Research Article

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Susceptibility to *Bt* proteins is not required for *Agrotis ipsilon* aversion to *Bt* maize

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Abstract

BACKGROUND: Although *Bacillus thuringiensis* (*Bt*) maize has been widely adopted in diverse regions around the world, relatively little is known about the susceptibility and behavioral response of certain insect pests to *Bt* maize in countries where this maize is not currently cultivated. These are important factors to consider as management plans are developed. These factors were investigated for *Agrotis ipsilon*, a global pest of maize, with Cry1F and Cry34Ab1/Cry35Ab1 maize.

RESULTS: *Agrotis ipsilon* demonstrated an initial, post-ingestive aversive response to Cry1F maize. Development and mortality were also affected – survival on Cry1F maize tissue was 40% and weight gain of survivors of Cry1F exposure was significantly reduced. A post-ingestive aversive response was also seen for Cry34Ab1/Cry35Ab1 maize; however, longer-term feeding, weight gain and survival were not affected.

CONCLUSION: *Agrotis ipsilon* showed aversion to both *Bt* treatments. Aversion to Cry34Ab1/Cry35Ab1 maize was unexpected because these proteins have no known insecticidal effect against Lepidoptera; however, results confirm that this aversion was temporary and did not affect growth or development. The Cry1F results suggest that *A. ipsilon* will abandon Cry1F maize in the field before any selection for resistance. These data support the use of refuge to delay Cry1F resistance development in *A. ipsilon* populations.

Keywords: event TC1507; event DAS-59122-7; larval movement; transgenic corn; antixenosis; refuge

1 INTRODUCTION

When *Bt* maize was first commercially grown in North America in the late twentieth century, it was intended to control primary pests of maize in the region, such as *Ostrinia nubilalis* (Hübner) and *Diatraea grandiosella* Dyar. Nearly two decades later, *Bt* maize is grown on five continents and many countries. The products that were developed for North American primary pests of maize are now applied to a variety of primary and secondary pests, such as *Ostrinia furnacalis* (Guenée) (Asia), *Sesamia nonagrioides* (Lefèbvre) (Europe), *Busseola fusca* (Fuller) (South Africa), *Spodoptera frugiperda* (JE Smith) (North and South America) and *Agrotis ipsilon* (Hufnagel) (ubiquitous).[1–4] As *Bt* maize was generally not developed to control these pests, current *Bt* maize events do not always provide 100% control of important pests in new geographies or pests of secondary economic importance.

Although a *Bt* maize event may not prevent all insect feeding, it may still be efficacious, protecting yield in the absence of high insect toxicity. Rejection of *Bt* maize owing to a behavioral response could contribute to efficacy in the field. Insect rejection of both toxic and non-toxic compounds is not uncommon.[5–10]

The initial rejection of a food source is not always permanent and could end in the eventual acceptance of that food source, i.e. loss of aversion.[11,12] Desensitization of the mechanism that causes the aversive response (e.g. taste mediated),[13] increased (or induced) detoxification of the aversive compound[12,14] or a combination of both desensitization and detoxification[11,15] could explain acceptance of a previously rejected food source such as *Bt* maize.

Agrotis ipsilon is an important global pest of maize, present on every continent where *Bt* maize is cultivated. Moths typically lay their eggs in weeds, and the larvae will move from feeding on weeds to feeding on corn when the weed host is destroyed.[16,17] *A. ipsilon* can cause significant damage to unprotected fields of maize by cutting off seedlings or tunneling into the base of an older plant and destroying the growing point.[18] A few commercial *Bt* maize events are efficacious against *A. ipsilon*; however, none provides complete control. A frequency of 5–10% cut plants due to *A. ipsilon* in a pure stand of Cry1F maize is not unexpected.[19] Since the commercialization of event TC1507 maize (expressing the Cry1F insecticidal protein) in the United States, the possibility of a behavioral response to the protein has been investigated,[20] but variability in results does not conclusively indicate rejection of Cry1F maize.

To continue to explore the possibility of a behavioral response to *Bt* maize, a series of laboratory studies were conducted with *A.*

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ipsilon larvae. Two separate experiments, of short and long durations, were designed to answer the following questions: (1) does *A. ipsilon* exhibit an initial aversive response to *Bt* maize?; (2) can *A. ipsilon* overcome aversion and develop normally on *Bt* maize?

2 METHODS

Methods are similar to those used by Binning *et al.*[21] and are described in detail here. Three types of maize (all Pioneer brand hybrids) were used for each experiment: (1) a hybrid that contained *Bt* event TC1507 (hereafter referred to as Cry1F maize); (2) a hybrid that contained *Bt* event DAS-59122-7, hereafter referred to as *Bt*-RW maize; (3) a non-*Bt* maize hybrid that was a near-isoline to both events and did not express any insecticidal proteins, hereafter referred to as non-*Bt* maize. Fully formed individual leaves were removed from plants to supply tissue for each experiment. Maize plants were grown in pots in a walk-in environmental growth chamber maintained using standardized parameters for maize production (17:7 L:D, 24 ± 3 °C). Leaves were removed at approximately growth stages V6 to V10. Each leaf was rinsed with tap water to remove surface debris and stored in resealable plastic bags in the refrigerator (~4 °C) or on wet ice until use, no longer than 48 h. Plant tissue was used instead of artificial diet to maximize the field relevance of the experiment and to reduce confounding effects that nutrition or water content might have on behavior.[22]

2.1 Short-duration study

For three replications, eggs from a susceptible laboratory population of *A. ipsilon* were obtained from a commercial source (Benzon Research, Inc., Carlisle, PA). For the remaining six replications, eggs from a susceptible laboratory population of *A. ipsilon* were obtained from a colony maintained by the USDA-ARS Corn Insect Crop Genetics Research Unit (Ames, IA). Larvae were maintained individually on non-*Bt* maize leaf clippings until they reached the third instar.

A 3 min exposure assay was utilized by Glendinning and Slansky[22] to determine whether *S. frugiperda* detected aversive compounds pre-ingestively or post-ingestively. The following short-duration study is modeled after their methods. The sequence of events for the short-duration study is outlined in Fig. 1a.

This study was divided into two phases – screening and testing. Starting with the screening phase, *A. ipsilon* within the first 24 h of the third stadium were individually removed from the rearing material, placed in an empty petri dish (100 × 25 mm, NUNC No. 4031) and deprived of food for 60 min. After this period of starvation, a cutting of non-*Bt* leaf (~4 cm^2) was placed within 2 cm of the larva's head. Initiation of feeding triggered the start of data collection. Time spent feeding was recorded for 3 min using the event tracking portion of a video tracking software program (EthoVision® XT; Noldus Information Technology, Wageningen, The Netherlands). After this short observation time, each

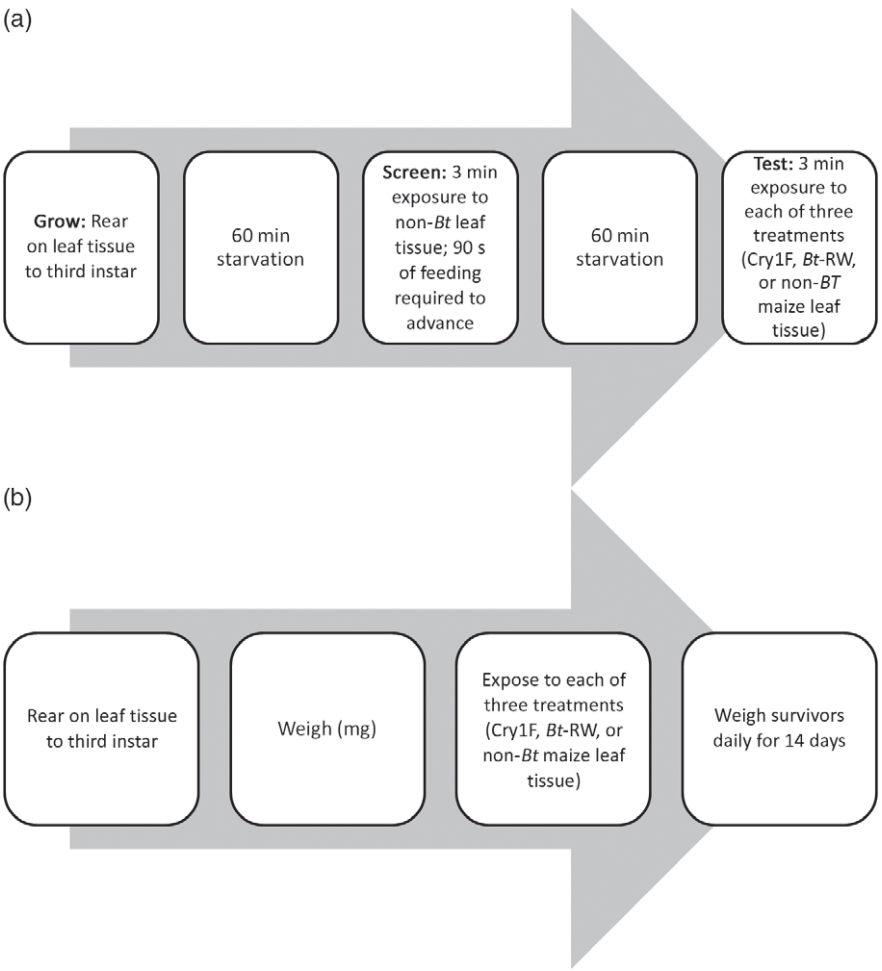

Figure 1. Sequence of events: (a) short-duration study; (b) long-duration study.

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larva was allowed to continue feeding on non-*Bt* leaf tissue for an additional 7 min to allow for a full feeding bout and avoid any potential for extreme hunger that might affect test results. Feeding times were recorded for each larva, and larvae that did not feed for at least 90 of the 180 s period of observation were discarded.

Following this screening phase, the testing phase began. Each larva was again food deprived in an empty petri dish for 60 min. Next, a leaf cutting from one of the three treatments (non-*Bt*, Cry1F or *Bt*-RW maize) was placed within 2 cm of the larva's head. Initiation of feeding triggered the start of data collection, and time spent feeding was recorded for 3 min. Nine larvae per treatment were tested in this phase. After the 3 min observation time, each larva was placed in an individual well of a six-well bioassay tray (BD Falcon No. 353046), and non-*Bt* leaf material was provided. After 72 h, larvae were checked for mortality.

To determine the validity of this test system, the amount of time spent feeding on non-*Bt* leaf tissue in the screening stage was compared with the amount of time spent feeding on non-*Bt* leaf tissue in the testing phase. If the time spent feeding in the testing phase was significantly shorter than the time spent feeding in the screening phase, this indicated that 60 min of starvation was not long enough to account for the normal gap between *A. ipsilon* feeding bouts on maize tissue.

If rejection of *Bt* maize was due (at least in part) to a deterrent, there should have been a rapid significant decrease in time spent feeding compared with non-*Bt* maize. Glendinning and Slansky[22] observed a shorter time spent feeding for *S. frugiperda* within the first 15–30 s of exposure to the deterrent compounds linamarin and caffeine. Even if deterrence was not observed, there could still be rejection related to a post-ingestive effect. Post-ingestive rejection of *Bt* would likely take longer than 60 s, especially if it were due to toxicity of *Bt*. The *Bt* protein must be ingested and passed through the foregut into the midgut where it must bind to receptors, insert into the membrane and finally form pores that lead to gut lysis and septicemia.[23] A delayed response (>60 s), such as that observed by Glendinning and Slansky[22] to nicotine hydrogen tartrate, would indicate that a reduction in feeding is due to a post-ingestive effect. If there is no rejection of *Bt* maize, the larvae should feed for the same amount of time as larvae exposed to non-*Bt* maize.

2.2 Long-duration study

The long-duration study was designed to investigate the susceptibility of *A. ipsilon* and larval ability to overcome aversion to *Bt* maize by monitoring daily growth and survival. The sequence of events for the long-duration study is outlined in Fig. 1b. The source of eggs for the long-duration study was a colony maintained by the USDA-ARS Corn Insect Crop Genetics Research Unit (Ames, IA). Larvae were individually maintained on non-Bt maize leaf cuttings until they reached the third instar. Third instars were chosen because of the tendency of *A. ipsilon* neonates to initiate feeding on weeds and move into maize as older instars.

Within the first 24 h of the third stadium, each larva was individually removed from the rearing material, placed in a well of a six-well bioassay tray and starved for 60 min. Next, each larva was individually weighed to the nearest 0.1 mg, returned to the well in the bioassay tray and provided with leaf cuttings from non-*Bt*, Cry1F or *Bt*-RW maize. The experiment ended on day 14 (day 1 being the day of infestation).

This experiment employed a randomized complete block design with 16 replications per treatment and two larvae per replication. Each maize plant provided leaf tissue for one replication per treatment. Mortality and weight of survivors were recorded daily. A shift from rejection to acceptance was indicated by larval survival and weight gain.

2.3 Data analysis

The statistical analyses in the short-duration study were conducted using SAS software, v.9.3, comparing the cumulative feeding time of *A. ipsilon* for the three treatments.[24] SAS PROC MIXED was utilized to fit the analysis of variance (ANOVA) model. A two-tailed *t*-test was conducted at each interval of 15 s. Owing to the multiple pairwise comparisons, a significant difference was identified if the *P*-value (of the *t*-test) for difference between treatments was less than 0.01, rather than 0.05.

The analysis of the long-duration study compared the total weight gain of *A. ipsilon* fed each of the three treatments. A heterogeneous variance model was utilized to compare treatment effects. SAS PROC MIXED was utilized to fit the model. A significant difference was identified if the *P*-value (of the *t*-test) for difference between treatments was less than 0.05.

3 RESULTS

3.1 Short-duration study

The average time spent feeding on non-*Bt* maize in the screening phase was no different from the time spent feeding on non-*Bt* maize in the testing phase ($t = 1.4$, df = 33, $P = 0.19$). This validates 60 min as an adequate gap between feeding bouts for *A. ipsilon* on maize leaf tissue.

In the testing phase, third-instar *A. ipsilon* spent significantly less time feeding on Cry1F than on non-*Bt* maize ($t = -2.58$, df = 21, $P = 0.02$) (Table 1). Mean time spent feeding on *Bt*-RW maize was also significantly different from non-*Bt* maize ($t = -3.68$, df = 21, $P = 0.001$), but not significantly different from Cry1F ($t = 1.10$, df = 21, $P = 0.29$). While this indicates that *A. ipsilon* initially rejects both Cry1F and *Bt*-RW maize, examination of the cumulative feeding was needed to evaluate whether this rejection was pre-ingestive or post-ingestive. Figure 2 compares the cumulative time spent feeding on Cry1F and non-*Bt* maize. A significant difference between Cry1F and non-*Bt* first occurs at 120 s ($t = -2.57$, df = 21.2, $P = 0.01$). Figure 3 compares the cumulative time spent feeding on *Bt*-RW and non-*Bt* maize. A significant difference between *Bt*-RW and non-*Bt* first occurs at 90 s ($t = -2.62$, df = 21.2, $P = 0.01$). These results indicate that *A. ipsilon* aversion to both Cry1F and *Bt*-RW maize is likely post-ingestive. There was no significant difference in cumulative time spent feeding on Cry1F and Bt-RW maize at any time point (Fig. 3). No mortality was observed for any treatment 72 h after exposure.

Table 1. Mean time *A. ipsilon* third instars spent feeding during a 3 min exposure to maize leaf tissue

Treatment	*n*	LS mean time feeding (s) (95% CI)[a]
Cry1F	9	112 (64–159) a
Bt-RW	9	87 (39–134) a
Non-*Bt*	9	174 (156–191) b

[a] CI: confidence interval. Treatments with different letters were statistically different ($P < 0.05$).

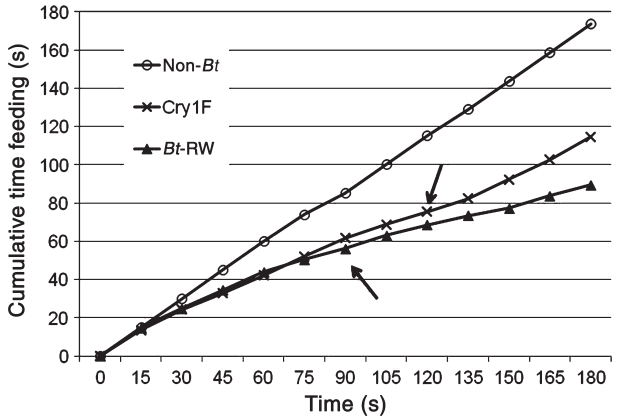

Figure 2. Cumulative time spent feeding by third-instar *A. ipsilon* on Cry1F, *Bt*-RW and non-*Bt* maize leaf tissue. The earliest significant difference between Cry1F and non-*Bt* was at 120 s ($P = 0.01$). The earliest significant difference between *Bt*-RW and non-*Bt* was at 90 s ($P = 0.01$). Each difference is indicated by an arrow.

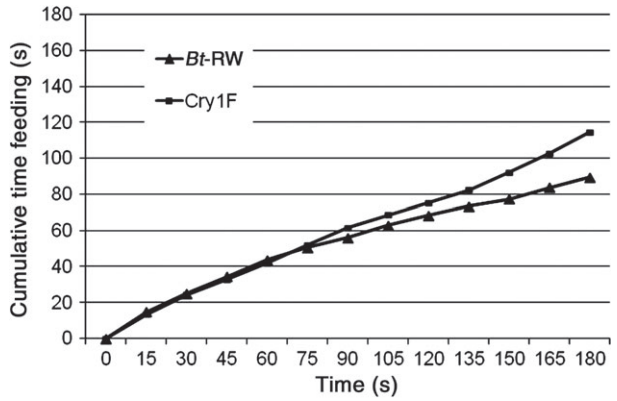

Figure 3. Cumulative time feeding by third-instar *A. ipsilon* on *Bt*-RW and Cry1F maize leaf tissue. There was no significant difference between *Bt*-RW and Cry1F at any time point, including 180 s ($P = 0.28$).

3.2 Long-duration study

Survival was low in the Cry1F maize treatment, with 40% (eight larvae) surviving for 14 days. Those survivors gained weight, although average total weight gain of survivors on Cry1F maize was significantly less than on either *Bt*-RW ($t = -6.65$, df = 56, $P < 0.0001$) or non-*Bt* maize ($t = -5.67$, df = 56, $P < 0.0001$) (Table 2). Those insects exposed to Cry1F maize that died before the end of the assay lived an average of 6.3 days, with a median of 6.5 days, and gained an average total of 4.0 mg before death, compared with the average of 162, 568 and 502 mg for larvae surviving exposure to Cry1F, *Bt*-RW and non-*Bt* maize respectively. The average total weight gain of insects exposed to *Bt*-RW maize was not significantly different from non-*Bt* maize ($t = -0.97$, df = 56, $P = 0.34$).

Frequency distributions of daily weight gain show that, on a daily basis, 48 and 52% of the weight gains for insects fed non-*Bt* and *Bt*-RW maize, respectively, were ≥21 mg (Fig. 4). The majority of daily weight gains were positive for all insects that were fed Cry1F leaf tissue (including those that lived and those that died), with 56% falling between 1 and 20 mg. Weight loss on Cry1F maize was less common, with 30% of daily weight gain ≤0 mg. The Cry1F treatment can be further separated into insects that survived and insects that did not survive exposure to Cry1F maize. For both groups, the majority of daily weight gains were between 1 and 10 mg (Fig. 5). Of the daily weight gains for those that did not survive Cry1F exposure, 46% were ≤0 mg and 3% were >10 mg (Fig. 5b). Conversely, 20% of the daily weight gains for Cry1F maize survivors were ≤0 mg, and 32% were >10 mg (Fig. 5a).

Weight change was averaged by treatment by day and is shown in Fig. 6. Insects exposed to non-*Bt* and *Bt*-RW maize showed similar trends in average daily weight change. Larvae stop eating and tend to lose weight when they molt, which would explain the two distinct losses of weight on days 9 and 13, and also a possible third molt on day 5 for non-*Bt* and on day 6 for *Bt*-RW maize (Fig. 6a). These three molts would suggest that the insects exposed

Table 2. Mean weight gain of surviving *S. frugiperda* larvae after 14 days of exposure to maize leaf tissue

Treatment[a]	*n*	Percentage mortality	LS mean weight gain (mg) (95% CI)[b]
Cry1F	20	60	162 (87–237) a
Bt-RW	20	0	568 (471–664) b
Non-*Bt*	20	5	502 (408–596) b

[a] Cry1F: event TC1507 maize; *Bt*-RW: event DAS-59122-7 maize; non-*Bt*: near-isoline non-*Bt* maize.
[b] CI: confidence interval. Treatments with different letters were statistically different ($P < 0.05$).

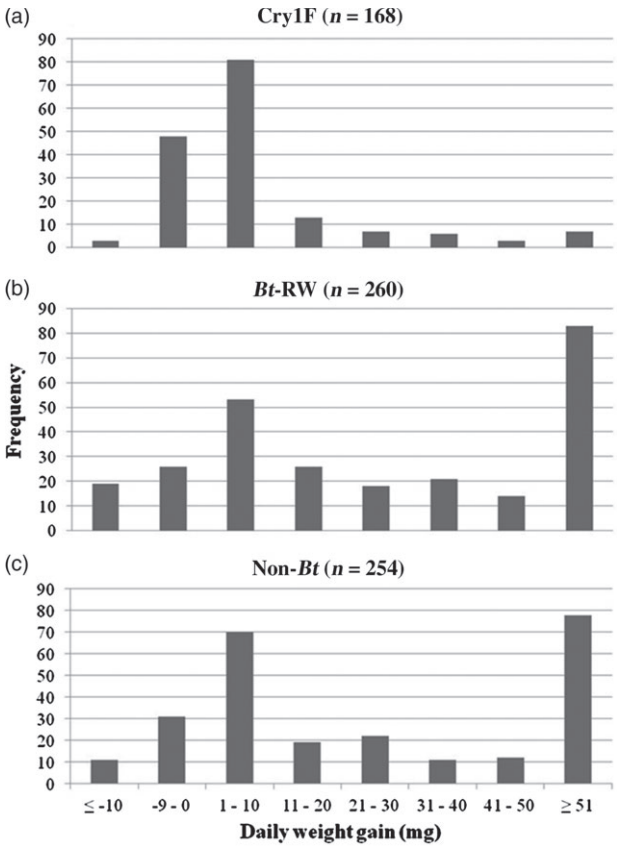

Figure 4. Frequency of weight gain values (mg) for *A. ipsilon* on each of three treatments: (a) Cry1F; (b) *Bt*-RW; (c) non-*Bt*. All observed weights from all insects were included. Insects that did not survive for the entire 14 days of exposure were weighed every day until death. Insects were not weighed after death. *N* is the total number of days that weight gain was measured across all insects.

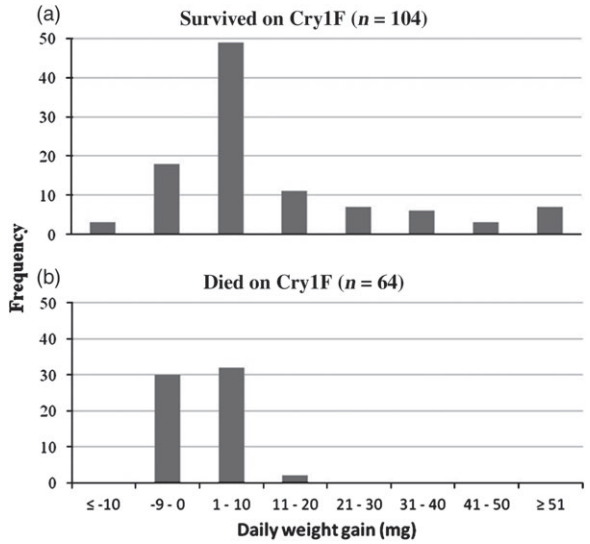

Figure 5. Frequency of weight gain values (mg) for *A. ipsilon* that survived (a) and did not survive (b) exposure to Cry1F leaf material. Insects that did not survive for the entire 14 days of exposure were weighed every day until death. Insects were not weighed after death. *N* is the total number of days that weight gain was measured across all insects.

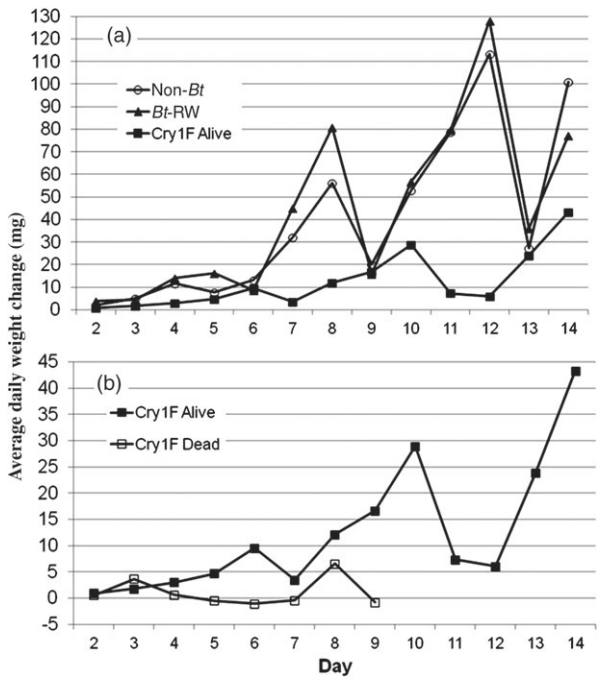

Figure 6. Average daily weight gain of *A. ipsilon* when exposed to *Bt*-RW, non-*Bt* and Cry1F maize. For Cry1F Alive, only weight gain of those insects that survived exposure to Cry1F for the entire length of the assay were included in the calculation. Cry1F Dead represents insects that did not survive for the duration of the experiment. Sample size varied from $n = 12$ to $n = 2$ across days for the Cry1F Dead line, and all insects were dead after day 9. $N = 8$ for all points on the Cry1F Alive line. Day 1 was the first day larvae were weighed, and therefore there is no weight change to report for that day.

starting at third instar to these treatments were sixth instars at the conclusion of the assay; however, larvae were not staged after initiation of the experiment. *A. ipsilon* typically experience 6–7 instars before pupation.[25] It is less clear from these data when the Cry1F survivors completed a molt (Figs 6a and 6b). Weight loss between days 6 and 7 and between days 10 and 11 suggest that ecdysis may have occurred near day 7 and 11. If there were two molts, then Cry1F survivors were fifth instars at the conclusion of this assay, demonstrating a developmental delay as a result of exposure to Cry1F maize. Average weight change of individuals that eventually died after Cry1F exposure was minimal, ranging from −1.1 to 3.7 mg, with 50% of the changes positive and 50% negative. There is no obvious indication before death of weight loss due to ecdysis for those that did not survive exposure to Cry1F maize.

4 DISCUSSION AND CONCLUSIONS

A. ipsilon third instars showed aversion to both *Bt* treatments in the short-duration study. Aversion to *Bt*-RW (event DAS-59122-7) maize was unexpected because the Cry34Ab1/Cry35Ab1 proteins expressed in event DAS-59122-7 maize are generally acknowledged to have no insecticidal effect against Lepidoptera. Results from the long-duration study confirm the absence of insect toxicity and the ability of the larvae to habituate the taste rejection response to *Bt*-RW maize (Table 2). Exposure to *Bt*-RW maize may stimulate receptors that cause a 'false alarm'.[26] *A. ipsilon* larvae frequently contact the soil, often burrowing through the soil and pulling cut plants below the soil surface to feed. *Bacillus thuringiensis* can also be found in the soil, and a possible explanation for false alarm deterrence could be that *A. ipsilon* evolved feeding deterrence to *Bt* as protection from any potential negative effects due to contact with insecticidal *Bt* varieties in the soil. Habituation to *Bt*-RW maize is an important mechanism, preventing the larvae from continuing to reject a non-toxic food source. Similar studies with other *Bt* proteins might be useful to understand whether the mechanism of aversion to Cry1F and Cry34Ab1/Cry35Ab1 is the same and applicable to *Bt* in general or unique to each protein or protein combination.

These results support current insect resistance management (IRM) plans. Third instars did not die after a short exposure to Cry1F maize. Additionally, experiments with whole plants in a greenhouse setting indicate that *A. ipsilon* larvae are more likely to sample, or taste, than cut a Cry1F plant (Binning RR, unpublished). Combined, these results suggest that *A. ipsilon* will abandon Cry1F maize in the field, and abandonment will occur before any selection for resistance. If non-*Bt* plants are available, selection may be completely avoided owing to this aversion. Abandoning Cry1F maize will also increase the risk of death by natural enemies.

Secondary or sporadic pests, such as *A. ipsilon*, are not typically considered when IRM plans are developed for *Bt* maize. However, the results reported in this paper indicate that both separate block or strip and blended refuge IRM plans for *Bt* maize would be effective at delaying resistance development for *A. ipsilon*.

The mean time to death after exposure for 6.3 days and the relatively low mortality in the Cry1F maize treatment suggest that mortality in the long-duration assay could be caused by starvation rather than toxicity; however, starvation and toxicity cannot easily be separated with these data. The larvae that survived Cry1F maize were significantly smaller and therefore less fit than those exposed to non-*Bt* or *Bt*-RW maize. The survivors gained weight and progressed through instars, indicating that the initial aversive response did not prevent all feeding, and 40% of the tested larvae were able at least partially to overcome any insecticidal effects of Cry1F. Detoxification, a heterogeneous genetic response, and natural variation in population susceptibility are possible explanations for the survival of third instars exposed to Cry1F

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maize for 14 days. The most likely explanation for survival on Cry1F maize is that these insects were on the lower end of the naturally occurring variation in susceptibility for this laboratory population. Combined with reduced feeding due to the aversive response, naturally lower susceptibility could account for survival, reduced growth and delayed development in this no-choice assay.

The benefits that Cry1F maize can provide by protecting maize plants from secondary pests can be very important in outbreak years, especially when pest presence and density is difficult to predict and insecticidal sprays may be largely ineffective. *Agrotis ipsilon* is a secondary, sporadic pest in maize-growing regions around the world. Some commercial *Bt* maize products provide some, but not 100%, protection from *A. ipsilon* feeding. Integrated pest management (IPM) practices that consider both primary and secondary maize pests can complement IRM plans that are designed around primary pests. Together, these pest management practices could extend the lifetime and utility of a *Bt* product against secondary pests such as *A. ipsilon*. These data support the use of refuge to delay Cry1F resistance development in *A. ipsilon* populations. Combined with IPM practices such as effective weed management, Cry1F maize will likely maintain its global utility against *A. ipsilon* for many seasons.

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