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Fall armyworm Spodoptera frugiperda and black cutworm Agrotis ipsilon susceptibility and avoidance to Bt maize, and implications for global insect resistance management

Rachel Renee Binning
Iowa State University

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Fall armyworm *Spodoptera frugiperda* and black cutworm *Agrotis ipsilon* susceptibility and avoidance to Bt maize, and implications for global insect resistance management

by

Rachel Renee Binning

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Entomology

Program of Study Committee:
Richard Hellmich, Co-Major Professor
Joel Coats, Co-Major Professor
Aaron J. Gassmann
Erin W. Hodgson
Jeffrey D. Wolt

Iowa State University
Ames, Iowa
2013

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DEDICATION

For my parents. Who not only let me keep caterpillars in jars on the windowsill, but bought me a book so I could identify them and feed them properly. Who didn’t cringe when I picked up snakes, snails, and cicadas. Who taught me what a taxonomic key is and how to use it in middle school. Who recognized my passion for science early and nurtured it continuously.
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Finally, thanks to my family for their encouragement and interest in this work. And, most of all, thanks to my husband for putting up with my absentmindedness, occasional grumpiness, decision fatigue, and for reminding me to feed the dogs.
ABSTRACT

*Bacillus thuringiensis* (Bt) maize (*Zea mays*) was developed primarily for North American pests such as European corn borer (*Ostrinia nubilalis*). However, most Bt maize products are also cultivated outside of North America, where the primary pests are different and often have lower susceptibility to Bt toxins. As these Bt maize products are commercialized in new geographies, insect resistance management (IRM) plans for those geographies need to consider these new pest and toxin combinations, instead of assuming the same refuge strategy applies to all pests in all geographies. Before implementing an IRM plan that includes size, placement and configuration of refuge, it is useful to understand the biology and susceptibility of the important pest(s) in each geography. Fall armyworm (*Spodoptera frugiperda*) and black cutworm (*Agrotis ipsilon*) are examples of global pests with unique biology and susceptibility to Cry1F (expressed in event 1507). The initial behavioral response of each species to Cry1F maize was tested by measuring the time naïve third instars spent feeding during a three-minute exposure. I also investigated whether these species had a behavioral and/or toxic response to Cry1F maize. Additional investigation of species susceptibility and ability to overcome an aversive response was conducted by exposing third-instars of each species to Cry1F maize and measuring weight gain and survival for 14 days.
Both *S. frugiperda* and *A. ipsilon* demonstrated an initial, aversive response to Cry1F maize. The response of *S. frugiperda* was post-ingestive, and few larvae survived a 14 d exposure. The response of *A. ipsilon* to Cry1F maize was post-ingestive, and 40% of the larvae survived a 14 d exposure. *A. ipsilon* also demonstrated an initial aversive response to Cry34Ab1/Cry35Ab1 (expressed in event 59122 maize) leaf tissue. However, all *A. ipsilon* larvae survived a 14 d exposure to Cry34Ab1/Cry35Ab1 maize. The interaction and significance of susceptibility and avoidance are discussed in the context of global IRM plan development for Bt maize products.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is organized into six chapters. Chapter 1 provides a general introduction including a description of the organization of the dissertation and a description of the rationale behind the research.

Chapter 2 is a literature review of the toxicity to insects of *Bacillus thuringiensis* (Bt), insect behavior upon exposure to Bt, and implications of the interaction of behavior and toxicity for insect resistance management. This chapter will be submitted to the Journal of Applied Entomology as a review article, with Joel Coats and Richard Hellmich as co-authors. Joel Coats and Richard Hellmich are co-major professors and contributed significantly to the review.

Chapter 3 describes two preliminary studies that helped define the design of the short and long duration assays presented in later chapters.

Chapter 4 summarizes short and long duration experiments with *Spodoptera frugiperda* larvae. The short duration experiment was designed to investigate the initial behavioral response of *S. frugiperda* to Bt maize and whether that response occurred pre- or post-ingestively. The long duration experiment was designed to investigate the effects of extended (14 d) exposure to Bt maize and whether *S. frugiperda* larvae could overcome the initial aversive response to grow and develop. Chapter 4 will be submitted to the Journal of
Economic Entomology, with Joel Coats, Xiaoxiao Kong, and Richard Hellmich as co-authors. Joel Coats and Richard Hellmich are co-major professors and contributed to the concept and design of the experiments. Xiaoxiao Kong contributed significantly to the statistical analysis.

Chapter 5 summarizes short and long duration experiments with *Agrotis ipsilon* larvae. The objectives are the same as described for Chapter 4. This chapter will be submitted to Pest Management Science with Joel Coats, Xiaoxiao Kong, and Richard Hellmich as co-authors. Joel Coats and Richard Hellmich are co-major professors and contributed to the concept and design of the experiments. Xiaoxiao Kong contributed significantly to the statistical analysis.

Finally, the general conclusions of this dissertation are presented in Chapter 6.

**Rationale**

The following projects were designed to investigate the behavioral responses of *S. frugiperda* and *A. ipsilon* to Cry1F (event 1507) maize. *Spodoptera frugiperda* is a significant pest of maize in South America, where blending refuge with Bt maize in the field is under consideration. Studies with *S. frugiperda* were intended to contribute to understanding of the plausibility of blended refuge for this pest. *Agrotis ipsilon* is a ubiquitous, but sporadic, secondary pest of maize. This pest was chosen because of its global distribution.
combined with the previous work indicating the existence of an aversive response to Cry1F maize (Richtman, 2006).

**REFERENCE**

CHAPTER 2. LITERATURE REVIEW

THE INTERACTION OF SUSCEPTIBILITY AND AVOIDANCE INFORMS THE REFUGE DEPLOYMENT STRATEGY FOR BT MAIZE

A Paper to be Submitted to the Journal of Applied Entomology
Rachel Binning, Joel Coats, and Richard Hellmich

Abstract

The susceptibility of insect pests to *Bacillus thuringiensis* (Bt) transgenic crops has been extensively studied, and the behavioral response of these pests is also often characterized. However, discussions regarding the relationship and interaction of antibiosis and antixenosis as protection mechanisms are less common. Pest avoidance of Bt maize (*Zea mays*) and the linkage, or lack of linkage, of avoidance to susceptibility could influence the design of insect resistance management (IRM) plans. This review examines insect pest susceptibility to and avoidance of Bt transgenic crops, the ability of insects to adapt to both antibiosis and antixenosis, and the impact of these factors on global IRM plans for Bt maize.

Introduction

*Bacillus thuringiensis* (Bt) is a common, entomopathogenic bacteria originally isolated from diseased silkworms in the early 20th century and has been used by farmers as an organic pesticide since the 1920s. In 1961, the United States (US) Environmental Protection Agency (EPA) registered Bt as a pesticide, active against lepidopteran pests. Over the next two decades, Bt
strains active against Diptera and Coleoptera were discovered. With the advent of transgenic technology, crops were engineered to express Bt for insect pest control. The EPA registered the first genetically modified Bt crop in 1995 and Bt maize has been grown commercially in the US since the introduction of lepidopteran-resistant maize in 1996. Since then, many other Bt maize products have been developed for resistance to both lepidopteran and coleopteran maize pests in North America. Examination of the toxicity of Bt as it relates to field crop pests, reports of pest avoidance to Bt, and adaptation of pests to toxicity and avoidance inform conclusions of how these two protection mechanisms may interact and influence insect pest management.

**Bt Toxicity**

The first Bt maize products commercialized in the US were highly toxic to the primary lepidopteran maize pests of the region – European corn borer (*Ostrinia nubilalis*) and Southwestern corn borer (*Diatraea grandiosella*). Both events MON810 and 1507, expressing the Cry1Ab and Cry1F insecticidal proteins, respectively, were classified as “high dose” against these primary pests. A Scientific Advisory Panel (SAP) defined high dose as “25 times the toxin concentration needed to kill a susceptible (SS) larva” and further indicated that this dose should kill 95% of heterozygous larvae with one resistance allele (RS) (US EPA, 1998b).
Field and laboratory experiments provide further evidence of the toxicity of these Bt proteins. Marcon et al. (1999) sampled 15 *O. nubilalis* populations from 10 states across the US and evaluated each population for susceptibility to Cry1Ab. In diet bioassay, neonates were highly susceptible, with LC$_{50}$ values ranging from 2.22-7.89 ng/cm$^2$. A similar study sampling 24 populations of *O. nubilalis* from 6 US states and 11 populations from Europe estimated neonate susceptibility to Cry1F ranged from 1.06-23.28 ng/cm$^2$ (Gaspers et al., 2011). Walker et al. (2000) measured survival of 3$^{rd}$ and 4$^{th}$ instar *O. nubilalis* on Cry1Ab maize (event MON810) in the field. After adjusting for control mortality, survival of these late instars on Cry1Ab maize ranged from 0.7-1.4%. Susceptibility to Bt typically declines as the insect grows, and very low survival of late instars on Bt maize is evidence that the event is likely high dose against the pest (ILSI 1999).

Even though Bt maize was historically developed for North American pests such as *O. nubilalis* and *D. grandiosella*, many Bt maize products are also cultivated outside of North America, where the primary pests may be different and have lower susceptibility to the toxins. For example, fall armyworm (*Spodoptera frugiperda*) susceptibility to Cry1F maize (event 1507) cannot be qualified as high dose (Storer et al. 2012). *S. frugiperda* is a significant pest of maize in Central and South America where growers use multiple insecticide applications for effective control in non-Bt maize fields. Although *S. frugiperda* cannot overwinter in the North American Corn Belt, this species migrates every
year and can cause significant damage to unprotected maize. Storer et al. (2012) reported >90% mortality for nine US field-collected populations of *S. frugiperda* neonates on Cry1F maize leaves. A dose-response bioassay using the same populations demonstrated Cry1F LC$_{50}$ values ranged from 1.0-30.3 ng/cm$^2$.

Western corn rootworm (*Diabrotica virgifera virgifera*) is a serious pest of maize in North America and certain regions of Europe. Bt maize events 59122, MON88017, and MIR604, express the Cry34Ab1/Cry35Ab1, Cry3Bb1, and mCry3A insecticidal proteins, respectively, for control of *D. v. virgifera* and other corn rootworm species. A recent review explored the effects of dose of these products against *D. v. virgifera* (Devos et al., 2013). Their conclusion, based primarily on adult emergence, was that these events are not high dose against *D. v. virgifera*. Estimates of adult emergence from Cry34Ab1/Cry35Ab1 maize plants range from <1% to approximately 4% (Lefko et al., 2008; Storer et al., 2006). Survival to adult for rootworms exposed to mCry3A maize plants was estimated to range from <1% to approximately 12% (Hibbard et al., 2010). Miehls et al. (2008) reported an average of approximately 1% rootworm survival on Cry3Bb1 maize in the field.
**Bt Avoidance**

**Avoidance in the Presence of Toxicity**

Although not all Bt maize products meet the definition of high dose, Bt maize may still be efficacious and protect yield. This could be a result of a lower, yet effective, level of toxicity and/or a behavioral response that causes the insect to reject the Bt maize as a food source. Insect avoidance of a toxic compound is not uncommon, and avoidance of toxic Bt plants or Cry proteins has been described for a wide variety of insect pest species (Table 1).

Insect pests of maize are well-represented in tests of Bt avoidance. Experiments with *O. nubilalis* suggest that neonates avoid high concentrations of Bt in diet (Mohd-Salleh and Lewis, 1982) and are more likely to abandon Bt maize than non-Bt maize (Goldstein *et al.*, 2010; Razze *et al.*, 2011). Neonate *Heliothis virescens* preferred diet without Bt, regardless of Bt concentration, in a choice test with each of three Bt formulations (Gould *et al.*, 1991). Choice tests for 4th and 5th instar *H. virescens* with the same treatments demonstrated concentration-dependent avoidance of Bt formulations, with greater avoidance at higher concentrations. Observations of *D. v. virgifera* on Bt maize event MON863, expressing the Bt protein Cry3Bb1, indicate a low level of avoidance (Clark *et al.*, 2006). Chapters 4 and 5 describe an initial aversive response of *S. frugiperda* and *Agrotis ipsilon*, respectively, to Cry1F maize leaf tissue in a short
duration assay, coupled with mortality and weight gain inhibition in a long duration feeding assay.

Cotton pests also have been studied extensively. Several experiments have shown avoidance of Bt plants by Helicoverpa armigera larvae. A study by Zhang et al. (2004) used choice tests to show that H. armigera neonates tend to avoid transgenic cotton leaf disks. Although fourth-instar larvae in their study did not obviously avoid transgenic cotton plants, they did show reduced consumption when compared to non-Bt plants. Another study measured feeding and movement frequency, indicating avoidance of Cry1Ac cotton plants (Men et al., 2005). Avoidance of Bt cotton also was documented with Trichoplusia ni (Li et al., 2006). Larvae appeared to sample Cry1Ac cotton leaves in a no-choice situation, moving on and off of leaves and living longer than unfed larvae. Another study tested various concentrations of two Bt toxins, Cry1Ac and Cry2Ab, against H. virescens and Helicoverpa zea in choice tests (Gore et al., 2005). Both species preferred untreated diet, regardless of Bt concentration. H. zea exhibited a dose-dependent response, tending to select diets with lower concentrations of Cry1Ac more often than higher concentrations.

Avoidance of Bt is not limited to maize and cotton pests. Choice tests indicated fewer Chilo suppressalis larvae were found on Cry1Ac, Cry9c, and Cry2A rice than on non-Bt rice after 72 hours of exposure (Chen et al., 2008). Spruce budworm (Choristoneura fumiferana) demonstrated avoidance that
increased with concentration after three days of exposure to Cry1A(a) (Ramachandran et al., 1993). Fall webworm (Hyphantria cunea) was tested in the same study and no avoidance response was observed. Gypsy moths (Lymantria dispar) also have been observed to avoid food containing Bt (Farrar and Ridgway, 1995).

Differences in larval response among Cry proteins indicate avoidance of Bt can be specific to an individual protein. Chen et al. (2008) suggest non-preference was stronger for the Cry1Ac and Cry9C rice varieties than for the Cry2A variety after 72 hours of exposure. Gore et al. (2005) described a stronger avoidance behavior by H. zea when exposed to Cry1Ac compared to Cry2Ab protein. Third-instar S. frugiperda and Agrotis ipsilon both exhibited an initial aversive response to Cry1F maize leaf tissue (Chapters 4 and 5). When exposed to Cry34Ab1/Cry35Ab1 maize, A. ipsilon exhibited a non-toxic, initial aversive response, but S. frugiperda did not have an aversive response. The response of A. ipsilon to both proteins (Cry1F and Cry34Ab1/Cry35Ab1) was characterized as post-ingestive, and the response of S. frugiperda to Cry1F was also characterized as post-ingestive. Avoidance of one protein and not another may be attributable to a post-ingestive avoidance response, indicating that the response may be induced by the Bt protein binding to a receptor or receptors in the midgut.
Avoidance in the Absence of Toxicity

Avoidance can occur in the absence of toxicity (Table 1). Gould et al. (1991) described an avoidance response of *H. virescens* larvae to both toxic and non-toxic concentrations of Bt. Prasifka et al. (2007) tested monarch (*Danaus plexippus*) larvae for behavioral changes when exposed to Cry1Ab maize anthers. Although they did not find a difference in movement parameters, they did note that larvae spent less time on milkweed with Cry1Ab anthers than on milkweed with non-Bt anthers or no anthers at all. Chapter 4 describes a significant initial aversive response of *A. ipsilon* to Cry34Ab1/Cry35Ab1 maize leaf tissue in a short duration assay, coupled with no mortality or weight-gain inhibition in a long duration feeding assay.

Adaptation

Adaptation to Bt Toxicity

Adaptation to Bt toxicity, or resistance, has occurred to sprayable formulations as well as transgenic crops. Field-evolved resistance to Bt sprays was first reported for diamondback moth (*Plutella xylostella*) (Tabashnik et al., 1990). Reports of field-evolved resistance to Bt maize resulting in loss of efficacy include *Busseola fusca* resistance to Cry1Ab maize in South Africa (van Rensburg, 2007) and *S. frugiperda* resistance to Cry1F maize in Puerto Rico (Storer et al., 2010). Numerous reviews exist exploring resistance to transgenic Bt crops in various contexts, including crop management and refuge strategy.
(Carrière et al., 2004; Devos et al., 2013; Gould, 1998; Ives et al., 2011; Tabashnik et al., 2008), population genetics and resistance mechanisms (Ferre and Van Rie, 2002; Gassmann et al., 2009; Gill et al., 1992; Griffitts and Aroian, 2005; Tabashnik, 1994; Tabashnik and Carrière, 2008), and selection for resistance in the lab and field (Gassmann, 2012; Liu et al., 2010; Storer et al., 2012; Tabashnik et al., 2003; Tabashnik et al., 2009). There are many circumstances and mechanisms that can contribute to insect resistance to Bt crops, and the question is not if resistance will occur, rather how long can resistance development be delayed while maintaining value for the farmer.

Loss of Aversion

Although insects may initially reject a food source, this response may change upon repeated exposure, resulting in acceptance of that food source (loss of aversion). Glendinning and Slansky (1995) demonstrated that S. frugiperda caterpillars that had initially reduced consumption of a toxic compound (indole 3-carbinol), increased their feeding to control levels after two days of continuous exposure. Another study exposed grasshoppers (Schistocerca americana) to three different deterrent alkaloids (Glendinning and Slansky, 1995). After three days of exposure, the grasshoppers lost their rejection response and fed normally on treated diet. Chapters 4 and 5 describe the initial rejection of Bt maize by S. frugiperda and A. ipsilon, followed by
apparent acceptance during 14 d of exposure based on development and weight gain of survivors.

Loss of aversion may be the result of desensitization of the mechanism that causes the aversive response (e.g., taste-mediated) (Glendinning et al., 2001b), increased (or induced) detoxification of the aversive compound (Glendinning and Slansky, 1995; Snyder and Glendinning, 1996) or a combination of both desensitization and detoxification (Glendinning and Gonzalez, 1995; Szentesi and Bernays, 1984). Glendinning et al. (2001a) reported the ability of *Manduca sexta* larvae to lose their aversive response to a non-toxic compound (salicin) within 12 h of exposure and they concluded that the loss of aversion was a result of the desensitization of a central nervous system mechanism. The same species was unable to overcome its aversive response to a toxic compound (aristolochic acid).

In situations where the Bt protein is expressed constitutively and the insect is highly sensitive, such as Cry1F maize and *O. nubilalis*, adaptation to the toxin would need to occur in advance of or concurrently with loss of aversion to allow insect survival on the plant (Glendinning and Slanksy, 1995; Lockwood et al., 1984). Differential behavioral responses between physiologically resistant and susceptible insects have been demonstrated. A colony of *O. nubilalis* selected in the lab for resistance to Cry1Ab were more likely to be found on Cry1Ab diet at lower concentrations when compared to susceptible lines in a
choice test (Prasifka et al., 2009). By increasing the concentration of Cry1Ab in the diet, the differences between the resistant and susceptible lines were eliminated, with both avoiding the Cry1Ab diet. A *Spodoptera exigua* colony selected for resistance to Cry1C spent more time feeding on Cry1C diet than the susceptible colony (Berdegue et al., 1996).

**Insect Resistance Management**

**Larval Movement**

Larval movement between non-Bt and Bt plants has long been identified as a significant disadvantage to blending refuge in the field (Davis and Onstad, 2000; Mallet and Porter, 1992; Parker and Luttrell, 1999). This is based on the survival advantage conferred to heterozygous resistant insects when movement occurs between a non-Bt plant and a Bt plant. Recent studies with Bt maize have measured larval movement and survival in a blended refuge scenario and used the results to inform modeling parameters. Larval movement and survival of sugarcane borer (*Diatraea saccharalis*) was measured in the field and greenhouse with Bt maize event MON 89034x88017 (Wangila et al., 2013). Significant larval movement was demonstrated in the field trials, however, the number of larvae surviving on non-Bt plants did not differ between pure stand and blended refuge scenarios. Binning et al. (2010) reported 100% survival of *D. virgifera* in the lab after 17 days of exposure to Cry34Ab1/Cry35Ab1 maize followed by forced movement to non-Bt maize. These data were later utilized in
modeling by Pan et al. (2011) for a predisperal tasting survival parameter. Their model compared the durability of block and blended refuge for D. v. virgifera, concluding that, in many cases, blended refuge can have equal or greater durability than block refuges for this pest. Carroll et al. (2013) measured movement and survival of D. grandiosella with MON 89034 in a blended refuge scenario and applied the results to a simulation model comparing block and blended refuge. When refuge compliance was considered, the amount of movement observed in the field trials did not significantly affect durability of the product deployed with blended compared to block refuge.

A variety of factors can influence the movement of herbivorous larvae in a maize field. Goldstein et al. (2010) reported that 42% of O. nubilalis neonates that hatched on a non-Bt plant had abandoned that plant after 24h. This type of pre-feeding dispersal may be an evolutionary advantage by reducing intraspecific competition for insects that lay eggs in masses on host plants rather than individually. Larvae that remain, or move and encounter a new plant, must assess the acceptability of the plant as a host. If the plant is unpalatable, larvae may move to a different location on the plant or abandon the plant completely.

Differential expression of the toxin within the plant may allow the larva to remain on the plant and simply move to a different location. Gould (1991) discussed insect behavior in response to differential concentration or placement of endogenous plant toxins and synthetic pesticides across plant tissues, and
implications for the development of behavioral resistance. A similar consideration could be made for Bt maize, which typically relies on promoters to determine the placement and concentration of the Bt protein across plant tissues. For example, excluding Bt protein from maize pollen is desired to reduce exposure for non-target insects. However, maize pests such as western bean cutworm (*Striacosta albicosta*), *H. zea*, and *O. nubilalis* may feed on pollen, silks and kernels. Efficacy of such traits could be lost if these pests can discriminately feed on pollen, silks, and low or non-expressing kernels. If, however, insects feed preferentially on non-expressing plant structures that are not important to yield, the plant itself could serve as a refuge for susceptible insect production (Berdegue *et al.*, 1996; Gould, 1998).

In cases where avoidance of unpalatable tissue is impossible, such as a pure stand of Bt maize, larvae may “selectively override” aversion of compounds that are deemed harmless enough to consume (Glendinning, 2002). This is a likely explanation of the results for *A. ipsilon* response to Cry34Ab1/Cry35Ab1 maize in Chapter 5. The Cry34Ab1/Cry35Ab1 maize leaf tissue was not toxic to third instar *A. ipsilon*, and the larvae were able to overcome their initial aversive response to feed and develop the same as larvae fed non-Bt maize.

**Implications for Refuge Deployment**

Refuge where susceptible insects can survive is an important component of an insect resistance management (IRM) plan (MacIntosh, 2009). The size and
placement of refuge maize is largely based on the high-dose refuge (HDR) strategy, which assumes that resistance will be functionally recessive and rare, mating of resistant and susceptible individuals will be random, and heterozygous resistant individuals will be killed by the high dose (Bates et al., 2005). Recently, the HDR strategy has been broadly applied to Bt transgenic crops in instances where the product may not be high dose against the primary pest(s) (Huang et al., 2011). Refuge strategy for new geographies, such as South America, needs to consider local pest biology and susceptibility, cultivation practices, landscape, and ease of implementation for farmers. IRM and integrated pest management (IPM) can work together to delay the development of resistance. Studies of larval movement and survival with each Bt maize product using local pests and local agricultural practices in each geography will help to determine whether or not blended refuge is a viable solution.

Conclusions

Bt proteins in transgenic field crops provide control against a wide variety of insect pests. Control may come in the form of antibiosis, antixenosis, or a combination. An adaptation to one or both of these protection mechanisms could decrease the utility of a Bt protein in transgenic crops, such as maize. History anticipates insect adaptation to Bt maize. To most effectively slow the development of resistance and/or behavioral adaptation, IRM may be implemented in conjunction with IPM everywhere Bt maize is planted, and is
most important in highest risk areas where Bt maize does not express a high
dose against primary pests and other conditions favor resistance and/or
behavioral adaptation, such as environmental and agricultural practices. Often, it
is these high risk regions where Bt maize delivers the most economic and
environmental benefit by reducing the number of insecticide sprays and
increasing yields under severe insect pressure.

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with different concentrations of Bacillus thuringiensis proteins. J. Econ.
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transgenes on Bt resistance in pink bollworm (Lepidoptera: Gelechiidae).
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no significant impact on survivorship of F1 progeny on MIR604. J. Econ.
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Table 1. Studies investigating avoidance of Bt toxins.

<table>
<thead>
<tr>
<th>Species</th>
<th>Bt Protein(s)</th>
<th>Avoidance</th>
<th>Concentration Dependent*</th>
<th>Toxic concentration**</th>
<th>Citation</th>
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<tr>
<td><em>C. fumiferana</em></td>
<td>Cry1A(a)</td>
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<td>yes</td>
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<td>Ramachandran <em>et al.</em> 1993</td>
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<td><em>H. cunea</em></td>
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<td>no</td>
<td>yes</td>
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<td>various Bt</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>Gould <em>et al.</em> 1991</td>
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<td>Gould <em>et al.</em> 1991</td>
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<td>yes</td>
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<td>Gould <em>et al.</em> 1991</td>
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<td>Cry1Ac</td>
<td>yes</td>
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<td>no</td>
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<td>yes</td>
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<td><em>P. gossypiella</em></td>
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<td>formulation)</td>
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<tr>
<td><em>T. ni</em></td>
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<td>yes</td>
<td>Li <em>et al.</em> 2006</td>
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<td>yes</td>
<td>plant</td>
<td>yes</td>
<td>Zhang <em>et al.</em> 2004</td>
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Table 1 (continued). Studies investigating avoidance of Bt toxins.

<table>
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<tr>
<th>Species</th>
<th>Bt Protein(s)</th>
<th>Avoidance</th>
<th>Concentration Dependent*</th>
<th>Toxic concentration**</th>
<th>Citation</th>
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<td><em>H. armigera</em></td>
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<td>yes</td>
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<td>yes</td>
<td>Rudeen and Gassmann 2012</td>
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<tr>
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<td>yes</td>
<td>Goldstein et al. 2010</td>
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<td>Razze et al. 2011</td>
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<td>yes</td>
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<td>yes</td>
<td>yes</td>
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<td>yes</td>
<td>yes</td>
<td>Mohd-Salleh and Lewis 1982</td>
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<td><em>A. ipsilon</em></td>
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<td>Chapter 5</td>
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<td>yes</td>
<td>plant</td>
<td>no</td>
<td>Chapter 5</td>
</tr>
</tbody>
</table>

* Utilization of plant material rather than purified protein is indicated with the designation of "plant" in this column.
** Avoidance of toxic and non-toxic concentrations is indicated with the designation of "both" in this column.
CHAPTER 3. PRELIMINARY STUDIES

Black Cutworm No-Choice Experiment with Maize Seedlings

Previous studies have investigated the possibility of a behavioral response of black cutworm (*Agrotis ipsilon*) to Cry1F maize (event 1507), which expresses the Cry1F insecticidal protein (Richtman 2006). Although these studies indicate that *A. ipsilon* may avoid Cry1F maize, the results were not definitive and did not assess whether the larvae tasted Cry1F maize before rejection. The following study further explores the behavioral response of *A. ipsilon* to Cry1F maize in a no-choice scenario.

**Materials and Methods**

**Greenhouse Experiment**

*A. ipsilon* diet (Southland Products, Inc, Lake Village, AR) was prepared following the manufacturer’s instructions. The diet was poured into ventilated rearing dishes, allowed to solidify, wrapped in plastic wrap and stored in a refrigerator set at approximately 4°C until used. Unused diet was disposed of after 7 days. The top of the diet was scored with a fork before infesting with neonate *A. ipsilon*. 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). Approximately 25-50 neonates were infested into diet dishes within 24 hours of hatch and maintained on artificial diet until they reached 3rd- 4th instar.
Maize plants were grown in flats in the Pioneer greenhouse (Johnston, IA) under typical greenhouse conditions (16:8 L:D, 31 ± 2°C). Twenty-five seeds of either Cry1F or non-Bt maize were planted at regular intervals in each flat. A completely randomized experimental design was utilized with 16 flats (replications) per treatment. Eight 3rd or 4th-instar *A. ipsilon* were infested into each flat when plants were at approximately growth stage V2. Tanglefoot® was placed around the rim of each flat to discourage larval escape. Plants were evaluated on day 2 and 4 and scored as cut, sampled, or not damaged. A plant was scored as cut when the stem was effectively cut all the way through. Cutting is the symptom of *A. ipsilon* infestation in the field that results in economic loss. A plant was scored as sampled when scarring or incomplete cutting was visible on the stem, but the plant was not cut. Sampling may be associated with the initial tasting of a plant that aids in host recognition. If neither symptom was observed, a plant was scored as not damaged.

**Data Analysis**

Statistical analyses were conducted using SAS software, Version 9.3 (SAS Institute Inc. 2011) to compare *A. ipsilon* damage to Cry1F and non-Bt plants. Plant damage was considered a nominal outcome. For each observation (day 2 or 4), Proc logistic was utilized to conduct a multinomial logistic regression with three levels (no damage, sampled, and cut) to compare the damage between Cry1F and non-Bt maize plants. A significant difference
between Cry1F and non-Bt maize was reported if the P-value of the chi-square test for the overall treatment effect is less than 0.05. In addition, an odds ratio was calculated using the formula 

\[(P_T / (1-P_T)) / (P_C / (1-P_C)),\]

where \(P_T\) is the proportion of Cry1F maize plants that were either cut or sampled and \(P_C\) is the proportion of non-Bt maize plants that were either cut or sampled. If the odds ratio is >1, then there were more Cry1F maize plants cut or sampled than non-Bt maize plants. If the odds ratio is <1, then there were more non-Bt maize plants cut or sampled than Cry1F maize plants. If the confidence interval overlaps with 1, then the difference between the treatments is not significant.

**Results**

On days 2 and 4, the overall treatment effect was significant (P<0.0001). On both sampling days, the odds ratio for cut plants was <1 and the odds ratio for sampled plants was >1 (Table 1). Since none of the confidence intervals overlap with 1, there were significantly more plants cut in the non-Bt flats compared to the Cry1F maize flats and significantly more plants sampled in the Cry1F maize flats compared to the non-Bt maize flats. The average percentage of plants cut in the non-Bt treatment was ~3x higher than in the Cry1F maize treatment on both days (Table 2). The opposite was true for sampling injury – the average percentage of plants sampled in Cry1F maize was ~3x higher than in the non-Bt maize treatment.
Conclusions

These results demonstrate a strong aversive response of *A. ipsilon* to Cry1F maize. The relatively high amount of sampling without cutting in Cry1F maize flats (3x that of non-Bt flats) indicates that *A. ipsilon* tastes plants before rejection. The results of this experiment led to the question of whether this rejection is preingestive or postingestive, thereby informing the design of the experiments described in Chapter 5.

Fall Armyworm Rearing Diet Experiment

Both the short and long duration studies described in Chapters 4 and 5 required that fall armyworm (*Spodoptera frugiperda*) and black cutworm (*A. ipsilon*) be grown (or reared) to 3rd instar before each experiment could begin. Two primary options exist for rearing lepidopteran larvae – artificial diet and plant material. Plant material – in this case, maize leaf tissue – is ecologically relevant for both pests. It also requires maize seed, greenhouse or field space, and plant maintenance. Artificial diet can be purchased in bulk and stored for extended periods of time. It is relatively simple to prepare, does not need to be replaced during the rearing process, and the antibacterial and antifungal ingredients help to prevent contamination. Considering only convenience, artificial diet would be preferred for rearing lepidopteran larvae. However, the nature of the short and long duration experiments would require the larvae to switch from artificial diet to maize leaf tissue. This host switch could affect initial
response to all treatments (short duration study) and long-term growth and development (long duration study).

This experiment was designed to investigate the effect of rearing diet on the susceptibility of *S. frugiperda* to Cry1F maize. The outcome of this study informed which rearing material was used for the short and long duration experiments.

**Materials and Methods**

This study is divided in two phases – rearing and evaluation. Figure 1 illustrates the sequence of the phases.

**Rearing Phase**

*S. frugiperda* diet (Southland Products, Inc, Lake Village, AR) was prepared following the manufacturer’s instructions. The diet was poured into plastic containers, allowed to solidify, wrapped in plastic wrap and stored in a refrigerator set at approximately 4°C until used. Unused diet was disposed of after 7 days. Non-Bt maize plants were grown in the Pioneer greenhouse (Johnston, IA) under typical greenhouse conditions. Leaves were collected at approximately plant growth stage V4 and on the same day they were introduced to larvae. Treatments for the rearing phase were:

1. Artificial diet, with manufacturer recommended antibiotic incorporated
2. Artificial diet, without antibiotic
3. Non-Bt leaf tissue, rinsed with deionized water
4. Non-Bt leaf tissue, surface sterilized

Surface sterilization consisted of a 70% ethanol wash for 1 minute followed by a wash in bleach (3% available chlorine) for 2 minutes and finally a 30 second rinse in 70% ethanol. In between each wash and following the final ethanol wash, leaves are rinsed in deionized water.

Eggs from a susceptible laboratory population of *S. frugiperda* were obtained from a commercial source (Chesapeake PERL, Inc, Savage, MD). *S. frugiperda* eggs were held in a growth chamber until hatch. Either a 1-cm² piece of non-Bt leaf or a 5 x 5 x 5-mm piece of artificial diet was placed in each well of a 128-well tray (CD International, Pitman, NJ) with 300 µl of agar in the bottom of each well. One neonate (<24h old) was placed in each well for a total of 128 larvae per rearing treatment per species. Food types were alternated every 4 wells and larvae were randomly assigned to wells. Trays were kept at 27°C and 60% relative humidity with 24h light. Insects were reared on each rearing treatment until they reached 3rd instar.

**Evaluation Phase**

Two maize types were used during the evaluation phase – leaf tissue from Cry1F (event 1507) and near-isoline non-Bt maize. Maize plants for both types were grown in the Pioneer greenhouse (Johnston, IA) under typical greenhouse conditions. Both Cry1F maize and non-Bt plants were individually
checked for the absence and presence, respectively, of event 1507 using event-specific PCR. Leaves were collected at approximately plant growth stage V4-V10 and on the same day they were introduced to larvae. Leaves were rinsed with water, patted dry with paper towels, and stored in labeled plastic bags in a refrigerator.

A 2-cm$^2$ piece of Cry1F maize leaf was placed in each well of a 32-well tray (CD International, Pitman, NJ). Each well had a layer of agar in the bottom to maintain moisture. Third instar \textit{S. frugiperda} were removed from their rearing phase treatments, individually weighed to the nearest 0.1 mg, and placed into each well on top of the leaf piece. Treatments were arranged in a randomized complete block design with 3 replications per treatment. Each 32-well tray was considered a replication, and there were 8 larvae per replication per treatment. The trays were sealed with lids and monitored for mortality daily. Leaf tissue was added daily.

A similar experimental design was utilized for evaluating larval performance on non-Bt leaf tissue, with one difference. Two replications, rather than three, were conducted with non-Bt maize leaf tissue due to a shortage of 3$^{rd}$ instars.

After 4 d (96 h), the experiment was terminated. A final count of mortality was made and surviving larvae were weighed to the nearest 0.1 mg.

Data analysis
To evaluate and compare mortality of *S. frugiperda* between different rearing treatments, statistical analyses were conducted using SAS software, Version 9.3 (SAS Institute Inc. 2011). A significant difference was identified within an evaluation treatment (Cry1F or non-Bt maize) if the P-value for difference between treatments was less than 0.05 using Fisher’s exact test.

To evaluate and compare the weight gain of *S. frugiperda* between different rearing treatments, statistical analyses were conducted using SAS software, Version 9.3 (SAS Institute Inc. 2011). SAS Proc mixed was utilized to fit the model. A significant difference was identified if the P-value for difference between treatments is less than 0.05.

**Results**

Mortality for insects fed non-Bt maize in the evaluation phase ranged from 0-6%, across all rearing phase treatments (Table 3). For the exposure phase, mortality for insects reared on diet with or without antibiotics and then exposed to Cry1F maize was not significantly different (P=0.56), nor was mean weight gain (t=0.98; df=18.6; P=0.34) (Table 3). There was also no significant difference for insects reared on rinsed leaves versus sterilized leaves followed by exposure to Cry1F maize for both mortality (P=0.46) and final weight (t=-2.25; df=7.36; P=0.06).

The highest mortality was 88% for insects that were reared to 3rd instar on rinsed leaves and then exposed to event 1507. This was significantly higher than
both artificial diet with and without antibiotic (P=0.02 and P=0.002, respectively) (Table 3). The lowest mortality for insects exposed to Cry1F maize was 42% for insects that were reared to 3\textsuperscript{rd} instar in diet without antibiotic. This was significantly lower than both sterilized and rinsed leaf rearing treatments (P=0.04 and P=0.002, respectively). Diet with antibiotic and sterilized leaf rearing treatments had mortalities after exposure to Cry1F maize that were not significantly different from each other (P=0.23). However rinsed leaf had significantly higher mortality than diet with antibiotic (P=0.02) and sterilized leaf had significantly higher mortality than diet without antibiotic (P=0.04) (Table 3).

The highest mean weight gain was 70.6 mg for insects that were reared to 3\textsuperscript{rd} instar on diet without antibiotic and then exposed to non-Bt maize (Table 3). This was not significantly different from artificial diet with antibiotic (t=-1.11; df=58; P=0.27); however, it was significantly higher than both sterilized (t=2.12; df=58; P=0.04) and rinsed leaf (t=2.38; df=58; P=0.02) rearing materials. Mean weight gain was not different between any of the other three rearing treatments after exposure to non-Bt maize leaf tissue.

The lowest weight gain was 14.3 mg for insects that were reared on diet without antibiotic and then exposed to Cry1F maize (Table 3). Mean weight gain was not significantly different between the two artificial diet treatments exposed to Cry1F maize (t=0.98; df=18.3; P=0.34). Sterilized leaves produced the highest weight gain (46.6 mg), which was significantly higher than both artificial diet
without antibiotic \( (t=-4.99; \text{df}=17.8; \text{P}<0.0001) \) and diet with antibiotic \( (t=-2.85; \text{df}=13.9; \text{P}=0.01) \), but not different from the rinsed leaf rearing treatment after exposure to Cry1F maize \( (t=-2.25; \text{df}=7.36; \text{P}=0.06) \).

**Conclusions**

Mortality in all four rearing treatments after exposure to non-Bt maize was low, ranging between 0 and 6%. This indicates the insects used in the assay were healthy and the assay design was appropriate for *S. frugiperda*. The only difference in mean weight gain within the non-Bt exposure was for the diet without antibiotic treatment, which was significantly higher than both leaf treatments but not different from the diet with antibiotic treatment. These results suggest that larvae that were reared on artificial diet were able to switch from diet to leaf material and were equally (diet with antibiotic) or better (diet without antibiotic) able to utilize non-Bt leaf material for growth.

Although the leaf rearing treatments had the highest mortality after exposure to Cry1F maize, the survivors of these treatments also had the highest weight gain on Cry1F maize. Artificial diet with antibiotic had the lowest mortality, but the lowest weight gain. These results are counterintuitive – mortality and weight gain are generally inversely correlated since both are measures of insect fitness. If leaf material was overall better suited for insect growth, then the mean weight gains for those reared on leaf material and exposed to non-Bt maize would have been greater than those reared on artificial diet. However, there was
no difference after exposure to non-Bt maize between the mean weight gain on leaf material and diet with antibiotic, and the mean weight gain on leaf material was significantly less than diet without antibiotic. These results suggest that *S. frugiperda* 3\textsuperscript{rd}-instars had a period of adjustment to the new rearing material that caused them to eat less and, therefore, consume a lower dose of Cry1F than those that were reared on leaf material.

Based on the differences in mortality and weight gain upon exposure to Cry1F maize after rearing *S. frugiperda* to 3\textsuperscript{rd} instar on leaf and artificial diet, and the ecological-relevance of leaf material, maize leaf tissue was selected as the rearing material for the short and long duration experiments described in Chapters 4 and 5.

REFERENCES


Evaluate susceptibility of 3rd instars on Cry1F leaf tissue

Feed neonates artificial diet with AB

Feed neonates artificial diet without AB

Feed neonates non-Bt leaf tissue with sterilization

Feed neonates non-Bt leaf tissue without sterilization

Figure 1. Experimental sequence of events. AB = Antibiotic.
Table 1. Odds ratio and confidence interval comparing the number of plants cut or sampled in flats of Cry1F maize to flats of non-Bt maize. If

| Sampling day | Cut Plants | | Sampled Plants | | |
| | Odds Ratio\(^1\) | 95% CI\(^2\) | Odds Ratio\(^1\) | 95% CI\(^2\) | |
| 2 | 0.30 | 0.18-0.50 | 3.14 | 2.05-4.81 | |
| 4 | 0.33 | 0.22-0.52 | 3.13 | 2.02-4.85 | |

\(^1\) If the odds ratio is >1, then there were more Cry1F plants cut or sampled than non-Bt plants. If the odds ratio is <1, then there were more non-Bt plants cut or sampled than Cry1F plants.

\(^2\) If the confidence interval overlaps with 1, then the difference between the treatments is not significant.

Table 2. Average percentage plants cut and sampled out of total stand in flats of Cry1F and non-Bt maize on days 2 and 4 of the experiment.

<table>
<thead>
<tr>
<th>% Cut (SE(^1))</th>
<th>% Sampled (SE(^1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>Day 4</td>
</tr>
<tr>
<td>non-Bt</td>
<td>18.1 (9.0)</td>
</tr>
<tr>
<td>Cry1F</td>
<td>5.1 (4.0)</td>
</tr>
</tbody>
</table>

\(^1\) SE = ± standard error of the mean
Table 3. Mortality and mean weight gain of *S. frugiperda* when reared to 3\textsuperscript{rd} instar on one of four treatments, then exposed to Cry1F or non-Bt maize leaf tissue for 4 days.

<table>
<thead>
<tr>
<th>Rearing phase treatment</th>
<th>Exposure phase treatment Cry1F</th>
<th>Exposure phase treatment non-Bt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Mortality*</td>
<td>% Mortality**</td>
</tr>
<tr>
<td>Artificial diet without antibiotic</td>
<td>42 a 14.3 (-2.6-31.3) a</td>
<td>0 70.6 (53.7-87.6) a</td>
</tr>
<tr>
<td>Artificial diet with antibiotic</td>
<td>54 ab 23.2 (3.5-42.8) ab</td>
<td>0 65.1 (48.1-82.0) ab</td>
</tr>
<tr>
<td>Leaf sterilized</td>
<td>75 bc 46.6 (27.9-65.3) c</td>
<td>6 59.8 (43.3-76.3) b</td>
</tr>
<tr>
<td>Leaf rinsed</td>
<td>88 c 34.9 (11.2-58.6) bc</td>
<td>0 58.6 (41.7-75.5) b</td>
</tr>
</tbody>
</table>

* Treatments with different letters were significantly different (P<0.05) within a column.

** No analysis was conducted for mortality of *S. frugiperda* after exposure to non-Bt maize leaf tissue.
CHAPTER 4. SUSCEPTIBILITY AND AVERSION OF *SPODOPTERA FRUGIPERDA* TO CRY1F BT MAIZE AND CONSIDERATIONS FOR INSECT RESISTANCE MANAGEMENT

A Paper to be Submitted to the Journal of Economic Entomology

Rachel R. Binning, Joel Coats, Xiaoxiao Kong, and Richard L. Hellmich

Abstract

*Bacillus thuringiensis* (Bt) maize (*Zea mays*) was developed primarily for North American pests such as European corn borer (*Ostrinia nubilalis*). However, most Bt maize products also are cultivated outside of North America, where the primary pests are different and often have lower susceptibility to Bt toxins. As these Bt maize products are commercialized in new geographies, insect resistance management (IRM) plans for those geographies need to consider relevant pest and toxin combination, instead of assuming the high-dose refuge strategy applies to all pests in all geographies. Before implementing an IRM plan that includes size and placement of refuge, it is useful to understand the biology and susceptibility of the primary pest(s) for each geography. Fall armyworm (*Spodoptera frugiperda*) is an important pest of maize in Central and South America where susceptibility to Cry1F (expressed in event 1507) is an example of a pest-by-toxin interaction that does not meet the high-dose definition. The behavioral and toxic response of *S. frugiperda* to Cry1F maize was investigated by measuring the percentage of time naïve third-instar spent feeding during a three-minute exposure. *S. frugiperda* also were exposed as
third-instars to Cry1F maize for 14 days to measure weight gain and survival. *S. frugiperda* demonstrated an initial, post-ingestive aversive response to Cry1F maize, and very few larvae survived a 14 d exposure. The role of susceptibility and avoidance are discussed in the context of global IRM refuge strategy development for Bt products.

**Introduction**

Bt maize has been grown commercially in the United States (US) since the introduction of lepidopteran-resistant maize in 1996. Since then, many other Bt maize products have been developed that confer resistance to lepidopteran and coleopteran maize pests in North America. The US Environmental Protection Agency (EPA) identified the preservation of Bt efficacy as “in the public good” (US EPA 1996, 1998a). At the request of the EPA, a Scientific Advisory Panel (SAP) considered the topic of insect resistance management (IRM) and refuge strategy as a means to extend the durability of Bt transgenic crops. At the time, all maize events on the market were highly toxic to the primary lepidopteran maize pests of North America – European corn borer (*Ostrinia nubilalis*) and Southwestern corn borer (*Diatraea grandiosella*). The SAP based their IRM recommendations on the “high dose” standard. The SAP defined high dose as “25 times the toxin concentration needed to kill a susceptible (SS) larva” and further indicated that this dose should kill 95% of heterozygous larvae with one resistance allele (RS) (US EPA 1998b). A
structured refuge of non-Bt maize was recommended based on the assumption that if the Bt maize product is high dose, resistance will be functionally recessive and rare. The SAP clearly identified size, configuration, and placement of the refuge relative to the Bt field as critical components of an IRM plan. The SAP’s recommendations were specific to a high-dose product; however, high-dose refuge (HDR) strategy has been broadly applied to Bt transgenic crops in instances where the product may not be high dose against the primary pest(s).

Bt maize is developed primarily for North American pests such as *O. nubilalis* and *D. grandiosella*. However, most Bt maize products are also cultivated outside of North America, where the primary pests are different and often have lower susceptibility to the toxins. As Bt maize products are commercialized in new geographies, it is important that IRM plans for those geographies consider relevant pest by toxin interactions, instead of assuming the HDR strategy applies to all pests in all geographies. Recent developments of field resistance to Bt maize by African maize stalk borer (*Busseola fusca*) in South Africa (Cry1Ab) and fall armyworm (*Spodoptera frugiperda*) in Puerto Rico (Cry1F) highlight the need to characterize the pest-by-toxin interaction (van Rensburg 2007, Matten *et al.* 2008). There are many important factors to consider when developing an IRM plan for a new geography, including the pest complex, cultivation and cultural practices, and crop biology (MacIntosh 2009). When considering only the pest-related factors of IRM, understanding the biology and susceptibility of the primary pest(s) for each geography and how a
pest might develop resistance to the toxin will help to develop an IRM plan with the appropriate size and placement of refuge.

Although a Bt maize product may not meet the definition of high dose, Bt maize may still be efficacious (i.e., protect yield). This could be a result of a lower, yet effective, level of toxicity and/or a behavioral response that causes the insect to reject the Bt maize as a food source. Insect rejection of a toxic compound is not rare (Zhang et al. 2004, Men et al. 2005, Li et al. 2006). However, rejection sometimes occurs in the absence of toxicity (Gould et al. 1991, Gore et al. 2005, Prasifka et al. 2007).

The initial rejection of a food source may be the beginning of a process that ends in acceptance of that food source, i.e., loss of aversion (Glendinning and Gonzalez 1995, Glendinning and Slansky 1995). Loss of aversion may be the result of desensitization to the mechanism that causes the aversive response (e.g., taste-mediated) (Glendinning et al. 2001), increased (or induced) detoxification of the aversive compound (Glendinning and Slansky 1995, Snyder and Glendinning 1996) or a combination of both desensitization and detoxification (Szentesi and Bernays 1984, Glendinning and Gonzalez 1995).

Fall armyworm susceptibility to Cry1F (event 1507) maize is an example of a pest-by-toxin interaction that does not meet the high dose definition (Storer et al. 2012). \textit{S. frugiperda} is an important pest of maize in Central and South America. And although \textit{S. frugiperda} cannot overwinter in the North American
Corn Belt, this species migrates every year and can cause significant damage to unprotected maize. Aversion to Cry1F by a maize pest such as *S. frugiperda*, and the ability of that pest to overcome aversion, may have implications for placement and size of a Bt maize refuge.

Accordingly, a series of laboratory studies were conducted to evaluate the behavioral response of *S. frugiperda* to the Cry1F protein as expressed in event 1507 maize. Two separate experiments, of short and long durations, were designed to examine 1) if *S. frugiperda* exhibit an initial aversive response to Cry1F maize, and 2) if *S. frugiperda* can overcome aversion and develop on Cry1F maize.

**Materials and Methods**

For both experiments, eggs from a susceptible laboratory population of *S. frugiperda* were obtained from a commercial source (Benzon Research, Inc, Carlisle, PA). Storer *et al.* (2010) report the LC$_{50}$ and GI$_{50}$ for the *S. frugiperda* Benzon colony as 428 and 19.7 ng Cry1F/cm$^2$ diet (surface overlay), respectively. Larvae were individually maintained on non-Bt maize leaf material until they reached the 3$^{rd}$ instar.

Three maize types were used for each experiment and were Pioneer brand hybrids. The hybrids included maize that contained Bt event 1507 (Cry1F maize), maize that contained Bt event 59122 (Bt-RW maize), and maize that
was a near-isoline non-Bt hybrid that did not express any insecticidal proteins (non-Bt maize). All maize tissues used for these studies were obtained by removing fully-formed individual leaves from plants (approximately growth stages V6-V10) 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 rinsed with tap water to remove surface debris and stored in resealable plastic bags in the refrigerator (approximately 4°C) or on wet ice until use, and not longer than 48 h. Insects were exposed to plant tissue 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 (Glendinning and Slansky 1994).

**Short duration study**

In order to identify how *S. frugiperda* detects Bt (preingestively or postingestively), Glendinning and Slansky (1994) utilized a three-minute (min) exposure assay. The short duration study described in this paper is modeled after their methods. The sequence of events for the short duration study is outlined in Figure 1.

This study is divided into two phases – screening and testing. For the screening phase, *S. frugiperda* that were within the first 24 h of the 3rd stadium were individually removed from the rearing material, placed in an empty petri dish (100x25-mm, NUNC #4031), and deprived of food for approximately 60
min. After starvation, a piece of non-Bt leaf material (approximately 3 cm$^2$) was placed within 1 cm of the larva’s head. Data collection began when the larva started feeding. Time spent feeding was recorded using the event tracking portion of a video tracking software program (EthoVision® XT, Noldus Information Technology) utilizing keystrokes to indicate when the larva stopped and started feeding. Observation continued for 3 min, after which time the larva was allowed to continue feeding for an additional 7 min to allow for a full feeding bout and avoid any potential for extreme hunger that might affect test results. At this point, larvae that had not fed for at least 90 of the 180 s observation period were discarded.

For the testing phase, the larva was food-deprived for a second time in an empty petri dish for 60 min. Then, a piece of leaf material from one of the 3 treatments (non-Bt, Cry1F, or Bt-RW) was placed within 2 cm of the larva’s head. Data collection began when the larva started feeding, and time spent feeding was recorded for 3 min. Twenty larvae per treatment were tested. Finally, each larva was placed in an individual well of a 6-well bioassay tray (BD Falcon #353046) and provided with non-Bt leaf material. Larvae were checked for mortality after 72 h.

Validity of this test system was determined by comparing the amount of time spent feeding on non-Bt leaf tissue in the screening stage to the amount of time spent feeding on non-Bt leaf tissue in the testing phase. If the time spent
feeding in the testing phase is shorter than the screening phase, this would indicate that 60 min of starvation is not long enough to account for the normal gap between *S. frugiperda* feeding bouts on maize tissue.

If rejection of Cry1F maize is due (at least in part) to a deterrent, there will be an immediate significant decrease in time spent feeding compared to non-Bt maize. Glendinning and Slansky (1994) observed *S. frugiperda* decreased time spent feeding within the first 15-30 s of exposure to the deterrent compounds linamarin, a cyanogenic glycoside, and caffeine. Even if deterrence is not observed, there may still be rejection related to a post-ingestive effect. Rejection due to a post-ingestive effect of Bt would likely take longer than 60 s, especially if it is due to toxicity of Bt. The Bt protein must be ingested, pass through the foregut into the midgut, bind to receptors, insert into the membrane, and finally form pores that lead to gut lysis and septicemia (Whalon and Wingerd 2003). Any delayed response (>60 s), similar to that observed by Glendinning and Slansky (1994) to nicotine hydrogen tartrate, will indicate that a reduction in feeding is due to a post-ingestive effect. If there is no rejection of Cry1F maize leaf disks, the larvae should feed for the same amount of time as larvae on the non-Bt leaf disk.

*Long duration study*

The long duration study was designed to investigate the ability of *S. frugiperda* to overcome aversion to Bt maize by monitoring daily growth and
survival. Third-instars were chosen because they are generally less susceptible to Bt, and will be more likely to survive the toxin long enough to show a loss of aversion. The sequence of events for the long duration study is outlined in Figure 2.

During the first 24 h of the third stadium, each larva was individually removed from the rearing material, placed in a well of a 6-well bioassay tray, and deprived of food for 60 min. Next, each larva was individually weighed to the nearest 0.1 mg, returned to the bioassay tray, and provided with leaf cuttings of non-Bt, Cry1F, or Bt-RW maize.

This experiment employed a randomized complete block design containing 16 replications per treatment, and two observations per replication. Each donor plant provided leaf tissue for one replication per treatment. Mortality and weight of survivors were recorded daily. The experiment ended on day 14, where day 1 was the day of infestation. A switch from rejection to acceptance was indicated by survival and weight gain.

Data analysis

For the short duration study, statistical analyses were conducted using SAS software, Version 9.3 (SAS Institute Inc. 2011) to compare the cumulative feeding time of S. frugiperda on the three treatments. SAS PROC MIXED was utilized to fit the analysis of variance (ANOVA) model. A two-tailed t-test was conducted at 15-s intervals, where a significant difference was identified if the P-
value (of the t-test) for difference between treatments was less than 0.01, due to multiple pair-wise comparisons.

For the long duration study, the total weight gain of S. frugiperda fed each of the three treatments was compared. A heterogeneous variance model was utilized to compare treatment effects. SAS PROC MIXED was utilized to fit the model. A two-tailed t-test was conducted and a significant difference was identified if the P-value (of the t-test) for difference between treatments was less than 0.05.

**Results**

**Short duration study**

Average time spent feeding on non-Bt maize in the screening phase was not different from time spent feeding on non-Bt maize in the testing phase (t=1.52; df=73; P=0.13). This validates 60 min as an adequate gap between feeding bouts for S. frugiperda on maize leaf tissue.

In the testing phase, mean time spent feeding on Bt-RW maize was not significantly different from non-Bt maize (t=-0.70; df=57; P=0.48) (Table 1). Third instar S. frugiperda spent significantly less time feeding on Cry1F maize compared to either non-Bt (t=-3.51; df=57; P=0.001) or Bt-RW (t=-2.80; df=57; P=0.01) maize. While this indicates that S. frugiperda reject Cry1F maize, examination of the cumulative feeding was needed to evaluate whether this
rejection was pre-ingestive or post-ingestive. Figure 3 compares the cumulative
time spent feeding on all three treatments. A significant difference between
Cry1F and non-Bt maize first occurs at 105 s (t=-2.61; df=57; P=0.01), indicating
that *S. frugiperda* aversion to Cry1F is likely post-ingestive. No mortality was
observed in any treatment 72 h after the short duration exposure study.

*Long duration study*

Mortality was high in the Cry1F treatment, with only 11% (2 larvae)
surviving for 14 d. Those survivors, however, did gain weight (Table 2). Average
total weight gain of survivors on Cry1F maize was significantly less than on
either Bt-RW (t=-6.54; df=54; P<0.0001) or non-Bt maize (t=-5.49; df=54;
P<0.0001). Those insects exposed to Cry1F that died before the end of the
assay lived an average of 4.3 d, with a median of 3.5 d, and lost an average total
of 2.2 mg before death.

Frequency distributions of daily weight gain show that 56% and 63% of
the weight gain for insects fed non-Bt and Bt-RW maize, respectively, was ≥31
mg per day (Figure 2). Conversely, 62% of the daily weight gain for insects fed
Cry1F leaf tissue were ≤0 mg for the entire cohort tested (including survivors
and those that died during the experiment). Of those fed Cry1F maize that did
gain weight on one or more days (38%), the daily weight gain was most often
(24%) between 1 and 10 mg. The Cry1F treatment also can be separated into
insects that survived and insects that did not survive exposure to Cry1F (Figure
3). Insects that survived exposure to Cry1F maize averaged a daily weight gain of 9.8 mg, and 77% of daily weight changes were >0 mg (Figure 3a). Of the daily weight changes for those that did not survive Cry1F exposure, 80% were ≤0 mg (Figure 3b).

When daily weight gain was averaged by treatment, insects exposed to non-Bt and Bt-RW maize showed similar trends in average daily weight change (Figure 6). All insects in these two treatments had pupated by day 8 and there was a distinct weight loss across days 6 and 7, suggesting that the larvae stopped eating in preparation for pupation. Average weight gain for larvae that survived Cry1F exposure was generally positive but relatively flat over time, although there was a distinct loss of weight on days 13 and 14 that appeared to mirror the pre-pupation weight loss for non-Bt and Bt-RW treatments on days 6 and 7. Average weight gain of individuals that eventually died after exposure to Cry1F was minimal, ranging from -1.6 to 10.5 mg, with 30% of the changes positive and 70% negative (Figure 6).

Discussion

The treatments of non-Bt and Bt-RW maize did not differ from each other in any analysis. Neither treatment caused an aversive response or significant mortality. This is expected since the proteins expressed in Bt-RW (59122) maize are generally acknowledged to have no toxic effect against Lepidoptera.
Mallet and Porter (1992) identify larval movement from Bt to non-Bt plants as a primary reason to avoid blending refuge in the field. This is based on the survival advantage blended refuge would confer to heterozygous resistant insects when movement occurs from a Bt plant to a non-Bt plant. However, if there is no selection for resistance (i.e., no mortality) before larval movement from Bt to non-Bt, then there is no heterozygote survival advantage. The short duration study indicates that the initial response of third-instar *S. frugiperda* to Cry1F maize is aversion (Table 1). The analysis shows that it takes 105 s for the response to be significant, suggesting that it is post-ingestive (Figure 3). Although the larvae are consuming Cry1F leaf tissue, the observation that all 20 insects in the short duration assay survived exposure to Cry1F indicates that the larvae are not consuming a toxic dose before rejection occurs. This is not the first study to conclude high survival after tasting exposure to Bt maize. Binning *et al.* (2010) described essentially 100% survival of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) larvae when exposed to Bt-RW maize for 17 d and then moved to non-Bt maize. These data were later utilized by Pan *et al.* (2011) to inform the predispersal tasting survival parameter in a simulation model to compare the durability of block and blended refuge for *D. v. virgifera*. Data from the study reported here could be used in a similar fashion. However, it is difficult to infer whether the larvae would abandon the maize plant after the initial aversive response demonstrated in the short duration study. Larval
movement and survival studies with whole plants could help address this question of host plant abandonment.

The alternative to host abandonment after initial rejection is that larvae remain on the Bt plant until they either 1) starve, 2) consume enough plant material to cause mortality, or 3) overcome both aversion and the toxic effect of Cry1F. The high mortality and median time to death after exposure of 3.5 d in the long duration experiment indicate that most *S. frugiperda* either starve or succumb to Cry1F toxicity (Table 2), however the two responses can’t be separated with these data. The insects that survive Cry1F maize are significantly smaller and therefore less fit than those fed non-Bt or Bt-RW maize. They did gain weight, however, indicating the initial aversive response did not cause permanent feeding cessation, and 11% of the tested larvae were able to at least partially overcome the toxic effects of Cry1F. Several possibilities could explain the survival of a few *S. frugiperda* on Cry1F maize, including detoxification or a heterogeneous genetic response. However, the simplest explanation is that these insects were on the lower end of the naturally occurring variation in susceptibility for the population. This, combined with reduced feeding due to the aversive response could account for survival plus reduced growth and development in this no-choice assay.

Larval movement is only one component of the insect-plant interaction that impacts the durability of blended refuge for Bt maize. Number and fitness of
susceptible insects produced from refuge plants, adult mating, dispersal, and oviposition are some of the additional parameters that may be considered before broad adoption of blended refuge strategy for Bt maize. Conclusions about larval movement after the initial aversive response cannot easily be drawn from the studies reported in this paper. Larvae may immediately abandon the host or move to a different part of the plant and continue to sample until it overcomes rejection or dies from toxicity. Additional studies are needed to investigate if host abandonment occurs, and if additional sampling after the initial tasting leads to selection for resistance. However, if the initial aversion does equate to host abandonment, then blended refuge could be a viable refuge deployment option for *S. frugiperda*. This could be critical information for countries outside of North America, where planting refuge is seldom required and *S. frugiperda* is a primary pest with continuous generations. Blending refuge seed in the bag could ensure that growers plant refuge despite an absence of refuge requirements. This study is one piece of evidence that can inform development of an effective IRM strategy for *S. frugiperda* outside North America to reduce selection pressure and extend the life of Bt traits such as Cry1F.

REFERENCES


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(US EPA) U. S. Environmental Protection Agency 1998b. Scientific advisory panel: subpanel on *Bacillus thuringiensis* (Bt) plant-pesticides and resistance management, February 9-10, 1998. Docket No. OPPTS-


Figure 1. Sequence of events for the short duration study.

Figure 2. Sequence of events for the long duration study.
Table 1. Mean time *Spodoptera frugiperda* 3rd-instars spent feeding during a 3-minute exposure to maize leaf tissue.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>LS-Mean Time Feeding (sec)</th>
<th>(95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1F</td>
<td>20</td>
<td>133 (117-149) a</td>
<td></td>
</tr>
<tr>
<td>Bt-RW</td>
<td>20</td>
<td>165 (149-181) b</td>
<td></td>
</tr>
<tr>
<td>Non-Bt</td>
<td>20</td>
<td>173 (157-189) b</td>
<td></td>
</tr>
</tbody>
</table>

*Treatments with different letters were statistically different (P<0.05).*
Figure 3. Cumulative time spent feeding by 3\textsuperscript{rd}-instar *S. frugiperda* on Cry1F, Bt-RW, and non-Bt maize leaf tissue. The earliest significant difference between 1507 and non-Bt is indicated by an arrow (P=0.01).
Table 2. Mean weight gain of surviving *S. frugiperda* larvae after 14 d exposure to maize leaf tissue. If pupation occurred before the end of the assay, final larval weight before the pre-pupal stage was used to calculate means.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n: larvae, pupae, dead</th>
<th>LS-Mean Weight Gain (mg) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1F</td>
<td>2, 0, 16*</td>
<td>127 (73-181) a</td>
</tr>
<tr>
<td>Bt-RW</td>
<td>0, 20, 0</td>
<td>317 (297-338) b</td>
</tr>
<tr>
<td>Non-Bt</td>
<td>0, 19, 1</td>
<td>311 (271-350) b</td>
</tr>
</tbody>
</table>

* Two individuals were missing from the Cry1F treatment at the end of the assay.
Figure 4. Frequency of weight gain values for each of three treatments (Cry1F, non-Bt, and Bt-RW). N is the total number of days that weight gain was measured across all insects. All observed weights from all live insects were included. Dead insects and pupae were not weighed, therefore are not represented in this figure.
Figure 5. Frequency of weight gain values for the insects that survived (a) and did not survive (b) exposure to Cry1F leaf material. N is the total number of days that weight gain was measured across all insects. Dead insects were not weighed, therefore are not represented in this figure.
Figure 6. Average daily weight gain of \textit{S. frugiperda} 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 varies from $n=16$ to $n=1$ across days for the Cry1F Dead line, and all insects were dead after day 11. N=2 for all points on the Cry1F Alive line. Day 1 was the first day larvae were weighed, therefore there is no weight gain to report for that day.
CHAPTER 5. SUSCEPTIBILITY TO BT PROTEINS NOT REQUIRED FOR AGROTIS IPSILON AVERSION TO BT MAIZE

A Paper to be Submitted to Pest Management Science

Rachel Binning, Joel Coats, Xiaoxiao Kong, and Richard L. Hellmich

Abstract

Although *Bacillus thuringiensis* (Bt) maize (*Zea mays*) has been widely adopted in diverse regions around the world, the response of many important insect pests to Bt maize has not been studied. The susceptibility and behavioral response of an insect pest to Bt maize are both significant factors to consider as management plans are developed for Bt maize in new regions. To address these questions for one global pest, the behavior and susceptibility of *Agrotis ipsilon* to events 1507 and 59122 maize were investigated by measuring the percentage of time naïve third-instars spent feeding during a three-minute exposure. Event 1507 maize expresses the insecticidal protein Cry1F, which is active against several lepidopteran pests of maize. Event 59122 maize expresses the binary insecticidal proteins Cry34Ab1/Cry35Ab1, which together, are active against several *Diabrotica* species (Coleoptera: Chrysomelidae). *A. ipsilon* were also exposed as third-instars to each Bt event for 14 days to measure weight gain and survival. *A. ipsilon* demonstrated an initial, pre-ingestive aversive response to Cry1F and Cry34Ab1/Cry35Ab1 maize. Survival on Cry1F and Cry34Ab1/Cry35Ab1 maize tissue was 40% and 95%, respectively, however weight gain of survivors of Cry1F exposure was significantly reduced.


Introduction

When Bt maize was first introduced in the United States in the late 20th century, it was intended to control primary pests of maize in the region such as *Ostrinia nubilalis* and *Diatraea grandiosella*. A few decades later, Bt maize is grown on five continents and dozens of 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* (Asia), *Sesamia nonagrioides* (Europe), *Busseola fusca* (South Africa), *Spodoptera frugiperda* (North and South America), and *Agrotis ipsilon* (ubiquitous). Since 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 toxicity. Rejection of Bt maize due to a behavioral response could contribute to efficacy in the field. Insect rejection of both toxic and non-toxic compounds is not uncommon (Gould et al. 1991, Zhang et al. 2004, Gore et al. 2005, Men et al. 2005, Li et al. 2006, Prasifka et al. 2007).

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 (Glendinning and Gonzalez 1995, Glendinning and Slansky 1995). Desensitization of the mechanism that causes the aversive response (e.g.,
taste-mediated) (Glendinning et al. 2001), increased (or induced) detoxification of the aversive compound (Glendinning and Slansky 1995, Snyder and Glendinning 1996) or a combination of both desensitization and detoxification (Szentesi and Bernays 1984, Glendinning and Gonzalez 1995) 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. *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. A few commercial Bt maize events are efficacious against *A. ipsilon*, however none provide complete control. A frequency of 5-10% cut plants due to *A. ipsilon* in a pure stand of Cry1F maize is not unexpected (McLeod and Butzen 2003). Since the commercialization of event 1507 maize (expressing the Cry1F insecticidal protein) in the United States, the possibility of a behavioral response to the protein has been investigated (Richtman 2006), 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. 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?, and 2) Can *A. ipsilon* overcome aversion and develop normally on Bt maize?

**Materials and Methods**

Three types of maize (all Pioneer brand hybrids) were used for each experiment: 1) a hybrid that contained Bt event 1507 (hereafter referred to as Cry1F maize), 2) a hybrid that contained Bt event 59122, hereafter referred to as Bt-RW maize, and 3) a near-isoline non-Bt maize hybrid that 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-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 48h. 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 (Glendinning and Slansky 1994).

**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 3rd instar.

A three-minute exposure assay was utilized by Glendinning and Slansky (1994) to identify whether *S. frugiperda* detected aversive compounds pre-ingestively or post-ingestively. The following short duration study that exposed *A. ipsilon* to Bt maize tissue is modeled after their methods. The sequence of events for the short duration study is outlined in Figure 1.

This study was divided into two phases – screening and testing. Starting with the screening phase, *A. ipsilon* within the first 24 h of the 3rd stadium were individually removed from the rearing material, placed in an empty petri dish (100x25 mm, NUNC #4031), and deprived of food for 60 minutes. 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). Keystrokes were used to indicate each time the larva stopped and started feeding. After this short observation time, each 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 calculated 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 6-well bioassay tray (BD Falcon #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 to 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 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 to non-Bt maize. Glendinning and Slansky (1994) observed lower 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 is due to toxicity of Bt. The
Bt protein must be ingested, 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 (Whalon and Wingerd 2003). A delayed response (>60 s), such as what was observed by Glendinning and Slansky (1994) 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.

**Long duration study**

The long duration study was designed to investigate 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 Figure 2. The only source of eggs for the long duration study was 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 3rd 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 6-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, where the
day of infestation is day 1.

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

Data analysis

The short duration study statistical analyses were conducted using SAS
software, Version 9.3 comparing the cumulative feeding time of *A. ipsilon* on the
three treatments (SAS Institute Inc. 2011). 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. Due to the multiple pair-wise comparisons, a significant
difference was identified if the P-value (of the t-test) for difference between
treatments is less than 0.01, rather than 0.05.

The long duration study analysis 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 is less than 0.05.
Results

*Short duration study*

Average time spent feeding on non-Bt maize in the screening phase was not different from 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 compared to 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 3 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 4 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 not a significant difference in cumulative time spent feeding on Cry1F and Bt-RW maize at any time point (Figure 5). No mortality was observed for any treatment 72 h after exposure.
Long duration study

Survival was low in the Cry1F maize treatment, with 40% (8 larvae) surviving for 14 d. 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 d, with a median of 6.5 d, and gained an average total of 4.0 mg before death. 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 (Figure 6). 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 (Figure 7). Of the daily weight gains for those that did not survive Cry1F exposure, 46% were ≤0 mg and 3% were >10 mg (Figure 7b). Conversely, 20% of the daily weight gains for Cry1F maize survivors were ≤0 mg and 32% were >10 mg (Figure 7a).
Weight change was averaged by treatment by day and is shown in Figure 8. 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 day 6 for Bt-RW maize (Figure 8a). These 3 molts would suggest the insects exposed starting at 3rd instar to these treatments were 6th instars at the conclusion of the assay, however larvae were not staged after initiation of the experiment. *A. ipsilon* experience a minimum of 6 instars before pupation. It is less clear from these data when the Cry1F survivors completed a molt (Figure 8a and 8b). Weight loss between days 6 and 7 and days 10 and 11 suggest that ecdisis may have occurred near day 7 and 11. If there were two molts, then Cry1F survivors were 5th 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 of weight loss due to ecdisis for those that died after exposure to Cry1F maize.

**Discussion**

*A. ipsilon* 3rd instars showed aversion to both Bt treatments in the short duration study. Aversion to Bt-RW (event 59122) maize was unexpected because the Cry34Ab1/Cry35Ab1 proteins expressed in event 59122 maize are generally acknowledged to have no toxic effect against Lepidoptera. Results
from the long duration study confirm absence of 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” (Glendinning 1994). *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 toxic 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, unique for each Bt variety (e.g., kurstaki or aizawai), or unique to each protein.

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 (Chapter 3). Combined, these results suggest *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 due to aversion. 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 of 6.3 d, and the relatively low mortality in the Cry1F maize treatment suggests mortality in the long duration assay could be caused by starvation rather than toxicity; however starvation and toxicity can’t 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 the initial aversive response was temporary and 40% of the tested larvae were able to at least partially overcome any toxic effects of Cry1F. Detoxification, a heterogeneous genetic response, and natural variation in population susceptibility are all possible explanations for the survival of 3rd-instars exposed to Cry1F maize for 14 d. 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 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, but not all, 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 pests like *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.

REFERENCES


Li, Y. X., S. M. Greenberg, and T. X. Liu. 2006. Effects of Bt cotton expressing Cry1Ac and Cry2Ab and non-Bt cotton on behavior, survival and development of Trichoplusia ni (Lepidoptera : Noctuidae). Crop Prot. 25: 940-948.


Figure 1. Sequence of events for the short duration study.
Figure 2. Sequence of events for the long duration study.

Table 1. Mean time *A. ipsilon* 3\textsuperscript{rd} instars spent feeding during a 3-minute exposure to maize leaf tissue.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>LS-Mean Time Feeding (sec) (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1F</td>
<td>9</td>
<td>112 (64-159) a</td>
</tr>
<tr>
<td>Bt-RW</td>
<td>9</td>
<td>87 (39-134) a</td>
</tr>
<tr>
<td>Non-Bt</td>
<td>9</td>
<td>174 (156-191) b</td>
</tr>
</tbody>
</table>

* Treatments with different letters were statistically different (P<0.05).
Figure 3. Cumulative time feeding by 3rd-instar *A. ipsilon* on Cry1F and non-Bt maize leaf tissue. The earliest significant difference between Cry1F and non-Bt is indicated by an arrow (P=0.01).
Figure 4. Cumulative time feeding by 3rd-instar *A. ipsilon* on Bt-RW and non-Bt maize leaf tissue. The earliest significant difference between Bt-RW and non-Bt is indicated by an arrow (P=0.01).
Figure 5. Cumulative time feeding by 3\textsuperscript{rd}-instar *A. ipsilon* on Bt-RW and Cry1F maize leaf tissue. There is no significant difference between Bt-RW and Cry1F at any timepoint, including 180 s (P=0.28).
Table 2. Mean weight gain of surviving *A. ipsilon* larvae after 14 d exposure to maize leaf tissue.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>% Mortality</th>
<th>LS-Mean Weight Gain (mg) (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1507</td>
<td>20</td>
<td>60</td>
<td>162 (87-237) a</td>
</tr>
<tr>
<td>59122</td>
<td>20</td>
<td>0</td>
<td>568 (471-664) b</td>
</tr>
<tr>
<td>Non-Bt</td>
<td>20</td>
<td>5</td>
<td>502 (408-596) b</td>
</tr>
</tbody>
</table>

* Treatments with different letters were significantly different (P<0.05).
Figure 6. Frequency of weight gain values (mg) for *A. ipsilon* on each of three treatments (Cry1F, Bt-RW, and non-Bt). N is the total number of days that weight gain was measured across all live insects. All observed weights from all insects were included. Dead insects were not weighed, therefore are not represented in this figure.
Figure 7. Frequency of weight gain values (mg) for *A. ipsilon* that survived (a) and did not survive (b) exposure to Cry1F maize leaf material. N is the total number of days that weight gain was measured across all insects. Dead insects were not weighed, therefore are not represented in this figure.
Figure 8. Average daily weight change of *A. ipsilon* when exposed to Bt-RW, non-Bt, and Cry1F. For a), only weight gain of those insects that survived exposure to 1507 for the entire length of the assay were included in the calculation for 1507 Survivors. For b), insects were split into those that survived 1507 (1507 Alive) and those that did not survive (1507 Dead). Sample size varies from n=12 to n=2 across days for the 1507 Dead line, and all insects were dead after day 9. N=8 for all points on the 1507 Alive line. Day 1 was the first day larvae were weighed, therefore there is no weight change to report for that day.
CHAPTER 6. GENERAL CONCLUSIONS

*Spodoptera frugiperda* larvae exhibited an initial, post-ingestive, aversive response to Cry1F maize. The dose of Cry1F in event 1507 maize is such that all larvae were able to survive a short (3-min) exposure. Those few that survived Cry1F exposure for 14 d were able to gain weight and progress through instars, indicating they were feeding and likely overcame their initial aversive response.

*Agrotis ipsilon* larvae exhibited an initial, post-ingestive, aversive response to Cry1F and Cry34Ab1/Cry35Ab1 maize. The dose of Cry1F in event 1507 maize is such that all larvae were able to survive a short (3-min) exposure. Those that survive Cry1F exposure for 14 d were able to gain weight and progress through instars, indicating they were feeding and likely overcame their initial aversive response. Cry34Ab1/Cry35Ab1 maize did not affect growth or development of *A. ipsilon* larvae in a 14 d assay, indicating they were able to quickly overcome their initial aversive response.

These results do not preclude the use of blended refuge for Cry1F maize.

**Future Research**

Although these studies provide critical new information regarding *S. frugiperda* and *A. ipsilon* susceptibility and behavioral response to Cry1F maize, more studies are needed to further explore this response and apply it to a refuge strategy recommendation.

The short duration study indicates that *S. frugiperda* show an initial aversive response to Cry1F maize. Typically, initial exposure to Cry1F maize will
be by neonates immediately after hatching. Therefore, repeating the short duration study with neonate *S. frugiperda* would help to understand if neonates exhibit the same aversive response and if they will ingest a lethal dose before ceasing feeding. Additionally, it is unclear from these data whether *S. frugiperda* will abandon the Cry1F plant or not. Further studies investigating larval movement with whole plants would be useful to understand the potential for larval movement in the field. Finally, investigating the link between resistance and loss of aversion (switching from rejection to acceptance of Cry1F maize) could be accomplished by repeating the short duration study with resistant colonies.

The long duration study indicates, for both insect pests, that survivors are able to grow and develop on Cry1F maize. To better describe whether growth and development is related to a loss of aversion, the short duration assay could be repeated with the individuals at certain time points during the long duration assay. If the cumulative feeding time after long term exposure to Cry1F maize is greater than naïve individuals and/or equivalent to larvae that were only exposed to non-Bt, then a loss of aversion could be concluded. A similar study was conducted by Glendinning *et al.* (2001). Additional experiments to understand whether that ability to overcome aversion is heritable could further inform IRM plans by helping to predict whether certain refuge configurations might select for resistance to both protection mechanisms – antibiosis and antixenosis. For A.
\textit{ipsilon}, this could be especially relevant since they are clearly highly mobile and likely to move from plant to plant.

REFERENCE

APPENDIX
ADDITIONAL INFORMATION

Exclusion of Replications from the *Agrotis ipsilon* Short Duration Assay

The short duration assay with *A. ipsilon* in Chapter 5 analyzed 9 replications. In reality, 13 replications were conducted over time in this experiment, however, replications 7-10 were excluded from the analysis. Exclusion of these replications was based on examination of the raw data grouped by insect batch followed by discussion with the rearing facility about potential insect quality issues.

Each replication in the short duration assay can take a significant amount of time to complete. Because of this time constraint, only 2-3 replications were completed per day. Each shipment of insects from the rearing facilities (USDA-ARS Corn Insect Crop Genetics Research Unit (Ames, IA) and Benzon Research, Inc, Carlisle, PA) was considered one batch. Shipments were received once a week and eggs were incubated such that they hatched over a period of two days. This allowed up to two days of experiments with the same batch of insects.

Treatments were examined by insect batch. Batches 1-5 were from the USDA-ARS rearing facility and batch 6 was from Benzon Research. Examination revealed that insect batches 4 and 5, representing two replications each, did not exhibit any aversive response to Cry1F maize (Figure 1). Other
insect batches had variation in their responses across replications. Batch 4 was tested on August 3, 2012 and Batch 5 was tested on August 12, 2012.

Figure 1. *A. ipsilon* time spent feeding on Cry1F maize leaf tissue sorted by insect batch.

Consequent conversations with the USDA-ARS research facility revealed that there was a significant personnel change during this time that likely affected insect quality (Richard Hellmich, personal communication). A decline in insect quality could easily explain a differential behavioral response within this experiment. Considering these factors, replications from insect batches 4 and 5 were dropped from the analysis.
Additional Tables and Figures

Table 1. Mean weight of 3rd-instar *S. frugiperda* exposed to one of 4 treatments (artificial diet with antibiotic (AB), artificial diet without antibiotic, leaf tissue rinsed, and leaf tissue sterilized) during the rearing phase described in Chapter 3. These were the starting weights prior to the testing phase where 3rd-instars were exposed to Cry1F or non-Bt maize.

<table>
<thead>
<tr>
<th>Rearing phase treatment</th>
<th>Mean 3rd instar weight (mg)</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial diet with AB</td>
<td>8.3</td>
<td>4.1</td>
<td>12.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Artificial diet without AB</td>
<td>10.8</td>
<td>4.6</td>
<td>48.4</td>
<td>8.3</td>
</tr>
<tr>
<td>Leaf rinsed</td>
<td>4.1</td>
<td>3</td>
<td>5.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Leaf sterilized</td>
<td>5.4</td>
<td>3.2</td>
<td>10.7</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 2. Mean and median number of feeding bouts per *S. frugiperda* 3rd instar as measured during the testing phase of the short duration assay (Chapter 4).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Treatment</th>
<th>Mean # of bouts per larva</th>
<th>SE</th>
<th>Median # of bouts per larva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding</td>
<td>Non-Bt</td>
<td>1.40</td>
<td>0.21</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Cry1F</td>
<td>1.85</td>
<td>0.32</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Bt-RW</td>
<td>1.40</td>
<td>0.17</td>
<td>1</td>
</tr>
<tr>
<td>Non-feeding</td>
<td>Non-Bt</td>
<td>0.45</td>
<td>0.21</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cry1F</td>
<td>1.40</td>
<td>0.35</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Bt-RW</td>
<td>0.55</td>
<td>0.18</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3. Mean and median number of feeding bouts per *A. ipsilon* 3rd instar as measured during the testing phase of the short duration assay (Chapter 5).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Treatment</th>
<th>Mean # of bouts per larva</th>
<th>SE</th>
<th>Median # of bouts per larva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding</td>
<td>Non-Bt</td>
<td>1.44</td>
<td>0.24</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Cry1F</td>
<td>2.78</td>
<td>0.66</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Bt-RW</td>
<td>2.56</td>
<td>0.38</td>
<td>3</td>
</tr>
<tr>
<td>Non-feeding</td>
<td>Non-Bt</td>
<td>0.44</td>
<td>0.24</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cry1F</td>
<td>2.00</td>
<td>0.75</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Bt-RW</td>
<td>2.22</td>
<td>0.46</td>
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</tr>
</tbody>
</table>

Figure 2. *A. ipsilon* time spent feeding on each of 3 treatments (Non-Bt, Cry1F, and Bt-RW maize leaf tissue) sorted by insect batch.
Table 4. *Spodoptera frugiperda* cumulative time feeding averaged at 15 second intervals during the screening phase and testing phase for each of 3 treatments (Cry1F, Bt-RW, and non-Bt maize). Larvae were exposed to non-Bt maize during the screening phase and one of the 3 treatments during the testing phase. These values were plotted in Figure 1, Chapter 4.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Testing phase treatment</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>75</th>
<th>90</th>
<th>105</th>
<th>120</th>
<th>135</th>
<th>150</th>
<th>165</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening Non-Bt</td>
<td>14.6</td>
<td>28.7</td>
<td>42.6</td>
<td>56.3</td>
<td>68.5</td>
<td>80.5</td>
<td>92.7</td>
<td>105.9</td>
<td>119.7</td>
<td>132.5</td>
<td>145.3</td>
<td>158.0</td>
<td></td>
</tr>
<tr>
<td>Testing Non-Bt</td>
<td>14.8</td>
<td>29.1</td>
<td>43.4</td>
<td>57.4</td>
<td>70.9</td>
<td>83.8</td>
<td>98.4</td>
<td>113.4</td>
<td>128.4</td>
<td>143.4</td>
<td>158.4</td>
<td>172.8</td>
<td></td>
</tr>
<tr>
<td>Screening Cry1F</td>
<td>14.2</td>
<td>27.3</td>
<td>40.8</td>
<td>54.9</td>
<td>68.3</td>
<td>81.8</td>
<td>95.0</td>
<td>108.2</td>
<td>121.8</td>
<td>135.3</td>
<td>148.8</td>
<td>162.3</td>
<td></td>
</tr>
<tr>
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<td>27.2</td>
<td>40.2</td>
<td>52.6</td>
<td>63.6</td>
<td>73.5</td>
<td>83.5</td>
<td>93.4</td>
<td>103.9</td>
<td>114.4</td>
<td>124.5</td>
<td>133.1</td>
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</tr>
<tr>
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<td>57.7</td>
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<td>87.5</td>
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<td>115.7</td>
<td>129.0</td>
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<td>168.5</td>
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<tr>
<td>Testing Bt-RW</td>
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<td>28.5</td>
<td>43.5</td>
<td>58.5</td>
<td>73.1</td>
<td>87.7</td>
<td>101.5</td>
<td>115.0</td>
<td>127.7</td>
<td>139.8</td>
<td>152.3</td>
<td>164.8</td>
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</tr>
</tbody>
</table>
Table 5. *Agrotis ipsilon* cumulative time feeding averaged at 15 second intervals during the screening phase and testing phase for each of 3 treatments (Cry1F, Bt-RW, and non-Bt maize). Larvae were exposed to non-Bt maize during the screening phase and one of the 3 treatments during the testing phase. These values were plotted in Figures 1-3, Chapter 5.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Testing phase treatment</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>75</th>
<th>90</th>
<th>105</th>
<th>120</th>
<th>135</th>
<th>150</th>
<th>165</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening</td>
<td>Non-Bt</td>
<td>15.0</td>
<td>30.0</td>
<td>43.9</td>
<td>58.9</td>
<td>73.9</td>
<td>88.9</td>
<td>103.9</td>
<td>118.1</td>
<td>132.4</td>
<td>146.7</td>
<td>161.1</td>
<td>175.7</td>
</tr>
<tr>
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<td>100.2</td>
<td>115.2</td>
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<td>143.7</td>
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<td>173.7</td>
</tr>
<tr>
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<td>Cry1F</td>
<td>15.0</td>
<td>27.8</td>
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<td>55.5</td>
<td>69.1</td>
<td>81.4</td>
<td>92.1</td>
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<td>115.1</td>
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<td>Cry1F</td>
<td>13.4</td>
<td>24.3</td>
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<td>52.1</td>
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<td>68.7</td>
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<td>82.4</td>
<td>92.2</td>
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<tr>
<td>Screening</td>
<td>Bt-RW</td>
<td>15.0</td>
<td>30.0</td>
<td>45.0</td>
<td>60.0</td>
<td>75.0</td>
<td>90.0</td>
<td>102.0</td>
<td>113.7</td>
<td>125.3</td>
<td>137.5</td>
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<tr>
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<td>25.0</td>
<td>34.5</td>
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<td>50.6</td>
<td>56.1</td>
<td>63.1</td>
<td>68.5</td>
<td>73.3</td>
<td>77.3</td>
<td>83.7</td>
<td>89.6</td>
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</table>
Figure 2. Box plot of weight gain for *S. frugiperda* larvae after 14 d exposure to Cry1F, non-Bt (Iso), and Bt-RW (RW) maize as described for the long duration experiment in Chapter 4. Statistics for the Cry1F treatment were separated into larvae that lived for the duration of the experiment (1F_alive) and larvae that died during the experiment (1F_dead).
Figure 3. Box plot of weight gain for *A. ipsilon* larvae after 14 d exposure to Cry1F, non-Bt (Iso), and Bt-RW (RW) maize as described for the long duration experiment in Chapter 5. Statistics for the Cry1F treatment were separated into larvae that lived for the duration of the experiment and larvae that died during the experiment.