

## Case Study

# An ecological risk assessment of Cry1F maize pollen impact to pale grass blue butterfly

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The intrinsic toxicity of lepidopteran-active *Bt* proteins necessitates assessment of non-target risks associated with environmental release of transgenic crops expressing these proteins. Principles of ecological risk assessment provide a means for assessing non-target risks when information regarding exposure to the toxin and species-specific effects are lacking. This is shown for the case of *Bt* Cry1F maize release in Japan, where off-field pollen dissemination and effect on butterfly species is of concern. The specific ecological entity of concern for the assessment of the non-target impact of Cry1F maize pollen was Yamato-shijimi (pale grass blue butterfly), *Pseudaeschnia maha* (Kollar), a commonly occurring, susceptible species. Yamato-shijimi is widely adapted in Japan where it occurs in both rural and metropolitan settings, corresponding to the distribution and habitat of katabami (*Oxalis corniculata* (L.)), the larval host plant. The northern extent of Yamato-shijimi habitat lies to the south of major maize production regions in Japan, but exposure may occur elsewhere where maize and Yamato-shijimi co-occur. Screening level assessment of potential adverse effects to Yamato-shijimi in the field environment considered the probability for spatial-temporal co-occurrence of the life stages of concern (1st and 2nd instars) and the stressor (Cry1F protein expressed in maize pollen) at environmentally relevant concentrations. In the event of exposure to maize pollen, early instars of Yamato-shijimi feed exclusively on the underside of katabami leaves, which further limits the portion of the butterfly population that would be exposed. Projected levels of exposure to Cry1F pollen are below the toxicity level of concern and, thus, indicate negligible risk. Most sensitive species characterization (intergenera sensitivity) similarly shows negligible risk to other Japanese butterfly species of concern when distributed beyond the maize field or field margin.

**Keywords:** *Bacillus thuringiensis* / *Bt* maize / Lepidoptera / non-target butterflies / *Pseudaeschnia maha* / Yamato-shijimi / *Oxalis corniculata*

## INTRODUCTION

Genetically-engineered maize (*Zea mays* (L.)) expressing Cry1 proteins from *Bacillus thuringiensis* (Berliner) (*Bt*) is increasingly used in global crop production to control lepidopteran pests. Because *Bt* maize may express the lepidopteran-active Cry1 proteins in pollen, its impact on non-target butterflies is a concern. In particular, the risk of *Bt* maize pollen to monarch butterfly (*Danaus plexippus* (L.)) in North America has been comprehensively studied from the standpoint of short-term exposures to individuals within the proximity of *Bt* Cry1Ab maize fields (Wolt et al., 2003), as well as implications of short-

term and long-term exposures to populations (Dively et al., 2004; Sears et al., 2001). These assessments couple quantitative ecological risk assessment methodologies with knowledge of non-target species biology to provide understanding of likely risk from *Bt* maize production systems.

Herculex<sup>TM</sup> I maize expresses the insecticidally active truncated Cry1F protein derived from *Bacillus thuringiensis* var. *aizawai* strain PS81I (NRRL B-18484) at levels efficacious for control of Asiatic corn borer (*Ostrinia furnicalis* (Guenee)). In addition to Asiatic corn borer, Herculex I (maize event DAS-Ø15Ø7-1, TC1507) provides

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resistance against European corn borer (*Ostrinia nubilalis* (Hübner)), black cutworm (*Agrotis ipsilon* (Hufnagel)), western bean cutworm (*Richia albicosta* (Smith)), and intermediate suppression of corn earworm (*Helicoverpa zea* (Boddie)). TC1507 maize expressing Cry1F protein has been subject to regulatory review and environmental approval in Japan as well as the United States, Canada, and Argentina (Agbios, 2005).

The non-target impact of Cry1F maize pollen flow is relevant to the overall risk assessment for Cry1F maize in Japan in terms of its importation and eventual cultivation (J-BCH, 2005). The Japanese Red List cites numerous butterflies of Japan that are endangered or vulnerable (Hamsen, 2001). Matsuo et al. (2002) evaluated these species and identified four with a larval stage corresponding to the period of maize anthesis. Three of these species are distributed far from maize fields, and the larvae of the fourth develops inside of a flower bud, thus exposure potential for these species is remote (MAFF, 2000). However, general effect and risk to other non-target butterflies is less certain.

Since, in Japan, no non-target butterfly analogous to monarch butterfly in North America has been identified as commonly occurring in and around maize fields, the Yamato-shijimi or pale grass blue butterfly (*Pseudozizeeria maha* (Kollar) (Lepidoptera: Lycaenidae)) has been viewed as an appropriate surrogate in risk assessments for *Bt* maize (Shirai and Takahashi, 2005). This species is widely distributed throughout Japan and is relatively more sensitive to *Bt* endotoxin in comparison to other representative Japanese butterflies (Matsuo et al., 2002).

An exposure and risk assessment was performed in accordance with ecological risk assessment principles (see for instance, USEPA, 1998). Environmental exposure of a stressor (maize-expressed Cry1F protein) was considered relative to effects on an ecological entity of concern (pale grass blue butterfly). Consideration of the potential impact of Cry1F maize pollen on the common and non-endangered species Yamato-shijimi provides insight as to how Cry1F maize may impact non-target butterfly species within Japan.

## ASSESSMENT APPROACH

Quantitative risk assessment methodology was used in conjunction with ecological considerations to characterize risk to Yamato-shijimi, consistent with the screening level approach of Wolt et al. (2003), to ascertain the degree of concern and the need or basis for more involved testing and assessment. The key aspects of this approach were

concise formulation of the problem addressed and its expression as a conceptual model; synthesis of a broad-based description of non-target impacts of Cry1F protein; and a focused characterization of exposure and effects specific to the ecological entity of concern. The results, along with conservatively projected effects values for a lepidopteran species of concern, were further bridged to a broader consideration of risk to non-target butterfly species in Japan.

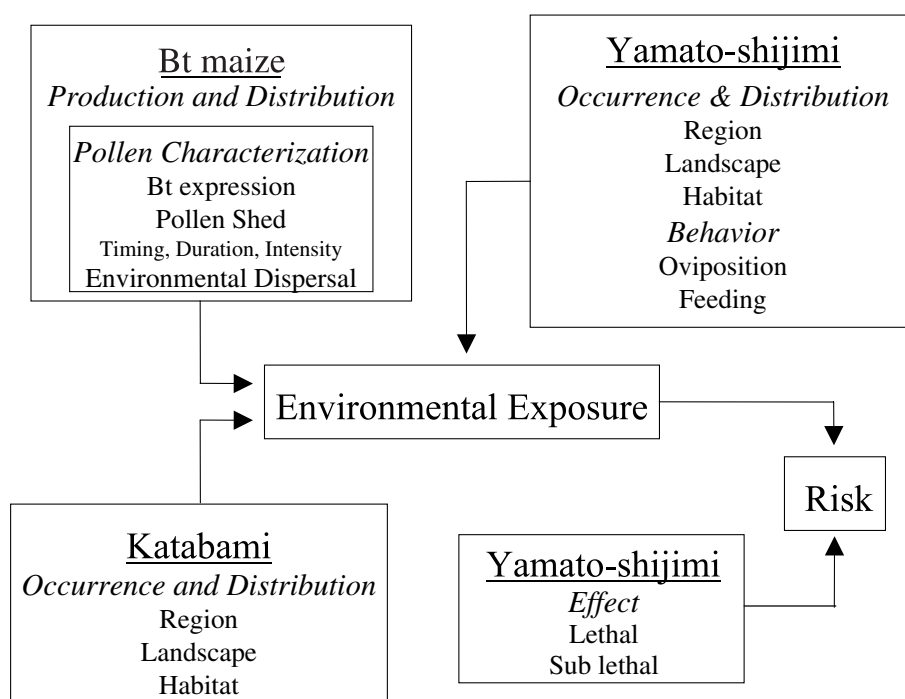
## Problem formulation

Off-field pollen dissemination is a potential route of exposure from maize-expressed Cry1F protein. Therefore, a consideration of the exposure of non-target species to maize pollen expressing Cry1F protein is relevant to the overall assessment of risks associated with the production of Cry1F maize. Yamato-shijimi is used in this assessment as a surrogate for additional non-target lepidopteran species of interest within Japan. As such, reasonable worst case assumptions regarding exposure concentrations are used. In accordance with a tiered assessment paradigm for risks to non-target insects (Romeis et al., 2006), evidence of negligible risk on the basis of these assumptions would constitute reasonable evidence for no harm. Conversely, evidence of significant risk at this stage of assessment warrants further detailed information concerning exposure probabilities, spectrum of effect, and the nature of the dose-response.

## Conceptual model

The conceptual model of Sears et al. (2001) for monarch butterfly risk assessment was adapted to guide the overall characterization of risk (Fig. 1). This conceptual model characterizes environmental exposure as a function of stressor source (*Bt* maize production and distribution), biology of the ecological entity of concern (Yamato-shijimi occurrence, distribution, and behavior), and food source (katabami, *Oxalis corniculata* (L.), occurrence and distribution). It then relates environmental exposure to effect (toxicity of Cry1F protein expressed in maize pollen to Yamato-shijimi) in arriving at the formulation of risk.

In addition, consideration of the intergenera sensitivity of lepidopteran insects to Cry1F protein was used to develop reasonable worst-case effects estimates for pollen exposure to a hypothetically sensitive butterfly species following the methodology of Wolt et al. (2003). A lower limit on the effect for a sensitive Lepidoptera was determined from the overall distribution of acute susceptibility for 16 lepidopteran species. The use of



**Figure 1.** Conceptual model for characterization of risk of Cry1F maize to pale grass blue butterfly (after Sears et al., 2001).

distributional analysis of effects in this manner to arrive at an effect endpoint is well established and has been described in SETAC (1994) and applied by Giesy et al. (1999). This methodology allowed for a conservatively based assessment of potential effect on other butterflies and for bridging to the risk findings for Yamato-shijimi.

## FINDINGS OF PREVIOUS NON-TARGET RISK ASSESSMENTS

### General effects on terrestrial invertebrates

*Bacillus thuringiensis* sbsp. *aizawai*, the bacterial source for Cry1F protein, is selectively active on the order Lepidoptera (Chambers et al., 1991). Toxicological studies on beneficial and non-target invertebrates using microbially-derived Cry1F protein or pollen from Cry1F maize plants support selectivity of maize-expressed Cry1F protein within Lepidoptera, given the margins of safety shown for other representative species (USEPA, 2001). These results are confirmed in observations from field abundance studies of beneficial and non-target insects associated with maize expressing Cry1F protein (USEPA, 2001).

### Specific effects on non-target Lepidoptera

Hellmich et al. (2001) measured the dose-response of neonate monarch butterfly larvae to purified insecticidal proteins from *B. thuringiensis* in artificial diet and found Cry1F protein was less potent than were Cry1Ab, Cry1Ac, or Cry9c proteins. Cry1F did not produce significant mortality at up to 30000 ng.ml<sup>-1</sup> diet. However, an increase in growth inhibition was observed at the highest concentration (30000 ng.ml<sup>-1</sup>). These authors additionally determined the effect of Cry1F on monarch larvae when exposed to Cry1F maize pollen on common milkweed (*Asclepias syriaca* (L.)) leaf disks (milkweed is the food source of monarch butterfly larvae). Under this more relevant exposure scenario, these studies showed no adverse effects of exposure to Cry1F maize pollen at doses as high as 1001–1600 grains.cm<sup>-2</sup> leaf (Hellmich et al., 2001), representing >5-fold the likely exposure within a maize field during pollen shed (171 grains.cm<sup>-2</sup>; Pleasants et al., 2001). Risk assessments of both short- and long-term effects of *Bt* maize pollen on monarch butterfly confirmed Cry1Ab protein expressed in maize pollen does not pose a serious threat to the monarch butterfly population (Dively et al., 2004; Sears et al., 2001). By extension, this suggests low risk from Cry1F; since even though expressed in pollen at relatively high levels, Cry1F protein lacks potency to monarch larvae.

In addition, the potential risk of *Bt* maize pollen to Karner blue butterfly (*Lycaeides melissa samuelis* (Nabokov)) was considered in the US registration for Cry1F maize events, and was not judged sufficient to warrant concern on the basis of limited exposure (USEPA, 2001).

## BIOLOGY AND ECOLOGY OF PALE GRASS BLUE BUTTERFLY

The biology, habitat, and distribution of Yamato-shijimi are described in detail by Fukuda et al. (1984). The following characterization of Yamato-shijimi draws on this source of information to establish the rationale for route of exposure and the relevance of the risk assessment to reproducing populations.

The larval host plant for Yamato-shijimi is katabami (yellow or creeping wood sorrel, *O. corniculata* (L.)), which is the only plant on which eggs are deposited and larvae feed. Shirôzu and Hara (1962) have definitively described life stages in Yamato-shijimi development. The adult generally lays eggs on the host plants in the sun during the spring and fall, when temperatures are cooler, but it often lays eggs on host plants in the shade during the midsummer. Eggs are deposited on the underside of leaves where the 1st and 2nd instars develop. The 1st and 2nd instars eat from the bottom side of katabami leaves, leaving the upper surface skin of the leaf intact. As they feed, the larvae move from leaf to leaf, leaving oval shaped leaf scars. The 3rd instars are usually found on host plant roots, stems, or nearby fallen leaves or pebbles. The final instars eat from the leaf margin, the leafstalk, the stem, and the fruit of katabami.

### Distribution and geographical variation

The pale grass blue is a common butterfly throughout much of Asia, where it is distributed mainly in subtropical and tropical zones. It is widely distributed in Iran, Pakistan, India, Myanmar, the Indochina Peninsula, the Philippines, south-central China, Taiwan, Korean Peninsula and the Japanese islands (Inayoshi, 1999; Savelle, 2003).

Yamato-shijimi is one of the most common butterflies of Japan; it is found in both rural and metropolitan areas. Yamato-shijimi is native to Honshu, Shikoku and Kyushu on the mainland of Japan, as well as the islands along the circumference of the mainland and the Nansei islands. Yamato-shijimi is not native to Hokkaido, and its occurrence there is only incidental, however, there is evidence for northward extension of native habitat from Tanohata-

Mura and Iwate-Ken into the Aomori Prefecture (Kudo et al., 2000).

### Life history

From 3 to 8 generations of Yamato-shijimi occur annually in Japan, with greater numbers of generations occurring in milder climates. Generally, the first adults appear between the end of March and the beginning of May in warmer regions, and in June in cooler regions. There is significant overlap in generations; thus, occurrence of early larval stages coincident with maize pollen shed would be expected where the butterfly and maize co-occur.

The Yamato-shijimi grows very quickly during the warm summer, with an observed egg term of 4 days, a larval stage of 16 days (final instars developing within 5 days) and a pupal stage of 8 days, totaling 28 days from egg to adults.

### Host plant characteristics

The reason for the wide adaptation of Yamato-shijimi is the distribution and habitat of the host plant, katabami. Katabami is a cosmopolitan perennial broadleaf weed common to disturbed open sites throughout the world. In Japan, katabami is widely distributed in agricultural settings and in metropolitan areas. It is a weed in lawn, garden, or greenhouse settings, but it does not commonly infest cultivated fields (Wilken and Hannah, 1998).

Katabami is a prostrate plant with branches that lie flat on the ground. Leaves are alternate, have hairy margins, and consist of three heart-shaped leaflets joined to a long petiole. The leaves often close and droop at night (Wilken and Hannah, 1998).

## EFFECTS CHARACTERIZATION

### Yamato-shijimi

Laboratory no choice studies have been used to assess toxicity of maize pollen expressing Cry proteins to Yamato-shijimi (Matsuo et al., 2002; Shirai and Takahashi, 2005). The laboratory assays involved exposure of first instars to pollen placed on the lower surface of katabami leaf segments. Leaf disks were exchanged daily with fresh leaf disks containing pollen throughout a seven-day exposure period. Results in studies similar to these showed increased mortality of Yamato-shijimi with time of exposure to Cry1F pollen

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**Table 1.** Acute sensitivity of lepidopteran species to Cry1F protein<sup>1</sup> (data are for 1st instars).

| Common Name             | Scientific name                         | LC <sub>50</sub> , µg.g <sup>-1</sup> |
|-------------------------|---|---------------------------------------|
| Diamondback moth        | <i>Plutella xylostella</i> Linnaeus     | 0.065                                 |
| European corn borer     | <i>Ostrinia nubilalis</i> Hübner        | 0.58                                  |
| Eastern cotton leafworm | <i>Spodoptera littoralis</i> Boisduval  | 0.99                                  |
| Tobacco budworm         | <i>Heliothis virescens</i> Fabricius    | 1.88                                  |
| Cabbage looper          | <i>Trichoplusia ni</i> Hübner           | 2.15                                  |
| Fall armyworm           | <i>Spodoptera frugiperda</i> Smith      | 2.49                                  |
| Beet armyworm           | <i>Spodoptera exigua</i> Hübner         | 7.8                                   |
| Sod webworm             | <i>Crambus</i> spp.                     | >10                                   |
| Common cutworm          | <i>Spodoptera litura</i> Fabricius      | 27                                    |
| Monarch neonate larvae  | <i>Danaus plexippus</i> Linnaeus        | >30 <sup>2</sup>                      |
| Bertha armyworm         | <i>Mamestra configurata</i> Walker      | >36                                   |
| Southwestern corn borer | <i>Diatraea grandiosella</i> Dyar       | >50                                   |
| Black cutworm           | <i>Agrotis ipsilon</i> Hufnagel         | 69.2                                  |
| Bollworm                | <i>Helicoverpa armigera</i> Hübner      | >100                                  |
| Spruce budworm          | <i>Choristoneura fumiferana</i> Clemens | 140                                   |
| Gypsy moth              | <i>Lymantria dispar</i> Linnaeus        | 410                                   |

<sup>1</sup> Diet incorporation studies are subject to rather wide variance depending on experimental conditions. Unless otherwise noted, data are representative values based only on exploratory assays, and do not necessarily represent definitive values (after Evans, 1998, Equivalency of microbial and maize expressed Cry1F protein; characterisation of test substances for biochemical and toxicological studies, unpublished technical report, Dow AgroSciences LLC).

<sup>2</sup> Definitively determined value for monarch neonate larvae (Hellmich et al., 2001).

relative to comparable isolines (J-BCH, 2005). For Yamato-shijimi larvae exposed for 5 days to Cry1F maize pollen, the LC<sub>50</sub> was 100 grains.cm<sup>-2</sup>, on the basis of the compensated mortality rate (J-BCH, 2005).

### Most sensitive species characterization

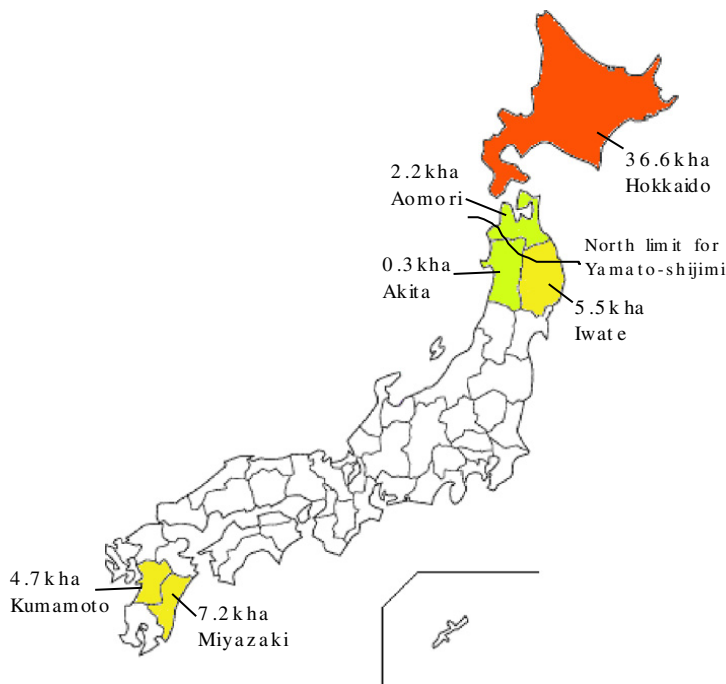
Effects characterization for a hypothetically exposed, sensitive non-target butterfly used a database for lepidopteran sensitivity to bacterially expressed Cry1F protein fed by dietary incorporation (Tab. 1). These LC<sub>50</sub> for 16 lepidopteran species demonstrate more than three orders of magnitude variation in sensitivity to Cry1F protein. The most sensitive species (diamondback moth, *Plutella xylostella* (L.)) is a target for insect control with the bacterium *Bacillus thuringiensis* ssp. *aizawai*, donor of the *cry1F* sequence. Notably, monarch larvae, the cause for concern regarding Cry protein hazard in North

America, are in the mid-range of Lepidoptera species sensitivity to Cry1F protein.

Lepidopteran species susceptibility (intergenera sensitivity) was estimated as the 5th centile cumulative effect (0.040 µg Cry1F.g<sup>-1</sup> diet) as determined from a cumulative probability plot of the LC<sub>50</sub> shown in Table 1. This value is >250-fold lower than the species geometric mean LC<sub>50</sub> (10.3 µg.g<sup>-1</sup>) and represents a worst case effect endpoint for the tier 1 risk assessment for a hypothetical sensitive species of concern. This effect level is approximately equivalent to 33 maize pollen grains.cm<sup>-2</sup> leaf on the basis of diet conversion factors and maize pollen density reported in Hellmich et al. (2001), and Cry1F pollen expression of 32 µg.g<sup>-1</sup> (USEPA, 2001). The calculation is (see Eq. below).

Thus, the effect level for a hypothetical non-target lepidopteran species of concern would be approximately 3-fold lower than measured for the Yamato-shijimi.

$$\frac{0.040 \mu\text{g Cry1F.g diet}^{-1} \times 0.0033 \text{ g diet.day}^{-1} \times 1500000 \text{ grains pollen.g pollen}^{-1}}{32 \mu\text{g Cry1F.g pollen}^{-1} \times 0.19 \text{ cm}^2 \text{ leaf.day}^{-1}}$$



**Figure 2.** Principal maize production regions of Japan relative to the northern extent of Yamato-shijimi distribution (MAFF, 2004). kha = 1000 ha. Highlighted prefectures account for 65% of total hectares.

## EXPOSURE CHARACTERIZATION

### Maize production

Maize production in Japan is almost exclusively for animal feeding (soiling maize). In 2004, 42% of maize in Japan was planted in Hokkaido (representing 36600 ha out of 87400 ha produced in Japan; MAFF, 2004). Concentrated maize production occurs principally north of the range of Yamato-shijimi distribution (Fig. 2).

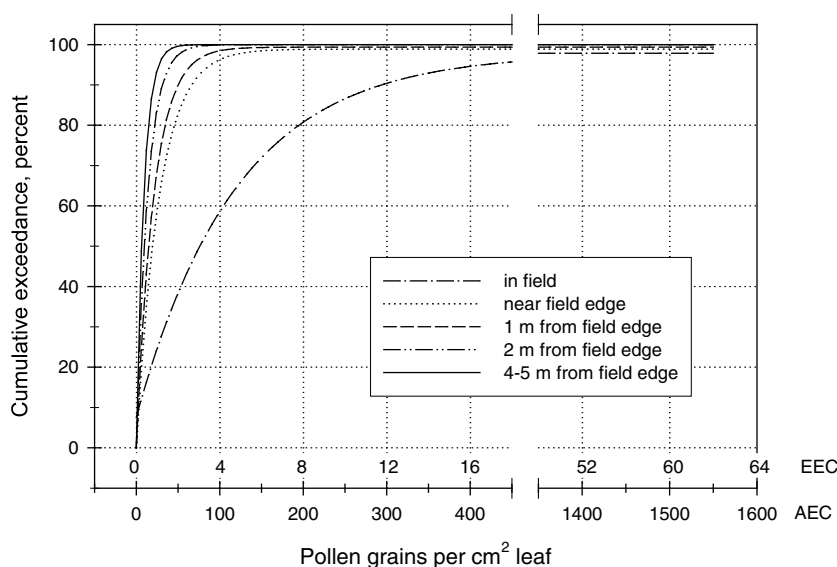
### Pollen exposure characterization

The potential for a butterfly of special concern to be exposed to Cry1F maize pollen will relate in large part to the probability of encountering Cry1F pollen deposited on the food source for larvae at a sensitive stage of growth. For the specific case of Yamato-shijimi, a reasonable worst case assumption is that some small, but definable, fraction of eggs will be laid on katabami occurring in or in near proximity to maize fields planted to Cry1F hybrids. A similar assumption has been made for monarch butterfly risk assessments (Dively et al., 2004; Sears et al., 2001) where measurements of the actual environmental concentration (AEC) of maize pollen on common milkweed (*Asclepias syriaca* (L.)) leaves in and around maize fields were used to determine exposure probabilities and consequent risk.

Maize pollen is large and once dehisced it will rapidly settle due to gravity. Patterns of maize pollen distribution, therefore, show marked declines with distance from the maize field edge. Pleasants et al. (2001) measured pollen concentrations on >1700 milkweed plants occurring in and around maize fields at locations throughout the North American Corn Belt and reported average pollen densities on the upper surfaces of milkweed leaves of 171 grains.cm<sup>-2</sup> within maize fields; 63 grains.cm<sup>-2</sup> at the immediate maize field edge, and 35, 14, and 8 grains.cm<sup>-2</sup> at distances of 1, 2, and 4–5 meters from the field edge. These measures of AEC are comparable to other, less extensive measurements of maize pollen deposition in Japan on the leaf surface of sunflower (*Helianthus annuus* (L.)) and black nightshade (*Solanum niger* (L.)) (J-BCH, 2005; Shirai and Takahashi, 2005) as well as to measurements made in Europe on the upper leaves of wild carrot (*Daucus carota* (L.)) (Lang et al., 2004). Differences in plant growth habit and leaf morphology would be expected to alter the degree of pollen interception and retention by a receptor plant. In the case of katabami, the nocturnal closure of leaves by drooping would likely limit maximum pollen accumulation; therefore, surrogate values from milkweed should be conservative.

Yamato-shijimi eggs are laid on the underside of leaves of katabami and larvae feed exclusively from the bottom side of the leaf during the 1st to 2nd instar (Fukuda et al., 1984). Consequently, the pollen concentrations

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**Figure 3.** Cumulative frequency distribution of maize pollen on leaves in and near maize fields. On the X-axis, the upper figures correspond to the actual environmental concentration (AEC) on the upper surface of milkweed leaves (Pleasants et al., 2001), and the lower figures correspond to the estimated environmental concentration (EEC) on the lower surface of katabami leaves ( $EEC = 0.04 \times AEC$ ).

measured on leaf surfaces (J-BCH, 2005; Lang et al., 2004; Pleasants et al., 2001; Shirai and Takahashi, 2005) overestimate exposure concentrations at early stages of development. Pleasants et al. (2001) determined that maize pollen density on the underside of milkweed leaves was 4% of that on the upper leaf surface. Therefore, a factor of 0.04 is used here to scale the pollen density distributions of Pleasants et al. (2001) to distributions of density for the lower leaf surface of katabami leaves (Estimated Environmental Concentration shown in Fig. 3). These estimates of pollen density on the lower leaf surface are used here as the surrogated values of EEC to which Yamato-shijimi are exposed while feeding on the underside of leaves.

### Spatial-temporal overlap

The area of most concentrated maize grain production within Japan, occurs north of the range for Yamato-shijimi. However, production will occur throughout Japan and for maize produced within the range of Yamato-shijimi distribution, there are generally two crops grown per year. For maize produced in the southern prefectures of the Japanese mainland, maize anthesis occurs in the period 20 to 30 June for the first crop and at the end of September for the second crop. In these areas, Yamato-shijimi generally produces 6 generations per year (but up to 8 generations may occur in certain years) (Fukuda et al., 1984). Because of the season-long abundance of Yamato-shijimi, there is some likelihood of butterfly occurrence in various life stages during maize pollen anthesis.

Spatial proximity of neonate Yamato-shijimi to maize fields, however, is less likely due to the uncommon association of *O. corniculata* to cultivated fields (Wilken and Hannah, 1998). Specific observations of abundance are not available for Japan, but in recent weed surveys of maize fields, *O. corniculata* occurred in less than 2% of observations (MDA, 2005).

## RISK CHARACTERIZATION AND ASSESSMENT

### Risk to pale grass blue butterfly

Spatial-temporal considerations largely limit the ability for Yamato-shijimi to be exposed to maize pollen. The small fraction of larvae at a sensitive growth stage that may be exposed to maize pollen comprises a negligible fraction of this species, which is very broadly distributed and which exhibits multiple generations per year in most habitats where it occurs. Even in the rare event where sensitive larval stages may occur within or at the near field edge of maize fields during pollen shed, the potential for exposure to pollen is low. This is because feeding of early instars occurs on the abaxial surface of katabami leaves, where the potential for pollen accumulation at exposure levels of significance is a remote possibility. If the laboratory-derived  $LC_{50}$  of approximately  $100 \text{ grains.cm}^{-2}$  is projected as the threshold of concern for Yamato-shijimi to Cry1F maize pollen, there is negligible risk to sensitive larvae should they occur in or near fields of Cry1F maize

during pollen shed. This is because the maximum level of projected exposure is  $64 \text{ grains.cm}^{-2}$  and the 90th percentile of exposure is  $\leq 12 \text{ grains.cm}^{-2}$ , even within the confines of the field where maximum pollen accumulation is possible (estimated environmental concentration, Fig. 3). These values range from 2- to 5-fold lower than the effect threshold developed from constant exposure no choice laboratory studies (J-BCH, 2005).

### Risk to other species of concern

The conservatively projected effect level used here for a hypothetical non-target species of concern is  $33 \text{ grains.cm}^{-2}$  leaf. Pollen exposure on upper leaf surfaces is not anticipated to exceed these levels beyond the near field edge. For instance, the Pleasants et al. (2001) data (actual environmental concentration, Fig. 3) show 90th percentile maize pollen densities on milkweed leaves of  $\leq 35 \text{ grains.cm}^{-2}$  at 1 meter, and rapid decrease over the next few meters.

### Uncertainties in the risk assessment

This risk assessment conservatively projects effects levels to Yamato-shijimi on the basis of laboratory studies where larvae have no choice as to food source and for which exposure to pollen is constant through early stages of larval development. Neither condition is anticipated in the field; thus, effects in the field environment should be less than estimated here. Actual pollen densities on the abaxial surface of katabami where early instars of Yamato-shijimi feed have not been determined, but are projected on the basis of extrapolation from a rich body of data for pollen on milkweed leaves. Extended duration studies that consider feeding habit and later stage instars feeding on upper leaf surfaces have not been conducted. Yamato-shijimi reproducing populations, however, are not commonly associated with maize fields, and effects to early instars of Lepidoptera species are generally sensitive indicators of effects to later generations (Peacock et al., 1998). Effect thresholds for a hypothetical threatened or endangered butterfly were based on reasonable worst case intergenera sensitivities and are therefore uncertain; but biology for species of concern (Matsuo et al., 2002) shows little opportunity for exposure, so estimates of risk remain conservative. A fully quantitative risk assessment would require specific data on the regional distribution of maize planting in Japan as well as improved characterization of katabami occurrence and distribution in and around maize fields.

### RISK CONCLUSIONS

While off-field pollen dissemination is a potential route of exposure from transgenic maize expressing Cry1F protein, previous studies and assessments establish that microbially-derived Cry1F protein or pollen from Cry1F maize plants is selectively active on lepidopteran species and exhibits wide margins of safety for other insects. A screening-level risk assessment consistent with the paradigm of ecological risk assessment was applied to non-target Lepidoptera in Japan.

Special emphasis is given to the Yamato-shijimi (pale grass blue butterfly), a commonly occurring, sensitive species that is widely distributed throughout Japan. Screening-level risk assessment methodology was used in conjunction with ecological considerations to characterize risk to Yamato-shijimi. Despite relatively widespread occurrence in Japan, coincident with occurrence of its food source, spatial-temporal considerations largely limit the ability for Yamato-shijimi to be exposed to maize pollen. Yamato-shijimi habitat lies to the south of major maize production regions in Japan, however, a northward shift in distribution as well as maize cultivation in southern regions of Japan affords a limited opportunity for co-occurrence of Cry1F pollen and Yamato-shijimi. The risk of an adverse effect to Yamato-shijimi being manifested in the field environment is especially low when considering the remote possibility for spatial-temporal co-occurrence of the life stages of concern (1st and 2nd instars) and the stressor (Cry1F expressed in maize pollen) at environmentally relevant concentrations.

Previous analyses have concluded that Lepidoptera appearing on the Japanese Red List are not at risk from exposure to *Bt* maize due to their spatial-temporal occurrence in relation to maize pollen shed. In addition, specific to Cry1F maize, there is negligible evidence for potential harm to other non-target lepidopteran species within Japan as determined from the worst case intraspecies effects estimate for a hypothetical non-target species of concern, and the low probability for exposure of sensitive species in maize fields or field margins during pollen shed.

### ACKNOWLEDGEMENTS

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