**Physiological impact of a *Bacillus thuringiensis* toxin on the black cutworm that enhances baculovirus pathogenicity**

by

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“Follow your bliss.”
-Joesph Campbell, The Power of Myth

I dedicate this to all those who have enabled me to follow my bliss.

# TABLE OF CONTENTS

# CHAPTER 1. General Introduction

## Thesis Organization

This thesis is divided into three chapters. Chapter 1 includes a literature review of the bacterium *Bacillus thuringiensis* (Bt) and Bt toxins with an emphasis on Cry1F, the black cutworm, *Agrotis ipsilon* (Hufnagel), Bt toxins and baculovirus interactions and insect gut physiology relevant to this research. The first chapter also includes the rationale behind the research and the research objectives. Chapter 2 describes a study of the physiological impact of a Bt toxin on the black cutworm which enhances baculovirus pathogenicity. The general conclusions of the thesis are in Chapter 3.

## Literature Review

### *Bacillus thuringiensis*

*Bacillus thuringiensis* (Bt) is a gram-positive, spore-forming bacterium, which is found mainly in the rhizosphere but can also be found in dust, marine sediments, on stored food products, animal skin and plant leaves (Maduell et al., 2008). Bt produces a parasporal crystal (Cry) toxin during sporulation, which has insecticidal properties (Gill et al., 1992). Upon ingestion by the insect, the bacterium travels to the insect midgut where a 130-kDa crystal is released. Midgut proteases, such as trypsin, chymotrypsin and elastase, cleave the crystal protoxin into a 60 to 70-kDa activated toxin (Oppert, 1999). For the protoxin to be converted to an activated toxin, the insect midgut must be at an appropriate pH, which is very alkaline from 9-11. The crystal is insoluble in other conditions, which makes it safe against non-target organisms which have a more acidic gut pH (Whalon and Wingerd, 2003). The

activated toxin, or δ-endotoxin, binds to cadherin-like or other receptor proteins, found on the brush border membrane on the surface of the midgut epithelial cells (Bravo et al., 2007). Toxin-receptor binding leads to toxin oligomerization. In some cases, oligomerization allows for increased binding affinity to other receptors, such as aminopeptidase-N, alkaline phosphatase or glycolipids, which is followed by insertion of the toxin into the membrane (Abdullah et al., 2006; Bravo et al., 2007; Chen et al., 2005). Insertion results in an ion imbalance through the formation of a pore channel, which ultimately results in cell lysis (Aronson and Shai, 2001). Susceptible insects can be classified into 3 types on the basis of toxin sensitivity. Type I insects are extremely sensitive to the toxin and exchange of hemolymph and gut contents leads to mortality in 1-7 hours. In Type II insects only gut paralysis is observed with death occurring in 2-7 days. In type III insects, mortality does not result from the toxin but from septicemia after spore germination in the gut (Heimpel and Angus, 1959). Larvae stop feeding and are likely killed by septicemia as bacteria enter the hemocoel (Schnepf et al., 1998). An alternative model suggests that Bt kills insects by activation of a magnesium-dependent cytotoxic event. This pathway is triggered when the toxin binds to a cadherin receptor which stimulates G protein α-subunit and adenylyl cyclase (AC). This stimulation leads to an increase in cAMP levels, which activates protein kinase A (PKA). The activation of the AC/PKA pathway triggers physical changes in the cell followed by pore formation and cell lysis, leading to septicemia (Zhang et al., 2006).

With Cry toxins, two types of binding can occur: reversible or irreversible. If a toxin forms a tight bond between itself and the receptor and a channel is formed, it will be referred to as irreversible (Valaitis et al., 1995). If a toxin is not able to bind to the brush border membrane to form an ion channel by inserting into the apical membrane, then the binding is

referred to as reversible. The reasoning for this distinction is that a toxin can have insecticidal potential for a certain species depending on whether or not the toxin has the ability to irreversibly bind (Rajamohan et al., 1995).

Cry toxins have three structural domains, which play different roles in the mode of action of the toxin (Aronson and Shai, 2001; Jurat-Fuentes and Adang, 2001). Domain I facilitates pore formation (Jurat-Fuentes and Adang, 2001). Domain II is involved in receptor binding. Domain III is involved with the stability of the toxin and specificity of binding (Schnepf et al., 1998). Different Cry toxins will bind to different receptors with binding sites on loops within domains II and III (Sangadala et al., 1994).

The presence of receptors determines toxin host specificity (Van Rie et al., 1990). Some insect species have only one binding protein, while other species can have multiple protein receptors. In *Sesamia nonagrioides* (Lepidoptera: Noctuidae), Cry1Aa, Cry1Ab, and Cry1Ac all used the same binding site, while Cry1Fa bound to a separate site (Gonzalez-Cabrera et al., 2006). The Cry1Fa binding site receptors are likely cadherin and aminopeptidase proteins of 170 and 110 kDa, which also bind Cry1Ab, Cry1Ac, Cry1J and Cry1Aa (Jurat-Fuentes and Adang, 2001). The HevCaLP protein, from cadherin gene *BtR4*, was proposed as a binding site for Cry1A and Cry1Fa in the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae), but was later shown to only serve as a receptor for Cry1A (Jurat-Fuentes and Adang, 2006).

Midgut bacteria may play a critical role in the insecticidal action of Bt. Removal of midgut bacteria by antibiotic treatment, reduced susceptibility of *Lymantria dispar, Vanessa cardui, Manduca sexta, Pieris rapae* and *H. virescens* larvae to Bt suggesting that the bacteria have a crucial role (Broderick et al., 2006; Broderick et al., 2009). The opposite was

seen in *Pectinophora gossypiella*, in which case larvae were more susceptible to Bt-induced mortality following antibiotic treatment (Broderick et al., 2009).

### *Agrotis ipsilon*

The black cutworm, *Agrotis ipsilon* (Lepidoptera: Noctuidae) feeds on a wide variety of cultivated plant and grass species and is a sporadic pest of corn, *Zea mays*, which makes it of economic importance (Busching and Turpin, 1977). Damage occurs in the foliage of seedling corn plants prior to the 4th instar. During the 4th- 6th instar, larval feeding results in cutting or tunneling into the stem, which disrupts xylem or phloem systems (Showers et al., 1983). If the plant is cut at or below the growing point, yield loss can result due to growth stunting or death of the plant (Whitford et al., 1989). Infestations by *A. ipsilon* larvae are sporadic and, in turn, difficult to predict because population densities vary yearly (Archer and Musick, 1977).

### Bt for control of *Agrotis ipsilon*

*Agrotis ipsilon* is resistant to Cry1Ba, Cry1Ac, Cry1Ca and Cry1J, although Cry1Ac and Cry1J have some toxicity to *A. ipsilon* neonates at very high doses. Cry1Ac, Cry1Ca and Cry1J have been shown to bind to the brush border membranes of the insect but do not have insecticidal activity, which could be attributed to reversible binding (Gilliland et al., 2002). The toxicity of Cry1Fb against *A. ipsilon* neonates was reported as an $LC_{50}$ of 4,530 (ng/cm$^2$) with protoxin and 3,110 (ng/cm$^2$) for the active toxin (de Maagd, 2003). In cotton, *H. virescens* neonate mortality by Cry1Fa2 was scored after 7 days at $LC_{50}$ of 4.44 ng/cm$^2$ (Blanco et al., 2008) compared to *A. ipsilon* which had an $LC_{50}$ of >16,000 ng/cm$^2$ (de

Maagd, 2003). Other toxins effective against *A. ipsilon* include Cry9Ca and Cry1Aa (de Maagd et al., 2003; Lambert et al., 1996).

*Bacillus thuringiensis* applied to crops in a spray formulation, has been used for control of lepidopteran pests for approximately 60 years (Romeis et al., 2006). For example DiPel® is a biological insecticide of *Bacillus thuringiensis* subspecies kurstaki in its natural form which expresses the five toxins of Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Bb. DiPel® also contains spores. This form of Bt can be very effective for pest control since multiple Cry forms are expressed. In recent years, transgenic corn and cotton plants have been modified to express insecticidal proteins from different subspecies of *Bacillus thuringiensis* and have revolutionized pest control of lepidopteran and coleopteran pests. Many transgenic corn lines have been developed to target lepidopteran pests. When tested against *A. ipsilon*, corn engineered to produce the Cry1Ab protein, had no insecticidal effects on the insect (Pilcher et al., 1997). Herculex® I [1] produces the Cry1Fa2 protein from *Bacillus thuringiensis* subspecies *azawai* and is the only commercially available Bt corn hybrid labeled for protection against the *A. ipsilon*, in addition to multiple other lepidopteran corn pests. In feeding assays, Herculex® I was only moderately toxic to *A. ipsilon* and larvae quickly recovered lost weight when placed on non- Herculex® I corn. In a bioassay comparing weights of third instar *A. ipsilon* after 7 days on Herculex® I and isoline (the same genetic line lacking the Cry protein), weights of those on Herculex® I were significantly lower than those on isoline (Richtman, 2006). Other insect species sensitive to Cry1F are listed in Table 1.

**Pathogens for control of *Agrotis ipsilon***

Pathogens have been investigated for control of *A. ipsilon*. Of 63 tested, seven viruses, five bacteria, two fungi and one protozoan of 63 tested caused 30% or greater mortality in *A. ipsilon* (Ignoffo and Garcia, 1979). When the baculoviruses *Rachiplusia ou* multiple nucleopolyhedrovirus *(*RoMNPV*)* and *Autographa californica* multiple nucleopolyhedrovirus *(*AcMNPV*)* were applied to seedling corn, a significant reduction in damage by *A. ipsilon* was reported (Johnson and Lewis, 1982). Baculoviruses have restricted host ranges generally infecting only a single species or a few species within a genus (Hunter-Fujita et al., 1998). Baculoviruses have been used since the 1890s for insect management. Although baculoviruses provide an attractive alternative to chemical control due to their high host specificity, their slow speed of kill and low field stability due to UV-degradation hinder their development for large scale pest control (Moscardi, 1999).

*Agrotis ipsilon* multiple nucleopolyhedrovirus (AgipMNPV) is highly pathogenic to *A. ipsilon*. First identified in 1999, AgipMNPV is far more pathogenic to *A. ipsilon* than AcMNPV or RoMNPV (Boughton et al., 1999). Other insect species in the family Noctuidae, the corn earworm, *Helicoverpa zea,* and the tobacco budworm, *Heliothis virescens* were shown to be somewhat susceptible to AgipMNPV but only at much higher doses than the *A. ipsilon* with the exception of the common cutworm, *Agrotis segetum*, which was more susceptible. The $LC_{50}$ for first instar *A. ipsilon* is 269 occlusion bodies (OB)/µl, 797 OB/µl for *H. virescens*, 7083 OB/µl for *H. zea* and 27 OB/µl for *A. segetum* (for neonates) (Boughton et al., 1999; El-Salamouny et al., 2003).

AgipMNPV, AcMNPV and RoMNPV affect *A. ipsilon* during its larval stages. The virus has two viral phenotypes: occlusion derived virus (ODV) for initial infection and

budded virus (BV) for infection of internal tissues (Entwistle and Evans, 1985). The occlusion bodies (OB) are ingested and the high pH of the insect midgut dissolves the polyhedrin matrix of the occlusion bodies releasing ODV. Proteases may also play a role in this early infection process (Wood, 1980). Following release of ODV from OB, ODV cross the peritrophic matrix, a layer of chitin and protein which separates the midgut lumen from epithelial cells, and bind to the microvilli of the epithelial cells. The receptor to which the virus binds on the microvilli has not been identified. After binding, nucleocapsids are released from the ODV and travel to the cell nucleus where viral DNA replication occurs (Federici, 1997). Newly produced BV spread to other cells and tissues. In the late stages of the infection process, OBs are produced in infected cells. When the larvae die, OBs will be released into the environment when the cuticle ruptures (Blissard and Rohrmann, 1990).

**Bt-virus interaction**

Bt and baculoviruses have been evaluated for combined use to improve crop protection. Table 2 lists specific Bt-virus combinations and the effect of combination for pest control. Bt types tested are either the bacterium in its natural state or the purified Bt toxin.

Some alternative strategies have been tried for control using the Bt/virus combination. Genes encoding Bt toxins have been engineered into baculovirus genomes as a strategy for improved pest control (Chang et al., 2003; Martens et al., 1995; Merryweather et al., 1990; Ribeiro and Crook, 1993). Unfortunately, none of these recombinant viruses showed a decrease in time to kill or a lethal dose/concentration over the wild-type. The incorporation of Bt genes in the baculovirus genome result in the synthesis of Bt toxins in the cell. Since this is an inappropriate target site, this may explain the lack of improvement over the wild-

type. Another construct has been made in which the polyhedrin protein is fused to a Bt protein and expressed in *E. coli* (Seo et al., 2005). Although this system shows an effective delivery system for Bt, it does not involve virus infection, since the construct only uses a baculovirus polyhedron protein and not the entire virus genome. Table 3 lists the engineered Bt-baculovirus combinations.

## Insect gut physiology

### Gut pH and proteases

Gut pH and proteases have a role in infection by both Bt and baculoviruses (Dow, 1992; Wood, 1980). Neither the gut pH nor protease composition of *A. ipsilon* has been documented. Lepidopteran gut pH can range from 8-12 in the midgut and can vary based on food intake and diet preferences (Chapman, 1998; Dow, 1992). In a study done with the cabbage butterfly, *Pieris brassicae,* larvae that were exposed to Bt had a lower midgut pH following exposure and had increased virus susceptibility, suggesting that the lowered pH may have been more favorable to the virus (Peters and Coaker, 1993). Gypsy moth, *Lymantria dispar*, which fed on acidic leaf foliage, had a lowered gut pH and a lowered susceptibility to gypsy moth nuclear polyhedrosis virus, showing the opposite effect (Keating et al., 1990).

Endopeptidases, like serine and cysteine proteases, produce peptides by cleaving proteins and are sometimes referred to more specifically as proteinases (Oppert, 1999). Exopeptidases, such as aminopeptidases, cleave individual amino acids from peptides (Chapman, 1998). In the bertha armyworm, *Mamestra configurata*, and tomato moth, *Lacanobia oleracea*, lepidopterans in the family Noctuidae, gut protease activity was found to be associated with serine proteases: trypsin, chymotrypsin and elastase-like proteases, but

no activity was associated with cysteine proteases (Gatehouse et al., 1999; Hegedus et al., 2003). Additionally, aminopeptidase activity was detected in midgut extracts (Hegedus et al., 2003).

Certain proteases are essential to activate Bt toxins and changes leading to a decrease in activity of these proteases serve as a mechanism of Bt-resistance (Oppert et al., 1997). Feeding on Bt decreased trypsin and chymotrypsin protease activity in the cotton bollworm, *H. zea* (Zhu et al., 2007) and was shown to inhibit serine protease expression and promote expression of serine protease inhibitors in spruce budworm larvae, *Choristoneura fumiferana* (van Munster et al., 2007). In the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), soluble and membrane-associated gut proteases were compared for Bt-resistant and susceptible insects. Trypsin-like proteases, in the soluble fraction of susceptible insects, were found to be the most active (Li et al., 2004), but in a similar study (Siqueira et al., 2004) no significant differences were found. In the Indianmeal moth, *Plodia interpunctella*, Bt-resistant and susceptible individuals were compared and a threefold lower activity level of chymotrypsin-like proteases was detected in resistant insects (Zhu et al., 1997). A similar trend was seen with Bt-resistant and susceptible strains of *H. virescens* with the trypsin-like proteases being less active in resistant insects (Karumbaiah et al., 2007). This reduction in protease activity may decrease the efficiency of conversion from protoxin to activated toxin.

**Peritrophic matrix**

The insect peritrophic matrix (PM) lines the gut and surrounds the food bolus. The PM is composed of chitin fibrils and glycoproteins and is produced by midgut cells (Chapman, 1998). The PM serves as a barrier to protect midgut epithelial cells from mechanical damage from ingested food particles as well as microorganisms such as bacteria

and viruses (Chapman, 1998; Wang and Granados, 1998) Due to its size, Bt cannot travel through the PM, but the toxins produced by the bacterium are small enough to go through (Yunovitz et al., 1986). Additionally, Bt has been shown to reduce or destroy certain glycoproteins in the PM (Rupp and Spence, 1985). Maize cysteine protease Mir1-CP damages the peritrophic matrix of the fall armyworm, *S. frugiperda* (Mohan et al., 2006). Damage to the PM decreases protection from virus infection (Derksen and Granados, 1988; Wang and Granados, 1997; Wang and Granados, 2000), Physical changes in the PM structure following feeding have been documented. Larvae fed on foliage developed a thicker PM than those fed on artificial diet, and those with the thicker PM had reduced infection by AcMNPV (Plymale et al., 2008). Enhancin, a *Trichoplusia ni* granulovirus protease protein, has the ability to degrade the PM facilitating an increase in baculovirus virulence (Lepore et al., 1996). The addition of enhancin has been shown to increase the toxicity of Bt in several lepidopteran species. This most likely occurs by altering permeability of the PM, which supports the hypothesis that the PM serves as a barrier to Bt toxin movement towards midgut epithelial cells (Granados et al., 2001).

Refractoriness to Bt toxins may result from toxin binding to the peritrophic matrix and subsequent proteolytic degradation of the toxin. The peritrophic matrix of *A. ipsilon* at 8 µm is unusually thick and highly glycosylated, when compared with those of other lepidopteran pest species, which may explain black cutworm resistance to many Cry toxins (Rees et al., 2009).

[1] Herculex® I Insect Protection Technology by Dow AgroSciences and Pioneer Hi-Bred. Herculex is a registered trademark of Dow AgroSciences LLC, Indianapolis, IN.

**Rationale**

Little work has been done in the area of insect/virus/Bt interaction, and currently there are no published papers on the physiological interaction between these three components. Previous research I conducted showed an interaction between Bt and virus that increased mortality by virus in *A. ipsilon* (Richtman, 2006). The research reported in this thesis was designed to identify physiological factors that would explain the increased mortality due to viral infection following feeding on Bt. Specifically, I chose to investigate whether alteration of gut pH, gut proteases and/or peritrophic matrix physiology occurred after Bt exposure and whether these changes could explain enhanced viral pathogenicity in *A. ipsilon*.

## Objectives

**General objective:** To determine the physiological mechanism(s) behind increased rates of mortality in *A. ipsilon* fed Bt followed by virus infection versus the isoline/virus combination.

**Specific objectives:**

1. **Determine if exposure to Bt results in a pH change or change in gut proteases that could contribute to increased infectivity of the virus.**

2. **Determine if exposure to Bt results in physical changes in the peritrophic matrix that could facilitate virus movement.**

Table 1. Pest insects in the order Lepidoptera susceptible to Cry1F.

| Insect | Reference |
|---|---|
| Black cutworm, *Agrotis ipsilon* | (de Maagd et al., 2003) |
| Western bean cutworm, *Striacosta albicosta* | (Eichenseer et al., 2008) |
| Tobacco budworm, *Heliothis virescens* | (Blanco et al., 2008) |
| Beet armyworm, *Spodoptera exigua* | (Luo et al., 1999) |
| Fall armyworm, *Spodoptera frugiperda* | (Luo et al., 1999) |
| Cotton bollworm, *Helicoverpa zea* | (Gao et al., 2006) |
| American bollworm, *Heliothis armigera* | (Liao et al., 2002) |
| Australian budworm, *Heliothis punctigera* | (Liao et al., 2002) |
| Cabbage looper, *Trichoplusia ni* | (Iracheta et al., 2000) |
| Soybean looper, *Pseudoplusia includens* | (Blanco et al., 2008) |
| European corn borer, *Ostrinia nubilalis* | (EPA, 2001) |
| Southwestern corn borer, *Diatraea grandiosella* | (EPA, 2001) |
| Lesser cornstalk borer, *Elasmopalpus lignosellus* | (EPA, 2001) |
| Sugarcane borer, *Diatraea saccharalis* | (EPA, 2001) |

Table 2. Bt-baculovirus combinations and the effect of combination for pest control.

| Reference | Year | Bt type | Virus type | Insect | Age | Administration | Effect |
|---|---|---|---|---|---|---|---|
| Peters et al. | 1993 | subspecies kurstaki | PbGV | *P. brassicae* | 2nd, 4th instars | Bt then virus | Increased susceptibility to the virus |
| Marzban et al. | 2009 | Cry1Ac | HaCPV | *H. armigera* | 1st, 3rd instars | Bt then virus | Increased susceptibility to the virus, had additive effect except at low Cry dose which had synergistic effects |
| Lipa et al. | 1976 | DiPel [a] | HaNPV | *S. exigua* | 3rd instars | simultaneous | synergistic effect |
| McVay et al. | 1977 | subspecies kurstaki | T. ni NPV | *T. ni* | 3rd instars | simultaneous | Additive effect |
| Bell and Romine | 1980 | commercial WP formulation | AcMNPV | *H. virescens* | from egg on | simultaneous | Higher plant yields when virus and Bt used for protection compared to virus alone |
| Matter and Zohdy | 1981 | subspecies thuringiensis Bactospeine | HaNPV | *H. armigera* | 1st, 3rd and 5th instars | simultaneous | 1st instars, lessened the effect to combine. 3rd instar and later, pathogens together has additive effect |
| Luttrell et al. | 1982 | DiPel [a] and Thuricide | H. zeaNPV AcMNPV | *H.zea* *H. virescens* | neonates, 2nd instars | simultaneous | Mortality greater than Bt alone but not virus alone |
| Bell and Romine | 1986 | DiPel [a] | HaNPV | *H. virescens* *H. Zea* | neonates | simultaneous | Virus and Bt resulted in mortality not greater than Bt alone and lowered mortality rate by virus when combined |
| Farrar et al. | 2004 | Cry1Ab corn | SfMNPV | *S. frugiperda* | 1st instars | simultaneous | Higher mortality with virus and Bt than isoline |
| Raymond et al. | 2006 | Cry1Ac | AcMNPV | *P. xylostella* | 3rd instars | simultaneous | Used Bt resistant and susceptible insects Antagonistic in both resistant and susceptible but stronger antagonism in susceptible insects |
| Pingel and Lewis | 1999 | subspecies kurstaki, aizawai | AfMNPV | *H. zea* *S. frugiperda* *O. nubilalis* | neonates | virus then Bt | Bt is equal to Bt + virus, virus $LC_{50}$ is lowered by addition on Bt |
| Street and Mulrooney | 2000 | subspecies kurstaki | Gem Star LC HzSNPV | *H. zea* | 2nd instars | virus then Bt | Additive effect |
| Young et al. | 1980 | DiPel [a] | T. ni NPV | *T. ni* | 3rd instars | virus then Bt, simultaneous | Additive effect, greater mortality for virus followed by Bt than simultaneous |
| Liu et al. | 2006 | Cry1Ac | HaNPV | *H. armigera* | 3rd instars | virus then Bt, simultaneous | Additive with virus and Bt together, Antagonistic when fed together |

[a] DiPel® is *Bacillus thuringiensis* subspecies kurstaki which expressed Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Bb.

Table 3. Engineered Bt-baculovirus combinations.

| Reference | Year | Bt type | Virus type | Insect | Age | Administration | Effect |
|---|---|---|---|---|---|---|---|
| Seo et al. | 2005 | Cry1Ac | AcMNPV-Polh | *P. xylostella* | 3rd instars | toxin-fusion protein | Fused Polh and Cry1Ac and expressed in *E. coli*, mortality rates similar to wild-type and Cry1Ac alone |
| Merryweather et al. | 1990 | subspecies kurstaki HD-73 | AcMNPV | *T. ni* | 2nd instars | Recombinant virus | Recombinant virus 2 fold higher $LD_{50}$ than wildtype |
| Ribeiro and Crook | 1993 | Cry1Ac | AcMNPV | Sf21 cells | n/a | Recombinant virus | $LT_{50}$ values not shorter than wildtype |
| Martens et al. | 1995 | Cry1Ab | AcMNPV | *S. exigua* | 2nd instars | Recombinant virus | No increase over wildtype |
| Chang et al. | 2003 | Cry1Ac | AcMNPV | *P. xylostella* | 2nd and 3rd instars | Recombinant virus | Recombinant virus killed better than wild-type virus but not better than Bt alone |

# CHAPTER 2. Physiological basis for increased *Agrotis ipsilon* multiple nucleopolyhedrovirus infection following feeding of *Agrotis ipsilon* larvae on transgenic corn expressing Cry1Fa2

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## Abstract

Larvae of the black cutworm, *Agrotis ipsilon* Hufnagel, were more susceptible to infection by *Agrotis ipsilon* multiple nucleopolyhedrovirus (AgipMNPV) after feeding on Herculex™ I, a transgenic corn hybrid expressing Cry1Fa2 compared to larvae fed on isoline corn. Experiments were conducted to investigate the physiological basis for increased susceptibility to virus infection following exposure to Herculex® I. Midgut pH, gut protease activity and peritrophic matrix structure are important factors for both Bt toxic action and baculovirus infection. No significant treatment differences were found in the pH of anterior midgut, central midgut or posterior midgut in larvae fed Herculex® I or isoline diets. Analysis of soluble and membrane-associated gut proteinase activities from larvae fed Herculex® I or isoline diets indicated that membrane-associated aminopeptidase activity and soluble chymotrypsin-like proteinase activity were significantly lower in Herculex® I -fed larvae compared to isoline-fed larvae. The number and relative molecular masses of soluble chymotrypsin-like proteinases did not differ. An experiment to determine whether chymotrypsin degradation of baculovirus resulted in reduced infection of larvae fed on isoline diet showed that baculoviruses are not susceptible to degradation by chymotrypsin. Analysis of the peritrophic matrices of Herculex® I -fed larvae and isoline-fed larvae by

scanning electron microscopy indicated that Herculex® I did not result in damage to the peritrophic matrix that could facilitate subsequent baculovirus infection. Further analyses are required to determine whether Bt toxin-induced epithelial cell sloughing enhances subsequent virus infection.

Keywords: *Agrotis ipsilon*, Herculex® I, AgipMNPV, chymotrypsin

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## 1. Introduction

The insecticidal toxins derived from *Bacillus thuringiensis* (Bt), a gram-positive bacterium, have been widely adopted for insect pest management. Rapid adoption of insect resistant transgenic crops that express Bt toxins have revolutionized lepidopteran pest control in recent years (Shelton, 2002). Following ingestion by the lepidopteran larva, the Bt crystal (Cry) protoxins are cleaved by midgut serine proteases, such as trypsin, chymotrypsin and elastase, and solubilized at high gut pH (> 8.0) into the 60 to 70-kD activated toxin (Andrews et al., 1985). The activated toxin binds to receptor proteins, such as cadherin-like proteins and exopeptidases such as aminopeptidase, found on the brush border membrane on the surface of the midgut epithelial cells (Gill et al., 1992; Hua et al., 2004). Binding and

conformational changes lead to pore formation and ion imbalance resulting in cell lysis (Schnepf et al., 1998).

There are numerous reports that exposure of lepidopteran larvae to Bt toxins results in increased susceptibility to baculovirus infection (Bell and Romine, 1980; Lipa et al., 1976; Marzban et al., 2009; Matter and Zohdy, 1981; Peters and Coaker, 1993; Streett and Mulrooney, 2000). For example, significantly more larvae of the fall armyworm, *Spodoptera frugiperda* reared on transgenic corn plants expressing the Bt toxin Cry1Ab, died following exposure to *S. frugiperda* multiple nucleopolyhedrovirus (*Sf*MNPV) compared to those fed on isoline corn (Farrar et al., 2004). However, the physiological basis for increased virus susceptibility following exposure to Bt toxins has not been investigated.

Herculex® I, which expresses the Cry1Fa2 protein from *Bacillus thuringiensis* subspecies *azawai,* has been marketed to protect against multiple lepidopteran corn pests, including the black cutworm, *A. ipsilon*. Larvae feed on a wide variety of cultivated plant and grass species, but are particularly damaging to corn. Damage is caused by larval feeding (also known as cutting) on stems of seedling corn at or below the growing point causing stand reduction. *A. ipsilon* infestations are sporadic and population densities variable (Archer and Musick, 1977).

When fed to *A. ipsilon* larvae, Herculex® I deterred larvae from feeding and provided moderate toxicity to larvae that consumed plant material. In a bioassay comparing the weights of third instar *A. ipsilon* after seven days on Herculex® I and isoline corn, the weights of larvae maintained on Herculex® I were significantly lower than those on isoline, but Herculex® I appeared to be non-lethal to *A. ipsilon* larvae (Richtman, 2006). *A. ipsilon* is not known to be susceptible to many Bt toxins and was not susceptible to Cry1Fa when

tested for insecticidal activity (de Maagd, 2003). Refractoriness to Bt toxins may result from toxin binding to the peritrophic matrix and subsequent proteolytic degradation; the peritrophic matrix of *A. ipsilon* is unusually thick and highly glycosylated, when compared with those of other lepidopteran pest species (Rees et al., 2009).

In this study, we sought to determine the physiological basis for increased susceptibility of *A. ipsilon* to infection by the baculovirus *Agrotis ipsilon* multiple nucleopolyhedrovirus (AgipMNPV) (Boughton et al., 1999) following exposure to Herculex® I corn. We tested the hypothesis that physiological changes in the midgut following exposure to Herculex® I facilitate subsequent virus infection. The common requirements for baculovirus infection and Bt toxicity by oral ingestion include activation by gut pH and proteases, navigation of the insect peritrophic matrix and binding to the midgut epithelial cells (Bonning, 2005; Schnepf et al., 1998) and hence we focused on midgut physiology. Here we report on the impact of feeding *A. ipsilon* larvae on Herculex® I on midgut pH, midgut proteinase composition and peritrophic matrix structure in comparison with larvae fed on isoline corn.

## 2. Materials and Methods

### *2. 1. Insects, plants and virus*

Larvae of the black cutworm, *A. ipsilon* and European corn borer, *Ostrinia nubilalis* (Hübner) were provided by the USDA-ARS Corn Insect and Crop Genetics Research Unit, Ames, IA. Larvae of the tobacco budworm, *Heliothis virescens* (Fabricius) were obtained from Bio-Serv, Inc. (Frenchtown, NJ). Larvae were reared on species specific diets from Southland Products (Lake Village, AR). Seeds of Herculex® I corn (Herculex® I Insect

Protection Technology by Dow AgroSciences and Pioneer Hi-Bred. Herculex is a registered trademark of Dow AgroSciences LLC, Indianapolis, IN) (Pioneer® 34M16) and isoline corn (Pioneer® 34M15) were planted in large pots of professional peat growing mix (Sunshine; Sun Gro Horticulture, Vancouver, British Columbia) with 12 seeds per pot. Plants were harvested and fed to larvae at the V2 stage (Ritchie et al., 1997).

The original isolate of *A. ipsilon* multiple nucleopolyhedrovirus (AgipMNPV) was acquired from infected black cutworm larvae collected by Dr. J. Maddox (Illinois Natural History Survey, Champaign, IL) and characterized by Boughton et al. (1999). For amplification, virus was applied to lepidopteran larval diet (Southland Co., Lake Village, AR) and fed to fifth-instars. After death, infected larvae were homogenized and virus was purified as described by Boughton et al. (1999). Wild-type *Autographa californica* multiple nucleopolyhedrovirus strain C6 (AcMNPV C6) was also used in this study.

*2.2. Impact of Herculex® I exposure on virus infection*

Third-instar *A. ipsilon* were fed diets of either Herculex® I corn, isoline corn, or no food followed by exposure to an equal dose of virus to test for percentage of larvae infected by treatment. Larvae were placed individually into a compartmental tray (Oliver Products Co., Grand Rapids, MI) and diets were randomly assigned to larvae. Larvae on a Herculex® I or isoline diet were fed two 2-cm leaf pieces and one 2-cm stem piece from corresponding plants and were allowed to feed for 24 hours. After 24 hours any remaining food pieces were removed from trays and larvae were starved for six hours. Any larvae that did not feed were discarded. A “no food” treatment was tested, where larvae were starved for 24 hours.

The $LD_{50}$ virus dose for third-instar *A. ipsilon*, 330 occlusion bodies (OB)/larva, (Boughton et al., 2001) was added to 2 $mm^3$ pieces of diet. Larvae that had been exposed to one of the three treatments (Herculex™ I, isoline corn or no food) were then transferred to new trays with one piece of the diet with virus and one larva in each compartment. After 24 hours, larvae that had not eaten the entire cube of diet were discarded. Larvae that had consumed the entire diet cube were placed on diet in individual compartments in trays (27˚ C). Larvae were observed on day 2 for non-virus death and those insects were discarded. On day 9, larvae were classified as infected and dead from virus based on virus symptoms of light body coloration, thin or ruptured cuticle and smaller body size than healthy larvae. Observations of polyhedra with a light microscope confirmed that these symptoms were indicative of virus infection. For the Herculex® I treatment, a total of 276 insects were evaluated on four different dates with 2-4 replicates per date. For the isoline corn treatment, a total of 308 insects were evaluated on four dates with 2-4 replicates per date. For the no food treatment, 44 insects were evaluated in two replicates.

In an additional experiment, third instar *A. ipsilon* were exposed to Herculex® I or isoline and after 24 hours were infected with AgipMNPV at a virus dose of 330 OB/larva, as described above. In this trial virus was given immediately following the 24 hour feeding period, rather than after a six hour starvation period as assayed above. At the same time, another set of insects were given the virus on a cube of diet for 24 hours followed by exposure to Herculex® I or isoline. Treatments were randomly assigned to larvae and the procedures described above were used in this experiment. For the Herculex® I and isoline treatment followed by virus, a total of 159 and 168 insects respectively were evaluated for virus induced mortality from four separate dates. For the virus followed by feeding on

Herculex® I or isoline, 164 and 133 insects respectively were evaluated from four dates. In both the experiment examining the effects of Herculex® I and virus exposure on the black cutworm, data from separate dates were pooled and analyzed using PROC LOGISTIC to determine differences between the treatments (SAS v. 9.1).

*2.3. Midgut pH measurements*

Fifth instar *A. ipsilon* were fed on a single 1 cm piece of stem and two 2 cm pieces of leaf from Herculex® I or isoline seedlings at the V2 stage for 24 hours. Larvae which did not feed during this 24 hour period were discarded. Larvae were individually pinned to a wax dish and dissected longitudinally exposing the digestive tract. Hemolymph was blotted with a KimWipe to avoid contamination with gut contents. The pH of the anterior, central and posterior section of the midgut was measured using a microelectrode (Microelectrodes, Inc., Bedford, NH). A small slit was cut in the area to be measured, the microelectrode and reference probe inserted, and a reading was taken. Readings were taken for 39 insects fed the Herculex® I diet and 38 insects fed the isoline diet. Treatment differences were analyzed using PROC MIXED (SAS v. 9.1).

*2.4. Gut proteinase assays*

Fifth instar *A. ipsilon* were fed for 24 hours on Herculex® I or isoline as described above. Larvae were mounted on a wax dish, opened longitudinally and the midgut was removed and submerged in 30 µl of deionized water in a 1.5 ml tube chilled on ice. Samples were vortexed for 2 minutes and then spun at 15,000 x g for 5 minutes at 4° C. The supernatant, containing gut contents, was then transferred into a clean tube. Total protein

concentration was measured (Bradford, 1976) with BSA as a protein standard. Membrane-bound gut proteinases were extracted by isolating brush border membrane vesicles (BBMV) from fifth instar *A. ipsilon* (Wolfsberger, 1987). Whole guts were dissected and frozen with liquid nitrogen and stored at -80 ° C. Thirty guts per treatment were combined for BBMV isolation. Prior to use in assays, BBMV were quantified and tested for quality by Leucine aminopeptidase assay using L-leucine ρ-nitroanilide (LρNA, Sigma) as a substrate.

Total proteinase activity was measured in the soluble and membrane fractions of the *A. ipsilon* midgut using fluorescently labeled casein with universal buffers at pH 8, 9, 10 and 11 (Frugoni, 1957). One mg/ml stock solution of BODIPY TR-X casein (Invitrogen) was prepared by adding 0.2 ml of 0.1 M sodium bicarbonate (pH 8.3) to a vial containing lyophilized material. Five microliters of soluble extract was added to each plate well containing 85 µl of universal buffer at each pH. A control was included that contained no enzyme. These mixtures were incubated with 10 µl of fluorescent labeled casein (10 µg/ml) at 37°C for 4 hours. After 4 hours, a measurement of relative fluorescence values at 584/620 nm was taken with a fluorescence plate reader (Spectramax M5, Molecular Devices, Sunnyvale, CA). Hydrolysis of casein was expressed as relative fluorescence per mg of protein. In this assay, six replicates per treatment (each using enzymes from an individual insect as one replicate) were measured for the soluble fraction for pH 8 to 11. For the membrane fraction, six replicates were measured and 30 guts per treatment were combined for BBMV isolation and an individual BBMV preparation represented a replicate.

The specific proteinase activity assay was run with soluble and membrane-associated enzymes. Samples were diluted with universal buffer (1.0 mg/ml) at pH 9, because this pH was nearest to the mean pH measurement for the midgut. Ten microliters of enzyme sample

were added to each plate well, which contained 40 µl of buffer. The substrates were N-succinyl-ala-ala-pro-phenylalanine ρ-nitroanilide (SAAPFρNA, Sigma) for chymotrypsin-like proteinases, N-succinyl-ala-ala-pro-leucine ρ-nitroanilide (SAAPLρNA, Sigma) for elastase-like proteinases and Nα-benzoyl-l-arginine ρ-nitroanilide (BAρNA, Sigma) and for trypsin-like proteinases. SAAPFρNA, SAAPLρNA, and BAρNA were diluted to 1.0 mg/ml with universal buffer. Fifty microliters of substrate solutions was added to individual wells containing enzyme. Control wells with substrate only (no enzyme) and enzyme only (no substrate) were included. Absorbance of nitroaniline was measured at 405 nm in 15-sec intervals for 5 min using a kinetic microplate reader (VMax, Molecular Devices, Sunnyvale, CA). In this assay, 40 replicates were measured per treatment (using enzymes from an individual insect as one replicate) for the soluble fraction. For the membrane fraction, six replicates were measured and 30 guts per treatment were combined for BBMV isolation and an individual BBMV preparation represented a replicate.

Aminopeptidase activity was measured using BBMV from the membrane fraction of the gut from fifth-instar larvae fed Herculex® I or isoline diets. One microgram of the BBMV preparation was added to a well containing 48 µl of buffer at pH 8, 9, 10 or 11. Fifty microliters of L-leucine ρ-nitroanilide (LρNA, Sigma) diluted in the corresponding buffer to 1.0 mg/ml was placed in the well with the BBMV preparation and buffer. Absorbance was measured at 405 nm for 5 min as described above. Twelve replicates were measured from six BBMV preparations, consisting of 30 guts per treatment, at pH 8 to11.

Proteinase activity blots were run for *A. ipsilon* and *Ostrinia nubilalis* using the soluble protein fraction. Enzyme samples (35 µg/lane total protein) were separated using 10-20% Tricine gradient gels with Tricine sample buffers at 4° C (Oppert et al., 1997).

Separated proteins were then transferred to nitrocellulose membranes in Tris-glycine transfer buffer (100V/ 60 minutes) with a cooling system to keep the temperature at approximately 4˚ C. The membrane was incubated in 5 ml of buffer A (200 mM Tris, pH 8.0, 20 mM $CaCl_2$) containing 2.5 mg of BAρNA for trypsin-like proteinases or SAAPFρNA for chymotrypsin-like proteinases placed directly on top of the membrane to liberate nitroaniline. The membrane was incubated for 20 minutes at 37° C with a plastic covering directly on top of the membrane and substrate. The membranes were then transferred to 0.1% $NaNO_3$ in 1.0 M HCl, 0.5% ammonium sulfamate in 1.0 M HCl, and then to 0.05% n-(1-naphthyl)-ethylenediamine in 47.5% ethanol, for 5 minutes each to diazotize the liberated nitroaniline. Blots were scanned immediately and then placed in plastic wrap for storage at -20° C.

In these proteinase assays, treatment differences at pH 9 were analyzed using PROC MIXED (SAS v. 9.1). Additionally, in the total proteinase activity assay and aminopeptidase activity assay, treatment differences at each pH were also compared.

*2.5. Impact of chymotrypsin on virus infectivity*

To determine the impact of chymotrypsin on baculovirus integrity, newly molted fourth instar *H. virescens* were starved for eight hours and then fed a diet cube (2 $mm^{3)}$ containing one of the following treatments: Virus in phosphate buffered saline (PBS) pH 9, PBS alone, virus pre-incubated for 2 to 4 hours with α-chymotrypsin from bovine pancreas (Sigma) at 700 µg/ml or at 3 mg/ml in PBS. Larvae that had eaten the entire diet cube after four hours were transferred to individual compartments with diet and placed in a growth chamber at 27 °C. Larvae were observed on day 2 for non-virus death (those insects were eliminated from the study) and then daily from day 6 until day 10 for virus-induced

mortality. Virus symptoms consist of light body coloration, thin or ruptured cuticle and smaller body size than healthy larvae. Previous observations of polyhedra with a light microscope confirmed that these symptoms were indicative of virus infection. For the chymotrypsin incubation treatments, a total of 56 insects were evaluated in four trials per treatment. For the virus only treatment, a total of 54 insects were evaluated in four trials. Percent virus mortality by treatment was analyzed. Data from separate trials were pooled and analyzed using PROC LOGISTIC to determine differences between the treatments (SAS v. 9.1).

*2.6. Scanning Electron Microscopy*

Fifth instar black cutworm larvae were fed on Herculex® I or isoline leaf pieces for 24 hours. Larvae were mounted on a wax dish, opened longitudinally and the midgut was removed and submerged in ice cold fixation buffer. The gut and matrix were preserved for scanning electron microscopy (Tang et al., 2007). The peritrophic matrix was visualized using standard procedures with a digital JEOL 5800LV scanning electron microscope and either 10 or 15 kV. Nineteen guts of insects fed isoline diets and 28 guts of insects fed Herculex® I diet were dissected out and preserved. Twelve guts from each treatment were randomly selected and examined using the scanning electron microscope.

## 3. Results

### *3.1. Agrotis ipsilon larvae were more susceptible to AgipMNPV following exposure to Herculex® I*

Exposure of *A. ipsilon* larvae to Herculex® I or isoline corn resulted in a significant difference in the extent of subsequent virus infection ($P < 0.0001$) (Fig. 1A). Of larvae fed on Herculex® I, 76% died after exposure to an $LD_{50}$ of AgipMNPV compared to 49% of larvae fed on the isoline diet. In addition, larvae starved for the equivalent amount of time as those fed the Herculex® I or isoline diets had lower rates of viral induced death (48%) than those fed the Herculex® I diet ($P <0.0001$). This result suggests that the higher virus mortality rate observed in insects fed on Herculex® I did not result from reduced fitness due to less feeding or starvation, but from another physiological mechanism.

The percentage infection of larvae exposed to virus and then fed on Herculex® I or isoline was not significantly different ($P > 0.05$) (Fig. 1B). There was also a significant difference between larvae fed Herculex® I and isoline diets and then exposed to virus ($P = 0.0015$).

### *3.2. Exposure to Herculex® I does not affect midgut pH*

The midgut pH values of larvae fed on isoline or Herculex® I were not significantly different ($P > 0.05$). The pH of the anterior midgut ranged from 7.3 to 10, while the central midgut pH ranged from 7 to 10.5 and the posterior midgut ranged from 6.5 to 8.5. Similar trends were seen in both treatments (Fig. 2).

*3.3. Chymotrypsin and aminopeptidase activity levels were lower in larvae fed Herculex® I*

There were no significant differences ($P > 0.05$) in the total proteinase activity levels for the soluble or membrane-bound protease fractions of *A. ipsilon* midgut between the isoline and Herculex® I treatments at any of the four pH levels tested (Fig. 3). In the soluble fraction, the caseinolytic value fluctuated, based on pH, between 8 and 10 in both treatments. In the membrane bound fraction, the caseinolytic value was the lowest at pH 8 and the highest at pH 11 in both treatments.

The specific proteinase activity of chymotrypsin-like proteinases in the soluble fraction was significantly lower in the Herculex® I -fed larvae than in those fed on the isoline ($P < 0.0001$). There were no significant differences between treatments in the activity level of the soluble fraction for trypsin-like or elastase-like proteinases ($P > 0.05$) (Fig. 4A). Elastase-like proteinase activity levels in the soluble fraction were very low so the membrane-bound fraction was not tested. The specific proteinase activity levels for the membrane bound fraction were lower than for the soluble fraction, and there were no significant differences between treatments (Fig. 4B).

Leucine aminopeptidase activity was tested in the membrane-bound proteinase fraction using LρNA as substrate at pH 8 to 11. Of those tested, the optimal pH for this assay was pH 8. A significant difference between treatments was seen with the isoline-fed insects showing higher aminopeptidase activity levels ($P < 0.0001$) (Fig. 5).

The number and molecular masses of trypsin-like proteinases (using BAρNA as substrate) and chymotrypsin-like proteinases (SAAPFρNA substrate) were visualized by proteinase activity blots. The elastase-like proteinase patterns were not examined due to the low activity detected in specific proteinase activity assays. When BAρNA was used as a

substrate, trypsin-like proteinase activity levels were low and bands were faint. When SAAPFρNA was used as substrate, bands were detected at 25 kDa, 37 kDa and >50 kDa in both the isoline-fed and Herculex® I -fed larvae, with more intense bands in the isoline-fed lanes (Fig. 6). In activity blots done with soluble fractions from *Ostrinia nubilalis* using SAAPFρNA as the substrate, bands were visible at 30 kDa and 50 kDa, as shown by Li et al. 2004.

*3.4. Chymotrypsin does not decrease baculovirus infectivity*

To assess whether increased chymotrypsin activity in isoline-fed larvae resulted in virus degradation, we tested the impact of chymotrypsin on virus integrity, using AcMNPV C6 and *H. virescens* larvae. There was no significant difference ($P > 0.05$) in mortality between *H. virescens* larvae exposed to virus incubated with chymotrypsin (700 µg/ml or 3 mg/ml) or virus only treatments ($P > 0.05$) (Fig. 7). Of larvae exposed to virus incubated with chymotrypsin (700 µg/ml or 3 mg/ml), 53% and 66% were infected, compared to 65% of larvae infected in the virus only treatment.

*3.5. Herculex® I does not appear to damage the peritrophic matrix*

There were no clear differences in the structure of the peritrophic matrix between Herculex® I -fed and isoline-fed larvae (Fig. 8). Minor variations in the appearance of the matrix were seen between images of the same treatment, which may result from different location within the gut or differing age of the matrix. Pores or tears seen in SEM images at 3000X magnification may be the result of sample processing for SEM or from insect processing of plant material.

## 4. Discussion

A considerable amount of research has been conducted to elucidate the mechanism of action of Bt-derived Cry toxins. Although several different hypotheses have been put forward (Soberon et al., 2009), a complete understanding of how these toxins work is lacking. Recent research suggests that Bt toxin binding to the peritrophic matrix may account for the lack of susceptibility of some lepidopteran species to Bt toxins, including *A. ipsilon* (Rees et al., 2009).

We have shown that exposure to Herculex® I, which expresses the Bt toxin Cry1Fa2, does not alter *A. ipsilon* midgut pH, overall gut proteinase activity or peritrophic matrix structure. All three of these components play a role in Bt toxin processing and toxic action in addition to in baculovirus infection. Lepidopteran midgut pH can range from 8 to12 and can vary based on food intake and diet preferences (Berenbaum, 1980; Dow, 1992). Gypsy moth, *Lymantria dispar*, which fed on acidic leaf foliage, had a lowered gut pH and a lowered susceptibility to gypsy moth nuclear polyhedrosis virus (Keating et al., 1990).

Serine proteinases are important in activation of Bt toxins in lepidopterans (Oppert, 1999). A decrease in the activity of these proteinases can serve as a mechanism of Bt resistance in several lepidopteran species (Karumbaiah et al., 2007; Li et al., 2004; Oppert et al., 1997; Zhu et al., 1997). Feeding on Cry1Ac decreased trypsin and chymotrypsin-like proteinase activity in the cotton bollworm, *H. zea* (Zhu et al., 2007), and that decrease could lead to a change in virus degradation or activation by proteases. Virus occlusion body matrix degradation by host proteases facilitates virus infection process of AcMNPV (Wood, 1980).

As shown for other noctuids (Chougule et al., 2008; Gatehouse et al., 1999; Hegedus et al., 2003) the primary *A. ipsilon* gut proteolytic activity results from serine proteases

(chymotrypsin, trypsin and elastase), with no cysteine protease activity detected. Chymotrypsin-like proteases predominated in *A. ipsilon* in the soluble fraction of the midgut. Aminopeptidase activity was found to be associated with the membrane fraction of the midgut, which was also detected in other noctuids (Hegedus et al., 2003).

On the basis that larvae fed on the isoline treatment consumed substantially more plant material than those fed on Herculex® I, the higher activity levels of chymotrypsin and aminopeptidase may result from the processing and digestion of plant material. Another explanation could be that lower chymotrypsin and aminopeptidase levels following exposure to Herculex® I could have resulted from the impact of the toxin itself on gene expression. When spruce budworm larvae, *Choristoneura fumiferana,* were exposed to small doses of Cry1Ab protoxin, serine protease inhibitors were upregulated, while serine proteases and some aminopeptidases were repressed (van Munster et al., 2007).

Unfortunately, our attempts to test the impact of high levels of chymotrypsin in the gut juice of isoline-fed insects on AgipMNPV viability were thwarted by the presence of the bacterial pathogen, *Serratia marcescens* in the *A. ipsilon* colony. *Serratia marcescens*, a common pathogen of insect colonies, causes inapparent infection until the colony is stressed. Exposure to a second pathogen can result in an epizootic (Inglis and Lawrence, 2001). Hence, we switched to a different baculovirus – host combination (*H. virescens* and AcMNPV) to test the ability of chymotrypsin to degrade baculoviruses. Our results showed no significant reduction in AcMNPV pathogenicity following chymotrypsin treatment, suggesting that high levels of chymotrypsin in the isoline-fed insects did not result in loss of infectious virus. This conclusion is supported by the fact that the expected numbers of larvae died following treatment with an $LD_{50}$ dose of virus.

Aminopeptidase is a probable binding site for Cry1Fa in *O. nubilalis* and *H. virescens* (Hua et al., 2001; Jurat-Fuentes and Adang, 2001). It is conceivable that binding of Cry1Fa2 to aminopeptidase reduced AgipMNPV exposure to aminopeptidase proteolytic activity, which could account for the increased virus mortality of *A. ipsilon* larvae fed Herculex™ I. We did not test the potential of aminopeptidase to inactivate baculovirus virions. Alternatively, Cry1Fa2 binding to aminopeptidase may result in a change in epithelial permeability or cell sloughing which facilitates virus entry into the epithelial cells. Larvae exposed to low doses of Bt toxin cease feeding, slough damaged cells and generate new cells (Boucias and Pendland, 1998; Spies and Spence, 1985). Cyt1Aa binding results in cell sloughing in *Chrysomela scripta*, which allows for greater susceptibility to Cry3Aa (Federici, 1998). Cell sloughing is also a common mechanism of resistance in lepidopterans to baculovirus infection (Hoover et al., 2000; Popham et al., 2004; Washburn et al., 2003) with complete loss of baculovirus infected cells and recovery by 60 hours after infection (Barrett et al., 1998). It has not been established whether cell sloughing resulting from virus or Bt toxin exposure facilitates subsequent virus infection. Indeed, the mechanism by which cells are sloughed is unknown, although an apoptotic mechanism may be involved (Brooks et al., 2002; Clem, 2005). The loss of epithelial cells by sloughing may result in physical gaps in the epithelium allowing for increased virus movement across the epithelium, or new cells may be more susceptible to virus entry. Increasing the time between feeding *A. ipsilon* larvae on Herculex® I or isoline and infection with virus to allow for recovery of the midgut epithelium, may help delineate whether cell sloughing contributes to increased infection by AgipMNPV.

Cry1Ac has been shown to bind to the peritrophic matrix of some lepidopterans including *A. ipsilon*. This binding and subsequent degradation of the toxin may account for reduced susceptibility of some insect species to Bt toxins (Rees et al., 2009). Cry1Fa2 binding to the peritrophic matrix could result in increased matrix permeability to AgipMNPV providing a possible explanation for increased virus infection in Herculex® I-fed larvae. However, given that the peritrophic matrix functions to protect the midgut epithelium from mechanical damage from ingested plant material (Tellam, 1996) and that we did not detect any damage resulting from Herculex® I exposure, it seems unlikely that Cry1Fa2 binding to the peritrophic matrix alone would result in cessation of feeding. A more likely scenario is that cell sloughing accounts for the reduction in feeding seen in *A. ipsilon* feeding on Herculex® I (Boucias and Pendland, 1998).

In this study, we have largely eliminated Herculex® I -induced modification of *A. ipsilon* midgut pH, gut proteinase activity or peritrophic matrix structure as mediators of enhanced AgipMNPV infection. Based on our findings, we suggest that exposure of *A. ipsilon* larvae to Herculex® I results in sloughing of midgut epithelial cells and consequent cessation of feeding. This sloughing of epithelial cells may increase the susceptibility of larvae to subsequent infection by AgipMNPV.

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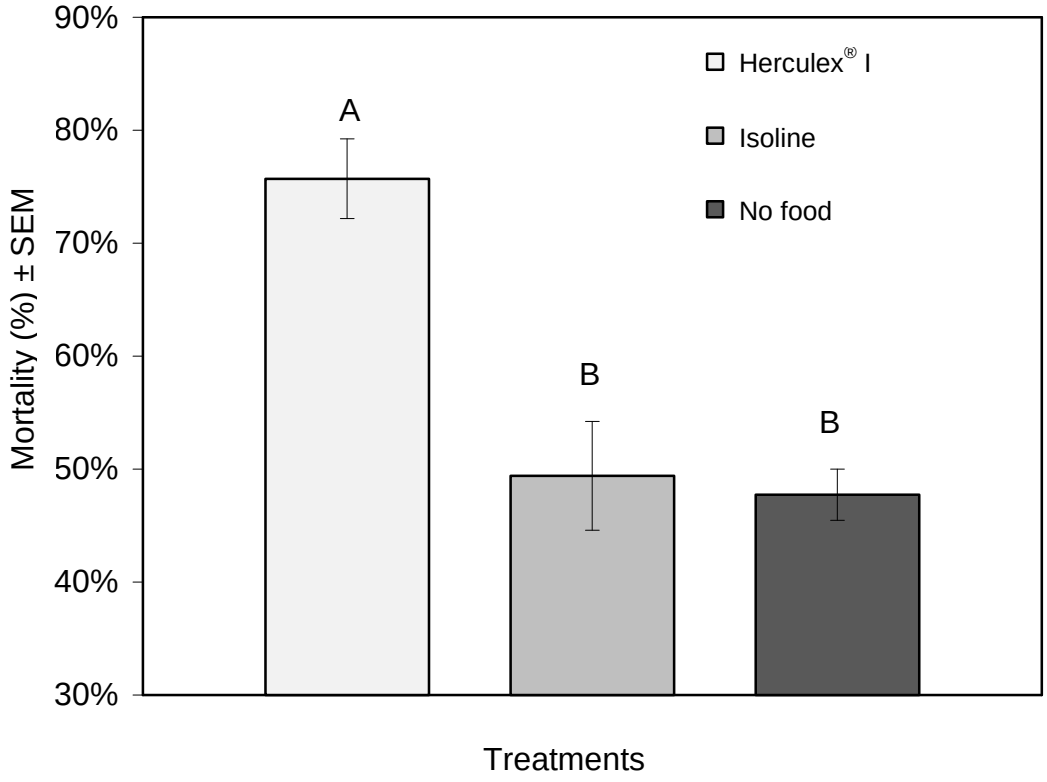
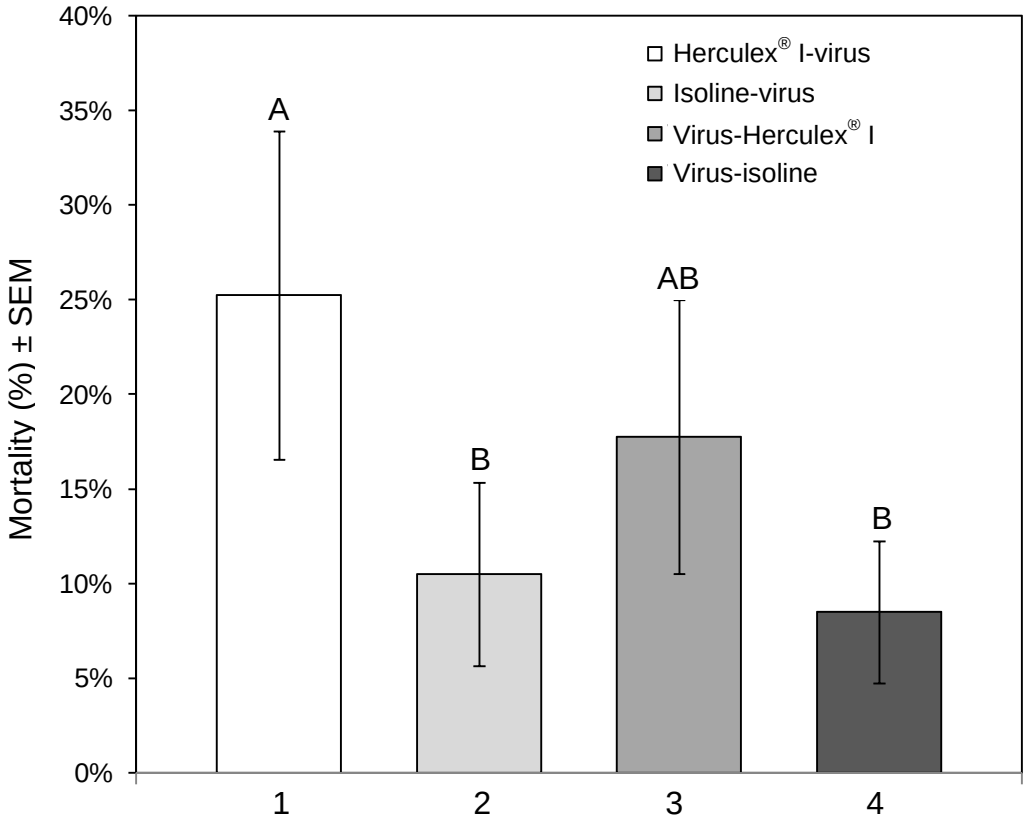



**Fig. 1.** Exposure of *Agrotis ipsilon* larvae to Herculex® I increases susceptibility to AgipMNPV.

A. AgipMNPV-induced mortality of *A.ipsilon* larvae following feeding on Herculex® I, isoline corn, or no food. Data are the mean per treatment replicate group ± S.E.M. Means with different letters are significantly different ($P \leq 0.0001$).




B. AgipMNPV-induced mortality of *A.ipsilon* larvae following feeding on Herculex® I, isoline corn, repeat of A (bars 1& 2). *Agip*MNPV treatment followed by exposure to Herculex® I or isoline showed no differences in mortality (bars 3 & 4). Larvae were infected at an $LD_{50}$ viral dose. Data are the mean per treatment replicate group ± S.E.M. Means with different letters are significantly different ($P \leq 0.0015$).

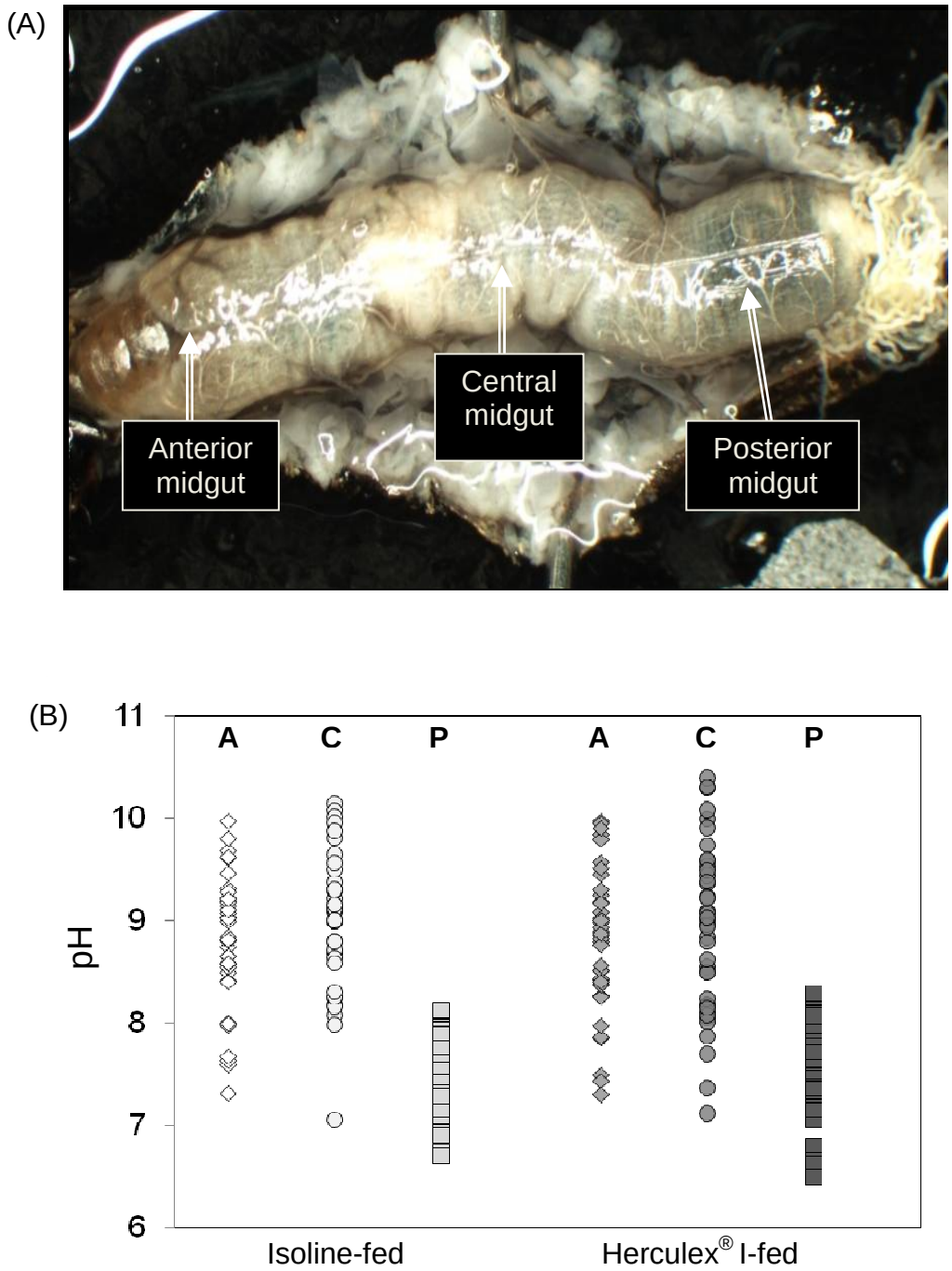



**Fig. 2.** Exposure of *Agrotis ipsilon* larvae to Herculex® I does not alter midgut pH.

A. Dissected larva of *A. ipsilon* shows gut regions used for comparison of pH between larvae fed on Herculex® I or isoline. B. The pH of the anterior (A), central (C) and posterior midgut (P) of *A. ipsilon* larvae fed on Herculex® I or isoline corn. Each symbol represents a pH reading from an individual insect. While the pH of the posterior midgut was more acidic than the central and anterior regions, the two treatments were not significantly different ($P > 0.05$).

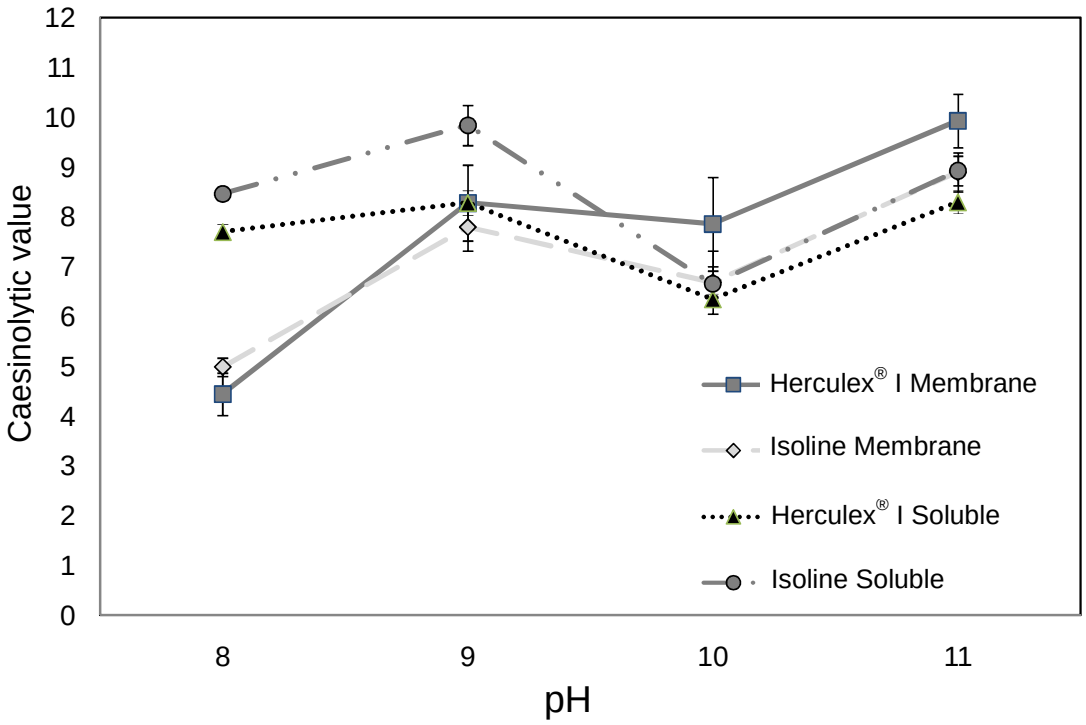



**Fig. 3.** Total *Agrotis ipsilon* gut proteinase activity of soluble and membrane-bound proteinase fractions from Herculex® I or isoline-fed larvae. Data are the mean ± S.E.M. Data, analyzed by fraction, are not statistically different from each other at any pH ($P > 0.05$).

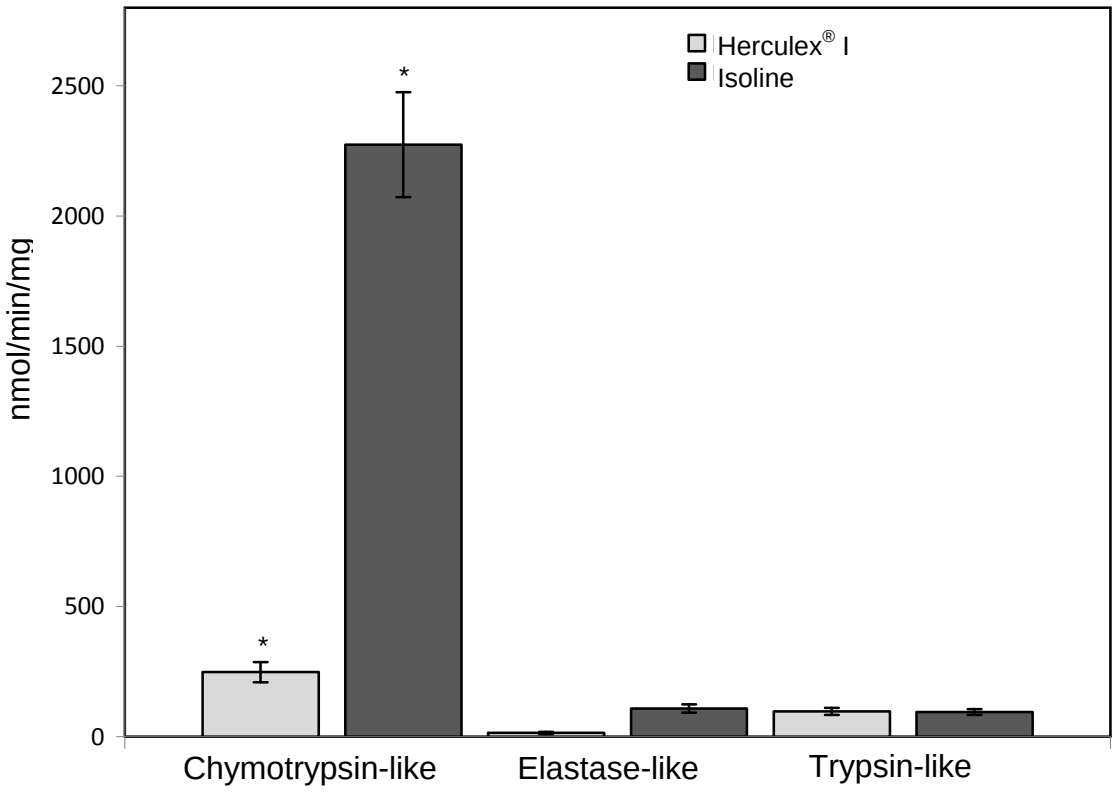
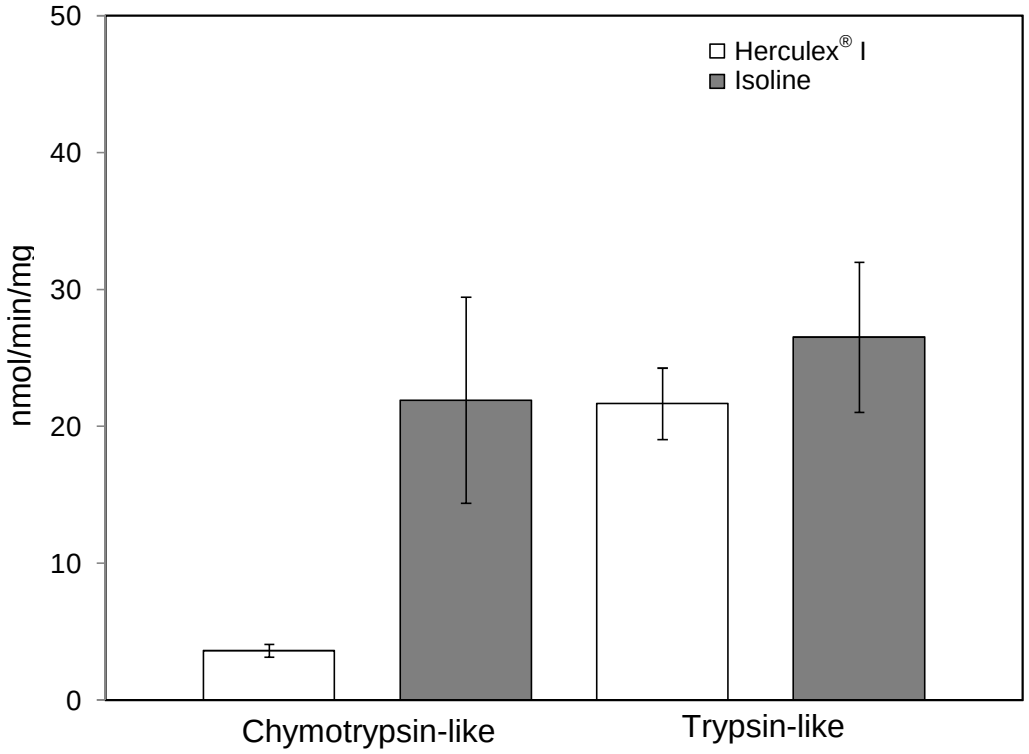



**Fig. 4.** Specific proteinase activities for the soluble and membrane-bound proteinases from *Agrotis ipsilon* larvae fed Herculex® I or isoline diets.

A. Specific activities (nmol/min/mg) using the soluble fraction of chymotrypsin- (using the substrate SAAPFρNA), elastase- (using the substrate SAAPLρNA) and trypsin-like (using the substrate BAρNA) proteinases from *A. ipsilon* larvae fed Herculex® I or isoline diets. Data are the mean ± S.E.M. Means with an asterisk are significantly different ($P < 0.0001$), as compared within each substrate.




B. Specific activities (nmol/min/mg) using the membrane fraction of chymotrypsin- (using the substrate SAAPFρNA) and trypsin-like (using the substrate BAρNA) proteinases from *A. ipsilon* larvae fed Herculex® I or isoline diets. Data are the mean ± S.E.M. Treatments are not statistically different from one another ($P > 0.05$).

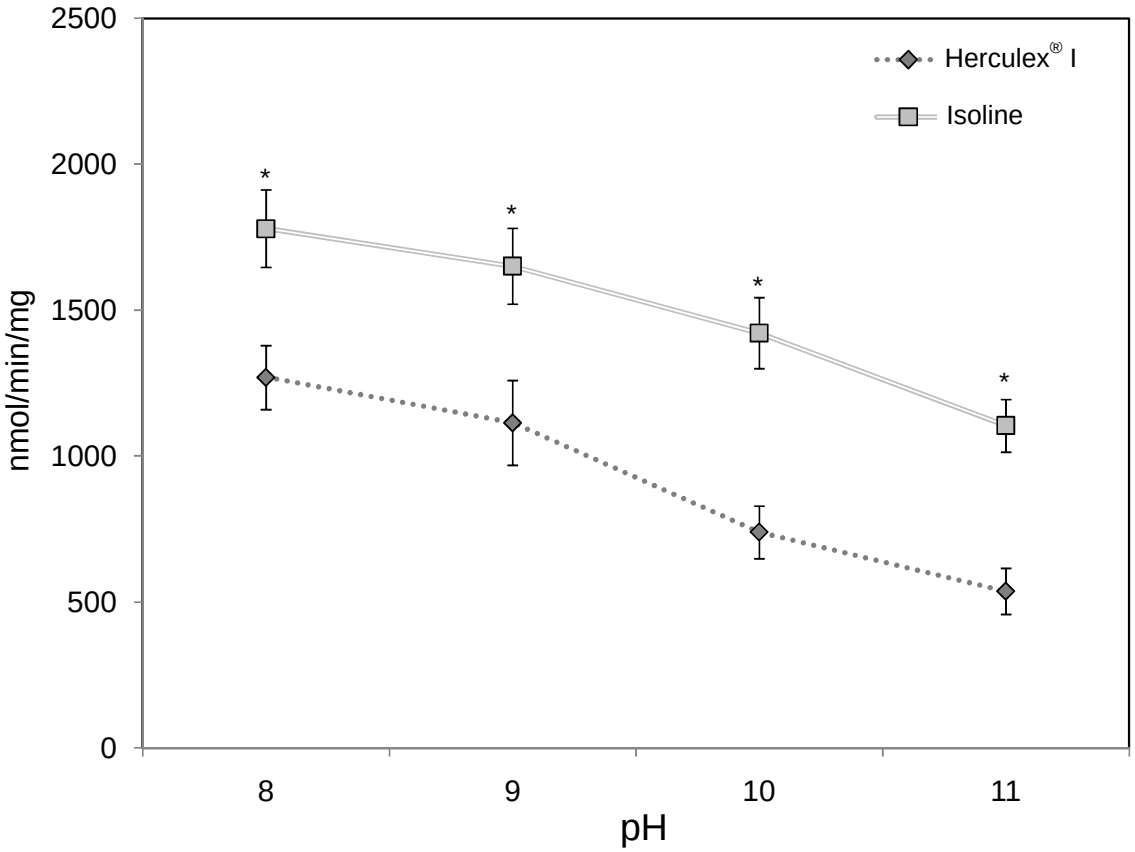



**Fig. 5.** Aminopeptidase activity is significantly higher in isoline-fed larvae of *Agrotis ipsilon*. Specific activities (nmol/min/mg) of aminopeptidases in membrane fractions from larvae of Herculex® I and isoline-fed *A. ipsilon* larvae. Data are the mean ± S.E.M. Means with an asterisk are significantly different ($P < 0.0001$), as compared by treatment within each pH.

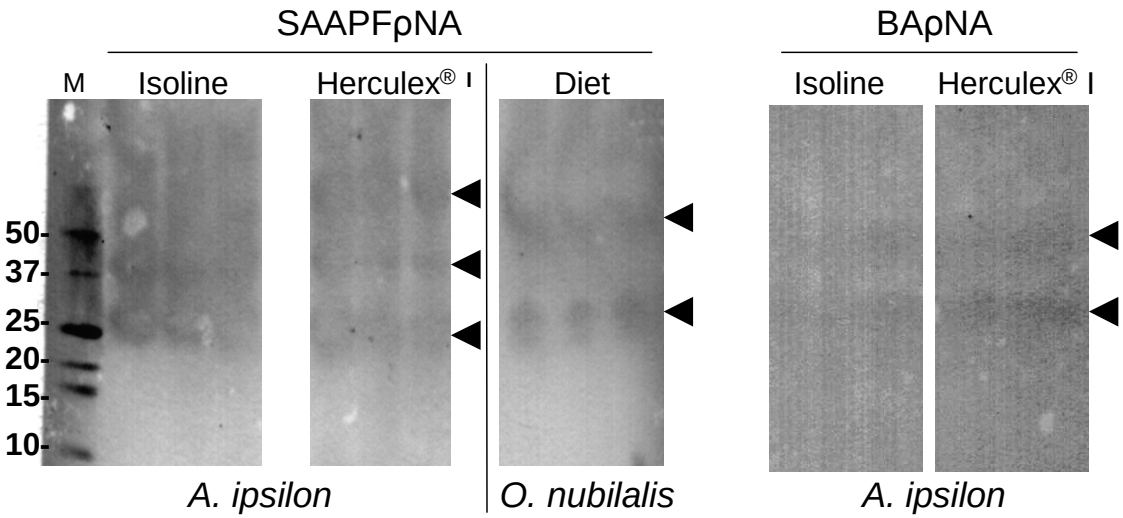



**Fig. 6.** Proteinase activity blots of chymotrypsin- (SAAPFρNA) and trypsin-like (BAρNA) proteinases in soluble fractions from *Agrotis ipsilon* larvae fed Herculex® I or isoline diets. Diet-fed *Ostrinia nubilalis* larvae were included for comparison. M represents the molecular mass marker in kDa.

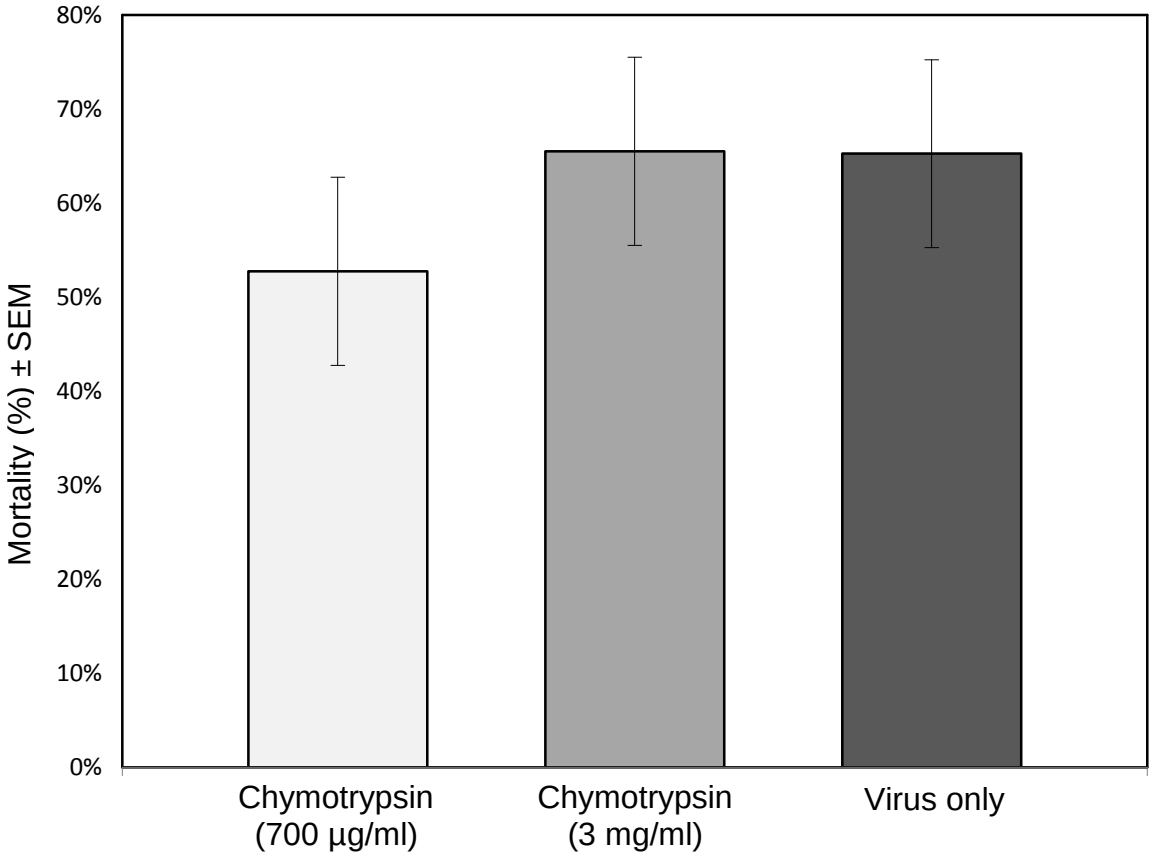



**Fig. 7.** Chymotrypsin does not degrade baculovirus occlusion bodies. Mortality of fourth instar *Heliothis virescens* exposed to AcMNPV incubated with two concentrations of chymotrypsin (700 µg/ml in PBS, or 3 mg/ml in PBS) and virus only. Data are the mean per treatment ± S.E.M. Treatments were not statistically different from one another ($P > 0.05$).

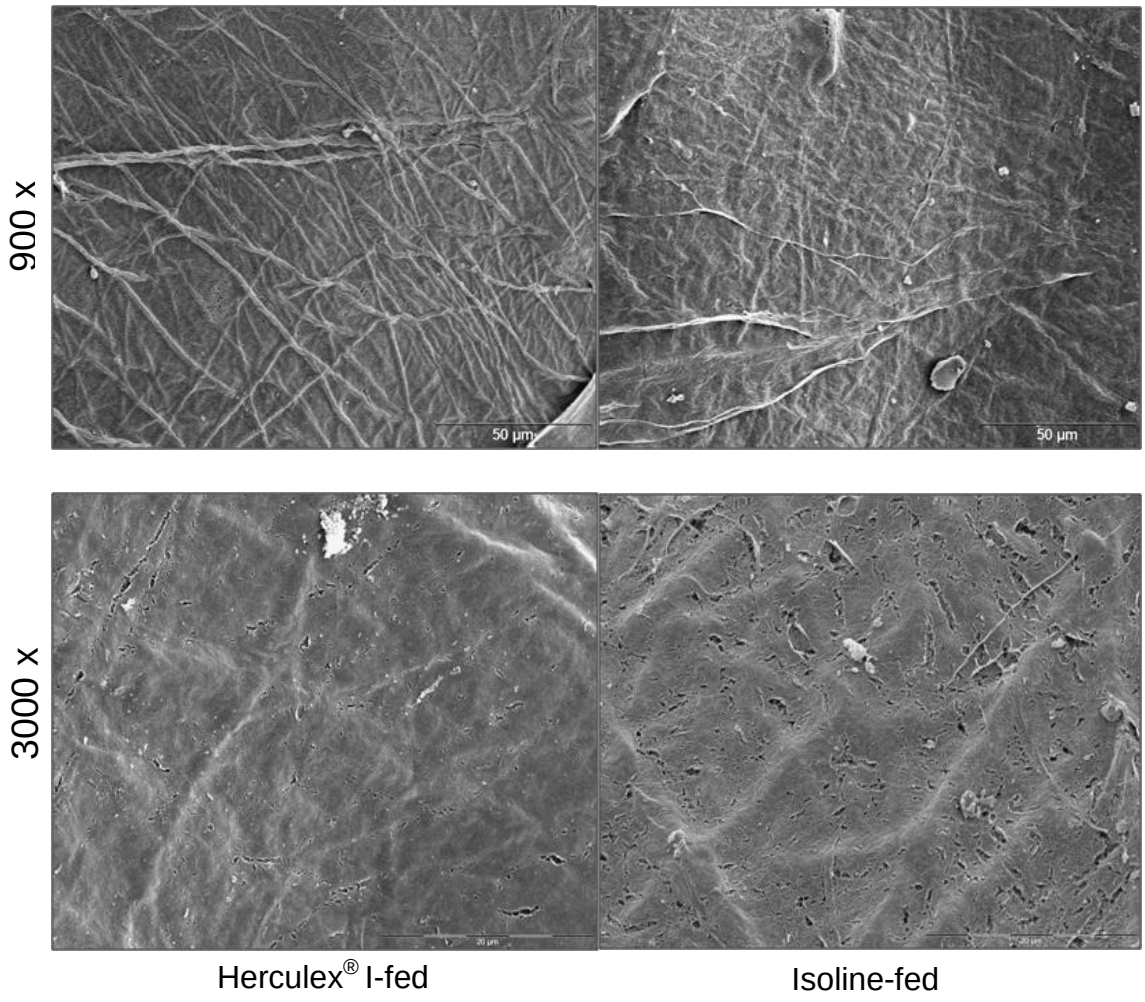



**Fig. 8.** Peritrophic matrix images taken by scanning electron microscope of *Agrotis ipsilon* larvae fed Herculex® I or isoline diets. Images at 900x and 3000x. There were no obvious treatment differences in appearance of the matrices. SEMs by Hailin Tang.

# CHAPTER 3. General conclusions

Transgenic crops expressing insecticidal toxins derived from *Bacillus thuringiensis* have been widely adopted for protection of agricultural crops (Shelton, 2002). Although models have been put forward to explain the mechanism of action of these toxins, their mode of action is incompletely understood (Soberon et al., 2009). Numerous researchers have observed that exposure to Bt toxins increases susceptibility of the host insect to subsequent infection by baculoviruses (Lipa et al., 1976, Matter and Zohdy, 1981, McVay et al., 1977, Peters et al., 1993, Streett and Mulrooney, 2000, Bell and Romaine, 1980, Farrar et al., 2004, Marzban et al., 2009). The goal of the research presented here was to determine the physiological basis for this increased virus susceptibility, which might also shed light on Bt toxin action. We used exposure of black cutworm, *Agrotis ipsilon*, larvae to Herculex ™ I corn which expresses the toxin Cry1Fa2. As a result of this research, we can draw the following general conclusions:

1. Larvae of *A. ipsilon*, feeding on Herculex® I, were significantly more susceptible to AgipMNPV than those feeding on isoline corn or no food treatments. When larvae were infected with virus first and then fed on Herculex® I or isoline, there were no significant treatment differences in virus-induced mortality. This result suggests that physiological changes resulting from exposure to the Cry1Fa2 toxin, facilitate infection with AgipMNPV.

2. *Agrotis ipsilon* larvae fed Herculex® I and isoline diets had no significant differences in gut pH or in overall gut proteinase activity. Aminopeptidase

activity and soluble chymotrypsin-like proteinase activity were significantly lower in Herculex® I -fed larvae than isoline-fed larvae. Bt toxins have been shown to repress expression of serine proteases and aminopeptidases, and upregulate expression of serine protease inhibitors (van Munster et al., 2007), which could account for these differences.

3. Based on similar larval infection rates in *Heliothis virescens* following ingestion of virus alone, or virus pre-incubated with chymotrypsin, we conclude that chymotrypsin does not degrade the model baculovirus, AcMNPV. Hence, higher levels of soluble chymotrypsin in isoline-fed larvae are unlikely to affect the integrity of AgipMNPV.

4. The peritrophic matrices of Herculex® I -fed larvae and isoline-fed larvae were compared using scanning electron microscopy imaging, but no physical differences were observed. Damage to or modification of the peritrophic matrix could facilitate virus entry in Herculex® I fed larvae. We conclude that feeding inhibition in larvae fed on Herculex® I did not result from damage to the peritrophic matrix.

## Future Research

We sought to provide an explanation for increased virus susceptibility following exposure to a Bt toxin. Future research could address whether exposure to Cry1Fa2 results in greater infectivity of the midgut epithelium by AgipMNPV. One approach would be to use the virus AgipMNPV-*lacZ* containing a *lacZ* reporter gene under the control of a heat shock promoter. Using this construct, the number of infected cells could be assessed by adding the

β-galactosidase substrate X-gal to produce a blue product allowing for quantification of viral foci in the midgut cells. Comparison of the number of foci between treatments of Herculex® I and isoline-fed larvae could then be quantified and compared. The virus AgipMNPV-*lacZ* could also be used to determine if exposure to Bt toxin results in more rapid spread of the virus into the hemocoel and body tissues, such as the gut, fat body, malpighian tubules and trachea (Li et al., 2007). Another approach would be to use the midgut epithelium tissue culture technique (Braun and Keddie, 1997) to evaluate the effect of Herculex® I feeding on the midgut epithelium using a system of artificial hemolymph to maintain a viable epithelium and dyes to visualize midgut lumen surface and epithelial cells. Examination of the columnar and regenerative cells using this approach, as well as testing differences in membrane permeability, in Herculex® I and isoline-fed larvae would indicate how the two treatments differ at the cellular level.

Of particular interest is whether cell sloughing induced by exposure to low levels of Cry1F could facilitate infection by AgipMNPV. Exposure to either a Bt toxin or an insect virus can result in sloughing of the affected gut epithelial cells (Brooks et al., 2002; Federici, 1998; Hoover, 2000; Popham et al., 2004; Washburn, 2003) but whether cell sloughing facilitates subsequent infection has not been addressed. It is conceivable that gaps left by sloughed columnar cells could facilitate virus movement through the gut epithelium, or expose underlying or new, more susceptible cells to virus. Indeed, sloughing of gut epithelial cells would result in feeding inhibition as observed for larvae fed on Herculex™ I.

One limiting factor for this project was the inability to use purified Cry1F. Access to Herculex® I seed was limited and access to purified Cry1F protein was not possible due to restrictive Intellectual Property rights. If products were made available to university

researchers without such company restrictions, a much larger scale project could be undertaken to provide valuable information to the benefit of companies, scientists and growers alike (Pollack, 2009).

Continued investigation of Bt toxin-virus interaction would benefit growers by providing greater understanding of Bt transgenic crops, a technology which has been so readily adopted by growers in recent years. This work is significant because increased understanding of this synergistic interaction may facilitate utilization of Bt in combination with baculoviruses for improved pest control. Since *A. ipsilon* is a sporadic pest of seedling corn, it is unlikely that a Bt toxin/virus combination approach would be useful in corn, but since *A. ipsilon* larvae feed on a large variety of other crops, including turf, the increase in susceptibility to virus may provide control tactics in other systems. AgipMNPV has shown potential for control of *A. ipsilon* on golf courses (Prater et al., 2006). Additionally, this phenomenon of increased virus susceptibility following Bt toxin feeding is not unique to *A. ipsilon* and AgipMNPV (Bell and Romine, 1986; Farrar et al., 2004; Lipa et al., 1976; Marzban et al., 2009; Matter and Zohdy, 1981; McVay et al., 1977; Peters and Coaker, 1993; Streett and Mulrooney, 2000). Bt toxin-virus combinations may be useful for control of other lepidopteran pest insects using other viruses in combination with Herculex® I or other Bt transgenic crops. For example, high populations of the pink bollworm, *Pectinophora gossypiella*, required spraying by a significant number of growers who planted Bollgard cotton in the United States in 1996. Use of an appropriate baculovirus on such pink bollworm populations may be a more environmentally sound approach than chemical insecticides (Kaiser, 1996).

Little work has been done in the area of insect/virus/Bt interaction, and currently there are no published papers on the physiological interaction between the three. This research

opens the door for a number of future research projects because many important questions remain unanswered. Further research could not only improve understanding of the Bt toxin-baculovirus interaction, but additionally could contribute to the specifics of the Bt toxin and/or baculovirus midgut infection processes.

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