the scientific assessment of the CBW alternative host data as effective refuge. No field data was collected for the eastern Texas region in which to estimate the relative productivity of CBW on non-cotton C_3 crops. For this region, the relative productivity on non-cotton C_3 crops was assumed to lower than in Mississippi. In Georgia and North Carolina, the non-cotton C_3 crop production is assumed to be a weighting of the acreage of peanuts and soybeans in these two regions and in the Mississippi region, the soybeans of maturity group 6 are assumed, conservatively, to have the same productivity as that of maturity group 5. The effective refuge calculations are affected by the uncertainties in the quantity and spatial production of susceptible CBW for each alternate. On a qualitative basis, the estimation of current and natural effective refuge is probably adequate.

BPPD agrees with the following assumptions Monsanto made for the three-gene model based on the mechanisms of activity of Bollgard, Bollgard II, and WideStrike cotton (i.e. expected receptor binding patterns of Cry1Ac, Cry1F, and Cry2Ab2 to CBW midgut membranes). It is assumed that the primary mechanism of Bt resistance is associated with modifications to the receptor binding site. Some level of cross-resistance between Cry1Ac and Cry1F associated with a common resistance mechanism of reduction in receptor binding would be expected. This issue is of particular concern for WideStrike cotton because it expresses both Cry1F and Cry1Ac and there is competition between Cry1F and Cry1Ac for receptor A. These general assumptions were evaluated by the June 2004 SAP for a spatially-explicit, stochastic CBW resistance model used by DAS for its WideStrike cotton product and found to be acceptable (SAP 2004).

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

Resistance associated with modification of the binding site receptor has been the primary Bt resistance mechanism reported to date (reviewed in Ferré & Van Rie 2002). Other Bt resistance mechanisms have been reported that are based on alterations in the proteases that cleave the protoxin, processing it into a smaller active toxin (Candas et al. 2003) and most recently, the discovery that esterases can bind and detoxify Bt toxins (Gunning et al. 2005). Monsanto's assumptions regarding the binding of Cry1Ac, Cry1F, and Cry2Ab2 to receptors A, B, and C are acceptable given the current understanding of the receptor binding patterns of Cry1Ac, Cry1F, and Cry2Ab2 to CBW midgut membranes.

The June 2004 SAP raised an issue as to how DAS calculated the expected mortality of different genotypes as a result of exposure to Cry1Ac, the factor "*Z*" (SAP 2004). Monsanto has modified the mathematical expression of the competition between Cry1F and Cry1Ac for receptor A and the calculation of the expected mortality of different genotypes as a result of exposure to Cry1Ac. The survival probabilities of all 27 CBW genotypes are found in **Tables 10**. To model for resistance, Monsanto assumed that each receptor has an alternate, fully resistant form whose structure is determined by a single, diallelic expression in a heterozygote is additive such that a heterozygote has 50% susceptible and 50% resistant receptors present in the gut. The concentration of the toxin-receptor complex is such a heterozygote is then half (given by **Equation 12**) and then this concentration is entered in **Equation 11** to determine the quantitative impact on the probability of survival due to that particular toxin-receptor combination. BPPD agrees with Monsanto's analysis of the expected mortality of the different CBW genotypes as a result of exposure to Cry1Ac, Cry1F, and Cry2Ab2 (see **Table 12**). These values differ from those reported by DAS for the same three products (SAP 2004) because of differences in the mathematical calculations.

BPPD agrees with the following other modeling assumptions:

- There are no fitness costs associated with the resistant forms of any of the three receptors. As Monsanto notes, there are recent studies in which Cry1Ac resistance is associated with fitness costs (e.g. Bird & Akhurst 2004; Carrière et al. 2001a, b).
- Resistance to each of the three Cry proteins was assumed to be complete.
- The CBW model was run for each of the four regions shown in Figure 2. This provides a snapshot in time in each region.
- The proportion of Bt cotton was varied for each of the three Bt cotton products across the four regions by "current" and "natural" refuge.
- For CBW there were six generations of the pest per year.
- The number of years until the frequency of each resistance allele exceeded 0.5 was recorded, up to a maximum of 30 years. The number of years to resistance and the relative efficacy remaining should only be interpreted on a qualitative basis.
- The model was run in a simple, deterministic manner, one region at a time. Spatially-explicit stochastic models would, however, offer a more nuanced and dynamic picture of the interactions of the three Bt cotton products in the landscape over time.

BPPD has some concerns with the following assumptions:

- Assumption that the initial resistance allele frequency for each of the three receptors is 0.002. Given that resistance management is more effective when the initial frequency of resistance is lower, an assumption of 0.002 should be a conservative assumption. Given the length of prior use of Bollgard cotton, 10 years, this estimation may or may not be optimistic. Monsanto's modeling does not account for any prior selection to Cry1Ac. Monitoring efforts have not detected any significant change in susceptibilities to the Bt proteins (BPPD 2005; Tabashnik et al. 2003). Given the limitations of the sampling, probability of detection based on the sensitivity of the discriminating dose (LC_{99}) bioassay (0.01), the current resistance allele frequency for Cry1Ac is estimated to be lower than 0.01, based onn the limit of detection of the discriminating dose (LC_{99}) bioassay (EPA 2001). The probability of detection of field resistance, therefore, would be relatively low unless resistance allele frequencies were high and the resistance was dominant. Given minimal adoption of WideStrike and Bollgard II and therefore minimal selection additional selection pressure for resistance to Cry1Ac, Cry1F, or Cry2Ab, it is likely that the initial resistance allele frequency of 0.002 is still a conservative assumption. Because the modeling simulations assume that all three Bt products are introduced as a mosaic, then an initial resistance allele frequency of 0.002 for Cry1Ac would be a conservative assumption. However, as noted above, given the adoption of Bollgard cotton and the selection pressure for CBW resistance to Cry1Ac, it is more likely that the resistance allele frequencies have increased and that a more relevant set of modeling scenarios would have examined prior selection (see discussion below).
- Assumption that resistance is recessive. In North Carolina, Burd et al. (2001) provide results from a reciprocal cross study that suggests CBW resistance to Cry1Ac and Cry2Ab is dominantly or incompletely inherited. These results suggest that the assumption that CBW resistance is recessive is perhaps incorrect. Burd et al. (2001) isolated nonrecessive Bt resistance genes present in CBW field populations in North Carolina and estimated the Cry1Ac resistance allele frequency for CBW as 0.0043 and the Cry2Aa resistance allele frequency for CBW as 0.00039.
- Assumption that there is no prior selection for resistance to Cry1Ac. Monsanto has presumed no prior selection for CBW resistance to Cry1Ac even though Bollgard has been commercially available for 10 years. Monsanto has modeled a mosaic of all three Bt cotton products rather than a sequential introduction of Bollgard II and WideStrike following introduction of Bollgard. Zhao et al. (2005) have shown that the concurrent use of one-and two-gene Bt broccoli plants resulted in control failure of both types of Bt plants for control of *Plutella xylostella* (diamondback moth) rather than the use of the two-gene plants by themselves. In addition, these researchers illustrated through their modeling efforts that resistance will evolve first to the toxin that is being used singly.

Given the assumptions of the three-gene model and its limitations, there is likely enough effective natural refuge to be sufficient to delay the evolution of resistance to Bollgard II cotton for more than 25 years (not a precise number of years) under all plausible scenarios in all four regions (**Table 14**). Based both on the intrinsic durability of each of the three Bt cotton products (**Figure 4**) and the three-gene modeling analyses for all three Bt cotton products together in the marketplace (**Table 14),** Bollgard II retained the highest level of efficacy against CBW in all scenarios (all regions). This is because of the relatively high mortality of individuals heterozygous to Cry1Ac resistance in the presence of Cry2A2, as compared to WideStrike; intermediate in many scenarios because of the shared binding receptor between Cry1F and CryAc and likelihood of cross-resistance, and Bollgard, weakest in all scenarios, no high dose for CBW and a single-gene product. Monsanto's models predict that CBW resistance to Bollgard cotton will evolve in less than the 30 year horizon in the Georgia, Mississippi, and E. Texas regions in most scenarios except for 2-C (Bollgard = 0.1; Bollgard II = 0.8; WideStrike = 0.1). Resistance always took at least 30 years to evolve for all three Bt cotton products in the North Carolina region in all scenarios, even the natural refuge scenarios. Monsanto's modeling does not presume any prior selection for Cry1Ac resistance.

When Bollgard cotton acreage is minimized, Bollgard II and WideStrike longevity is maximized **(Table 14)**. Selection pressure for resistance to Cry1Ac is reduced and the relative value of the unique proteins, Cry1F and Cry2Ab2 is enhanced. Large amounts of Bollgard II cotton in the marketplace increased the durability of both Bollgard and WideStrike **(Table 14)**.

TBW

In Gustafson and Head (2005), Monsanto addressed BPPD's (2004b) and the SAP's (2004) previous concerns regarding the calculation of the effective refuge size (weighted per host per generation), inclusion of alternate hosts (three sub-compartments for TBW vs. two-compartments), increased spatiality (four regions), impact of multiple Bt cotton products (three gene model and scenarios varying the level of adoption of each product) and larval mortality. This means that Monsanto's current TBW modeling analysis (Gustafson & Head 2005) is more robust than the one previously reviewed by the Agency (BPPD 2004b) and the SAP (2004). As noted above, the modeling analysis provides a regional snapshot of the population dynamics of TBW and thus is a limited picture of what might be happening in the landscape (although these snapshots may represent the worst case scenario). Making an extrapolation over many years based on just a few snapshots in time, given the variability of the data, is uncertain.

Monsanto combined pooled, county-level estimates of the percent cotton-reared TBW moths with county-level landcover information to estimate the current effective refuge and the natural refuge for each county by month (see Head & Gustafson 2005, discussed in Section II and III above). The landscape is divided into three sub-compartments to estimate TBW current and natural effective refuge: Bt cotton, non-Bt cotton, non-cotton area (**Figure 1**). The current effective refuge for TBW is defined as the proportion of TBW moths actually produced in the effective refuge compartment prior to selection by Bt cotton (**Equation 9**). The potential natural refuge for TBW is given by **Equation 10**; it is conservatively assumed that all current non-Bt cotton acres would contain *cry1Ac* so the term, A_{NBTC}, is removed. **Table 9** contains the current and natural effective refuge values used in the TBW modeling. These were the averages of all available 2004 and 2005 values for the selected counties where the number of moths collected during that month were at least 10. BPPD agrees with Monsanto's method for calculating the relative TBW productivity on each prospective alternate host. However, uncertainties in the pheromone sampling, gossypol analyses, and spatial landscape mapping affect the estimation of the current and natural effective refuge. These issues were discussed in Sections II and III of the review.

BPPD agrees with the following assumptions Monsanto made for the three-gene model based on the mechanisms of activity of Bollgard, Bollgard II, and WideStrike cotton (i.e. expected receptor binding patterns of Cry1Ac, Cry1F, and Cry2Ab2 to TBW midgut membranes). It is assumed that the primary mechanism of Bt resistance is associated with modifications to the receptor binding site.

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

As noted earlier, these general assumptions were evaluated by the June 2004 SAP for a spatially-explicit, stochastic CBW resistance model used by DAS for its WideStrike cotton product and found to be acceptable (SAP 2004). See discussion above regarding Bt resistance mechanisms.

BPPD has some concern about whether these assumptions are still valid for TBW. BPPD reviewed the relevant literature and determined that three-site model proposed by Jurat-Fuentes & Adang (2001) for binding of Bt Cry1 toxins to sites in the TBW midgut membrane is relevant to this discussion. Based on the ligand-blot analyses, the Cry1Ac toxin was able to recognize three receptors: A, B, and C; while the Cry1Fa toxin was able to recognize receptor A and perhaps a second predicted, but not determined site. Receptor C is only recognized by the Cry1Ac toxin. Neither Cry1Ea nor Cry2a competed with any of the labeled Cry1A toxins. Cry1Ac competed with high affinity for Cry1Ab binding sites. Cry1Aa, Cry1Fa, and Cry1Ja also competed for Cry1Ab binding sites. Cry1Ab competed for labeled Cry1Ac binding sites with a higher affinity than did Cry1Aa, Cry1Fa, and Cry1Ja. It was noted by the study authors that it was inherently difficult to label Cry1Fa. One can conclude from Fuentes and Adang (2001) that Cry1F and Cry1Ac have shared binding affinity for Receptor B (in their model) which is analogous to what was proposed by Monsanto in its three-gene model (competition is for receptor Ai in this model) (Gustafson & Head 2005). Some level of cross-resistance between Cry1Ac and Cry1F associated with a common resistance mechanism of reduction in receptor binding would be expected. This issue is of particular concern for WideStrike cotton because it expresses both Cry1F and Cry1Ac and there is competition between Cry1F and Cry1Ac for a shared receptor. Competition binding experiments indicate that perhaps 40% of the labeled-Cry1Ac binding to brush border membrane vesicles was reduced by competition with Cry1Fa, although a saturation concentration of Cry1Fa was not reached. Based on the data provided in Jurat-Fuentes & Adang (2001), Monsanto's assumptions regarding the binding patterns of Cry1Ac, Cry1F, and Cry2Ab2 to receptors A, B, and C on TBW midgut membranes are acceptable with one caveat. Additional competition binding studies using TBW brush border membrane vesicles at saturable concentrations of Cry1Fa are recommended. Based on the results of additional competition binding studies, the survival probabilities of all 27 genotypes may need to be recalculated and the modeling redone. Uncertainty in the shared binding affinity of the Cry1Ac protein to receptor A and B (in Monsanto's model) would impact the modeling predictions for all three Bt cotton products because the Cry1Ac protein is common to all of them. Monsanto's modeling does not assume any prior selection for Cry1Ac resistance. The degree to which the modeling predictions would be impacted is unknown

The June 2004 SAP raised an issue as to how DAS calculated the expected mortality of different genotypes as a result of exposure to Cry1Ac, the factor "*Z*" (SAP 2004). Monsanto has modified the mathematical expression of the competition between Cry1F and Cry1Ac for receptor A and the calculation of the expected mortality of different genotypes as a result of exposure to Cry1Ac. The survival probabilities of all 27 TBW genotypes are found in **Tables 11**. To model for resistance, Monsanto assumed that each receptor has an alternate, fully resistant form whose structure is determined by a single, diallelic expression in a heterozygote is additive such that a heterozygote has 50% susceptible and 50% resistant receptors present in the gut. The concentration of the toxin-receptor complex is such a heterozygote is then half (given by **Equation 12**) and then this concentration is entered in **Equation 11** to determine the quantitative impact on the probability of survival due to that particular toxin-receptor combination. These values differ from those reported by DAS for the same three products (SAP 2004) because of differences in the mathematical calculations.

BPPD agrees with the following other modeling assumptions:

- There are no fitness costs associated with the resistant forms of any of the three receptors. As Monsanto notes, there are recent studies in which Cry1Ac resistance is associated with fitness costs (e.g. Bird & Akhurst 2004; Carrière et al., 2001a, b).
- Resistance to each of the three Cry proteins was assumed to be complete.
- The proportion of Bt cotton was varied for each of the three Bt cotton products across the four regions by "current" and "natural" refuge.
- For TBW, the model was run for the "worst-case" county in each state (indicated by shading in **Tables 7 and 8**).
- For TBW were six generations of the pest per year.
- The number of years until the frequency of each resistance allele exceeded 0.5 was recorded, up to a maximum of 30 years. The number of years to resistance and the relative efficacy remaining should only be interpreted on a qualitative basis.
- The model was run in a simple, deterministic manner, one region at a time. Spatially-explicit stochastic models, however, would offer a more nuanced and dynamic picture of the interactions of the three Bt cotton products in the landscape over time.

BPPD has additional concerns regarding the assumptions for: initial resistance allele frequency for each of the three receptors and prior selection for Cry1Ac resistance. These were discussed above in the context of CBW modeling but, in general, are applicable also to the TBW modeling with the addition of the following comments.

- Assumption that the initial resistance allele frequency for each of the three receptors is 0.002. Gould et al. (1997) estimated the TBW resistance allele frequency as 0.001. Given the length of prior use of Bollgard cotton, 10 years, this estimation may or may not be optimistic. Monitoring efforts have not detected any significant change in susceptibilities to the Bt proteins (BPPD 2005; Tabashnik et al. 2003). As noted above, given the limitations of sampling and sensitivity of detection (0.01), the probability of detection of field resistance, therefore, would be relatively low unless resistance allele frequencies were high and the resistance was dominant.

- Assumption that resistance is recessive. Because Cry1Ac, Cry2Ab2, and Cry1F are all expressed at a "high dose" in all three Bt cotton products, it is expected that resistance will be conferred by rare, recessive alleles and most resistant adults from Bt crops will mate with susceptible adults from refuges. In a recent review, Tabashnik et al. (2003) comment that "…inheritance of resistance to transgenic plants with high concentrations of Bt toxins is recessive in all cases examined to date involving pests that have high inherent susceptibility to the toxins produced…".
- Assumption of no prior selection to Cry1Ac. See earlier BPPD comments on CBW modeling.

From a qualitative perspective, modeling indicates the intrinsic durability of Bollgard II is greater than WideStrike which is greater than Bollgard. The intrinsic durability of all three Bt cotton products is much greater for TBW than for CBW because of the "high dose" of Cry1Ac for TBW expressed in all three products. In virtually all cases, all three products retained their efficacy (i.e. no resistance) for more than 30 years (maximum time for the simulation) even if all cotton in a region is planted to that product and no structured refuge is required (i.e. all natural refuge) (**see Figure 5**). The only exceptions occur for Bollgard cotton in Tennessee and Mississippi. Given the assumptions of the three-gene model and its limitations, there is likely enough effective natural refuge to be sufficient to delay the evolution of resistance to Bollgard II cotton for more than 30 years (i.e. the time horizon of the model, not to be interpreted as a precise number of years) under all plausible scenarios in all four regions (**see Table 15**). This is due to the extremely high efficacy of Cry1Ac against TBW, and the fact that Cry1Ac is present in all three Bt cotton products. In the state with the lowest natural refuge for TBW, Mississippi (see **Table 13**), resistance to Cry1Ac and Cry1F evolved after 21 years in scenario 1-N if the structured refuge requirements for Bollgard and WideStrike cotton were removed. Uncertainties in the pheromone captures, gossypol analyses, spatial analysis, effective refuge calculation, degree of shared binding affinity of Cry1Ac to receptor A and B, effect of prior selection for Cry1Ac resistance, and other modeling assumptions affect the precision and accuracy of the modeling predictions.

Caprio (2006) used a spatially-explicit, stochastic, two-locus resistance model to investigate the likelihood that TBW would not evolve resistance to Bollgard II within a 15-year time horizon. He used two different scenarios, one with a 4% structured refuge and 1% wild hosts (5% untreated refuge), and the second with no structured refuge and a 1% wild host refuge. The model simulated 1024 cotton patches, each ca. 100 acres in size. The model assumed that only Bollgard cotton was used initially and simulated a gradual transition to Bollgard II over five years. Mortality rates for this cotton with nine different genotypes were calculated. The initial gene frequency was assumed to be 0.001. The model assumes four generations of TBW develop on cotton per year. Generations outside of cotton were not incorporated into the model. A carrying capacity of 80,000 larvae (eggs/patch) for early pre-bloom cotton, 800,000 for peak bloom cotton, and 500,000 larvae (eggs/field) for flowering cotton were used in the modeling. Fitness costs were not included in the model. The model parameters were not varied. Thirty runs of the simulation for each of the two scenarios were made and changes in resistance allele frequencies at both resistance loci were evaluated. In 30 runs of the simulation with a structured 5% untreated refuge, resistance was not observed at either locus. The mean rate of increase in resistance allele frequency for the Cry2A2 locus was 1.032 (SD =0.151), while the mean rate of increase for the Cry1Ac locus was 1.0567 (SD=0.169). In 30 runs of the simulation with no structured refuge and a 1% unstructured refuge, resistance was observed to occur in three cases to the Cry1Ac trait in Bollgard II. The mean rate of increase in resistance allele frequency for the Cry2Ab2 locus was 0.8984 (SD =0.391), while the mean rate of increase for the Cry1Ac locus was 85.88 (SD=262.53; three outlier populations). These results suggest that the risk of TBW resistance in a 15-year time horizon with or without a structured refuge is relatively small if the estimates of mortality of TBW larvae and other components of the model are reasonably accurate. The results do suggest much higher risk of TBW resistance to the Cry1Ac trait if the structured refuge is removed. The predictions of Caprio's spatially-explicit, stochastic, two-locus TBW resistance model are similar to those predicted by Monsanto's three-gene, deterministic model (Gustafson and Head, 2005). That is, structured and natural refuge delay TBW resistance effectively to the two proteins, Cry1Ac and Cry2Ab2, expressed in Bollgard II.

Overall Conclusions and Discussion

Overall, the modeling suggests that Bollgard II cotton should have more than 25 years of durability for the control of CBW and TBW in all regions with natural refuge as the only source of susceptible insects. The presence of larger amounts of Bollgard II in the marketplace also increases the durability of other Bt cotton products that are present but the intrinsic durability of Bollgard II cotton is much greater than Bollgard or WideStrike cotton. However, there are uncertainties in the modeling parameters and assumptions which could impact the modeling output.

Modeling suggests that the overall durability of Bollgard II cotton can be enhanced if Bollgard cotton is removed from the marketplace. This conclusion is supported by other researchers who examined the benefit of managing resistance evolution to two toxins with dissimilar modes of action using a pyramided approach (Zhao et al. 2005; Roush 1998; Livingston et al. 2004; Hurley 2000; Caprio 2006). On the other hand, the concurrent use of single- and two-gene Bt plants can offer exposed populations a "stepping stone" to develop resistance to both proteins. That is why it is important to consider the removal of single, less durable, Bt cotton products from the marketplace in favor of more durable, two-gene (assuming independent modes of action) products. In Australia, the use of the single-gene Bt cotton product (i.e., Bollgard) and the pyramided Bt cotton product (i.e. Bollgard II) was permitted only for the first two years after the introduction of Bollgard II, but now only Bollgard II is permitted. In the U.S., Bollgard II cotton has been in the marketplace for four years (registered in December, 2002) and WideStrike cotton has been in the marketplace for two years (registered in September, 2004). In 2004, Bollgard cotton acreage accounted for >95% of all Bt cotton acreage in the U.S. (see Head et al.

2005, MRID# 467172-03). Ten years of selection pressure for resistance to Cry1Ac has already occurred. Field resistance to Cry1Ac places additional selection pressure on the Cry2Ab2 component of Bollgard II cotton. Monsanto's modeling does not assume any prior selection for Cry1Ac resistance either by TBW or CBW. Encouraging the adoption of Bollgard II will increase the overall durability of all three Bt cotton products. From an insect management point of view, removal of Bollgard cotton from the marketplace should be encouraged as quickly as possible.

Researchers from Australia (Mahon et al. 2004) have reported that one population of *Helicoverpa armigera* (a close relative of *Helicovera zea*, CBW, in the U.S.) collected from maize plants exhibited resistance to Cry2Ab. These researchers note that there is no evidence of cross-resistance to Cry1Ac and that inheritance of resistance is recessive. An explanation as to why *H. armigera* had unexpected high levels of Cry2Ab2 resistance levels is unknown, but research is ongoing to evaluate this resistance. A better understanding of background levels of resistance to Cry2Ab toxins in *H. armigera* populations and opportunities for survival of resistant genotypes on Bollgard II is needed. What this means for either TBW or CBW resistance to Cry2Ab toxins in Bollgard II in the U.S. is not known, but we should be aware of the possibility that Cry2Ab resistance may be more common than expected.

V. Proposed Revisions to the Bollgard II IRM Program

Monsanto's proposed revisions to the IRM program to use natural refuge for Bollgard II cotton are contained in the volume titled "Scientific and Economic Justification for Not Requiring Structured Cotton Refuges for Bollgard II Cotton in the U.S. Cotton Belt from Texas to the East Coast" (MRID# 467172-03). This report summarizes the alternate TBW host studies, TBW effective refuge, and resistance modeling previously discussed in this review. In addition, Monsanto has included sections describing cross resistance potential, CBW considerations, economic and environmental benefits of the proposed IRM revision.

a) Natural Refuge Proposal

As described in the Background section, the terms and conditions of registration for Bt cotton varieties (including Bollgard and Bollgard II cotton) required the planting of a structured, non-Bt cotton refuge. For TBW, the refuge strategy is supported by "high dose" expression of Bt toxin(s) by the registered hybrids. For CBW, although there is not high dose expression, the pest is highly polyphagous and experimental data have shown that it makes use of alternate, non-cotton hosts (see discussion in the Background section). The IRM plan for Bt cotton also includes resistance monitoring requirements for both TBW and CBW on all registered toxins. As of the 2004 growing season, no documented cases of resistance of significant shifts in pest susceptibility to Cry1Ac (the toxin in Bollgard) have been detected (see BPPD 2005).

Monsanto has proposed to replace the structured refuge requirements for Bollgard II cotton with a natural refuge approach (a similar proposal for Bollgard cotton has not been proposed).

Growers would no longer be required to plant a non-Bt cotton refuge and could plant up to 100% Bollgard II cotton. The natural refuge strategy would be applicable to the southeastern cotton growing regions including Texas (western cotton regions, in which pink bollworm is the primary target pest, are not included). This proposal is based on a number of supporting factors: 1) Bollgard II expresses two distinct Bt toxins which offers an IRM advantage over single toxin products; 2) Bollgard II offers better control of the target pests (TBW and CBW) than Bollgard cotton; 3) The alternate host data for TBW (reviewed in this memorandum) and CBW (previously reviewed - see Background section) indicate that adequate natural refuge exists independent of non-Bt cotton refuges.

Monsanto's submission described the potential resistance management benefits of using multiple toxins (also known as "pyramiding" toxins). A number of models (Roush 1994, 1998 and Caprio 1998b) indicate that the deployment of multiple toxins in a transgenic crop can delay the onset of pest resistance longer than a single toxin crop. The advantages of pyramided toxins are contingent on several factors: high efficacy of each toxin against the target pest and distinct modes of action for each toxin (i.e. lack of cross resistance). In the case of Bollgard II, the Cry1Ac and Cry2Ab2 Bt toxins are expressed (the event was created by inserting the Cry2Ab2 gene into existing Bollgard cotton). Both Cry1Ac and Cry2Ab2 each are known to have high activity against TBW and together the toxins were shown to increase the overall efficacy against TBW by 3.5 fold. For CBW, Cry2Ab2 was shown to have high activity (close to "high dose"), while Cry1Ac has less activity and is not considered high dose.

b) Cross Resistance

The IRM benefits of a multiple gene product could be reduced if there is cross resistance between the expressed toxins. Cross resistance can result if a pest develops resistance to one toxin, which then confers a degree of resistance to the second toxin. Monsanto's submission summarizes information to support the contention that there is no cross resistance potential between Cry1Ac and Cry2Ab2. These summarized data to discuss cross resistance were originally submitted by Monsanto with the initial registration application for Bollgard II (MRID# 455457-01).

Structurally, the Cry1A and Cry2A groups of Bt toxins are divergent, with only 20% similarity between amino acid sequences. In addition, polyclonal antibody assays showing an absence of cross-reactivity of anti-Cry2Ab2 antibodies with Cry1Ac protein (and vice-versa) demonstrate that the tertiary structure of Cry2A proteins differs significantly from Cry1A proteins. Given the lack of structural similarities, Monsanto concluded that the insecticidal mechanisms of Cry1Ac and Cry2Ab2 are distinct and unlikely to result in cross resistance.

Once ingested by the target pests, the Cry1Ac and Cry2Ab2 toxins behave differently. During trypsin digestion, Cry1Ac forms a stable core protein that is not produced by Cry2Ab2 under similar conditions. Also, in the insect midgut, the two toxins bind to the gut membranes in different manners: Cry1Ac interacts with brush border membrane proteins to open ion channels whereas Cry2Ab2 interact with other proteins to open unique ion channels. The ion channels created by Cry2Ab2 are larger and have reduced ion selectivity than those created Cry1Ac, which may explain the increased potency observed with the toxin. As with the structural differences between the two toxins, these differences in membrane interaction suggest low cross resistance potential between for the Bollgard II toxins.

Further evidence to document the lack of cross resistance potential was shown in studies utilizing Cry1Ac-resistant colonies of TBW and CBW. When challenged on Cry2Ab2 (Bollgard II plant material), Cry1Ac-resistant TBW remained susceptible to the protein with few survivors (none of the survivors progressed beyond the first larval instar). A similar experiment with CBW resulted in less than 5% survival on Bollgard II cotton plants.

BPPD Review (Cross Resistance)

Cross resistance data were previously submitted to BPPD by Monsanto for the initial registration of Bollgard II. These data (the same data set described above) were reviewed in detail by BPPD (see BPPD 2002a) prior to the issuance of the registration. In this review, BPPD agreed with Monsanto's overall conclusions that the cross resistance potential between Cry1Ac and Cry2Ab2 in Bollgard II should be low for both TBW and CBW based on structural and binding characteristics.

However, since Bollgard II has been registered, additional research has been conducted into the cross resistance potential of Cry1 and Cry2 proteins. In one study, Jurat-Fuentes et al. (2003) investigated resistance to Cry1Ac and Cry2Aa in TBW. Several strains of TBW that had been selected on and developed resistance to Cry1Ac were found to also be resistant to Cry2Aa. Because these two toxins are not known to share binding sites in TBW, the authors suggested that another mechanism may be involved in the observed resistance. This alternate resistance mechanism could be one that affects a shared process in the mode of action of the two toxins. As was described by Monsanto, the structure and binding properties of Cry1A and Cry2A toxins are different at the membrane level. However, both toxins have similar activation processes in the midgut before membrane interaction; an alteration of the toxin activation process (i.e. differential midgut protease activity) could lead to common resistance to both proteins. The authors also suggested "midgut epithelium regeneration" as a potential mechanism (in which the midgut recovers from exposure to cry toxins), although this area requires additional research.

Though Cry2Aa is not a toxin expressed in Bollgard II, the work of Jurat-Fuentes et al. (2003) illustrate the need to consider alternate resistance mechanisms other than structure or binding when evaluating cross resistance potential. Other potential resistance mechanisms could include metabolic changes (e.g. protease inhibition, gut recovery) or behavioral adaptations. Such alternate mechanisms may be complex and difficult to evaluate in the context of transgenic crops (most cross resistance evaluations have been conducted in the laboratory). BPPD previously discussed some of the theoretical considerations for cross resistance in transgenic crops as part of the 2001 Bt crops reassessment (see EPA 2001), concluding that additional study is needed to full understand the ramifications for IRM.

For Bollgard II, the data evaluated to date on protein structure and midgut binging seem to indicate that the cross resistance potential may be relatively low for Cry1Ac and Cry2Ab2. However, based on the work of Jurat-Fuentes et al. and the limited overall knowledge base on the subject, cross resistance cannot be entirely eliminated as a possibility with Bollgard II. Another important consideration is the presence of Bollgard cotton (or other single gene varieties expressing Cry1Ac) in the marketplace with Bollgard II. If resistance to Cry1Ac were to develop on Bollgard, dual gene Bollgard II would essentially become a single toxin (Cry2Ab2) product. In this case, TBW would only require a single resistance mechanism to Cry2Ab2, negating the benefits of Bollgard II as a pyramided crop. Monsanto's natural refuge proposal made no specific commitment to withdraw Bollgard from the market should the proposal be accepted, so it is likely significant acreage of Bollgard will remain in place.

c) Alternative Hosts/Natural Refuge - CBW

CBW is commonly known as a highly polyphagous pest that feed on a wide range of plant hosts, including cultivated crops, wild host plants, and weeds in addition to cotton. To evaluate and quantify these alternate hosts as potential natural refuge, Monsanto conducted a large scale experiment during 2002 and 2003. This study involved an aerial mapping project of cropping patterns in cotton growing states, a survey of CBW production on alternate hosts, and a bioassay designed to detect the type of host plant used for development of sampled CBW (a "C_3/C_4" analysis of carbon isotopes). The C_3/C_4 bioassay is described in the Background section of this review.

Overall, Monsanto concluded that these studies showed that there are substantial areas of alternate host crops (corn, peanut, sorghum, and soybean) available in cotton-growing areas and that CBW production is high on these crops. The C_3/C_4 assays revealed that C_4 alternative hosts (primarily corn and sorghum) make a significant contribution to the CBW adult population throughout the season. These data have been previously reviewed by BPPD (see BPPD 2004a) and are fully discussed in the Background section. BPPD previous reviewed Monsanto's model as part of the June 2004 SAP meeting on the effective of natural refuge for CBW resistance management in Bollgard and Bollgard II cotton (SAP 2004). Monsanto revised its current and natural effective refuge calculations and model based on the June 2004 SAP recommendations (SAP 2004). Based on Monsanto's modeling of intrinsic durability and three-gene multiple product durability modeling, there is likely enough effective natural refuge to be sufficient to delay the evolution of CBW resistance to Bollgard II cotton for more than 25 years (not a precise number of years) under all plausible scenarios in all four regions. Bollgard II retained the highest level of efficacy against CBW in all scenarios (all regions) because of the relatively high mortality of individuals heterozygous to Cry1Ac resistance in the presence of Cry2A2, as compared to WideStrike; intermediate in many scenarios because of the shared binding receptor between Cry1F and CryAc and likelihood of cross-resistance, and Bollgard, weakest in all scenarios, no high dose for CBW and a single-gene product. The presence of greater amounts Bollgard II cotton in the marketplace increased the durability of both Bollgard and WideStrike cotton. Conversely, the presence of substantial amounts of Bollgard cotton in the marketplace tended to decrease the longevity of Bollgard II and WideStrike cotton. Monsanto's modeling did not assume prior selection for Cry1Ac resistance. A detailed discussion of the CBW modeling is found in Section IV of this review.

d) Alternative Hosts/Natural Refuge - TBW

Like CBW, TBW is known as a polyphagous pest that utilizes a number of crops and wild hosts for development. To further analyze the potential of alternate hosts to serve as refuge, Monsanto provided a literature review of TBW biology, a description of a two year TBW sampling effort/gossypol bioassay to determine host plant development, a calculation of TBW "effective" refuge, and the results from an aerial mapping project to investigate cropping patterns. From these efforts, Monsanto has concluded that a large portion of the TBW population in cotton-growing regions originates from alternate (non-cotton) hosts, resulting in an effective refuge that exceeds 20%. Monsanto's submitted materials and conclusions are reviewed and discussed in detail in sections I, II, III, and IV of this document.

e) Modeling – TBW and CBW

As part of the analysis of alternate host utilization and the impact on IRM/refuge, Monsanto ran model simulations using the alternative host data obtained for CBW and TBW. Simulations were run for distinct cotton-growing regions (CBW) or states (TBW) with scenarios accounting for both natural refuge only and effective refuge (natural refuge plus Bt cotton refuges and other non-Bt cotton acreage). For TBW, "worst case" counties (those with the lowest amount of natural refuge) were modeled. For CBW, the four regions were modeled. Based on the model results, Monsanto concluded that Bollgard II cotton would have more than 25 years without CBW or TBW resistance using the natural refuge strategy. None of three Bt cotton products lost efficacy against TBW during the 30 year time horizon of the modeling using any current or natural refuge scenario except scenario 1-N, if the structured refuge requirements for Bollgard and WideStrike cotton were removed. CBW modeling predicted that higher amounts of Bollgard II enhanced the durability of both Bollgard and WideStrike. Conversely, higher amounts of Bollgard reduced the durability of both Bollgard and WideStrike. The modeling and results are discussed in detail in section IV of this review.

f) Economic Benefits

Monsanto's submission concluded that replacing the structured refuge requirements for Bollgard II will result in economic benefits to growers. Overall, Bollgard II cotton provides higher yields and less input costs compared with non-Bt (conventional) cotton treated with insecticides. Under the current IRM requirements (5% or 20% non-Bt cotton refuges), growers experience refuge-related costs including reduced productivity in refuge acres in addition to refuge maintenance requirements (time and labor).

For the 20% refuge requirement, growers must plant the required non-Bt cotton acreage within one mile of the Bollgard II acreage and may treat the refuge as needed with insecticides to control lepidopteran pests. Comparative studies summarized by Monsanto have shown that the pest control costs are smaller (under moderate to high pest pressure) and crop yields are higher with Bt cotton relative to conventional cotton. For example, data from 1995-2002 showed an average increase in yield (lint) of 6% with Bollgard cotton (another set of data showed a 10% average lint increase during 1995-1999). Since refuge acres are essentially conventional cotton that is managed with insecticides, growers do not experience these benefits of Bt cotton on at least 20% of their cotton acres. One study cited by Monsanto (Banerjee and Martin 2005) demonstrated that Bt cotton had a $38 per acre benefit over conventional (refuge) cotton plantings. Other data sets tabulated by Monsanto (1995-2002) and a third party study (1995-1999) indicated that growers received an average net dollar return of $39.35/acre and $49.80/acre respectively with Bollgard. Based on these results, Monsanto estimated that Bt cotton provides a $40/acre benefit over the 20% treatable refuge acreage, not including other refuge management costs (e.g. scouting, labor, time, etc.). Therefore, grower using this refuge option will lose $40 per acre on 20% of their cotton acreage; when averaged across all cotton acres (Bt and refuge), the cost amounts to $8 per acre. For a typical grower planting 1,800 acres (the average size farm in the Delta region) the 20% refuge option results in a total cost of about $14,400 (1,800 acres x $8 per acre cost).

With the 5% refuge options (embedded or external unsprayed), Monsanto estimated that growers can lose up to $150 per acre relative to their Bollgard acreage. Another estimate from a cotton researcher estimated losses of $67 per acre with the 5% options (Martin et al. 2006). The losses can be attributed primarily to reduced yields and are particularly acute for the 5% unsprayed option, where as much as 100% of the crop can be lost due to pest infestation. Monsanto calculated that growers planted an unsprayed refuge will yield almost 250 pounds per acre less than conventionally grown (insecticide treated) cotton. For the 5% refuge, the total cost when averaged across all cotton acres (5% refuge and 95% Bt cotton) is $7.50/acre (similar to the $8/acre cost for the 20% refuge). Considering a typical 1,800 acre cotton farm, the overall cost is approximately $13,500 (1,800 acres x $7.50 per acre cost).

Monsanto's submission also discussed other non-quantified refuge-related costs to growers (i.e. non-yield and treatment related). In much of the cotton-growing area, farms are becoming larger and more complex. Cotton plantings may include many fields over large areas, with some acreage falling in multiple counties. The need to plant refuges can limit the flexibility of these operations by requiring in-depth land use planning. During planting, refuges add logistical impediments, such as the use of additional labor and the need to clean planter boxes between fields.

BPPD Review (Economic Benefits)

As part of the evaluation of the Bollgard II registration, BPPD reviewed benefits information related to the product (BPPD 2002b), including an assessment of the economic benefits of the crop. BPPD estimated the per acre benefit of Bollgard II to be $27.63 per acre, considering the (projected) technology fee, adoption rate, improved efficacy/pest spectrum, and reduced insecticide costs for secondary pests (e.g. beet armyworm). This figure differs from Monsanto's calculations, which were $40 to $150 per acre, depending on the refuge option employed. It is noted that BPPD's assessment was conducted prior to registration using projections for many of the inputs, while Monsanto used or cited data obtained from farm surveys of growers planting Bollgard. Regardless of the specific per acre benefit, BPPD agrees that Bollgard and Bollgard II provide significant economic benefits to growers relative to conventional and refuge non-Bt cotton.

On the other hand, short term economic benefits to growers may be compromised should resistance to Bt cotton occur. The primary objective of IRM and structured refuges has been to preserve the effectiveness of Bt toxins and maintain long term benefits to growers. If resistance were to occur, growers will likely experience costs related to reverting to other (still efficacious) Bt varieties or conventional (insecticide-treated) cotton. Additional costs could result from implementing the remedial action plan, which requires (among other measures) the use of conventional insecticides to control outbreak pest populations. Monsanto's submission did not address the potential effects of resistance on the cost/benefit analysis for Bollgard II, though quantifying such costs would likely be difficult and involve imprecise estimates.

g) Environmental Benefits

In addition to direct economic benefits to growers, Monsanto's submission described a number of environmental benefits resulting from adoption of Bollgard II cotton. These benefits include improved resistance management, reduced use of conventional insecticides, and prolonged efficacy of conventional insecticides.

In terms of IRM benefits, Bollgard II provides a two toxin approach to controlling the target pests. Models have shown that a dual dose product can delay resistance relative to single dose products, although this advantage can be curtailed if significant acreage of single toxin products also coexists with the dual toxin product in the landscape. Single gene products (e.g. Bollgard cotton) can provide a "stepping stone" to resistance, increasing the frequency in which resistance develops in the dual gene transgenic crop. To date, Bollgard cotton has much greater adoption among cotton growers than Bollgard II, largely due to economic reasons (i.e. Bollgard II has no clear advantage over Bollgard for most growers). However, Monsanto has contended that should the natural refuge proposal be accepted, growers will have a clear incentive to adopt Bollgard II cotton on a wider scale (i.e. no required structured refuge, increased economic benefits). If this proves to be the case, the transition from Bollgard to Bollgard II across the landscape will improve IRM by reducing selection pressure to Cry1Ac found in single toxin Bollgard hybrids.

Further insecticide use reduction benefits could be obtained through the adoption of the natural refuge proposal (allowing Bollgard II on 100% of growers' acreage). A survey cited by Monsanto showed that 22% of cotton growers utilized the 20% refuge option, which allows for the use of conventional insecticides to treat pests in the refuge. By planting 100% Bollgard II, growers would no longer need to treat those 20% refuge acres for lepidopteran pests. Based on the 22% adoption figure, Monsanto estimated that 385,000 total refuge acres could be saved from insecticide treatment. Monsanto also noted that Bollgard II cotton has the potential to reduce up to three insecticide treatments per season in comparison to conventional cotton. The resulting decrease in pesticide use has potential benefits for non-target species, including predatory arthropods that would avoid exposure to non-specific insecticides. Additional benefits could result from reduced fuel use and the need to dispose of fewer pesticide containers.

In conjunction with overall reduced pesticide usage, Monsanto has proposed that the efficacy of conventional insecticides could be preserved for longer periods of time. Less use of pesticides would mean less exposure to pest populations and ultimately less selection pressure for resistance. Pest resistance has been problematic for a number of chemicals used in cotton, most notably the pyrethroid insecticides and TBW.

BPPD Review (Environmental Benefits)

BPPD's assessment of the benefits for Bollgard II identified both resistance management and reduced insecticide use as positive environmental advantages of the product. For IRM, it is clear that a two gene product with functional high doses for the target pests is a better option than a single gene product for the same pests. However, as Monsanto has stated in their report, a landscape containing a mixture of single gene and dual gene products can compromise the IRM benefits of the two gene crop. This can occur if resistance develops to the single gene crop (Bollgard/Cry1Ac in this case); the dual gene product (Bollgard II) essentially becomes a single gene product expressing Cry2Ab2. Monsanto's natural refuge proposal provides no details or plan for increasing adoption of Bollgard II (and phasing out Bollgard) other than the incentive to forgo structured refuge with Bollgard II. It is not clear how quickly Bollgard II acreage will increase and whether sufficient hybrids will be available with the Bollgard II genes. Given the wide discrepancy between Bollgard and Bollgard II acreage, it is highly probable that significant acreage of Bollgard will remain in place even if the natural refuge proposal is implemented. Since large acreage of Bollgard could present a resistance risk for Bollgard II, it is recommended that Monsanto provide details as to how the adoption of Bollgard II/phase out of Bollgard will be facilitated.

BPPD agrees with Monsanto regarding the benefits of reduced pesticide use. Bt cotton has clearly decreased the need for insecticide treatments for lepidopteran pests (refer to the benefits assessment in EPA 2001). The use of 100% Bollgard II, with high efficacy against the major target pests and some secondary pests, presumably would eliminate the need for treatment of the 20% structured refuge option. The exact amount of insecticide reduction would be dependent on the scale of adoption of Bollgard II, an issue that remains unclear. It should be noted that, like the economic and IRM benefits, is pest resistance occurs to Bollgard II, the pesticide use reduction may be compromised. In fact, resistance would likely result in a substantial increase in the amount of pesticide used, as growers would either need to treat Bollgard acreage (as part of remedial action) or resume planting conventional cotton varieties and regular pest treatments.

BPPD Review (Overall Natural Refuge Proposal for TBW)

Taken together, the TBW sampling, gossypol analyses, and effective refuge determinations clearly demonstrate that a significant portion of the TBW population is derived from non-cotton hosts. This conclusion supports what is commonly known in the literature: that TBW is a polyphagous pest that utilizes a number of cultivated crops, wild hosts, and weeds for larval development. However, questions remain on the larger issue of whether these alternate hosts can serve as unstructured refuge for TBW for Bollgard II Bt cotton. Within the Cotton Belt, the gossypol data differed greatly by state/region. For some areas (i.e. North Carolina/Georgia), the proportion of non-cotton-origin TBW was consistently high throughout the season and may support the natural refuge proposal. In other regions (i.e. the Mississippi Delta, eastern Texas) the data were variable and more difficult to interpret. Given the assumptions of the Monsanto model, TBW resistance to Bollgard II is predicted not to evolve over the 30-year time horizon of the model for any scenario (current or natural effective refuge) in any region. On the other hand, there are a number of unresolved issues with the data, effective refuge, modeling, and interpretation of the results (detailed below). Should these issues be resolved, BPPD could support the use of natural refuge alone for Bollgard II in the southeastern U.S. cotton region. The strongest case for support of a natural refuge for TBW resistance management exists for the North Carolina and Georgia regions.

While Monsanto utilized the same gossypol methodology in both 2004 and 2005, there was no statistical analysis of the gossypol results between the two years, likely because of differences in sampling for each season. Therefore, it is not possible to compare the results between seasons except on a qualitative basis. Though many of the state trends were similar in both seasons, it is not known if there was a definitive correlation between the data sets.

As previously stated, the results from the gossypol bioassays varied by region. Perhaps the strongest case for the use of natural refuge can be made in North Carolina and Georgia. In these states, the data were more consistent and clearly showed that the large majority (>90%) of TBW originated from non-cotton sources. This trend was clear throughout the entire cotton growing season in both 2004 and 2005 (see table 3 in this review or Monsanto figure 1 in appendix 1). These states are known to contain substantial acreage of alternate crops (peanut, tobacco, and soybean) that are known to be preferred TBW hosts. Modeling predicted that resistance to Bollgard II would not evolve for any natural or current effective scenarios for either the North Carolina or Georgia region.

For the other sampled states, the data more variable both within each growing season and between seasons. In the Delta states (Mississippi, Louisiana, and Arkansas), the general trend was high proportions of non-cotton TBW early in the season, followed by a decline after June (when cotton became available as a host). During this period, the percentage of cotton-origin frequently dipped below 50%, though it was almost always at least 20% (see table 3). In Texas, during the cotton season (July-October) a majority of TBW were found to be cotton-origin, with the proportion exceeding 80% in some individual counties. These results seem to indicate that cotton is a much more important host for TBW in these states then in the east coast states (North Carolina and Georgia) and that fewer alternate hosts may be available during the cotton season.

BPPD has identified several issues with the TBW sampling and gossypol methodology. First, the use of pheromone traps for sampling resulted in the collection of only males. As such, no females were included in the bioassays. It is unclear whether inferences about female behavior (i.e. host utilization) can be made from data obtained solely from males. BPPD also questions whether the two year testing period is adequate for an experiment of this magnitude. For both Tennessee and Texas, only one year of data were compiled. Though relatively consistent, cropping and landscape patterns can change over time -- a factor that may not be reflected in a study of limited duration. Given the inherent variability in natural and agronomic ecosystems, a two year study may present only a “snapshot” of the host availability and productivity for TBW that may not be representative of future conditions. It is recommended that Monsanto address both of these issues in a future submission.

In addition to the sampling questions, BPPD noted that for a number of counties, trapping locations, and trapping periods there were low trap captures of TBW. For many traps, few (or no) moths were collected. For example, for Mississippi during July, 2004 (the height of the cotton season), in half of the sampled counties (6 of 12) less than 10 moths were collected. In Tennessee, TBW trap captures were so few that no gossypol analyses were conducted during 2004. Low TBW numbers have also been observed with Bt cotton resistance monitoring efforts (see BPPD 2005), possibly due to a suppressive effect from the widespread adoption of transgenic cotton. Data from collection sites with few captures (i.e. <10) were included in the effective refuge calculations, but not in the modeling (where worst case scenarios were used). It is unclear if the low trap captures in some areas may have affected the reliability of the results. There is also concern that should a resistant population emerge locally from a Bt cotton field, there will be insufficient susceptible TBW available to mitigate the threat.

Overall, the modeling suggests that Bollgard II cotton should have more than 30 years of durability for the control of TBW in all regions with natural refuge as the only source of susceptible insects. The predictions of the Caprio’s spatially-explicit, stochastic, two-locus TBW resistance model (Caprio 2006) are similar to those predicted by the Monsanto’s three-gene, deterministic model (Gustafson & Head 2005). From a qualitative perspective, modeling indicates the intrinsic durability of Bollgard II is greater than WideStrike which is greater than Bollgard. The presence of larger amounts of Bollgard II in the marketplace also increases the durability of other Bt cotton products that are present but the intrinsic durability of Bollgard II cotton is much greater than Bollgard or WideStrike cotton. However, there are uncertainties in the modeling parameters and assumptions which could impact the modeling output. Modeling suggests that the overall durability of Bollgard II cotton can be enhanced if Bollgard cotton is removed from the marketplace. Uncertainties in the pheromone captures, gossypol analyses, spatial analysis, estimation of effective refuge calculation, degree of shared binding affinity of Cry1Ac to receptor A and B, genetics of resistance, resistance mechanism, initial resistance allele frequency, and other modeling assumptions affect the precision and accuracy of the modeling predictions. Additional competition binding studies using TBW brush border membrane vesicles at saturable concentrations of Cry1Fa are recommended. Uncertainty in the shared binding affinity of the Cry1Ac protein to receptor A and B (in Monsanto’s model) would impact the modeling predictions for all three Bt cotton products because the Cry1Ac protein is common to all of them. Monsanto’s modeling also assumes no prior selection to Cry1Ac even though Bollgard cotton has been on the market since 1996. The degree to which the modeling predictions would be impacted is unknown.

It is important to consider that Bollgard II (Cry1Ac and Cry2Ab2) exists in a mosaic with single gene Bollgard (Cry1Ac). Modeling work has shown that in such a scenario, the pyramided product can be at risk for resistance if there is significant acreage of a single gene variety (Zhao et al. 2003, 2005; Hurley 2000). If resistance develops to the single gene product (Cry1Ac in this case) the dual gene product would effectively become a single gene product (Cry2Ab2). The single gene variety may in a sense act as a "stepping stone" for resistance to the pyramided product. Zhao et al. (2005) have shown that the concurrent use of one-and two-gene Bt broccoli plants resulted in control failure of both types of Bt plants for control of *Plutella xylostella* (diamondback moth) rather than the use of the two-gene plants by themselves. In addition, these researchers illustrated through their modeling efforts that resistance will evolve first to the toxin that is being used singly. Monsanto's natural refuge proposal contained no details on how this mosaic might be managed. There was no discussion as to whether Monsanto will "phase out" Bollgard to facilitate the adoption of Bollgard II. Rather, it was suggested in the submission that if there is no requirement to plant structured refuge a strong incentive will exist for growers to switch to Bollgard II. However, considering the large acreage of Bollgard currently in the marketplace (relative to Bollgard II), it is highly likely that a significant percentage of cotton acreage will remain planted with Bollgard varieties for at least several years.

The potential use of natural refuge for Bollgard II represents a switch from structured refuge (i.e. refuge planted and managed by growers) to an unstructured (i.e. unmanaged) refuge. There have been three major considerations to designing and deploying structured refuge for Bt crops. These are 1) production of sufficient numbers of susceptible insects to dilute any potential resistance genes, 2) proximity of the refuge to the Bt field to ensure random mating between susceptible and resistant insects, and 3) synchrony of the refuge with the Bt fields to ensure overlapping emergence between susceptible moths and any resistant survivors of the Bt crop. When evaluating the functionality of natural (unstructured) refuge, it is useful to consider these topics.

In terms of susceptible production, the goal of structured refuge for Bt crops has historically been a ratio of 500 susceptible insects for every resistant insect that could emerge from the Bt field (see EPA 2001). Monsanto's TBW sampling/gossypol experiments did not look at susceptible insect production from a numerical perspective per se; rather the proportion of moths originating on cotton/non-cotton sources was assessed (all trapped moths can be assumed to be Bt susceptible). The experiments clearly showed that a portion of the TBW develops on cotton, while another portion develops on non-cotton crops and wild hosts. However, as discussed above, the numbers collected at individual traps were highly variable, with some traps collecting few or no moths and others trapping over one hundred. It is noted that the sampling was conducted under the current IRM strategy that mandates a non-Bt cotton structured refuge which should (theoretically) have supplied a source of susceptible moths. On the other hand, should the natural refuge strategy be employed, the acreage of Bollgard II cotton could significantly increase while overall non-Bt cotton acreage may decrease. Given this scenario, it is conceivable that the portion of TBW developing on cotton (as determined from Monsanto's experiments) could be reduced, leaving mostly non-cotton-origin moths. It is not clear whether reducing the cotton-origin portion of the susceptible TBW population would have a measurable effect on the ability to dilute emerging resistance genes.

Both refuge proximity and developmental synchrony criteria were directly addressed by Monsanto's experimental design. For proximity, the TBW traps were placed adjacent to cotton fields (both Bt and non-Bt cotton). Though the traps were baited with a pheromone attractant that could attract moths from surrounding areas, the sampling likely presented an accurate portrayal of the TBW population around the cotton field trap sites. Developmental synchrony was also examined by collecting samples throughout the growing season. Data were assessed for each month of the cotton season and a statistical analysis showed that in the majority of cases the cotton/non-cotton proportions did not significantly differ within the individual sampling months. At variable proportions, the results showed that TBW developing on non-cotton hosts overlapped TBW from cotton during the major part of the cotton growing season (July- September) (see figure 1 attached to this review). These results were not surprising: though TBW may develop on different plant hosts at slightly different rates, there are four to six generations per year that likely create a natural overlap (discussion in Benedict 2004).

BPPD generally agrees with Monsanto that a natural refuge strategy (allowing 100% Bollgard II) will result in economic and environmental benefits. These benefits will result largely from yield increases and reduced insecticide use on cotton acreage previously planted as refuge (i.e. conventional cotton). However, these benefits will likely be compromised should the lepidopteran target pests develop resistance to the Bollgard II toxins. In such a case, there could be significant costs to growers that might have to switch to conventional (insecticide treated) cotton.

Under the current terms and conditions of registration for approved Bt cotton products, registrants are required to monitor for pest resistance to the expressed toxins. To date, BPPD has reviewed the annual monitoring reports through the 2004 growing season and found no evidence of resistance to Cry1Ac or Cry2Ab2 in either TBW or CBW (see BPPD 2005). Should the natural refuge proposal ultimately be accepted, it is highly recommended that these monitoring efforts continue for both Cry1Ac and Cry2Ab2. Monsanto's submission did not discuss any proposed changes to the monitoring requirements for either Bollgard product. However, considering that replacement of structured refuge with natural refuge may increase the Bollgard II acreage (and selection pressure for resistance), it may be justified to increase the monitoring efforts for Cry2Ab2 (i.e. increased sampling sites and collections).

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MRID# 467172-01: "Production of Tobacco Budworm from Alternate Host Plants and the Role of These Host Plants as Natural Refuge for Bollgard II Cotton." Submitted December 20, 2005.

MRID# 467172-02: "Modeling the Impact of Natural Refuge on the Evolution of Tobacco Budworm and Cotton Bollworm Resistance to Bollgard II Cotton." Submitted December 20, 2005.

MRID# 467172-03: "Scientific and Economic Justification for Not Requiring Structured Cotton Refuges for Bollgard II Cotton in the U.S. Cotton Belt from Texas to the East Coast." Submitted December 20, 2005.

April 3, 2006: Letter to Dennis Szuhay containing supplemental information requested in support of natural refuge amendment request for Bollgard II cotton. Supplemental to MRID# 467172-01.

April 7, 2006: Letter to Dennis Szuhay containing supplemental information requested in support of natural refuge amendment request for Bollgard II cotton. Supplemental to MRID# 467172-02.

May 10, 2006: Letter to Dennis Szuhay containing supplemental information requested in support of natural refuge amendment request for Bollgard II cotton. Supplemental to MRID# 467172-02.

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