PLANT-MEDIATED EFFECTS ON INSECT-VIRUS INTERACTIONS

A Thesis in
Entomology
by
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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2006
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Abstract

The effects of host plants on baculoviral efficacy have been well documented; consumption of particular host plants often renders insect herbivores less susceptible to viral disease. Ingestion of cotton (Gossypium hirsutum L.) foliage in the first through third instars prior to oral viral inoculation in the fourth instar significantly reduced mortality of Heliothis virescens F. by Autographa californica nucleopolyhedrovirus (AcNPV). A construct of AcNPV that expresses lacZ under the control of the hsp70 promoter of Drosophila (AcNPV-hsp70/lacZ) was used to examine viral infection in vivo. Larvae that consumed cotton foliage in the first 8 h of the fourth instar prior to viral inoculation experienced a significant reduction in both mortality and percentage of larvae with foci of infection, compared with larvae fed artificial diet. Although cotton ingested prior to inoculation significantly reduced the percentage of larvae with midgut infection foci (indicated by lacZ signaling), there was no difference in the percentage of larvae possessing systemic tracheal infection foci, compared with larvae fed artificial diet. These results support the hypothesis that ingested cotton foliage hinders establishment of midgut infections in H. virescens, perhaps by modifying midgut physiology or by direct antagonism between virions and foliage components. This effect may not be limited to cotton, since other host plants of H. virescens (tobacco and oakleaf lettuce) also significantly reduced larval susceptibility to AcNPV when consumed prior to viral inoculation.

The peritrophic matrix (PM) of herbivorous insects lines the midgut, protecting it from damage by ingested plant material. A significantly thicker PM was observed in cotton-fed larvae compared with artificial diet-fed larvae, suggesting that this thickened
PM protects the midgut cells from damage by ingested foliage and secondarily provides protection from ingested pathogens. Disruption of the PM by the metalloprotease enhancin in cotton-fed larvae restored larval susceptibility to the level of diet-fed larvae. When viral pathogenesis was examined using AcNPV-hsp70/lacZ to indicate AcNPV infection, lacZ signaling appeared in larvae fed artificial diet+enhancin significantly earlier and in a greater proportion of larvae, compared with larvae fed untreated artificial diet. Thus, the PM appears to be a barrier to baculoviral infection in cotton-fed H. virescens larvae and may be a significant factor determining pathogen success by influencing establishment of midgut infections.

Although the ability of host plants to influence larval susceptibility to baculoviral infection is well-known, the influence on viral pathogenicity of inducing systemic acquired resistance in host plants has not been previously reported. SAR was induced in cotton foliage by the plant elicitor benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH). BTH application significantly increased foliar peroxidase activity and total phenolic levels, but did not alter catecholic phenolic levels. Moreover, consumption of SAR-induced foliage did not affect H. virescens pupal mass or larval mortality by AcNPV as compared to consumption of untreated foliage. Thus, activators of SAR like BTH and baculoviruses are likely to be compatible components of an IPM system.

Further, the colony source of H. virescens significantly influenced larval mortality. We examined the influence of food consumed prior to viral inoculation on larval mortality of H. virescens from three different lab colonies, designated as NCSU97, AGR and NCSU02. The NCSU97 colony had not had field-collected individuals added since its establishment five years prior. In contrast, the AGR and NCUS02 colonies had
field-collected individuals added within a year of experimentation. Larvae from the AGR and NCSU02 colonies fed artificial diet were significantly less susceptible to AcNPV infection than artificial diet-fed NCSU97 larvae; the mechanism regulating this decreased susceptibility appears to be restricted to the midgut environment.

This is the first report detailing the influence of ingested cotton foliage on baculoviral pathogenesis in *H. virescens*. The observations that ingestion of cotton foliage alters PM structure and reduces midgut viral infections suggest possible mechanisms of host plant influence on pathogens that infect through the midgut. Understanding the interactions between ingested foliage and insect pathogens is likely to become more important as pathogens are used more widely as biocontrol agents. Further, these results showcase the ability of the PM to protect insects from ingested pathogens and indicate that the PM is a potentially important pest control target.
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List of Abbreviations

AcNPV: *Autographa californica* nucleopolyhedrovirus

AGR: *Heliothis virescens* colony from the Agripest company

BTH: benzothiadiazole, benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester

BV: budded virus

C: cotton

CEB: cytoskeletal extraction buffer

CHA: chlorogenic acid

D: diet

E_h: redox potential

GV: granulovirus

H: hours

Hpi: hours post-inoculation

HzSNPV: *Helicoverpa zea* single nucleopolyhedrovirus

IIM: invertebrate intestinal mucin

IPM: integrated pest management

L: lettuce

LdMNPV: *Lymantria dispar* multiple nucleopolyhedrovirus

M: multiple or midgut

M+T: midgut plus tracheal

MNPV: multiple nucleopolyhedrovirus

NCSU02: *Heliothis virescens* colony from the North Carolina State University, established 2002
NCSU97: *Heliothis virescens* colony from the North Carolina State University, established 1997

NPV: nucleopolyhedrovirus

OB: occlusion body

ODV: occlusion-derived virion

PM: peritrophic matrix

POD: peroxidase

PPO: polyphenol oxidase

S: single

SAR: systemic acquired resistance

SEM: scanning electron microscopy

T: tracheal

TEM: transmission electron microscopy

TnGV: *Trichoplusia ni* granulovirus

TnSNPV: *Trichoplusia ni* single nucleopolyhedrovirus

X-gal: 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
Acknowledgements

I would like to thank my advisor, Kelli Hoover for her guidance, and the lab technician, Mike Grove, for teaching me the practical side of studying pathogens and for help with the majority of my experiments. The members of my committee, Diana Cox-Foster, Gary Felton, A. Daniel Jones and Kelly Johnson, provided valuable input and steered me away from nonproductive experiments.

A large number of undergraduate students worked on this project; I am very thankful for their efforts: Tony Pomicter, Bri Reed, Mariana Amaral, Brandon Arce, Laura Behrendt, Ryan Bissot, Jeff Chen, Melody Conklin, Sean Duerr, Matt Gardner, Matt Gordon, Jo Lynne Harenza, Djamila Harouaka, Jon Knowles, Kassi Miller, Rebecca Miller, Yeji Park, Becky Pundiak, Julie Reddinger, Dave Thomas, Owen Thompson and Meghan Turk. I would also like to thank my fellow graduate students Randa Jabbour, Jim McNeil and Scott Geib for their help with the \textit{lacZ} experiments and their overall support. Other individuals also contributed to this work: Anthony Boughton, Missy Hazen, Michelle Peiffer, Jo Ann Snyder and Jean Voigt. I would like to thank Dave Love for his efforts in keeping the growth chambers running.

This work was funded by the National Science Foundation Integrated Organismal Biology Program, Grant No. IBN-0077710.

Finally, I would like to thank my parents, Gary and Andie Plymale, and my sisters, Mary and Sarah, for their encouragement, moral support and prayers during my tenure at Penn State.
Epigraph

"All I have seen teaches me to trust the creator for all I have not seen."

~Ralph Waldo Emerson
Chapter 1. Introduction

An herbivore’s resistance to disease is influenced by genetic and environmental factors. Diet is an environmental factor with the possibility for a substantial impact because what an herbivore eats mediates the expression of its genetic factors. The potential for diet to influence disease is showcased in the model system comprised of the larvae of tobacco budworm, *Heliothis virescens*, fed cotton foliage and challenged with a baculovirus, *Autographa californica* nucleopolyhedrovirus. In this case, larvae fed cotton foliage experience significantly less mortality than cohort larvae fed artificial diet or lettuce foliage (Hoover et al. 1998c). Other scientists have made substantial progress toward explaining the influence of cotton foliage on viral mortality in this system, but some aspects remain unclear, particularly in the interactions between insect tissues, ingested foliage and virus particles. The following sections summarize other researchers’ findings and detail my strategy for further investigation into the fine points of these interactions.

Insect: *Heliothis virescens*.

*Heliothis virescens*, a lepidopteran in the family Noctuidae, is found throughout North and South America (Fitt 1989). *H. virescens*, commonly called the tobacco budworm and geranium budworm and included in the cotton bollworm complex, is a major agricultural pest (Neunzig 1969). While the insect will feed on plants from 14 families, the primary host plants are in the families Asteraceae, Fabaceae, Malvaceae and Solanaceae (Fitt 1989).
The *H. virescens* life cycle lasts from 35 to 50 days. There are three generations per year in temperate climates, five per year in subtropical climates and up to 11 annually in tropical climates. During nocturnal flights, gravid female moths lay white eggs singly on host plant buds, flowers, fruit or leaves (Fitt 1989). The eggs hatch within two to five days. Larvae develop through five instars within fourteen to twenty-five days. Larvae prefer to consume the more nutritious reproductive tissue of host plants; leaves may be eaten by small larvae or if reproductive tissues are not available. The color of *H. virescens* larvae varies greatly; larvae may be green, red, brown or yellow. *H. virescens* may be distinguished from the closely related *Helicoverpa zea* by the presence of a small tooth on the inside of each mandible in *H. virescens*. Mature larvae burrow into the soil to pupate. Pupae normally develop into adults within twelve to sixteen days, but may diapause during extreme temperatures (Fitt 1989). Adults are cream to brown and possess three olive stripes on each forewing.

**Baculovirus description and pathogenesis.**

Baculoviruses are large, enveloped, double stranded DNA viruses that infect arthropods. The family Baculoviridae contains two genera: the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs) (van Regenmortel et al. 2000). NPVs infect primarily lepidopterans; GVs infect exclusively lepidopterans (Granados 1980). NPVs and GVs are characterized by proteinaceous occlusion bodies (OB). NPV occlusions are large, often polyhedral, made of polyhedrin protein and contain several enveloped virions. Each GV occlusion is small, oval, made of granulin protein and contains one enveloped virion. There are two types of NPVs, designated S and M. The single (S)
NPV virion consists of a single enveloped nucleocapsid; the multiple (M) NPV virion may comprise one to many enveloped nucleocapsids. Although pathogenesis of SNPVs and MNPVs are very similar, there are slight differences (Washburn et al. 2003b). Only the MNPV life cycle will be described here.

All NPVs have two distinct morphotypes, each with a specific role in the infection process. The polyhedral occlusion body (OB) contains several enveloped occlusion-derived virions (ODV), each containing one to many nucleocapsids. The OB provides some environmental protection for the virions; viable virus particles persist in the environment for at least 24 hours (Young and Yearian 1974). The ODV is the viral form responsible for horizontal transmission of the virus, infecting the midgut of target insects. Budded virus (BV) is the second phenotype of NPVs; particles are enveloped virions containing one nucleocapsid. Budded virus particles are specialized for spreading infection throughout the insect, but do not infect the midgut (Granados 1980). This tissue specificity is mediated by distinctive surface proteins (Volkman 1997). ODV envelopes contain p74; BV envelopes are characterized by gp64 protein spikes.

Upon ingestion, the OB is broken down in the midgut of the target insect by exposure to the alkaline environment and possibly also by alkaline proteases, releasing the enveloped ODV (Granados and Lawler 1981; Pritchett et al. 1982). The ODV must penetrate the peritrophic matrix (PM), which lies between the food bolus and the midgut cells (Ryerse et al. 1992). Once the ODV cross this potential barrier, each ODV particle must enter a midgut columnar epithelial cell to initiate infection (Keddie et al. 1989; Granados and Lawler 1981). Entry is accomplished by fusion of the virion envelope to the microvillar membrane of an epithelial cell, followed by admission of the
nucleocapsids into the cell (Granados and Lawler 1981). Receptors on the microvillar membrane are thought to mediate this fusion event, but no receptors have been specifically identified (Haas-Stapleton et al. 2004). Some of the nucleocapsids are directed to the nucleus, where uncoating, transcription and viral replication occur (Granados and Lawler 1981). Other nucleocapsids move to the basal region of the columnar cell, where they bud through the plasma membrane, acquiring gp64, as budded virus particles (Washburn et al. 2003a).

Nuclear hypertrophy is the first obvious sign of virus infection (Harrap 1972). Host chromosomes are relocated to the periphery of the nucleus and a dense region of viral replication, the virogenic stroma, appears in the center (Granados and Lawler 1981). The first progeny virions produced leave the nucleus by membrane budding, and proceed to the basal portion of the cell (Granados and Lawler 1981). The basal plasma membrane now contains protein spikes, or peplomers, of the virally-encoded glycoprotein gp64, a characteristic budded virus protein (Keddie et al. 1989; Washburn et al. 2003a). The nucleocapsids bud out through this membrane near a tracheole, becoming BV particles. Progeny produced later usually remain in the nucleus, although this is rare in midgut cells. These late progeny are enveloped in a de novo membrane and a polyhedrin occlusion body forms around them. The nuclear and plasma membranes rupture late in infection, releasing the OBs.

BV enter the tracheoles associated with midgut cells and infect tracheal epidermal cells (tracheoblasts) by receptor-mediated endocytosis (Engelhard et al. 1994). Progeny from these infections are packaged as BV and released, infecting tracheoblasts and hemocytes (Washburn et al. 1995). Once the infection is established outside of the
midgut, it spreads quickly through the tracheal system and hemocoel by BV particles
(Keddie et al. 1989; Engelhard et al. 1994). Most insect tissues can become infected and
produce large quantities of OB prior to tissue break down. The insect then succumbs to
the infection within a few days. The cuticle disintegrates, releasing millions of OB into
the surrounding environment (Hawtin et al. 1997).

**Midgut oxidative stress.**

Midgut contents have a unique potential to influence the success of a baculoviral
infection since ingested food interacts both with virus particles and susceptible midgut
epithelial cells. Virus particles may be altered through interactions with ingested plant
material; these changes may produce reduced mortality in the insect host. Interactions
between plant material and virus particles may be mediated by plant-mediated generation
of reactive oxygen species such as hydrogen peroxide, hydroxyl radicals and superoxide
radicals (Hoover et al. 1998a). Foliar phenolics have been implicated in plant-insect
interactions and are being investigated as a potentially important component of plant-
virus interactions (Felton et al. 1987; Keating et al. 1988; Felton and Duffey 1990;

Phenolics are found in all plants; one putative role is in plant defense against
insects and pathogens (Appel 1993). Phenolics are distinguished by one or more
hydroxyl groups attached to an aromatic ring. This is the only unifying character;
phenolics are otherwise a diverse group, encompassing alkaloids, flavonoids, terpenoids
and glycosides (Appel 1993). The reactions a phenolic may participate in are determined
in part by the oxidative status of the compound (Appel 1993). Phenolics may occur in
several oxidative states. Phenolic refers to the fully protonated state; an oxidized state,
the quinone, and intermediate states, including semiquinone free radical intermediates, also form (Appel 1993). The oxidative status of a particular phenolic is largely determined by attributes of the surrounding physiochemical environment, including pH, oxygen availability, and relative redox potentials of other chemicals (Appel 1993; Duffey and Stout 1996). For example, chlorogenic acid, an ortho-dihydroxyphenolic ubiquitous among higher plants, may act as a prooxidant or an antioxidant. Ingestion of an artificial diet containing chlorogenic acid increased oxidative stress in *H. zea* midguts (Summers and Felton 1994), but application of a chlorogenic acid/rutin mixture to cotton foliage decreased foliar free radical generating capacity (Hoover et al. 1998a).

The oxidative status of the midgut lumenal environment is impacted by ingested phenolics (Appel 1993). Midgut pH and redox potential ($E_h$) both determine midgut oxidative status. A phenolic may be transformed to a quinone through autoxidation or by oxidative enzymes found in both plants and insects (Summers and Felton 1994). Autoxidation of phenolics is most likely to occur in an oxidizing alkaline environment (Appel 1993). Midgut pH of *H. virescens* larvae fed cotton foliage was measured at 9.3 (Johnson and Felton 1996). *H. virescens* larval midgut $E_h$ varies, depending on the host plant ingested (Johnson and Felton 1996). *H. virescens* larvae that consume cotton foliage have a weakly reducing midgut environment of -5 mV; lower $E_h$ values indicate a more reducing environment (Johnson and Felton 1996).

Two common oxidative enzymes which co-occur in plant tissues with phenolics and may be involved in their enzyme-catalyzed oxidation are polyphenol oxidase (PPO) and peroxidases (POD). Two electrons are transferred during PPO-catalyzed oxidation but POD-catalyzed oxidation proceeds in a stepwise fashion, with one electron removed.
from the phenolic at a time (Summers and Felton 1994). The electron may be transferred to a number of other compounds; a superoxide radical is generated if the electron is accepted by molecular oxygen. Formation of a superoxide radical may lead to production of other reactive oxygen species, including hydrogen peroxide (Summers and Felton 1994). Hydrogen peroxide is able to freely cross cell membranes, unlike other ROS, and may, in conjunction with peroxidase and/or ferrous ions, stimulate production of potentially damaging reactive oxygen species in new locations (McKersie 1996; Passardi et al. 2005).

Insects possess a suite of antioxidants to protect their tissues from oxidants. However, production of large amounts of reactive oxygen species may overwhelm the regenerative capacity of antioxidants, allowing damaging oxidative reactions such as lipid peroxidation and oxidation of DNA and proteins to proceed (Summers and Felton 1994). The PM, situated between the ingested food and the midgut epithelium, may curb oxidative damage, scavenging hydroxyl radicals and decreasing hydroperoxide formation (Summers and Felton 1996). However, the antioxidant capacity of this matrix can also be overwhelmed. While some phenolics display antioxidant function (Felton and Johnson 2001), larval midgut tissues may still be subjected to oxidative stress, since they are in close proximity to ingested phenolics and the associated reactive oxygen species.

**Factors influencing baculovirus infection.**

The success of a particular baculoviral infection is mediated by many variables, including environmental factors, host plant constituents and insect physiology. Outside of the host insect, occlusions are usually found on plant surfaces, deposited there by disintegration of virally-infected hosts. Liberated virus particles are particularly
susceptible to environmental stresses. Virus particles are inactivated by ultraviolet radiation and environmental exposure may lessen virus efficacy (Young and Yearian 1974; McLeod et al. 1977). The plant that the virus is placed on may also impact the vigor of virus particles. For example, cotton leaves produce an alkaline exudate that releases ODV from the protective OB (McLeod et al. 1977). The released ODV are quickly inactivated by environmental stresses.

The effect of host plant on baculoviral efficacy has been well documented; consumption of certain host plants makes insects less susceptible to virus attack (Duffey et al. 1995; Cory and Hoover 2006). *Lymantria dispar* larvae fed a high dose of *L. dispar* multiple NPV (LdMNPV) on disks of red maple or red or black oak foliage experienced significantly less mortality than cohort insects fed the same dose on disks of pitch pine or quaking or bigtoothed aspen (Keating and Yendol 1987; Keating et al. 1990). *H. virescens* larvae that ingested *Autographa californica* multiple NPV (AcNPV) occlusions on cotton disks were significantly less susceptible to mortal infection than those fed inoculum on iceberg lettuce disks (Hoover et al. 1998c). Host plant foliage ingested with viral inoculum has also been observed to mediate viral mortality in *H. zea, Spodoptera littoralis, S. exigua* and *S. frugiperda* (Richter et al. 1987; Forschler et al. 1992; Santiagoalvarez and Ortizgarcia 1992; Ali et al. 1998; Farrar and Ridgway 2000).

Virus particles ingested by a target insect must overcome a number of potential obstacles before successfully establishing a lethal infection. These impediments to viral success may come from aspects of insect physiology, ingested host plant foliage or the interactions between insect tissues and plant material. The first potential hurdle to establishing a lethal infection is ODV release. Midgut pH is the primary determinant of
successful ODV release (Pritchett et al. 1982). Midgut pH of larval lepidopterans varies significantly with insect species (Johnson and Felton 1996). Ingested foliage significantly altered *L. dispar* midgut pH; insects fed more acidic, more strongly buffered red oak foliage had a four-tenths pH unit lower average midgut pH than those fed less acidic, weakly buffered bigtooth or quaking aspen (Keating et al. 1990). Conversely, ingested foliage did not alter midgut pH of *H. zea, H. virescens, Pseudoplusia includens* or *Hyphantria cunea* fed geranium, cotton, clover or soybean foliage (Johnson and Felton 1996). Thus, the impact of host plant on viral efficacy through changes in midgut pH is uncertain and might vary by insect species. However, the host plant may influence ODV release by other mechanisms. For example, solubility and infectivity of OBs combined with the plant phenol chlorogenic acid (CHA) and the oxidative enzyme PPO *in vitro* were significantly reduced compared to untreated OBs (Felton and Duffey 1990). Examination of these occlusions revealed covalently bound chlorogenoquinone, the reaction product of CHA and PPO. Covalent binding of this quinone to the OB protein may prevent normal interaction between the protein and the alkaline midgut environment, reducing ODV release (Felton and Duffey 1990).

Once the occlusion-derived virions are released, they must make their way to and infect midgut columnar epithelial cells. Relieved of their protective protein occlusion, ODV are susceptible to degradation in the alkaline midgut environment (Pritchett et al. 1984). The PM may also pose a possible barrier to infection. The PM was not originally considered a major impediment to virus infection because the virus is able to establish lethal infections with the PM present (Granados and Lawler 1981). However, closer examination of the PM and its interaction with virus particles demonstrated its potential
as an obstacle to infection in some hosts. Inoculation of *Trichoplusia ni* larvae with AcNPV or *T. ni* single nucleopolyhedrovirus (TnSNPV) and subsequent examination of the PM revealed that these viruses are able to alter PM structure by eliminating one glycoprotein (Derksen and Granados 1988). The previously-formed PMs of insects exposed to *T. ni* granulovirus (TnGV) were missing three glycoproteins. The factor responsible for this alteration was found in the granulin fraction of TnGV. Co-inoculation of AcNPV and crude TnGV granulin significantly increased *AcNPV* mortality in *T. ni*, suggesting that the PM may be a barrier to baculovirus infection (Derksen and Granados 1988). Enhancin, a metalloprotease, purified from TnGV, significantly increased *Anticarsia gemmatalis* multiple nucleopolyhedrovirus mortality in *T. ni*, TnSNPV mortality in *T. ni* and AcNPV mortality in *T. ni*, *H. zea*, *S. exigua* and *Pseudaletia unipuncta* (Gallo et al. 1991; Wang et al. 1994; Lepore et al. 1996; Wang and Granados 1998). Exposure to enhancin was also documented to increase permeability of *T. ni* and *P. unipuncta* PMs to dextran beads (Peng et al. 1999). Thus, the PM is an obstacle to viral infection in some hosts, albeit one that viruses can partially overcome.

The host plant has been suggested to influence PM structure, and therefore, permeability, but no experimental evidence has been reported to support this hypothesis.

Infection of midgut columnar epithelial cells does not guarantee a mortal viral infection. Infections must be established beyond the midgut to ensure that the insect succumbs. The longer the virus remains in the midgut cell, the better its chances of spreading infection to other tissues. The insect can protect itself from disease and other challenges by removal of damaged or infected midgut epithelial cells. The midgut is cleared of all virally-infected cells when the insect molts (Engelhard and Volkman 1995).
Infected cells may also slough off prior to the molt (Washburn et al. 1998). Host plants may influence viral success by increasing the sloughing rate of infected cells (Hoover et al. 2000). Increased production of prooxidant phenolics and oxidative enzymes in insect-damaged plants may increase lipid peroxidation, damaging midgut cell membranes and possibly increasing sloughing rates (Summers and Felton 1994; Bi and Felton 1995; Bi et al. 1997). It has been speculated, but not shown, ingested host plant foliage may increase oxidative stress in the midgut, causing infected cells to be sloughed before viral infection is established outside the midgut (Hoover et al. 1998a).

**Interactions among *H. virescens*, AcNPV and cotton foliage.**

Through this work, I seek to further elucidate specific biological mechanisms of host plant interference with successful virus infection. *H. virescens* has been used as a model to examine the attenuation of AcNPV infection by ingested cotton foliage. Ingestion of cotton foliage significantly reduces susceptibility of larval *H. virescens* to AcNPV received *per os*, compared with ingestion of artificial diet or iceberg lettuce (Hoover et al. 1998c; Hoover et al. 2000). This decrease in viral efficacy is not observed if the virus is delivered directly into the hemocoel, bypassing the midgut (Hoover et al. 2000). Thus, the influence of ingested cotton foliage on viral success in *H. virescens* is restricted to the midgut environment.

Ingested foliage may hinder viral success through the action of destructive free radicals on midgut cells or virus particles. Cotton foliar peroxidase levels are negatively correlated with viral mortality, suggesting that oxidative status of the midgut may be an important factor in determining the outcome of an infection (Hoover et al. 1998b; Hoover
et al. 1998c). Peroxidase may influence viral success through enzymatic oxidation of phenolics; higher peroxidase activity may lead to increased phenolic oxidation, resulting in greater production of semiquinone free radical intermediates and/or reactive oxygen species (Hoover et al. 1998c). In fact, foliar free radical production increased linearly with foliar peroxidase activity in a previous report (Hoover et al. 1998a). The idea that oxidative stress may be a primary determinant of infection success was examined by altering oxidative status of cotton foliage through application of pro- or antioxidants to the leaf surface (Hoover et al. 1998a). Viral success was significantly influenced by these directed changes in foliar oxidative state; AcNPV mortality was negatively correlated with both cotton foliar peroxidase activity and free radical generating capacity, measured in vitro (Hoover et al. 1998a). Thus, the ability of cotton foliage to interfere with viral infection may involve foliar free radical generating capacity, regulated in part by oxidative enzymes.

Free radicals generated in macerated foliage may prevent infection by disabling ingested virus particles or midgut epithelial cells. Midgut epithelial cells damaged by free radicals may be more likely to be sloughed from the midgut environment. Calcofluor White M2R is a fluorescent brightener reported to decrease apoptosis and sloughing of baculovirus-infected midgut cells (Washburn et al. 1998; Dougherty et al. 2006). *H. virescens* larvae fed cotton and dosed with AcNPV alone or AcNPV plus M2R were dissected twenty-four hours after receiving the virus; those that had been given M2R displayed significantly more infected midgut cells (Hoover et al. 2000). Viral mortality of cotton-fed insects was also significantly increased when M2R was added to the viral inoculum (Hoover et al. 2000). These results suggest that increased sloughing of
midgut epithelial cells due to free radical damage may be one mechanism whereby ingested foliage interferes with a successful viral infection. Although M2R has been shown to prevent apoptosis of baculovirus-infected midgut cells in vitro, it also has been demonstrated to inhibit peritrophic matrix formation; thus, increased sloughing should not be viewed as a definitive cause of enhanced viral infection (Wang and Granados 2000; Dougherty et al. 2006).

**Objectives**

Despite these insights into how host plant may influence viral success, the specific mechanisms by which infection is hindered remain unclear. This work attempted to provide insights into potential mechanisms by examining interactions among larval *H. virescens*, AcNPV and ingested cotton foliage that occur within the insect midgut environment through the following objectives:

- Verify that ingested cotton foliage suppresses AcNPV efficacy through interactions in the midgut environment of *H. virescens* larvae.
- Determine where in the infection process cotton foliage intervenes to prevent establishment of a successful infection.
- Determine if ingested cotton foliage mediates viral infection by altering the peritrophic matrix.
- Establish if increasing foliar peroxidase by treating cotton foliage with Actigard® influences *H. virescens* larval mortality.
- Resolve the influence of other *H. virescens* host plants on larval susceptibility to AcNPV.
Significance

The work outlined above will contribute to the existing literature on tritrophic interactions. The influence of nutrition on disease outcome is a well-established phenomenon in animals (reviewed in Duffey et al. 1995; reviewed in Smith et al. 2005). Although it has been suggested that cotton-mediated inhibition of virus disease involves multiple mechanisms, the biological bases for inhibition of disease are currently unknown (Hoover et al. 1998b; Hoover et al. 2000). This work will contribute to our understanding of how pathogenesis is altered in the insect midgut when cotton foliage is ingested along with viral inoculum.

This project will also contribute to resolving controversy concerning the pathway of infection from the midgut to the hemocoel. Although baculoviral infection has been shown to proceed from the midgut directly into the tracheal system in T. ni and H. virescens, some researchers continue to argue that viral progeny can pass directly from the midgut cell through the basal lamina into the hemocoel (Engelhard et al. 1994; Federici 1997). My findings will document the pathway of infection by AcNPV in diet and cotton-fed fourth instar H. virescens, confirming that transmission from the midgut to the trachea is a critical part of infection success. Thus, my observations of pathogenesis will contribute to the basic understanding of insect-virus interactions.

Roles attributed to the peritrophic matrix (PM) include protection against physical abrasion and chemical ultrafiltration. However, more recent reports suggest that the PM may have additional functions such as serving as a trap for free radicals; suggesting that the extent of the PMs contributions to midgut physiology have not been fully realized (reviewed in Lehane 1997; reviewed in Barbehenn 2001). The results of the proposed
research will confirm that the PM is a protective barrier against ingested pathogens. Furthermore, although food has been hypothesized to influence PM structure, there is yet no direct evidence of food-mediated structural changes to the PM. This project will show the extent of food-dependent changes in PM structure of *H. virescens* larvae and may indicate whether these structural changes enhance the PM’s protection against pathogen ingress.

Baculoviruses are attractive biological control candidates for insect pests and are used world-wide to protect crops against some lepidopteran and hymenopteran pests (Mishra 1998; reviewed in Moscardi 1999). Because baculoviruses are highly host specific and do not leave behind toxic residues, they overcome many of the objections to the use of chemical pesticides. While the host plant has been shown to influence baculoviral efficacy in many lepidopterans, the mechanisms responsible remain largely unknown. An adequate understanding of host plant influences on entomopathogens is essential for designing an effective integrated pest management (IPM) program. A successful IPM system would also benefit from understanding how plant defensive compounds influence entomopathogens. These findings may increase the utility of baculoviruses in IPM systems world-wide.
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Evidence that the stilbene-derived optical brightener M2R enhances *Autographa
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Chapter 2. Impact of Ingested Cotton Foliage on Pathogenesis of a Baculovirus in Heliothis virescens (Lepidoptera: Noctuidae)

Abstract

The effects of host plants on baculoviral efficacy have been well documented; consumption of particular host plants often renders insect herbivores less susceptible to viral disease. Ingestion of cotton (Gossypium hirsutum L.) foliage in the first through third instars prior to oral viral inoculation in the fourth instar significantly reduced mortality of Heliothis virescens F. by Autographa californica nucleopolyhedrovirus (AcNPV). A construct of AcNPV that expresses lacZ under the control of the hsp70 promoter of Drosophila (AcNPV-hsp70/lacZ) was used to examine viral infection in vivo. Larvae that consumed cotton foliage in the first 8 h of the fourth instar prior to viral inoculation experienced a significant reduction in both mortality and percentage of larvae with foci of infection, compared with larvae fed artificial diet. Although cotton ingested prior to inoculation significantly reduced the percentage of larvae with midgut infection foci (indicated by lacZ signaling), there was no difference in the percentage of larvae displaying systemic tracheal infection foci, compared with larvae fed artificial diet. These results support the hypothesis that ingested cotton foliage hinders establishment of midgut infections in H. virescens, perhaps by modifying midgut physiology or by direct antagonism between virions and foliage components in the midgut lumen.
Introduction

The diet an herbivore consumes has long been known to be an important factor in herbivore-pathogen interactions. In particular, considerable attention has been paid to the influence of ingested foliage on the performance of pathogens of larval lepidopterans (reviewed in Duffey et al. 1995; Cory and Hoover 2006). Host plants can influence the efficacy of fungal, bacterial and viral pathogens (Hare and Andreadis 1983; Keating and Yendol 1987; Farrar et al. 1996; Peng et al. 1997; Farrar and Ridgway 2000; Ali et al. 2002; Ali et al. 2004). Interactions between plants and baculoviruses have been studied most extensively because of the potential of these pathogens as highly selective biological control agents (reviewed in Duffey et al. 1995; Cory and Hoover 2006).

Baculoviruses are orally infectious double-stranded DNA viruses specific to arthropods, which comprise the two genera Granuloviruses and Nucleopolyhedroviruses (NPV) (van Regenmortel et al. 2000). NPVs infect primarily lepidopteran larvae and occur either as one nucleocapsid per occlusion-derived virion (ODV) (designated as SNPVs), or as multiple nucleocapsids per ODV (often designated as MNPVs). NPVs exist outside of the host as environmentally-resistant, protein-encased occlusion bodies (OBs). Upon consumption by a susceptible larva, the OBs dissolve in the host’s alkaline midgut, releasing the ODV contained within (Pritchett et al. 1982). ODV pass through the peritrophic matrix (PM) and fuse with midgut epithelial cells, releasing the nucleocapsids (Granados and Lawler 1981), which are then transported to the nucleus where viral replication occurs (Volkman 1997; reviewed in Bonning 2005). Infection of a midgut epithelial cell is termed a primary infection. Nucleocapsids not involved in viral replication may be repackaged as budded virus (BV) particles, moving out of the cell to
spread infection systemically without waiting for production of progeny virus (Granados and Lawler 1981; Washburn et al. 2003a; Washburn et al. 2003b). BV are distinguished from ODV by the presence of gp64 peplomers embedded in the outer membrane (Volkman 1997; reviewed in Bonning 2005). Repackaged and progeny BV particles spread from the midgut to adjacent tracheolar cells to produce secondary (systemic) infections (Engelhard et al. 1994). After a systemic infection is established, BV spread throughout the insect, infecting tracheal epidermal cells, hemocytes, fat body, muscle and nervous tissue, salivary glands and the epidermis (Vail and Vail 1987; Volkman 1997; reviewed in Bonning 2005).

Although the impact of diet on pathogen efficacy has been well established in larval lepidopterans, the specific biological mechanisms responsible are not fully understood. *Heliothis virescens* F. larvae fed cotton (*Gossypium hirsutum* L.) foliage are less susceptible to mortal infection by *Autographa californica* multiple nucleopolyhedrovirus (AcNPV) received *per os* than larvae fed iceberg lettuce or artificial diet (Hoover et al. 1998a). This decrease in viral efficacy is not observed if the virus is delivered directly into the hemocoel, bypassing the midgut (Hoover et al. 2000). Thus, the influence of ingested cotton foliage on viral success in *H. virescens* is restricted to the midgut environment. Further, cotton-mediated resistance has been correlated with increasing foliar peroxidase activity and free radial generating capacity, measured *in vitro*, suggesting that midgut oxidative status may be an important factor regulating infection success (Hoover et al. 1998a).

Ingested cotton foliage may decrease baculoviral mortality in *H. virescens* larvae by preventing primary midgut infections or interfering with establishment of secondary
infection (systemic tracheal infections), perhaps through more rapid sloughing of infected midgut cells. Midgut cell sloughing is responsible for developmental resistance to mortal infection by baculovirus in *H. virescens* and *Trichoplusia ni* larvae as larvae age within an instar (Washburn et al. 1998). This mechanism has also been suggested as the basis for baculoviral resistance in cotton-fed *H. virescens* larvae (Hoover et al. 2000). Here, we demonstrate that the type of food ingested in the instars before or in the same instar immediately prior to viral inoculation is most predictive of viral mortality. Additionally, we describe the pathogenesis of *H. virescens* larvae fed artificial diet or cotton foliage. Our results strongly suggest that ingested foliage hinders establishment of initial midgut infections.

**Materials and Methods**

**Insects.**

*Heliothis virescens* eggs were acquired from the North Carolina State University Insectary, from a colony started in 1997 (Raleigh, NC). Larvae were fed in groups through the third instar on semisynthetic diet (Southland Products Inc., Lake Village, AR) in wax-lined paper cups (Xpedx, Harrisburg, PA) at 25°C and a 16L:8D photoperiod.

**Plants.**

Cotton, *Gossypium hirsutum*, cv. Acala SJ2 seeds were donated by the California Planting Cotton Seed Distributors (Bakersfield, CA). Seeds were sown in sterile soil in plastic pots and fertilized at planting with slow-release 14-14-14 fertilizer. Plants were
grown in a growth chamber under a day: night temperature regime of 29:24°C and a 16L:8D photoperiod. Plants were grown to the six to eight leaf stage; at that time, the upper two or three leaves were harvested for use in experiments.

**Virus Preparation.**

Two different viral constructs were used in these experiments. AcNPV-hsp70/lacZ (C6 parent), provided by Suzanne Thiem (Michigan State University, East Lansing, MI) and AcNPV-hsp70/lacZ (E2 parent) provided by Loy Volkman (University of California, Berkeley, CA) (Thiem unpublished, Engelhard et al. 1994). Viruses were amplified in *H. virescens* larvae. The E2 recombinant was used for the pathogenesis experiments because although potency of the C6 recombinant had not changed over time, we observed that some cells infected with this recombinant failed to express lacZ despite the presence of occlusions. Thus, we conducted the pathogenesis experiments (requiring visualization of lacZ expression in infected cells) using the E2 recombinant of AcNPV. Occlusion bodies (OB) from both viruses were harvested from virus-killed cadavers as described previously (Hoover et al. 1995), quantified using a hemacytometer, and stored at 4°C in neutrally buoyant 60% glycerol with 0.002% sodium azide (Engelhard et al. 1994).

**The Impact of Timing of Foliage and Virus Delivery on Disease Outcome.**

*H. virescens* larvae were reared in groups from neonate to late third instar on artificial diet (D) or cotton foliage (C) at 25°C under a 16L:8D photoperiod. Third instars displaying head capsule slippage (premolt to the fourth instar) were held at 28°C and
observed frequently for molting. Newly molted fourth instars (designated as 40s) were placed individually in 29.5 milliliter plastic cups and fed artificial diet (D) or pieces of excised cotton foliage (C). Larvae were allowed to feed for eight h (now designated as 48s), then microinoculated per os with OBs using a microapplicator (PAX 100, Burkard Scientific, Middlesex, UK) holding a 1 milliliter tuberculin syringe fitted with a 32 gauge blunt needle (Popper Precision Instruments, Inc., Lincoln, RI). Eight h post-molt was used because it was the earliest time point at which the midgut was full of plant material or artificial diet for all larvae, based on the time at which frass was produced (data not shown). In summary, larvae were fed either artificial diet or cotton foliage from neonate to the fourth instar, in the fourth instar for the first 8 h before virus challenge, or in the fourth instar following virus challenge, yielding eight treatment groups in a complete randomized crossed design (Table 1). Each larva received 108 or 145 OBs of AcNPV-hsp70/lacZ (C6) in a 1 μliter aliquot. The number of OBs delivered was consistent within each experiment, but varied between experiments.

Inoculated larvae were placed individually in 29.5 milliliter plastic cups and fed artificial diet (D) or cotton foliage (C), creating eight experimental groups of 30 to 45 larvae per treatment group (Table 1). Controls consisted of an additional cohort treated in the same way but not inoculated; larvae that died within three days post-inoculation were discarded as handling deaths. Larvae were maintained at 28°C in the dark and checked daily for mortality or pupation (light exposure does not influence larval mortality). Cotton foliage was added to larval cups as needed. All larvae were transferred to artificial diet after molting to the fifth instar (5th) for ease of handling. This transfer does not influence mortality because larvae clear midgut infections during the
first molt following virus challenge (Washburn et al. 1995). This experiment was repeated twice, once at each viral dose.

**Influence of Foliage on AcNPV Pathogenesis.**

Newly-molted fourth instar *H. virescens* were placed in 29.5 milliliter clear plastic cups containing artificial diet or fresh cotton foliage. These larvae were allowed to feed for eight hours, then microinoculated *per os* with OBs as described above. Each larva received 50 to 180 OBs of AcNPV-hsp70/lacZ (E2) in a 1 μl aliquot. The number of OBs delivered was consistent within each experiment, but varied between experiments. Following inoculation, larvae were returned to their respective diets. Thirty larvae per treatment were inoculated and monitored for mortality as internal controls.

At various times post-inoculation, 15 to 30 larvae were dissected as whole mounts. The time points ranged from 12 to 60 h post-inoculation (hpi) among all experiments; a subset of time points was tested within each experiment. Artificial diet-fed larvae develop more quickly than cotton-fed larvae. By 60 hpi, the artificial diet-fed larvae were either premolts or had molted to the fifth instar. Midgut lacZ signaling has been reported to decrease at the molt; thus, these larvae were excluded from the experiment (Washburn et al. 1995). For each experiment, 30 uninoculated larvae were dissected at the early and late time points as controls for false positive lacZ signaling.

Larvae to be dissected were surface sterilized in 70% ethanol and the dorsal cuticle was slit from posterior to anterior. Wholemounts were processed for lacZ expression as described previously (Engelhard et al. 1994) with some modifications.
Wholemounts were fixed in 2% paraformaldehyde in cytoskeletal extraction buffer (CEB: 10mM PIPES, 60mM sucrose, 100mM KCl, 5 mM Mg(OAc)$_2$, 1 mM EGTA, pH 6.8) for 18 h at 4°C and rinsed with CEB. The food bolus was removed, then wholemounts were incubated with X-gal for 12 to 24 h at room temperature in the dark.

Wholemounts were examined and scored using a Nikon dissecting microscope (6.7 to 80X); the number and type of lacZ positive cells (designated as infection foci) were recorded as described in Washburn et al. (1995). Foci were recorded as M, indicating infection of a midgut cell only; M+T, indicating infection of a midgut cell and the tracheolar cell servicing that midgut cell; or T, indicating infection of a tracheolar cell only. Similarly, each larva was classified as follows: larvae possessing only infected midgut cells were designated as M, larvae with any combination of infected midgut and tracheolar cells were designated as M+T, and larvae in which only tracheolar cells were infected were designated as T. Larvae were considered systemically infected if they possessed any tracheal infections, either as M+T, or T only. Tracheal infections cannot be sloughed and thus, once even a single tracheal signal is observed, *H. virescens* larvae ultimately succumb to infection (Washburn et al. 1995).

The proportion of larvae with primary (midgut) infections was calculated by dividing the number of larvae possessing only infected midgut cells by the total number of larvae dissected. Likewise, the proportion of larvae with systemic infections was calculated by combining the number of larvae possessing midgut plus tracheal infections with the number of larvae possessing tracheal only infections; this sum was divided by the total number of larvae dissected.
Statistics.

Data were analyzed using SAS (SAS Institute, v. 9.1.3). Analysis of variance for categorical data (proc CATMOD) was used to assess the effect of virus dose, food, and their interaction on larval mortality. Logistic regression (proc LOGISTIC) was used to evaluate the effect of virus dose, food, h post-inoculation, and their interactions on the presence or absence and progress of larval infection (Zar 1999).

Results

The Impact of Timing of Foliage and Virus Delivery on Disease Outcome.

The food consumed from neonate to the fourth instar or in the 8 h of the fourth instar before viral inoculation was most predictive of larval mortality from virus (Fig. 1). Insects fed artificial diet during either of these periods were significantly more likely to die than those fed cotton foliage (neonate to fourth instar $\chi^2 = 8.41, p = 0.0037$; 8 h before inoculation $\chi^2 = 9.60, p = 0.0019$). Although the food larvae ate after virus challenge did not significantly influence mortality ($\chi^2 = 0.16, p = 0.6924$), the combined effect of food consumed during all three time periods, indicated by a significant interaction ($\chi^2 = 4.51, p = 0.0337$), was an important predictor of mortality, as seen by the 45% decrease in percent mortality in the treatment group fed cotton foliage from neonate to the fifth instar (CCC; Fig. 1).

Influence of Foliage on AcNPV Pathogenesis.

Three time course experiments were performed in which the influence of ingested artificial diet or cotton foliage on viral pathogenesis in H. virescens larvae was
characterized. Hereafter, experiments will be referred to by virus dose used in each replicate (i.e., 85, 90 or 100 OBs of AcNPV-hsp70/lacZ strain E2). In total, 208 larvae were used to determine larval mortality, while 472 larvae were dissected, processed, and examined for lacZ signaling, indicative of viral infection. H. virescens mortality was significantly influenced by virus dose ($\chi^2 = 27.27, p < 0.0001$; Fig. 2). Food ingested also affected larval mortality ($\chi^2 = 17.36, p < 0.0001$); larvae fed cotton foliage were experienced a 35% reduction in mortality when inoculated with 85 or 90 OB, compared with larvae fed artificial diet (Fig. 2). Moreover, ingestion of cotton foliage significantly reduced the proportion of lacZ-positive larvae at any given time point, compared with larvae fed artificial diet ($\chi^2 = 51.57, p < 0.0001$; Fig. 2). In most cases, this reduction was two-fold or greater. As expected, virus dose and h post-inoculation (hpi) were positively associated with pathogenesis.

LacZ signaling was observed in both artificial diet and cotton-fed larvae at 12 hpi, the earliest time point sampled (Fig 2). However, throughout the early time points (12-20 hpi), the proportion of artificial diet-fed larvae that were lacZ-positive was consistently two to four-fold greater than the proportion of lacZ-positive cotton-fed larvae, suggesting that consumption of cotton foliage inhibited the ability of the virus to infect the midgut. At 12 hpi, 73% of artificial diet-fed larvae and 17% of cotton-fed larvae were lacZ-positive; the proportion of lacZ-positive artificial diet-fed larvae was approximately four-fold greater than the proportion of lacZ positive cotton-fed larvae.

Over time, the proportion of lacZ-positive artificial diet-fed larvae possessing systemic foci ranged from 84 to 95%, while the proportion of lacZ-positive cotton-fed larvae with systemic foci ranged from 50 to 100% (Fig 3). Each systemic tracheal
infection results from a primary midgut infection; consequently, the proportion systemically-infected reflects virus transmission efficacy from the midgut to the tracheal system. Therefore, the similarity in proportion of systemically-infected artificial diet and cotton-fed larvae suggests that ingested cotton foliage did not appear to influence the spread of infection beyond the midgut. Similar results were observed at the late time points, 30 to 60 hpi. The proportion of lacZ-positive artificial diet-fed larvae at these later time points was 1.5 to 2 times greater than the proportion of lacZ-positive cotton-fed larvae. At the later time points, all infection foci observed were systemic, regardless of food consumed (Fig 3).

Interestingly, in the 100 OB dose experiment, there was not a substantial increase in the proportion of artificial diet-fed larvae signaling from 12 to 20 hpi, indicating that the majority of infections had already established by 12 hpi. (Figs. 2 and 3) The opposite was true of cotton-fed larvae; the number of infection foci from 12 to 20 hpi doubled, suggesting that feeding on cotton generates marked obstacles to establishment of initial infections. The lack of plant-mediated resistance in cotton-fed larvae inoculated with 100 OB is not entirely unexpected; higher viral doses have previously been shown to overwhelm effects of ingested foliage (Hoover et al. 1998c).

Although infection of midgut cells was significantly influenced by food consumed prior to viral inoculation ($\chi^2 = 51.57$, p $< 0.0001$), food did not significantly affect the spread of virus from primary to secondary infection foci in H. virescens ($\chi^2 = 2.48$, p = 0.1152; Fig. 3). Types of foci were recorded only in the 85 and 100 OB experiments. In the 85 OB experiment, primary foci were observed in cotton-fed larvae, but not in larvae fed artificial diet. In the 100 OB experiment, systemic foci were observed in both
artificial diet and cotton-fed larvae at 12 hpi. This result is in line with a previous report of the earliest midgut foci in artificial diet-fed *H. virescens* in the fourth instar as 4 hpi and the first systemic foci at 6 hpi (Trudeau et al. 2001).

**Discussion**

Current hypotheses of foliar interference with baculoviral infection may be divided into two groups of biological mechanisms as (1) prevention of primary midgut infections or (2) decreased establishment of systemic infections. Primary infections may be hindered if virions released from occlusions are unable to successfully interact with midgut cells. For example, interactions with oxidized foliar phenolics can reduce solubility and infectivity of occlusion bodies *in vitro* (Felton and Duffey 1990). Moreover, ingestion of cotton foliage may result in increased generation of reactive oxygen species and other radical species within the midgut, potentially altering the structure of the peritrophic matrix (PM), a putative antioxidant (Summers and Felton 1996; Barbehenn and Stannard 2004). An altered PM may function as a greater barrier to virions, lessening the chance of a successful primary infection. In addition to influencing the establishment of primary infections, ingested foliage may also interfere with virus transmission out of the midgut cells, affecting establishment of systemic infections. Generation of free radicals and other oxidatively-active compounds during ingestion and digestion of cotton foliage may damage midgut cells, leading to increased sloughing and clearing of the infection before it is established in the tracheal system (Hoover et al. 1998a). Midgut cell sloughing is an important mechanism of developmental resistance to baculovirus in *H. virescens* and *Trichoplusia ni* larvae (Washburn et al. 1998) and has
been suggested as the basis for baculoviral resistance in cotton-fed *H. virescens* larvae in previous reports (Hoover et al. 2000).

In this study, the ability of ingested cotton foliage to reduce the proportion of *lacZ*-positive larvae at any given time point, compared with larvae fed on artificial diet, indicates that ingested cotton foliage decreases viral efficacy by interfering with establishment of primary midgut infections. However, cotton foliage did not interfere with spread of the virus beyond the midgut. Also viral mortality was only affected by foliage if there was pre-conditioning of the host’s midgut environment prior to virus challenge. Feeding on cotton foliage after virus challenge did not interfere with viral efficacy. Taken together, these data suggest that cotton foliage inhibits mortality from baculovirus either through direct interactions with virions and/or by altering midgut physiology thereby reducing the ability of the virus to establish infections in the midgut, not by increasing sloughing of infected midgut cells.

This finding contradicts a previous report in which increased sloughing rates of infected midgut cells was proffered as a potential mechanism of cotton-mediated inhibition of baculoviral disease in *H. virescens* (Hoover et al. 2000). In this previous study, a greater number of infection foci were observed at 24 hpi in cotton-fed larvae treated with the optical brightener M2R than in cotton-fed larvae in the absence of M2R. The working hypothesis for the effect of M2R was that it blocked sloughing of infected midgut cells. However, the authors did not rule out alternative explanations. The literature indicates that M2R has multiple effects on the midgut. A recent report supports the hypothesis that M2R blocks sloughing of midgut cells (Dougherty et al. 2006), but other reports show that M2R inhibits chitin synthetase *in vitro* and prevents peritrophic
matrix formation (Bartnickigarcia et al. 1994; Wang and Granados 2000). Thus, results observed by Hoover et al. (2000) could be explained by an M2R-mediated disruption of the peritrophic matrix rather than (or in addition to) an M2R-mediated decrease in midgut cell sloughing.

Ingestion of foliage simultaneously with baculovirus exposure has been shown to reduce viral efficacy in several larval lepidopterans (reviewed in Duffey et al. 1995). A variety of mechanisms have been suggested whereby ingested foliage could decrease susceptibility to viral infection. Decreased viral mortality was predicted by increased peroxidase activity and free radical generation measured by in vitro testing of foliage (Hoover et al. 1998a; Hoover et al. 1998b; Hoover et al. 1998c). While foliar phenolics may have antioxidant capacity (Johnson and Felton 2001; Johnson 2005), redox cycling of phenolics may trigger production of free radicals within the midgut (Summers and Felton 1994). Free radicals are highly chemically reactive and appear to be an important predictor of viral resistance in cotton-fed larvae (Hoover et al. 1998a).

Our results substantiate the hypothesis that ingested foliage interferes with establishment of midgut infections. Cotton foliage ingested in the instars before and/or in the 8 h prior to viral inoculation significantly reduced *H. virescens* larval mortality by virus. In contrast, larvae that received cotton foliage only within the same instar after viral challenge, did not benefit from the protective effects of cotton against baculovirus, nor did foliage influence movement of the virus beyond the midgut. Together, these findings support interactions between ingested foliage and the midgut environment and/or virus particles as the most likely scenarios of cotton-mediated interference in disease by baculovirus in *H. virescens*. Although cotton foliar influence on baculoviral infection is
examined here, these results may have implications for interactions between insects and other pathogens that gain entrance to the host through the midgut. Larvae of *Helicoverpa* *zea*, *Spodoptera exigua* and *Pseudoplusia includens* fed cotton foliage exhibit reduced susceptibility to *Bacillus thuringensis* and increased survival time, compared with larvae fed soybean or tomato (Ali et al. 2004). The mechanisms whereby pathogen ingress into midgut cells is hindered by ingestion of foliage is the subject of further investigation by our laboratory.
References Cited


Hare, J. D., and T. G. Andreadis. 1983. Variation in the susceptibility of Leptinotarsa decimlineata (Coleoptera: Chrysomelidae) when reared on different host plants to the fungal pathogen, Beauveria bassiana in the field and laboratory. Environmental Entomology 12: 1892-1897.


Table 1. Experimental treatment groups used to determine the timing of host plant effect on viral efficacy. *H. virescens* larvae were fed either artificial diet or cotton foliage during three developmental periods: neonate to newly molted fourth instar, for the first 8 h following molting to the fourth instar, and from 8 h post-molt to freshly molted fifth instar. The $4^x$ notation describes larvae that are $x$ h post-molt to the fourth instar, i.e., $4^0$ are newly-molted fourth instars. Similarly, the $5^0$ notation denotes newly molted fifth instars. Larvae were inoculated with AcNPV as $4^8$s and then transferred to the final diet treatment until they molted to the fifth instar, at which time all larvae were fed on artificial diet until they died or pupated.

<table>
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<th>Insect Stage</th>
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<td>Neonate to $4^0$</td>
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<td>$4^8$ to $5^0$</td>
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Figure 2.1. Influence of artificial diet or cotton foliage ingested at difference times relative to viral inoculation on *H. virescens* larval mortality. Mean percentage mortalities of *H. virescens* larvae fed one of the diet treatments in Table 1 and inoculated with 108 or 145 OBs of AcNPV-hsp70/lacZ (C6). Mortalities are represented as the percentage difference in mortality from larvae fed artificial diet from neonate to the newly molted fifth instar (5\(^0\)) (DDD larvae). Significantly more larvae died when fed artificial diet from neonate to fourth instar or in the fourth instar before inoculation (\(\chi^2 = 8.41, \text{df} = 1, p = 0.0037\) and \(\chi^2 = 9.60, \text{df} = 1, p = 0.0019\), respectively). Although food consumed after inoculation did not significantly influence larval mortality (\(\chi^2 = 0.16, \text{df} = 1, p = 0.6924\)), there was a significant interaction of the three time periods in their impact on mortality (\(\chi^2 = 4.51, \text{df} = 1, p = 0.0337\)). Larval mortality was also significantly
influenced by virus dose and the interaction of dose*food consumed in the fourth instar before inoculation. ($\chi^2 = 15.16, \, df = 1, \, p < 0.0001$ and $\chi^2 = 5.68, \, df = 1, \, p = 0.0172$, respectively).
Figure 2.2. Influence of artificial diet or cotton foliage on *H. virescens* larval mortality or viral infection at times post inoculation. Percentage of *H. virescens* larvae that died or were *lacZ*-positive following consumption of artificial diet or cotton.
foliage and inoculated with AcNPV-hsp70/lacZ (E2). Larval mortality was significantly influenced by virus dose and food ($\chi^2 = 27.27$, df = 2, $p < 0.0001$ and $\chi^2 = 17.36$, df = 1, $p < 0.0001$, respectively). Consumption of cotton foliage was negatively associated with mortality. Viral infection, or presence of lacZ signaling, at a given time post-inoculation was significantly influenced by virus dose, food, and h post-inoculation ($\chi^2 = 16.58$, df = 2, $p = 0.0003$; $\chi^2 = 51.57$, df = 1, $p < 0.0001$; $\chi^2 = 19.98$, df = 1, $p < 0.0001$, respectively). As with mortality, consumption of cotton foliage was negatively associated with viral infection.
Figure 2.3. Influence of artificial diet or cotton foliage on percentage of *H. virescens* larvae with primary or systemic infections at times post inoculation. Percentage of *H. virescens* larvae fed artificial diet or cotton foliage that have primary or tracheal (systemic) infections as a function of time post-inoculation with AcNPV-hsp70/lacZ (E2). Progression of infection from primary to systemic foci was significantly influenced by time ($\chi^2 = 5.79, df = 1, p = 0.0162$). Food ingested did not significantly influence spread of infection beyond the midgut ($\chi^2 = 2.61, df = 1, p = 0.1060$)
Chapter 3. Impact of the Peritrophic Matrix on Baculoviral Pathogenesis in *Heliothis virescens*

**Abstract**

The peritrophic matrix (PM) of herbivorous insects lines the midgut, protecting it from damage by ingested plant material. Ingested food has the potential to influence PM structure and there has long been interest in the influence of plant foliage on PM structure. In this report, we characterize the PM of *Heliothis virescens* larvae fed artificial diet or cotton foliage. In addition to protecting the midgut from food, the PM has also been hypothesized to protect herbivorous insects from ingested pathogens. Here, we examine the influence of the PM, ingested cotton foliage, and their interaction on *Autographa californica* nucleopolyhedrovirus (AcNPV) pathogenesis in *H. virescens*. Larvae fed cotton foliage were less susceptible to AcNPV infection than artificial diet-fed larvae. Furthermore, disruption of the PM by the metalloprotease enhancin in cotton-fed larvae restored larval susceptibility to the level of diet-fed larvae. When viral pathogenesis was examined, lacZ signaling, indicative of AcNPV infection, appeared in larvae fed artificial diet+enhancin significantly earlier and in a greater proportion of larvae, compared with larvae fed untreated artificial diet, although, by 18 hours post-inoculation, the proportion of lacZ positive larvae was similar in the two treatments. Thus, the PM appears to be a barrier to baculoviral infection in cotton-fed *H. virescens* larvae and may be a significant factor determining pathogen success by influencing establishment of primary infections.
Introduction

The midgut of insect herbivores is the primary site of direct contact between insect tissues and ingested plant material. In order for the insect to thrive, the midgut must be well protected against the physical and chemical threats posed by ingested plant material (Hammerschmidt and Schultz 1996). The semipermeable peritrophic matrix (PM) lines the midgut and surrounds the food bolus in most insects, separating it from the midgut epithelium (reviewed by Terra 2001). The PM consists of a mesh of chitin microfibrils overlaid with a gel comprised of proteoglycans, proteins, and glycoproteins (Ryerse et al. 1992; reviewed by Lehane 1997).

The PM is purported to have many functions. Originally, its primary role was thought to be protecting the midgut epithelium from physical abrasion by ingested plant material (Sudha and Muthu 1988). Additional protective roles have since been described. Due to its semipermeable nature, the PM acts as an ultrafilter, separating tannins and other allelochemicals from the midgut epithelium, while allowing passage of nutrients and digestive enzymes (reviewed by Lehane 1997 and Barbehenn 2001). The PM is also thought to present a barrier to microorganisms, preventing them from gaining access to the midgut epithelium, presumably by ultrafiltration (reviewed by Lehane 1997, Peng et al. 1999). Moreover, this structure may adsorb ingested chemicals, effectively removing them from the midgut environment (reviewed by Lehane 1997). The PM may also serve as a functional antioxidant in some insects, protecting the midgut epithelial cells from significant oxidative damage (Summers and Felton 1996; Barbehenn and Stannard 2004). Despite these studies, the ability of ingested foliage to influence PM physical structure has only been documented in one system (Pechan et al. 2002; Mohan et al. 2006).
Baculoviruses are double-stranded DNA viruses of arthropods, which comprise two genera—the Granuloviruses (GVs) and the Nucleopolyhedroviruses (NPVs) (van Regenmortel et al. 2000). Baculoviruses are orally infectious; NPV infection begins when a susceptible lepidopteran larva ingests one or more environmentally-resistant proteinaceous occlusion bodies (OBs). The OBs disintegrate in the larva’s alkaline midgut fluids, releasing occlusion-derived virions (ODV) (Pritchett et al. 1982). The ODV pass through the PM, and then attach to and infect midgut columnar epithelial cells (Granados and Lawler 1981; reviewed by Volkman 1997 and Bonning 2005). This infection of a midgut epithelial cell is termed a primary infection. Baculoviruses are distinguished from other viruses by possessing two morphologically distinct but genetically identical virion phenotypes. ODV initiate infection in the midgut epithelial cells; conversely, budded virions (BV), which infect multiple cell types except midgut cells, spread infection throughout the insect body (reviewed by Volkman 1997 and Bonning 2005). Progeny and/or repackaged BV move out of the midgut via tracheal epidermal cells (Granados and Lawler 1981; Engelhard et al. 1994; reviewed by Bonning 2005). The infection of tracheae and other non-midgut tissues is termed a systemic infection. The movement of virus from the midgut into the tracheal system, establishing a systemic infection, is essential for NPV success. Infected midgut cells may be sloughed, removing viral inoculum from the insect. Conversely, the insect cannot slough tracheal infections (Washburn et al. 1995). Once in the tracheal system, the virus spreads throughout the insect by infecting the hemocytes, fat body cells, muscle and nervous tissue, salivary glands and epidermis (Vail and Vail 1987; reviewed by Volkman 1997 and Bonning 2005).
The PM has the potential to impact baculoviral infection, since it separates ingested virions from susceptible midgut cells. The PM is a semipermeable barrier. PM pore size has been estimated in larval lepidopterans at 21-29 nm in diameter, which is smaller than the ODV of *Autographa californica* nucleopolyhedrovirus (AcNPV), which are 50-100 nm X 250-300 nm (Barbehenn and Martin 1995; reviewed by Bonning 2005). Consequently, the PM may be a barrier to ODV, thereby hindering initial midgut infections.

Recent reports support the hypothesis that the PM provides some protection from baculoviral infection. Enhancin, a metalloprotease isolated from the granules of the *Trichoplusia ni* granulovirus (TnGV), degrades the PM structural protein invertebrate intestinal mucin, increasing PM permeability (Lepore et al. 1996; Wang and Granados 1997; Peng et al. 1999). Simultaneous exposure to enhancin and AcNPV significantly increased mortality of artificial diet-fed *T. ni*, *Helicoverpa zea*, *Spodoptera exigua* and *Pseudaletia unipuncta*, compared with mortality caused by AcNPV alone (Derksen and Granados 1988; Wang et al. 1994). Despite these findings, the ability of the PM to provide protection from baculoviruses remains uncertain (Washburn et al. 1995).

Lepidopteran larval susceptibility to baculoviral infection is influenced by the host plant consumed prior to baculoviral exposure (reviewed by Cory and Hoover 2006). Previous reports state that *Heliothis virescens* larvae fed cotton foliage prior to AcNPV inoculation were more resistant to mortal infection than larvae fed iceberg lettuce or artificial diet (Hoover et al. 1998b). This resistance has been correlated with increased foliar peroxidase activity and free radical generation (Hoover et al. 1998a). However, the specific mechanisms through which larval resistance is achieved remain unknown.
Ingested plant material may influence larval mortality by interacting with the insect midgut environment, including the PM, and/or with virus particles. Here, we examine the role of the PM in baculoviral pathogenesis of *H. virescens* larvae fed cotton foliage or artificial diet.

**Materials and Methods**

**Insects.**

*Heliothis virescens* eggs were acquired from the North Carolina State University Insectary, from a colony started in 1997 (Raleigh, NC). Larvae were fed in groups through the third instar on semisynthetic diet (Southland Products Inc., Lake Village, AR) in wax-lined paper cups (Xpedx, Harrisburg, PA) at 25°C and a 16L:8D photoperiod. Late third instar larvae exhibiting head capsule slippage were collected and refrigerated for one to four days to synchronize larval development. This procedure does not alter larval growth or susceptibility to baculovirus (data not shown).

**Plants.**

Cotton, *Gossypium hirsutum*, cv. Acala SJ2 seeds were donated by the California Planting Cotton Seed Distributors (Bakersfield, CA). Seeds were sown in sterile soil in plastic pots and fertilized with slow-release 14-14-14 fertilizer. Plants were grown under a day: night temperature regime of 29:24°C and a 16L:8D photoperiod. For the PM degradation bioassay, plants were grown to the six to eight node stage; at that time, the upper two or three fully-expanded leaves were harvested for use. Due to growth chamber
constraints, plants were at the four node stage when the upper two leaves were harvested for use in the pathogenesis experiment.

**Virus production.**

AcNPV-hsp70/lacZ, provided by Loy Volkman (University of California, Berkeley, CA), was amplified in *H. virescens* larvae (Engelhard et al. 1994). Occlusion bodies (OB) were harvested from virus-killed cadavers and partially purified as previously described (Hoover et al. 1995). OBs were quantified using a hemacytometer and stored at 4°C in neutrally buoyant 60% glycerol with 0.002% sodium azide (Washburn et al. 1995).

TnGV, provided by Ping Wang, (Cornell University Experiment Station, Geneva, NY) was amplified in *T. ni* larvae (Wang et al. 1994). Granules were harvested from virus-killed cadavers, washed with 1% SDS and ultrapure water, quantified using a spectrophotometer, and stored at 4°C in ultrapure water (Wang et al. 1994).

**Effect of PM degradation on larval mortality.**

Discs of artificial diet and SJ2 cotton foliage were prepared; artificial diet discs measured 4 mm in diameter and 1.5 mm high, while cotton leaf discs measured 10 mm in diameter. Half of the discs were treated with 1X10^{12} TnGV granules in a 1 μl aliquot. Intact TnGV granules were used because of the difficulties experienced in purifying enhancin. In this case, intact TnGV is equivalent to purified enhancin in that *H. virescens* larvae are not susceptible to TnGV, which was verified in a preliminary bioassay of *H. virescens* larvae. We observed no viral deaths from TnGV, even at the very high doses...
used here. Untreated and enhancin-treated food discs were placed individually in a 24-well plate.

Newly-molted fourth instar *H. virescens* larvae were transferred individually to a 24-well plate containing an untreated or enhancin-treated disc of artificial diet or cotton foliage. These larvae were allowed to feed for eight hours, then microinoculated *per os* with 30 OBs of AcNPV-hsp70/lacZ in a 1 µl aliquot. Eight hours post-molt was used because it was the earliest time point at which the midgut was full of artificial diet or plant material for all larvae, based on the time at which frass was produced. Larvae that had not consumed their entire food disc within the eight hours were removed from the experiment.

Larvae were dosed using a PAX 100 microapplicator (Burkard Scientific, Middlesex, UK) holding a 1 mL tuberculin syringe fitted with a 32 gauge blunt needle (Popper Precision Instruments, Inc.; Lincoln, RI). Following inoculation, larvae were fed untreated artificial diet or cotton foliage. Thirty larvae were inoculated per treatment. Insects were maintained at 28°C and checked daily for mortality or pupation. Larvae that died prior to day four were considered handling deaths (mortality by virus does not occur in fourth instar *H. virescens* before day 4, data not shown). All cotton-fed larvae were transferred to artificial diet after molting to the fifth instar for ease of handling. This transfer does not influence mortality, since larvae clear midgut infections during the first molt following viral inoculation (Washburn et al. 1995; Hoover et al. 2000).
Electron microscopy of the PM.

_H. virescens_ larvae were fed untreated food discs or discs treated with $2 \times 10^7$ TnGV granules as described above. These larvae were not inoculated with AcNPV. After allowing larvae to feed for eight hours, those that had consumed the entire food disc were dissected. Intact midguts were removed and stored in primary fixative at 4°C (1.5% formaldehyde and 2.5% gluteraldehyde in 0.1M phosphate buffer, pH 7.4) (Dykstra 1992). Two to four midguts per treatment group were further processed for electron microscopy. Selected midguts were rinsed three times for five minutes each at room temperature (0.1M cacodylate buffer, pH 7.4). Samples were secondarily fixed in 1% osmium tetroxide for one hour, rinsed three times in cacodylate buffer for five minutes each, and dehydrated through an ethanol series (Hunter 1984).

Following ethanol dehydration, samples for scanning electron microscopy were freeze fractured in liquid nitrogen and the sample fragments underwent critical point drying (Baltec CPD-030 Critical Point Dryer, Techno Trade, Manchester, NH) (Dykstra 1992; Echlin 1992). Sample pieces were then mounted on aluminum stubs using double sided sticky carbon dots and sputter-coated with 10 nm of gold-palladium (Baltec SCD-050 Sputter Coater, Techno Trade, Manchester, NH) (Dykstra 1992). Prepared samples were viewed using a scanning electron microscope (SEM) (JSM 5400, JEOL, Peabody, MA) at an accelerating voltage of 20 kV. Digital images were captured using image archiving software (IMIX-PC v. 10, PGT, Princeton, NJ).

Samples for transmission electron microscopy were further dehydrated in acetone, then embedded in eponate resin and cut into 70nm sections (Dykstra 1992). Sections were mounted on uncoated copper grids and stained with uranyl acetate and lead citrate.
(Dykstra 1992). Prepared samples were viewed using a transmission electron microscope (TEM) (JEM 1200 EXII, JEOL, Peabody, MA) at an accelerating voltage of 80 kV. Digital images were captured using a TEM high resolution camera (F224, Tietz, Gauting, Germany). A minimum of three measurements of PM width were taken from each digital image, using ImageJ (v. 1.36b, National Institutes of Health). Care was taken to measure the thickest and thinnest regions of every image, to accurately represent the range of PM widths observed within each treatment.

**Influence of foliage and PM degradation on pathogenesis of AcNPV.**

*H. virescens* larvae were fed untreated food discs or discs treated with 2X10⁷ TnGV granules and inoculated with 26 OBs of AcNPV-hsp70/lacZ. Thirty larvae per treatment were monitored for mortality as internal controls. Additional inoculated larvae were dissected as wholemounts from 3 to 18 hours post-inoculation (hpi), with 21 to 30 larvae dissected per time point. Thirty control uninoculated larvae were dissected at 3 and 18 hpi as controls for false positive lacZ signaling.

Larvae to be dissected were surface sterilized and the dorsal cuticle was slit from posterior to anterior. Wholemounts were processed for lacZ expression as described previously (Engelhard et al. 1994) with some modifications. Wholemounts were fixed in 2% paraformaldehyde in cytoskeletal extraction buffer (CEB) for 18 hours at 4°C, rinsed with CEB, and then incubated in X-gal for 12 to 24 hours at room temperature in the dark.

Wholemounts were examined and scored using a Nikon dissecting microscope (6.7 to 80X); the number and type of lacZ positive cells (infection foci) were recorded as
described in Washburn et al. (1995). Foci were recorded as M, indicating infection of a midgut cell only; M+T, indicating infection of a midgut cell and the tracheolar cell servicing that midgut cell; or T, indicating infection of a tracheolar cell only. Similarly, each larva was classified as follows: larvae possessing only infected midgut cells were designated as M, larvae with any combination of infected midgut and tracheolar cells were designated as M+T, and larvae in which only tracheolar cells were infected were designated as T. Larvae were considered systemically infected if they possessed any tracheal infections, either as M+T, or T only. The proportion of larvae with primary midgut infections was calculated by dividing the number of larvae possessing midgut only infections by the total number of larvae dissected. Likewise, the proportion of larvae with systemic infections was calculated by combining the number of larvae possessing midgut and tracheal infections with the number of larvae possessing tracheal only infections; this sum was divided by the total number of larvae dissected.

**Statistics.**

Data were analyzed using SAS (v. 9.1.3, Cary, NC). Analysis of variance for categorical data (proc CATMOD) was used to assess the effect of enhancin consumption, food, and their interaction on larval mortality. The general linear model (proc GLM) was used to evaluate the effect of enhancin consumption and food on PM width. Logistic regression (proc LOGISTIC) was used to determine the effects of enhancin consumption, food, hours post-inoculation, and their interactions on the presence or absence and progress of lacZ signaling (foci of infection). Regression (proc REG) was used to
characterize the relationship between PM width and larval lacZ signaling at 18 hpi (Zar 1999).

Results

Effect of PM degradation on larval mortality.

_H. virescens_ larval mortality was recorded following ingestion of untreated or enhancin-treated artificial diet or cotton foliage and inoculation with AcNPV OB. Larvae fed untreated cotton foliage experienced a significant reduction in viral mortality, compared with larvae fed untreated artificial diet (χ² = 6.46, p = 0.0110; Fig 1). Further, larvae fed enhancin prior to AcNPV inoculation experienced significantly higher mortality than those fed untreated food (χ² = 5.47, p = 0.0193; Fig 1). Interestingly, there was a nearly significant interaction between food and enhancin treatment (χ² = 3.49, p = 0.0617). While enhancin consumption did not significantly alter the mortality of diet-fed larvae (χ² = 0.11, p = 0.7421), it did significantly increase that of cotton-fed larvae (χ² = 8.85, p = 0.0029).

Electron microscopy of the PM.

Scanning and transmission electron micrographs were taken of the PM from _H. virescens_ larvae fed untreated or enhancin-treated artificial diet or cotton foliage (Figs 2 and 3). PMs isolated from larvae fed untreated artificial diet or cotton foliage were fairly robust, with at least two layers (Figs 2). PMs isolated from artificial diet and cotton-fed larvae have similar surface characteristics; yet, the PM of artificial diet-fed larvae was observed to be significantly thinner than that of cotton-fed larvae (F = 111.72, p <
Mean PM width from artificial-diet fed larvae was 353 nm, within the 300-500 nm range previously reported for *H. virescens* larvae fed artificial diet (Fig 4, Ryerse et al. 1992). Alternatively, mean PM width from larvae fed cotton foliage was 765 nm, more than twice the width of PM from artificial diet-fed larvae. Regardless of whether a larva consumed artificial diet or cotton foliage, ingestion of enhancin resulted in a thinner, disrupted PM ($F = 31.55$, $p < 0.0001$; Figs 2 through 4). *H. virescens* larvae fed diet+enhancin were found with a much thinner and highly disrupted or altogether absent PM, compared with larvae fed untreated artificial diet. Larvae fed cotton+enhancin displayed a 26% thinner, highly disrupted PM, compared with larvae fed untreated cotton foliage (Figs 2 through 4).

**Influence of foliage and PM degradation on pathogenesis of AcNPV.**

The influence of enhancin and food on AcNPV mortality and pathogenesis was characterized in *H. virescens* larvae. In contrast to results described above, in the pathogenesis experiment, *H. virescens* mortality of larvae used in the internal control bioassay was not significantly influenced by food ($\chi^2 = 0.65$, $p = 0.4191$) or enhancin consumption ($\chi^2 = 2.20$, $p = 0.1382$; Fig 5). A 1000-fold lower enhancin dose was used for the pathogenesis experiment than for the PM degradation experiment. Although this lower dose of enhancin altered PM structure (Figs 2 through 4) and increased mortality by virus, the effect on mortality was not statistically significant.

Despite not significantly influencing *H. virescens* larval mortality, food ingested prior to viral inoculation did significantly influence viral infection at given times post inoculation, manifested as *lacZ* signaling ($\chi^2 = 7.57$, $p = 0.0060$). At 12 hpi, the first
infection foci were observed in artificial diet-fed larvae. From 12 to 18 hpi, the proportion of artificial diet-fed larvae that were lacZ positive was 2.3-fold greater than the proportion of cotton-fed larvae that were lacZ positive. In both artificial diet and cotton-fed larvae, primary and systemic infection foci were observed at 12 and 18 hpi. Hours post-inoculation also significantly influenced lacZ signaling, with a higher percentage of larvae expressing lacZ at later times ($\chi^2 = 86.41$, $p < 0.0001$; Fig 5).

In artificial diet-fed larvae, lacZ signaling was significantly influenced by the interaction of enhancin consumption with hpi ($\chi^2 = 5.85$, $p = 0.0155$; Fig 5A). LacZ positive infection foci were observed in larvae fed enhancin seven hours before they were seen in larvae inoculated with AcNPV in the absence of enhancin. Primary infection foci were first observed in artificial diet+enhancin larvae at 5 hpi and systemic infection foci were observed at 8 hpi (Fig 6). In contrast, infection foci were not observed in NPV inoculated larvae fed artificial diet without enhancin until 12 hpi; at 12 hpi, both primary and systemic foci were seen. From 5 to 12 hpi, the proportion of lacZ positive enhancin-fed larvae was up to 20 times greater than the proportion of lacZ positive larvae inoculated with AcNPV in the absence of enhancin. By 18 hpi, the proportion of lacZ positive larvae was the same in both groups.

Enhancin did not have as dramatic an effect on AcNPV infection in cotton-fed larvae. Infection foci were first observed at 8 hpi in cotton-fed larvae inoculated with AcNPV in the absence of enhancin and in cotton+enhancin-fed (Fig 5B). Although consumption of enhancin did not result in earlier infection of cotton-fed larvae, the proportion of enhancin-fed larvae that were lacZ positive at 8 hpi was 2.5 times the proportion of larvae fed untreated foliage that were lacZ positive (Fig 5B). Interestingly,
by 12 hpi, the proportion of \( lacZ \) positive enhancin-fed larvae had decreased to the level of larvae treated with NPV without enhancin. The types of infection foci observed were similar in both groups (Fig 6).

There was a strong negative relationship between mean PM width and \( lacZ \) signaling (\( R^2=90.4, p = 0.049 \)) at 18 hpi (the last time point of the pathogenesis experiment) (Fig 7). Larvae with a thicker PM, cotton-fed larvae in this instance, were less likely to show evidence of infection (\( lacZ \) signal) at 18 hpi.

**Discussion**

Host plant-mediated changes to the PM of *H. virescens* larvae may provide larvae some protection from viral infection. The PM of cotton-fed *H. virescens* larvae is significantly thicker than that of diet-fed larvae. We suggest that this thickened PM is formed to protect the midgut cells from damage by ingested foliage. *H. virescens* larvae possess a Type II PM, which is secreted from the anterior midgut and surrounds food as it is ingested (Ryerse et al. 1992). We propose four potential mechanisms whereby ingested foliage may influence PM structure. First, ingested foliage may stimulate increased synthesis of PM components, leading to a thicker PM. Second, ingested foliar phenolics may inhibit serine proteases (Jedinak et al. 2006), potentially decreasing PM proteolysis and resulting in a thicker PM, compared with artificial diet-fed larvae. Third, a decreased rate of food movement in cotton-fed larvae, compared with larvae fed artificial diet, may result in physical accumulation (thickening) of the PM if the PM is produced at a constant, food-independent rate. Fourth, ingested foliage may alter the structure of previously-formed PM through cross-linking between foliar and PM.
components. For instance, oxidized phenolics, lipid peroxides, free radicals and/or reactive oxygen species generated in the midgut during phenolic redox cycling and/or tannins found in ingested foliage may cross-link PM proteins (Johnson and Felton 1996; Summers and Felton 1996; Hoover et al. 1998a; Barbehenn and Stannard 2004).

While protecting the H. virescens larval midgut from damage by ingested plant material, a thickened PM may secondarily protect the larva from ingested pathogens. In this study, resistance of cotton-fed H. virescens larvae to baculovirus was reduced when the PM was disrupted by enhancin prior to AcNPV inoculation, suggesting that the PM of cotton-fed larvae is a major factor in baculoviral resistance. This conclusion was corroborated by the pathogenesis experimental findings.

Disruption of the PM resulted in earlier infection in artificial diet+enhancin-fed larvae, compared with larvae fed untreated artificial diet. In larvae fed artificial diet+enhancin, the putative PM barrier would be disrupted by enhancin before AcNPV inoculation, allowing AcNPV virions immediate access to susceptible midgut cells. Despite significantly reducing the time of first infection, enhancin did not influence the spread of infection out of the midgut in artificial diet-fed larvae. In cotton-fed larvae, consumption of enhancin resulted in a higher proportion of lacZ positive larvae at 8 hpi, compared with larvae fed untreated cotton. This difference in proportion of infected larvae may be credited to PM disruption by enhancin. Interestingly, by 12 hpi, the proportion of lacZ positive enhancin+AcNPV larvae had decreased to the level of the AcNPV-only larvae. This decrease in the proportion of lacZ positive larvae may reflect loss of primary infection foci due to midgut cell sloughing. Sloughing of infected midgut cells has been proposed as the basis for baculoviral resistance of cotton-fed H. virescens
larvae in a previous report (Hoover et al. 2000). Interestingly, there was no difference in spread of infection beyond the midgut between larvae fed untreated cotton foliage and those fed cotton foliage+enhancin. Among all treatments, the proportion of lacZ positive larvae at 18 hpi was negatively related to PM width. Together, these findings provide compelling evidence that plant-mediated changes in PM structure influence the success of baculoviral infection in *H. virescens* larvae.

Although cotton foliage was observed to reduce *H. virescens* baculoviral mortality, compared with artificial diet-fed larvae, in the PM degradation bioassay and in previously reported pathogenesis experiments, ingested cotton foliage did not significantly reduce larval mortality in this pathogenesis experiment (Ch 2). Cotton plants used in the pathogenesis experiment were smaller than those used in the PM degradation experiment because of growth chamber constraints. This difference in plant development likely affected foliar chemistry, which, in turn, influenced larval mortality.

While the results described here strongly suggest that the PM influences baculoviral infection, the PM is only one of many factors determining pathogen success. Plant-mediated inhibition of disease likely acts at multiple points in the infection pathway. In addition to altering PM structure, ingested foliage may influence virion integrity. Foliar phenolics may interact with viral OBs, reducing OB dissolution and infectivity (Felton and Duffey 1990; Feldman et al. 1999). Further, ingested foliage may contribute to increased midgut oxidative stress, potentially leading to sloughing of primary midgut infections before systemic infections are established (Hoover et al. 1998a; Hoover et al. 2000). Although we have provided persuasive evidence that the PM is one mechanism whereby ingested cotton foliage influences baculoviral infection in *H.*
virescens, additional mechanisms likely exist and further work is needed to clarify these interactions between insect tissues, ingested plant foliage and viral inoculum.
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Figure 3.1. Influence of artificial diet or cotton foliage an enhancin on *H. virescens* larval mortality. Percentage mortality of *H. virescens* larvae fed untreated or enhancin-treated artificial diet or cotton foliage prior to inoculation with occlusions of AcNPV-hsp70/lacZ (N = 25 to 28 larvae per treatment). Untreated food significantly influenced larval mortality; larvae fed untreated artificial diet were more likely to die than larvae fed untreated cotton foliage ($\chi^2 = 6.46$, df = 1, $p = 0.0110$). Overall, larval mortality was significantly associated with enhancin ($\chi^2 = 5.47$, df = 1, $p = 0.0193$); larvae fed enhancin prior to AcNPV inoculation were more likely to die than larvae fed untreated food. The interaction between food and enhancin was nearly significant, suggesting that food influenced enhancin efficacy ($\chi^2 = 3.49$, df = 1, $p = 0.0617$). In fact, enhancin significantly increased mortality of cotton-fed larvae, but not artificial diet-fed larvae ($\chi^2 = 8.85$, df = 1, $p = 0.0029$; $\chi^2 = 0.11$, df = 1, $p = 0.7421$, respectively).
Figure 3.2. Scanning electron micrographs showing the influence of artificial diet or cotton foliage and enhancin on the *H. virescens* peritrophic matrix. A = artificial diet-fed, B = artificial diet+enhancin-fed, C = cotton-fed, D = cotton+enhancin-fed. Midgut is designated by mg, food by f, and the PM by small triangles. The PM lines the midgut, separating it from the food. The PM of larvae fed artificial diet appears slightly thinner than that of larvae fed cotton foliage, but no difference in surface texture is observed. The PM of larvae fed artificial diet+enhancin appears substantially thinner and more porous than that of larvae fed untreated artificial diet. The PM of larvae fed cotton+enhancin appears slightly thinner than that of larvae fed untreated cotton, but no difference in surface texture is observed. Magnification of large pictures is 5,000X; scale
bars represent 5 μm. Small squares delineate the region shown in the 15,000X insets. N = 4 to 6 larval midguts per treatment.
Figure 3.3. Transmission electron micrographs of the PM from *H. virescens* larvae fed untreated or enhancin-treated artificial diet or cotton foliage. A = artificial diet-fed, B = artificial diet+enhancin-fed, C = cotton-fed, D = cotton+enhancin-fed. The PM of larvae fed artificial diet appears slightly thinner, compared with that of larvae fed cotton foliage. The PM of larvae fed artificial diet+enhancin appears thinner and disrupted, compared with that of larvae fed untreated artificial diet. The PM of larvae fed cotton+enhancin appears thinner and disorganized, compared with that of larvae fed untreated cotton. Magnification is 20,000X; scale bars represent 500 nm. N = 6 to 10 midgut sections per on treatment group.
Figure 3.4. The influence of artificial diet or cotton foliage and enhancin on width of *H. virescens* peritrophic matrix. Mean PM width, measured from TEM images, of *H. virescens* larvae fed untreated or enhancin-treated artificial diet or cotton foliage. PM width is significantly influenced by food and enhancin consumption ($F = 111.72$, df = 1, $p < 0.0001$; $F = 31.55$, df = 1 $p < 0.0001$, respectively). Error bars represent one standard error of the mean (N ranged from 6 to 10 midgut sections, depending on treatment group).
Figure 3.5. Influence of artificial diet or cotton foliage and enhancin on *H. virescens* larval mortality or viral infection at times post-inoculation. Percentage of *H. virescens* larvae that died or were lacZ positive following consumption of untreated or enhancin-treated food and inoculation with AcNPV. The results from artificial diet-fed larvae are shown in (A), while the results from cotton-fed larvae are shown in (B). In artificial diet-fed larvae (A), consumption of enhancin increased mortality, but the change
was not significant ($\chi^2 = 0.59, \text{df} = 1, p = 0.4435$). Viral infection of artificial diet-fed larvae, indicated by presence of lacZ signal, is significantly influenced by hours post-inoculation (hpi) ($\chi^2 = 65.29, \text{df} = 1, p < 0.0001$). While enhancin did not significantly influence lacZ signaling, the interaction between hpi and enhancin was significant ($\chi^2 = 2.95, \text{df} = 1, p = 0.0858; \chi^2 = 5.85, \text{df} = 1, p = 0.0155$, respectively). As expected, enhancin efficacy decreases with time since consumption. As with artificial diet-fed larvae, consumption of enhancin increased mortality in cotton-fed larvae (B), but the change was not significant ($\chi^2 = 1.70, \text{df} = 1, p = 0.1924$). Viral infection of cotton-fed larvae, indicated by presence of lacZ signal, is significantly influenced by hpi, but not by enhancin ($\chi^2 = 24.37, \text{df} = 1, p < 0.0001; \chi^2 = 0.58, \text{df} = 1, p = 0.4483$, respectively).

Mortality bioassay N=107 larvae, dissection and lacZ visualization N= 529 larvae.
Figure 3.6. Influence of artificial diet or cotton foliage and enhancin on percentage of *H. virescens* larvae with primary or systemic infections and times post-inoculation. Percentage *H. virescens* larvae that have primary or systemic infection foci at a given hour post-inoculation (hpi) following consumption of untreated or enhancintreated artificial diet or cotton foliage and inoculation with AcNPV occlusions. In both artificial diet and cotton-fed larvae, spread of infection from primary to systemic infection foci was significantly influenced by hpi ($\chi^2 = 8.15$, df = 1, p = 0.0043; $\chi^2 = 8.04$, df = 1, p = 0.0046, respectively). Neither food nor enhancin significantly influenced infection spread beyond the midgut ($\chi^2 = 0.42$, df = 1, p = 0.5164; $\chi^2 = 0.18$, df = 1, p = 0.6737, respectively). N = 21 to 30 larvae per time point per treatment.
Figure 3.7. Relationship between *H. virescens* peritrophic matrix width and infection at 18 hours post viral inoculation. PM width predicts the percentage of *H. virescens* larvae that are *lacZ* positive at 18 hours post-inoculation (hpi); larvae with a thicker PM are less likely to become infected. Presence of *lacZ* infection at 18 hpi = 72.698 – (0.064 * PM width in nm); $R^2 = 0.904$, $p = 0.049$. 
Chapter 4. The Influence of Foliage from Four Host Plants on Baculoviral Efficacy in *Heliothis virescens*

**Abstract**

The ability of ingested plant material to influence baculoviral success in larval lepidopterans is well-established. We examined how ingestion of iceberg lettuce, cotton, tobacco or oakleaf lettuce prior to oral inoculation with *Autographa californica* nucleopolyhedrovirus (AcNPV) influenced mortality of developmentally-matched fourth instar *Heliothis virescens* larvae, compared with ingestion of artificial diet. While consumption of iceberg lettuce foliage in the fourth instar prior to AcNPV inoculation did not influence *H. virescens* larval mortality, consumption of cotton, tobacco or oakleaf lettuce foliage significantly reduced larval mortality, compared with artificial diet. Further, oakleaf lettuce foliage fed at any time prior to viral inoculation, from neonate to the fourth instar or in the fourth instar before viral inoculation, significantly decreased *H. virescens* larval mortality, compared with artificial diet fed during the same period.

**Introduction**

Baculoviruses are orally-infectious pathogens of arthropods, chiefly lepidopteran larvae. Baculoviruses are found in the environment as proteinaceous occlusion bodies (OBs). Upon consumption by a susceptible host, the OBs dissolve in the midgut, liberating occlusion-derived virions (ODV) (Pritchett et al. 1982). The ODV pass through the peritrophic matrix, fusing with and infecting midgut epithelial cells (Granados and Lawler 1981); baculoviral replication in the nucleus of infected cells
(reviewed in Volkman 1997). Early in the infection cycle, progeny virions bud from infected midgut cells and infect associated tracheolar cells, spreading the infection out of the midgut (Engelhard et al. 1994). Late in the infection cycle, progeny virions remain in the nucleus and are occluded within a protein matrix, forming OBs (reviewed in Volkman 1997). This infection cycle repeats, spreading infection throughout the larva’s body and eventually killing it (reviewed in Volkman 1997). OBs formed in infected tissues are released from the cadaver as it ruptures from the action of virally-encoded proteases and chitinases, contaminating the surrounding environment.

The susceptibility of an individual larva to mortal viral infection may be influenced by several factors, including food ingested in conjunction with viral occlusions (reviewed in Cory and Hoover 2006). Plant foliage consumed prior to, or in conjunction with, viral inoculum can modulate viral efficacy, often reducing the susceptibility of host larvae to viral infection (Duffey et al. 1995). *Heliothis virescens* F., the tobacco budworm, is an agricultural pest insect for which the affect of host plant foliage on viral efficacy has been reported.

*H. virescens* is a significant pest of cotton, but also feeds on several other host plants, including tobacco and wild lettuce (Harding 1976; Martin et al. 1976; Fitt 1989). The influence of selected host plants on *H. virescens* susceptibility to baculoviral infection has been previously described (Ali et al. 1998; Hoover et al. 1998). *H. virescens* larvae fed cotton or soybean foliage treated with *Helicoverpa zea* single nucleopolyhedrovirus (HzSNPV) occlusions were less susceptible to mortal infection than larvae fed virus-treated crimson clover or Carolina geranium foliage (Ali et al. 1998). Similarly, cotton foliage treated with *Autographa californica*
nucleopolyhedrovirus occlusions (AcNPV) provided the greatest degree of protection against mortal infection, followed by romaine lettuce, iceberg lettuce and artificial diet (Hoover et al. 1998).

However, in the experiments referenced above, researchers did not control for developmental age within the instar and, thus, developmental age could have been a confounding factor in the relative susceptibility to virus in these studies. Lepidopteran larvae differing in age by no more than a few hours become increasingly resistant to mortal infection by baculoviruses (Engelhard and Volkman 1995; Washburn et al. 1995; Kirkpatrick et al. 1998). Further, in the above experiments, larvae were dosed using leaf disks to which virus had been applied. This method, while resembling the natural situation, likely allows for differences in virus acquisition rates, since larvae consume foliage from different plants at different rates (Plymale, personal observation). Herein, we examined how ingestion of iceberg lettuce, tobacco or oakleaf lettuce foliage by larvae of equivalent developmental age prior to oral inoculation with AcNPV influenced *H. virescens* mortality, compared with ingestion of cotton foliage or artificial diet.

**Materials and Methods**

**Plants.**

Seeds of lettuce, *Lactuca sativa* L., cv. Oakleaf, were sown in sterile soil in plastic pots and fertilized with slow-release 14-14-14 fertilizer. Plants were grown under a day:night temperature regime of 29:24°C and a 16L:8D photoperiod. Plants were grown for three weeks; at that time, all leaves were harvested for use in experiments. Cotton, *Gossypium hirsutum* L., cv. Acala SJ2, and tobacco, *Nicotiana tabacum* L., cv.
K-326, were grown under the same conditions. Cotton was grown to the six to eight leaf stage; at that time, the upper two or three fully-expanded leaves were harvested for use in experiments. Tobacco was grown to the four leaf stage; at that time, the upper two fully-expanded leaves were harvested for use in experiments. Organic iceberg lettuce was purchased from a local grocery store; the outer three leaves were not used.

**Virus preparation.**

AcNPV-hsp70/lacZ (C6 parent), provided by Suzanne Thiem (Michigan State University, East Lansing, MI) and AcNPV-hsp70/lacZ (E2 parent) provided by Loy Volkman (University of California, Berkeley, CA) (Thiem unpublished; Engelhard et al. 1994) were used in these experiments. We switched to the E2 recombinant because, although potency of the C6 recombinant had not changed, we observed that some cells infected with this recombinant failed to express lacZ despite the presence of occlusions. Although lacZ expression was not required for these experiments, it was essential for other concurrent experiments. Occlusion bodies (OB) from both viruses were harvested from virus-killed cadavers as described previously (Hoover et al. 1995), quantified using a hemacytometer, and stored at 4°C in neutrally buoyant 60% glycerol with 0.002% sodium azide (Engelhard et al. 1994).

**Influence of foliage from different host plants on larval mortality.**

To determine whether a particular host plant consumed prior to viral inoculation influences mortality of developmentally-matched *H. virescens* larvae, fourth instar larvae were fed artificial diet, iceberg lettuce, cotton, tobacco or oakleaf lettuce foliage before
oral viral inoculation. *Heliothis virescens* eggs were acquired from the North Carolina State University Insectary (Raleigh, NC). Larvae were grown in groups through the third instar on semisynthetic diet (Southland Products Inc., Lake Village, AR) in wax-lined paper cups (Xpedx, Harrisburg, PA) at 25°C and a 16L:8D photoperiod. Late third instar larvae exhibiting head capsule slippage were collected for use in experiments.

Newly molted fourth instars were transferred to one ounce clear plastic cups containing artificial diet, iceberg lettuce, cotton, tobacco or oakleaf lettuce foliage. Larvae were allowed to feed for eight hours, then microinoculated *per os* with OBs in a 1 μL aliquot using a microapplicator (PAX 100, Burkard Scientific, Middlesex, UK) holding a 1 mL tuberculin syringe fitted with a 32 gauge blunt needle (Popper Precision Instruments, Inc., Lincoln, RI). Eight hours post-molt was used because it was the earliest time point at which the midgut was full of plant material or artificial diet for all larvae, based on the time at which frass was produced. Larvae fed iceberg lettuce foliage received 180 OBs of AcNPV-hsp70/lacZ (E2 recombinant). Larvae fed cotton foliage were inoculated with 50, 90 or 180 OBs of AcNPV-hsp70/lacZ (E2 recombinant). Larvae fed tobacco foliage were inoculated with 124 or 165 OBs of AcNPV-hsp70/lacZ (C6 recombinant). Larvae fed oakleaf lettuce received 95 OBs of AcNPV-hsp70/lacZ (C6 recombinant). Larval cohorts fed artificial diet were inoculated with one of each of the above doses to permit comparisons with plant-fed larvae. The number of OBs delivered was consistent within each experiment, but varied between experiments. More than one viral dose was tested for cotton, tobacco and oakleaf lettuce-fed larvae because the degree of inhibition of viral disease in cotton-fed *H. virescens* can be inversely related to dose (Hoover et al. 1998).
Twenty-five to 55 larvae were inoculated per treatment. Following inoculation, larvae were returned to their respective diets, maintained at 28°C, and checked daily for mortality or pupation. This experiment was repeated once with iceberg lettuce-fed larvae, four times with cotton-fed larvae, and twice each with tobacco or oakleaf lettuce-fed larvae. The effect of ingested iceberg lettuce on *H. virescens* larval mortality has been previously reported (Hoover et al. 2000); the single replicate using iceberg lettuce-fed larvae was to enable comparison with this previous work.

**The impact of timing of foliage and virus delivery on disease outcome.**

In order to determine the timing of the impact of foliage on disease outcome, *H. virescens* larvae were fed artificial diet or oakleaf lettuce foliage for different blocks of time before and after oral viral inoculation. Larvae were reared from neonate to late third instar on semisynthetic diet (Southland Products Inc., Lake Village, AR) or oakleaf lettuce foliage in wax-lined paper cups (Xpedx, Harrisburg, PA) at 25°C and a 16L:8D photoperiod. Third instars displaying head capsule slippage (premolt to the fourth instar) were held at 28°C and observed frequently for molting. Newly molted fourth instars (designated as 4⁰s) were placed individually in one ounce plastic cups and fed artificial diet or oakleaf lettuce foliage. Larvae were allowed to feed for eight hours (now designated as 4⁸s), then microinoculated per os with 95 OBs of AcNPV-hsp70/lacZ (C6 recombinant) in a 1 μL aliquot as described above. After inoculation, larvae were placed individually in one ounce plastic cups and fed artificial diet (D) or oakleaf lettuce foliage (L), creating seven treatment groups of 20 to 45 larvae per group in a randomized crossed design (Table 1).
Controls consisted of an additional cohort treated in the same way but not inoculated; larvae that died within three days post-inoculation were discarded as handling deaths. Larvae were maintained at 28°C and checked daily for mortality or pupation. All larvae were transferred to artificial diet after molting to the fifth instar (5⁰) for ease of handling. This transfer does not influence mortality because larvae clear midgut infections during the first molt following virus challenge (Washburn et al. 1995). This experiment was replicated twice.

Statistics.

Data were analyzed using SAS (SAS Institute, v. 9.1.3). Analysis of variance for categorical data (proc CATMOD) was used to assess the effect of food, viral dose and their interaction on larval mortality (Zar 1999).

Results

Influence of foliage from different host plants on larval mortality.

The susceptibility of *H. virescens* larvae fed iceberg lettuce, cotton, tobacco or oakleaf lettuce foliage to mortality from AcNPV was assessed. Ingestion of iceberg lettuce prior to inoculation did not significantly influence larval mortality compared with larvae fed artificial diet ($\chi^2 = 0.76$, $p = 0.3822$). In contrast, cotton, tobacco and oakleaf lettuce foliage significantly reduced larval mortality when consumed prior to viral inoculation compared with artificial diet (cotton $\chi^2 = 35.79$, $p < 0.0001$; tobacco $\chi^2 = 10.21$, $p = 0.0014$; oakleaf lettuce $\chi^2 = 16.98$, $p < 0.0001$; Fig 1).
The impact of timing of foliage and virus delivery on disease outcome.

Developmentally-matched fourth instar *H. virescens* larvae fed on a combination of artificial diet or oakleaf lettuce displayed no apparent differences in larval size among the treatment groups. However, lettuce fed larvae required, on average, one additional day to develop from neonate to the fourth instar. In addition, there were no differences in observable size between larvae fed artificial diet or lettuce foliage for the 8-hour period of the fourth instar before viral inoculation.

The food consumed from neonate to the fourth instar or in the 8 h of the fourth instar before viral inoculation was most predictive of larval mortality (neonate to fourth instar \( \chi^2 = 6.74, p = 0.0094 \); 8 h before inoculation \( \chi^2 = 23.19, p < 0.0001 \); Fig 2). Insects fed foliage during either of these periods were significantly less likely to die than those fed artificial diet. Although the food larvae consumed after viral inoculation did not significantly influence mortality (\( \chi^2 = 1.51, p = 0.2199 \)), there was a significant interaction between the food consumed from neonate to fourth instar and food consumed after inoculation (\( \chi^2 = 11.89, p = 0.0006 \); Fig 2).

**Discussion**

The host plant consumed prior to baculoviral exposure has been shown to modulate susceptibility of lepidopteran larvae to baculoviral infection in numerous systems (reviewed in Cory and Hoover 2006). The host plant has previously been reported to influence mortality, both when viral inoculum was consumed over time along with foliage and when viral inoculum was administered in a pulse following foliage consumption (Ali et al. 1998; Hoover et al. 1998; Hoover et al. 2000). Herein, while
controlling for larval developmental age and rate of viral acquisition, we show that ingestion of cotton, tobacco and oakleaf lettuce foliage significantly reduced susceptibility of *H. virescens* larvae to AcNPV compared with artificial diet. In contrast, iceberg lettuce consumed prior to viral inoculation did not have a significant effect on larval mortality by virus, compared with artificial diet, which is consistent with a previous report (Hoover et al. 1998). These results indicate that host plant influence on baculoviral efficacy can occur even when the confounding effect of developmental resistance is removed as a source of variability, which can occur as the result of differences in the rate of foliage/inoculum acquisition.

Cotton and tobacco foliage contain relatively high levels of phenolics (cotton: total phenolics 32-60 μmol/g fresh foliage; tobacco total phenolics 13-24 mg/g dry foliage, chlorogenic acid 0.7-1 mg/g fresh foliage, flavonoid phenols 0.4 mg/g fresh foliage), oakleaf lettuce moderate levels (chlorogenic acid 0.2-0.35 mg/g fresh foliage, flavonoid phenols 33 mg/g fresh foliage) and iceberg lettuce low levels (flavonoid phenols 0.3 mg/g fresh foliage) (Andersen et al. 1979; Bi et al. 1997; Hoover et al. 1998; DuPont et al. 2000; Nicolle et al. 2004). Further, these plants may differ in other aspects, including chlorophyll levels, total photosynthate, and nitrogen content. We speculate that the physiological response of larvae to phenolics and other foliar compounds may alter their susceptibility to baculoviral infection (Ojala et al. 2005; Lee et al. 2006).

The influence of oakleaf lettuce on larval mortality was further characterized by varying the timing of foliage ingestion with respect to viral inoculation. Foliage consumed at any time prior to viral inoculation significantly decreased *H. virescens* larval mortality, compared with artificial diet fed during the same period, but foliage consumed
after viral challenge had no effect. We also observed a significant interaction between oakleaf lettuce foliage consumed from neonate to the fourth instar and foliage consumed after inoculation. This result suggests that prolonged exposure to host plant foliage may alter larval physiological status, influencing future interactions between the larva, ingested food and the virus. The above results are consistent with a previous report in which the same experimental design was used to examine the timing of the influence of cotton foliage on mortality of *H. virescens* by AcNPV (Ch 2). Ingested cotton foliage was previously reported to decrease efficacy of AcNPV inoculated into the midgut, but did not influence efficacy of AcNPV injected directly into the hemocoel, suggesting that cotton foliage acts through the midgut environment (Hoover et al. 2000; Ch 6).

Ingested foliage may influence midgut physiology in a number of ways. Foliage consumed in the same instar as viral inoculation may interact directly with virus particles, potentially reducing occlusion body dissolution and, thus, occlusion-derived virion release (Felton and Duffey 1990; Feldman et al. 1999). Ingested plant material may also alter peritrophic matrix structure, reducing interactions between virions and midgut cells (Ch 3). Possession of a thicker peritrophic matrix has been correlated with to be decreased susceptibility to mortal baculoviral infection (Piubelli et al. 2006; Ch 3). Further, sloughing rates of midgut cells may be accelerated, removing viral inoculum from the midgut before the virus is able to establish a systemic infection (Hoover et al. 2000). Beyond the midgut, ingested foliage may also influence the larva’s immune response to viral infection (Ojala et al. 2005).

In conclusion, we have demonstrated that a diversity of host plants reduce susceptibility of developmentally-matched *H. virescens* larvae to AcNPV. The ability to
influence viral pathogenicity appears to be widespread among *H. virescens* host plants (Ali et al. 1998); and ingested foliage has recently been reported to affect efficacy of *Bacillus thuringiensis* to several lepidopteran larvae (Ali et al. 2004). Thus, further work is needed to characterize the specifics of interactions between host plants, *H. virescens* larvae and pathogens, because insight into these interactions may increase the utility of insect pathogens as bioinsecticides.
References


Table 4.1. Experimental treatment groups used to determine the timing of host plant effect on viral efficacy. *H. virescens* larvae were fed either artificial diet or oakleaf lettuce foliage during three developmental periods, i.e., neonate to newly molted fourth instar, for the first 8 h following molting to the fourth instar, and from 8 h post-molt to freshly molted fifth instar. The $4^x$ notation denotes larvae that are $x$ h post-molt to the fourth instar, i.e., $4^0$ are newly-molted fourth instars. Similarly, the $5^0$ notation denotes newly molted fifth instars. Larvae were inoculated with AcNPV as $48^s$ and then transferred to the final food treatment until they molted to the fifth instar, at which time all larvae were fed on artificial diet until they died or pupated.

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Figure 4.1. Influence of host plant foliage on *H. virescens* larval mortality. Mean percentage mortality decrease of *H. virescens* larvae fed iceberg lettuce, cotton, tobacco or oakleaf lettuce foliage, compared with larvae fed artificial diet, for 8 h prior to oral inoculation with AcNPV-hsp70/*lacZ* occlusions. Results are presented as percentage decrease from artificial diet-fed mortality to allow results from different viral doses to be combined. Asterisks are below the bars that were significantly different than artificial diet. Ingestion of iceberg lettuce prior to inoculation did not significantly affect larval mortality compared with larvae fed artificial diet ($\chi^2 = 0.76$, df = 1, $p = 0.3822$). In contrast, larvae fed artificial diet were more likely to die than larvae fed cotton, tobacco or oakleaf lettuce foliage (cotton $\chi^2 = 35.79$, df = 1, $p < 0.0001$; tobacco $\chi^2 = 10.21$, df = 1, $p = 0.0014$; oakleaf lettuce $\chi^2 = 16.98$, df = 1, $p < 0.0001$). Although viral dose significantly influenced mortality of cotton-fed larvae, there was no interaction between viral dose and food ($\chi^2 = 123.08$, df = 2, $p < 0.0001$; $\chi^2 = 1.29$, df = 2, $p = 0.5259$).
respectively). Error bars represent one standard error of the mean; N = 25-55 larvae per treatment per replicate. Experiments were performed once for iceberg lettuce-fed larvae, four times for cotton-fed larvae, and twice each for tobacco and oakleaf lettuce-fed larvae.
Figure 4.2. Influence of artificial diet or lettuce foliage ingested at different times relative to viral inoculation on *H. virescens* larval mortality. Mortalities of *H. virescens* larvae fed one of the food treatments in Table 1 and orally inoculated with AcNPV-hsp70/lacZ occlusions in a 1μL pulse. Significantly fewer larvae died when fed lettuce foliage from neonate to fourth instar or in the fourth instar before inoculation compared with larvae fed artificial diet ($\chi^2 = 6.74$, df = 1, $p = 0.0094$ and $\chi^2 = 23.19$, df = 1, $p < 0.0001$, respectively). Although food consumed after inoculation did not significantly influence larval mortality ($\chi^2 = 1.51$, df = 1, $p = 0.2199$), there was a significant interaction between the food consumed from neonate to fourth instar and food consumed after inoculation ($\chi^2 = 11.89$, df = 1, $p = 0.0006$). Error bars represent one standard error of the mean; N = 20-45 larvae per treatment per experiment. The experiment was replicated twice.
Chapter 5. Induction of systemic acquired resistance by benzothiadiazole in cotton foliage does not adversely affect baculovirus pathogenicity to Heliothis virescens

Abstract

Baculoviral efficacy against lepidopteran larvae is substantially impacted by the host plant consumed along with, or prior to, viral inoculum. Here, we characterize how baculoviral pathogenesis to cotton-fed Heliothis virescens larvae is affected by induction of systemic acquired resistance (SAR). SAR induced by the plant elicitor benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) has been shown to protect against plant pathogens; in contrast, reports of SAR effects on chewing herbivores have been negligible. The influence of SAR on natural enemies of herbivores has not been reported. In this study, BTH application significantly increased foliar peroxidase activity and total phenolic levels, but did not alter catecholic phenolic levels. Consumption of SAR-induced foliage did not influence H. virescens pupal mass or larval mortality by the microbial control agent Autographa californica nucleopolyhedrovirus any more than did consumption of untreated foliage. Thus, activators of SAR, including BTH, and baculoviruses are likely to be compatible components of an IPM system.

Introduction

Systemic acquired resistance (SAR) is a long-lasting response typically induced in host plants in reaction to pathogen infection (reviewed in Durrant and Dong 2004). SAR involves induction of multiple plant defensive compounds, including PR-1, β-1,3-
glucanases, chitinases, and peroxidases (Gaffney et al. 1993; reviewed in Van Loon and Van Strien 1999). The induction of SAR essentially “inoculates” the plant, protecting against future infection by pathogens (Friedrich et al. 1996). The plant elicitor benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), also known as acibenzolar-S-methyl and Actigard®, is a functional analog of salicylic acid that activates SAR in both monocots and dicots (Gorlach et al. 1996; Lawton et al. 1996). Foliar application of BTH induces pathogen response genes typically associated with SAR and provides protection against an array of bacterial, fungal and viral plant pathogens (Friedrich et al. 1996; Inbar et al. 1998; Wendehenne et al. 1998; Inbar et al. 2001; Maxson-Stein et al. 2002; Bokshi et al. 2003; Zhu et al. 2003). Further, foliar application of BTH to tomato foliage has been shown to decrease leafminer and whitefly insect densities (Inbar et al. 1998; Nombela et al. 2005) and aphid population growth (Boughton et al. 2006); however, SAR activation by BTH in tomato or cotton foliage does not appear to influence larval survival or growth rate of *Helicoverpa zea* or *H. armigera* (Stout et al. 1999; Inbar et al. 2001).

Because of the efficacy of BTH in protecting plants against pathogen infection in several cropping systems, this elicitor, along with other SAR activators, may be used with increasing frequency in IPM systems (reviewed in Vallad and Goodman 2004).

One effect of SAR induction is inhibition of chewing herbivore responses mediated by jasmonic acid, referred to as the jasmonic acid-dependent wound pathway (reviewed in Felton and Korth 2000; Thaler et al. 2002; Cipollini et al. 2004). Lepidopteran larvae feeding on naïve plants induce a variety of directly toxic and/or antinutritive compounds which can reduce larval growth rate (reviewed in Duffey and Stout 1996; Bi et al. 1997; Chen et al. 2005; reviewed in Felton 2005). Since SAR
activation may be antagonistic with induction of jasmonate-dependent wound responses, plants previously challenged by pathogens may be more suitable hosts for lepidopteran larvae than plants previously damaged by chewing herbivores (Stout et al. 1999; Inbar et al. 2001). While chewing herbivores can induce plant responses that reduce larval growth, plant responses to damage by herbivores can modulate susceptibility of these same herbivore species to pathogens, by increasing or decreasing susceptibility, depending on the system (Ali et al. 1998; Hoover et al. 1998b). However, the compatibility of SAR with natural enemies of lepidopterans has not been previously reported.

Baculoviruses are a group of orally-infectious, dsDNA viruses of arthropods, most commonly infecting lepidopteran larvae (reviewed in Bonning 2005). The family Baculoviridae is divided into the genera Granulovirus and Nucleopolyhedrovirus (NPV) (van Regenmortel et al. 2000). NPVs are found in nature as environmentally-resistant proteinaceous occlusion bodies (OB) (reviewed in Bonning 2005). Susceptible larvae become infected when they consume at least one OB, usually on host plant foliage. Upon ingestion, the OB is degraded in the alkaline midgut of the host (Pritchett et al. 1982), releasing occlusion-derived virions, which pass through the peritrophic matrix and infect midgut epithelial cells (Granados and Lawler 1981). NPVs replicate in the nucleus of infected cells; progeny virions produced early in the infection cycle bud out from infected midgut cells (Granados and Lawler 1981) and enter associated tracheolar cells, spreading the infection out from the midgut (Engelhard et al. 1994). In lepidopteran hosts, most tissues become infected (Vail and Vail 1987) and the virus eventually kills the larva (reviewed in Volkman 1997).
Most baculoviruses are highly host specific; infecting, at the extremes, from one to forty lepidopteran species (reviewed in Cory and Myers 2003). Because of their high degree of host specificity, baculoviruses have been considered candidate pest control agents (reviewed in Szewczyk et al. 2006). The potential of baculoviruses as bioinsecticides has not been fully realized, due in part to the high cost of production and their slow speed of kill. As both of these issues are addressed, baculoviruses are likely to be implemented more frequently as a part of integrated pest management (IPM) strategies. Thus, it is important to assess how baculoviral efficacy is influenced by other components of IPM. In this report, we characterize how baculoviral pathogenesis to cotton-fed Heliothis virescens larvae is affected by induction of systemic acquired resistance.

Herein, we characterized changes in cotton foliar peroxidase and phenolics following SAR induction by BTH and assessed the influence of SAR induction on efficacy of the insect pathogen AcNPV to H. virescens larvae. Although foliar peroxidase activity and total phenolics were significantly increased following SAR induction, consumption of induced foliage did not affect H. virescens pupal weight or susceptibility to viral infection, compared with untreated foliage.

**Materials and Methods**

**Insects.**

_Heliothis virescens_ eggs were acquired from the North Carolina State University Insectary, from a colony started in 1997 (Raleigh, NC). Larvae were grown in groups through the third stadium on semisynthetic diet (Southland Products Inc., Lake Village,
AR) in wax-lined paper cups (Xpedx, Harrisburg, PA) at 25°C and a 16L:8D photoperiod.

**Plants.**

Cotton, *Gossypium hirsutum* cv. Maxxa, seeds were donated by the California Planting Cotton Seed Distributors (Bakersfield, CA). Seeds were sown in sterile soil in plastic pots, fertilized at planting with slow-release 14-14-14 fertilizer, and grown in an Conviron® environmental chamber under a day:night temperature regime of 29:24°C and a 16L:8D photoperiod.

BTH (Actigard®) was provided by Syngenta/Novartis (Greensboro, NC). A 0.056 g per L solution of BTH (Actigard® 50WG) in ultrapure water was sprayed to run-off on upper leaf surfaces of six-node cotton plants. Control plants were left untreated, sprayed with ultrapure water or sprayed with a 0.28 g per L solution of diatomaceous earth. Diatomaceous earth is an inactive ingredient of the BTH wettable granule formulation; the latter treatment is equivalent to 50% the concentration of 0.56 g per L BTH, a 10 fold higher concentration of BTH than was applied to foliage. Plants to be treated were removed from the chamber for application of solutions to prevent drift to control plants. BTH is not labeled for use on cotton; 0.056 g per liter was chosen based on the tomato label rate of 0.14 g per liter per hectare.

Four days after treatment, the top two fully-expanded leaves of plants were removed. A sub-sample of intact leaves from all treatment groups was frozen in liquid nitrogen and stored at -80°C for peroxidase activity and phenolic assays. Remaining foliage from untreated and BTH-treated plants was fed to larvae, to determine the influence of SAR activation on larval mass and mortality.
**Foliar assays.**

Peroxidase activity was measured in BTH-treated, diatomaceous earth-treated and untreated cotton foliage using a guaiacol/hydrogen peroxide substrate by measuring the change in absorbance at OD$_{470}$, as described previously (Bi et al. 1997), except the assay was modified for use with a microplate reader. Peroxidase activity is reported as change in absorbance, with one unit of activity = 0.001 $\Delta$OD$_{470}$ per mg foliage per minute. Total phenolics were measured using the Folin-Ciocalteau reagent with caffeic acid as the standard at OD$_{720}$ (Singleton and Rossi 1965). Catecholic phenolics were measured using 0.5% diphenylborate reagent and chlorogenic acid and rutin as standards at OD$_{390}$ and OD$_{440}$, respectively, on a SpectraMax 190 (Molecular Devices, Sunnyvale, CA) (Broadway et al. 1986; Hoover et al. 1998a).

**Influence of SAR induction on larval weight gain.**

Newly-molted fourth instar *H. virescens* larvae were transferred individually to 30 mL plastic cups containing artificial diet or foliage from BTH-treated or untreated cotton plants and allowed to feed *ad libitum*; larvae were maintained at 28°C and fresh foliage was provided as needed. Mass was recorded at pupation; 31-38 larvae were weighed per treatment group.

**Virus preparation.**

AcNPV-hsp70/lacZ, provided by Suzanne Thiem (Michigan State University, East Lansing, MI) was amplified in *H. virescens* larvae (Thiem unpublished). Occlusion bodies (OB) were harvested from virus-killed cadavers and partially purified as described.
previously (Hoover et al. 1995), quantified using a hemacytometer, and stored at 4°C in neutrally buoyant 60% glycerol with 0.002% sodium azide (Engelhard et al. 1994).

**Influence of SAR induction on viral mortality in *H. virescens*.**

Newly-molted fourth instar *H. virescens* larvae were transferred individually to a 30 mL plastic cup containing artificial diet or foliage from BTH-treated or untreated cotton plants and allowed to feed for eight hours. Eight hours post-molt was used because it was the earliest time point at which the midgut was full of plant material or artificial diet for all larvae, based on the time at which frass was produced. The susceptibility of *H. virescens* larvae to fatal viral infection decreases as larvae age within an instar, so controlling for age post-molt is essential (Washburn et al. 1995). Larvae were then microinoculated *per os* with 95 OBs of AcNPV-hsp70/lacZ in a 1 μl aliquot using a PAX 100 microapplicator (Burkard Scientific, Middlesex, UK) holding a 1 mL tuberculin syringe with 32-gauge blunt needle (Popper Precision Instruments, Inc.; Lincoln, RI). Following inoculation, larvae were fed artificial diet or foliage from BTH-treated or untreated cotton plants. Twenty-five to 50 larvae were inoculated per treatment group. Insects were maintained at 28°C and checked daily for mortality or pupation. All cotton-fed larvae were transferred to artificial diet after molting to the fifth instar for ease of handling. This transfer does not influence mortality, since larvae clear midgut infections during the first molt following viral challenge (Washburn et al. 1995). Two trials were performed with artificial diet and BTH cotton-fed larvae; one trial was performed with larvae fed untreated cotton.
Statistics.

Data were analyzed using SAS (SAS Institute, v. 9.1.3). The general linear model (proc GLM) was used to assess the influence of SAR induction on foliar peroxidase activity, phenolics and pupal weight. Analysis of variance for categorical data (proc CATMOD) was used to assess the effect of food, SAR induction and their interactions on larval mortality (Zar 1999).

Results

Application of BTH increased foliar peroxidase activity 2.5 fold (F = 4.70, p = 0.0261; Fig 1) and total phenolics 1.3 fold (F = 21.13, p = 0.0442; Table 1), compared with untreated plants, but did not affect the concentration of catecholic phenolics (F = 0.62, p = 0.5143; Table 1).

Consumption of foliage from the beginning of the fourth instar to pupation reduced pupal mass six fold (F = 95.24, p < 0.001), compared with artificial diet, but there was no influence of BTH treatment on pupal weight (Fig 2). Ingestion of cotton foliage prior to inoculation with AcNPV reduced larval mortality 1.3 fold, compared with ingestion of artificial diet ($\chi^2 = 13.97, p = 0.002$), but BTH treatment had no effect ($\chi^2 = 1.19, p = 0.2761; Fig 3$).

Discussion

Consistent with previous reports, SAR activation by foliar application of BTH increased foliar peroxidase activity in cotton and consumption of induced foliage did not affect *H. virescens* pupal mass, compared with untreated foliage (Inbar et al. 2001). In
our study, however, induction of SAR did not interfere with performance of the microbial control agent, AcNPV. This finding suggests that the use of baculoviral pathogens against lepidopteran larvae is compatible with the induction of SAR.

To our knowledge, changes in foliar phenol  ic levels following activation of the SAR pathway by BTH have not been previously reported. We observed that induction of SAR significantly increased total foliar phenolics, but did not alter the level of catecholic phenolics. Efficacy of baculoviral pathogens to lepidopteran larvae may be influenced by foliar phenolics (Felton et al. 1987; Felton and Duffey 1990; Ali et al. 1999); in particular, catecholic phenolics have been shown to decrease baculoviral infectivity in vitro (Felton and Duffey 1990). Thus, the absence of an SAR-induced effect on *H. virescens* larval mortality is consistent with the absence of a change in catecholic phenolics following BTH application.

Despite measuring a significant increase in foliar peroxidase following BTH application, we did not observe a reduction in *H. virescens* larval survival, pupal mass or susceptibility to baculoviral infection (Dowd and Lagrimini 1997; Behle et al. 2002). This result contrasts with previous reports in which cotton foliar peroxidase activity was an important predictor of baculoviral pathogenicity (Hoover et al. 1998a; Hoover et al. 1998b). However, several differences exist in the methods used between our study and these previous reports. In particular, Hoover et al. increased foliar peroxidase activity either by topical application of a crude extract of peroxidase from cotton foliage (1998a) or by induction in response to prior herbivory (1998b). In this study, peroxidase activity was increased by activating SAR. Although induction of peroxidase activity (measured as the ability to oxidize guaiacol/H₂O₂) was shown in each of these studies, the specific
isozymes induced in response to herbivore feeding or as part of SAR could be different, contributing to differences in experimental outcome (Allison and Schultz 2004). In addition, SAR and the JA-dependent wound pathways are likely to induce a different suite of phytochemicals in addition to peroxidases, which, acting together, produce significantly different outcomes in tritrophic systems.

In summary, the inability of foliage from BTH-induced cotton plants to significantly influence viral mortality suggests that higher foliar peroxidases may not necessarily modulate baculoviral efficacy. Application of BTH is unlikely to adversely affect the performance of baculoviruses; activators of SAR, including BTH, and baculoviruses are likely to be compatible components of an IPM system.
References


Table 5.1. Phenolic concentrations in control (water) and BTH-treated cotton foliage. Mean (SEM) concentrations of catecholic and total phenolics measured at 96 hours post spray. Application of BTH did not significantly alter the amount of catecholic phenolics ($F = 0.62$, df = 1, $p = 0.5143$), but did significantly increase total foliar phenolics ($F = 21.13$, df = 1, $p = 0.0442$); N = two plants.

<table>
<thead>
<tr>
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<th>Catecholic phenolics (μmol/g foliage)</th>
<th>Total phenolics (μmol/g foliage)</th>
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<tbody>
<tr>
<td>Water treated</td>
<td>11.47 (0.91)</td>
<td>24.07 (1.22)</td>
</tr>
<tr>
<td>BTH treated</td>
<td>12.22 (0.26)</td>
<td>31.26 (0.98)*</td>
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**Figure 5.1. Influence of diatomaceous earth and BTH application on cotton foliar peroxidase activity.** Mean peroxidase activity measured at 96 hours post spray, shown as change in activity per mg of cotton foliage per minute. Application of BTH significantly increased foliar peroxidase activity ($F = 4.70$, df = 2, $p = 0.0261$), compared with no treatment. Error bars represent one standard error of the mean; $N =$ two plants. Small letters indicate significant differences between treatments.
Figure 5.2. Influence and untreated and BTH-treated cotton foliage on *H. virescens* pupal mass. Mean pupal mass of *H. virescens* fed artificial diet or foliage from untreated or BTH-treated cotton plants from fourth instar to pupation. Food significantly influenced pupal weight; insects fed artificial diet were heavier than those fed foliage from untreated or BTH-treated cotton plants (F = 95.24, df = 2, p < 0.001). Consumption of foliage from BTH-treated plants did not differentially influence pupal weight, compared with foliage from untreated plants. Error bars represent one standard error of the mean; N = 31-38 larvae per treatment. Small letters indicate significant differences between treatments.
Mean percentage mortality of *H. virescens* larvae fed artificial diet or foliage from untreated or BTH-treated cotton plants prior to AcNPV inoculation. Food consumed before viral inoculation significantly influenced larval mortality; insects fed artificial diet were more likely to die than those fed foliage from untreated or BTH-treated cotton plants ($\chi^2 = 13.97$, df = 1, $p = 0.002$). Consumption of foliage from BTH-treated plants did not significantly affect larval mortality, compared with foliage from untreated plants ($\chi^2 = 1.19$, df = 1, $p = 0.2761$). Error bars represent one standard error of the mean; N = 25-50 larvae per treatment per experimental trial. Experiments were replicated twice each for artificial diet and BTH cotton-fed larvae and once for untreated cotton-fed larvae.
Chapter 6. Intraspecific variation in *Heliothis virescens* susceptibility to *Autographa californica* nucleopolyhedrovirus

Abstract

Baculoviruses are orally-infectious pathogens of arthropods; all lepidopteran larvae are susceptible to at least one species of baculovirus. Differences in susceptibility to mortal baculoviral infection have been described among lepidopteran species, but have not been reported among unselected conspecifics from different colonies, despite the potential for genetic variability in pathogen resistance within a species. We examined the influence of food consumed prior to viral inoculation on mortality caused by *Autographa californica* nucleopolyhedrovirus (AcNPV) in *Heliothis virescens* larvae from three different lab colonies, designated as NCSU97, AGR and NCSU02. The NCSU97 colony did not have field-collected individuals added since its establishment five years prior, making this colony substantially inbred. In contrast, the AGR and NCUS02 colonies had field-collected individuals added within a year of experimentation. We propose that genetic differences among these colonies influenced larval susceptibility to baculoviral infection. Larvae from the AGR and NCSU02 colonies fed artificial diet were significantly less susceptible to AcNPV infection than artificial diet-fed NCSU97 larvae; the mechanism regulating this decreased susceptibility appears to be restricted to the midgut environment. Further, while cotton-fed NCSU97 were less susceptible to AcNPV infection than artificial diet-fed NCSU97 larvae, the mortality of AGR and NCSU02 larvae was not influenced by cotton foliage, under our experimental conditions. We
suggest that these differences in larval susceptibility among colonies reflect differences in larval midgut physiology.

**Introduction**

Although the pathogenicity of baculoviral strains is known to vary within a given insect host species (Hodgson et al. 2001), variability in susceptibility to baculoviruses among different insect strains has not been described, except in cases where insects have been selected for resistance to NPV (Piubelli et al. 2006). The family Baculoviridae includes the granuloviruses (GV) and nucleopolyhedroviruses (NPV) (van Regenmortel et al. 2000). NPVs occur in nature as environmentally-resistant polyhedral occlusion bodies. A susceptible lepidopteran larva becomes infected when it consumes an NPV occlusion body (OB). The proteinaceous OB degrades in the alkaline larval midgut, releasing occlusion-derived virions (ODV) (Pritchett et al. 1982). These virions pass through the peritrophic matrix, fusing with and infecting midgut epithelial cells (reviewed by Bonning 2005). The virus replicates within the nucleus of the infected cell, producing progeny virions (Granados and Lawler 1981). These virions, called budded virions, bud out through the cell membrane and infect the tracheolar cells associated with the infected midgut cell, spreading infection throughout the host and eventually killing it (Engelhard et al. 1994, reviewed by Bonning 2005).

Successful baculoviral infection is contingent upon a variety of host plant and insect factors. For example, viral efficacy decreases when larvae are fed particular host plants prior to, or in conjunction with, viral OBs (reviewed by Duffey et al. 1995 and Cory and Hoover 2006). Also, insect hosts can thwart viral infection at multiple points in
the infection pathway. The peritrophic matrix may act as a barrier to released ODV, decreasing successful interactions between virions and midgut cells (Ch 3). Larvae can slough infected midgut cells, removing viral inoculum from the midgut (Washburn et al. 1998). Further, once infection is established outside of the midgut, hemocytes of resistant larvae may actively clear or prevent the spread of infection (Trudeau et al. 2001).

Differences in susceptibility to mortal baculoviral infection have been described among lepidopteran species. However, despite the potential for variability in pathogen resistance within a species, similar differences in susceptibility to viral infection have not been reported between conspecifics from different colonies, except in the case of selection for viral resistance (Gonzalez-Cabrera et al. 2001; Ali et al. 2006; Piubelli et al. 2006). We evaluated susceptibility of *Heliothis virescens* larvae from three lab colonies to *Autographa californica* multiple nucleopolyhedrovirus (AcNPV). The colonies examined had been maintained for different periods of time, with and without field-collected individuals added. Also, because previous work showed that susceptibility of *H. virescens* larvae to viral infection is influenced by the host plant consumed along with viral inoculum (Ali et al. 1998; Hoover et al. 1998a), we tested how ingestion of cotton foliage prior to baculoviral inoculation influences mortality of larvae from these colonies.

**Materials and Methods**

**Insects.**

*Heliothis virescens* from three colonies were used in this study. NCSU97 eggs were obtained from the North Carolina State University Insectary (Raleigh, NC) from a
colony established in 1997 without addition of field-collected individuals. AGR eggs were obtained from the Agripest Company (Zebulon, NC) from a colony established in 1996 with field-collected individuals added every two years. NCSU02 eggs were obtained from the North Carolina State University Insectary (Raleigh, NC) from a colony established in 2002 from field-collected insects. The NCSU97 colony had gone through 60 generations, the AGR colony 70 generations, and the NCSU02 colony 5 generations when the experiments were performed. Larvae were grown individually on semisynthetic diet (Southland Products Inc., Lake Village, AR) in 30 mL plastic cups (Xpedx, Harrisburg, PA) at 25°C and a 16L:8D photoperiod.

Plants.

Cotton, *Gossypium hirsutum*, seeds were donated by the California Planting Cotton Seed Distributors (Bakersfield, CA). Seeds were sown in sterile soil in plastic pots, fertilized at planting with slow-release 14-14-14 fertilizer, and grown in an environmental chamber under a day: night temperature regime of 29:24°C and a 16L:8D photoperiod. When plants reached the six to eight leaf stage, the upper two or three leaves were harvested for use in experiments.

Virus preparation.

AcNPV-hsp70/lacZ, provided by Suzanne Thiem (Michigan State University, East Lansing, MI) was amplified in *H. virescens* larvae (Thiem unpublished, Engelhard et al. 1994). Occlusion bodies (OB) were harvested from virus-killed cadavers and partially purified as described previously (Hoover et al. 1995), quantified using a hemacytometer,
and stored at 4°C in neutrally buoyant 60% glycerol with 0.002% sodium azide (Engelhard et al. 1994). Budded virus particles (BV) were harvested from cell media following infection of Sf21 cells grown in TnMFH+9% FBS, and were quantified by plaque assays of Sf21 cells (O’Reilly et al. 1994).

Influence of cotton foliage and H. virescens colony on viral mortality.

Newly-molted fourth instar H. virescens larvae were transferred individually to a 30 mL plastic cup containing artificial diet or cotton foliage and allowed to feed for eight hours. Eight hours post-molt was used because it was the earliest time point at which the midgut was full of plant material or artificial diet for all larvae, based on the time at which frass was produced. The susceptibility of H. virescens larvae to viral infection decreases as larvae age within an instar, so controlling for age post-molt is essential (Washburn et al. 1995). Larvae were then microinoculated per os with 80 OBs or injected intrahemocoelically with 0.2 PFUs of BV of AcNPV-hsp70/lacZ (C6), in a 1 μl aliquot using a PAX 100 microapplicator (Burkard Scientific, Middlesex, UK) holding a 1 mL tuberculin syringe. A 32-gauge blunt needle was used for OB inoculations and a 32-gauge sharp needle for BV injections (Popper Precision Instruments, Inc.; Lincoln, RI).

Following inoculation, larvae were fed artificial diet or cotton foliage. Thirty larvae were inoculated per treatment, except for one replicate for which 60 larvae were inoculated per treatment. Insects were maintained at 28°C and checked daily for mortality or pupation. Larvae that died prior to day four were considered handling deaths. Mortality by virus does not occur in fourth instar H. virescens before day 4,
based on autopsies of insects that died prior to day 4 post-inoculation (Plymale, unpublished data). All cotton-fed larvae were transferred to artificial diet after molting to the fifth instar for ease of handling. This transfer does not influence mortality, since larvae clear midgut infections during the first molt following viral challenge (Washburn et al. 1995). One to three experimental replicates were performed of OB inoculations, while one or four replicates were performed of BV injections. The susceptibility of NCSU02 larvae fed cotton foliage and injected with BV was not assessed. Larvae from different colonies were assayed in experiments on the same day or within one week, using the same virus preparation.

Statistics.

Data were analyzed using SAS (SAS Institute, v. 9.1.3). Analysis of variance for categorical data (proc CATMOD) was used to assess the effect of *H. virescens* colony, food and their interactions on larval mortality (Zar 1999).

Results

Using *H. virescens* larvae from three lab colonies, we assessed the influence of food consumed prior to viral inoculation on larval mortality by virus. There was a significant interaction between colony and food ingested when larvae were inoculated with OBs ($\chi^2 = 8.04, p = 0.0179$). NCSU97 larvae fed cotton foliage prior to oral virus inoculation experienced a 30% reduction in mortality, compared with larvae fed artificial diet ($\chi^2 = 19.66, p < 0.0001$; Fig 1). In contrast, food consumed prior to oral virus inoculation did not significantly influence mortality of AGR or NCSU02 larvae (AGR $\chi^2$
However, mortalities from virus of AGR and NCSU02 larvae fed either artificial diet or cotton foliage were equivalent to those of cotton-fed NCSU97 larvae (Fig 1). Neither insect colony nor food consumed prior to viral challenge influenced mortality when larvae were injected with budded virus (BV) (colony $\chi^2 = 3.40, p = 0.1826$; food $\chi^2 = 3.19, p = 0.0741$; Fig 2).

**Discussion**

AGR and NCSU02 larvae fed artificial diet displayed significantly lower mortality than artificial diet-fed NCSU97 following inoculation with AcNPV occlusions, suggesting that the AGR and NCSU02 larvae possess a level of inherent resistance to baculoviral infection. In addition, the NCSU97 colony experienced lower mortality in cotton-fed insects, while the other two colonies did not show this effect of foliage on susceptibility to virus. Insects from the AGR and NCSU02 colonies were likely less inbred than those from the NCSU97 colony. Both the AGR colony and NCSU97 colonies were established several years prior to these experiments, but the AGR colony was refreshed every two years with new field-collected individuals, whereas the NCSU97 colony was not. The NSCU02 colony was established from field-collected insects less than a year before these experiments were performed.

The combination of genetic variability in an insect colony population and variability in the food source ingested at the time of infection may interact to produce a greater range of responses to exposure to virus as variability in these factors increase. The three colonies had a similar response to exposure to virus when fed on cotton, but the NCSU97 colony experienced higher mortality when fed on artificial diet. If we assume
that the NCSU97 colony is less genetically diverse and that artificial diet is a relatively homogenous food source, and if this colony is more susceptible to the virus in the first place, then the observed higher percentage mortality on artificial diet may result from a unilateral response to the virus on an invariant food source. In contrast, adding variability in plant quality to the system (inherent differences in phytochemical levels from leaf to leaf and plant to plant) would result in variability in individual responses of the NCSU97 colony to virus infection. Given the ability of cotton to reduce mortality by virus, this would manifest as a lower overall percentage mortality. Thus, the assumed higher genetic variation within the AGR and NCSU02 colonies would generate a range of susceptibilities to viral infection, manifested as decreased viral susceptibility, even on the more homogenous food source artificial diet.

Differences in virus-insect colony-plant interactions appear to be related to differences in midgut physiology. Inoculation with budded virus, which bypasses the midgut, eliminated colony and plant influences on host response to virus infection. Decreased susceptibility of the AGR and NCSU02 colonies could be the result of differences in peritrophic matrix structure and permeability to virions, differences in the molecular structure of midgut cell receptors, or subtle differences in midgut pH or enzymes. Also, since the AGR and NCSU02 larvae may be genetically more similar to field insects, their midgut physiology may already be “primed” to deal with plant secondary compounds, and thus, their midgut environment may not be as influenced by ingested plant material.

Midgut cell sloughing and peritrophic matrix structure are two aspects of midgut physiology known to be influenced by ingested plant material. Increased midgut cell
sloughing in cotton-fed *H. virescens*, compared with larvae fed artificial diet, has previously been proposed as a potential mechanism of baculoviral resistance (Hoover et al. 2000). The midguts of AGR and NCSU02 larvae may have a higher basal rate of cellular turnover, removing viral inoculum from the larvae before infections can establish outside of the midgut. The midguts of NCSU97 larvae may have a lower base cell turnover rate, but this sloughing rate may be increased to a rate similar to that of AGR and NCSU02 larvae following ingestion of oxidatively-active cotton foliage (Johnson and Felton 1996). In addition to potentially increasing midgut cell sloughing, ingested foliage may increase peritrophic matrix (PM) thickness, decreasing interactions between occlusion-derived virions and midgut cells (Ch 3). After several generations in culture on artificial diet, the PM of NCSU97 larvae may be thinner than that of AGR or NCSU02 larvae.

Although food did not influence mortality of AGR and NCSU02 larvae in this instance, field and greenhouse-grown plant material have repeatedly been documented to affect viral mortality of *H. virescens* (Ali et al. 1998; Hoover et al. 1998b; Hoover et al. 2000). These previous studies used *H. virescens* from the University of Arkansas colony or the USDA Insectary in Stoneville, MS; the history of both colonies is unknown. In addition, the methods used in these previous studies differed from our method in many respects. Developmental resistance within the instar was not controlled for in previous studies and the method of virus delivery was different. These authors used surface contamination of foliage with viral occlusions and, thus, plant surface/virus interactions that can influence viral persistence (McLeod et al. 1977; Young et al. 1977) and prolonged ingestion of foliage with inoculum were factors in these previous studies.
Although our method of virus delivery as a pulse after several hours of ingestion of foliage does not reflect how virus is acquired in the field, it was necessary to permit us to control for developmental resistance in order to ascertain inherent colony differences in susceptibility. In *H. virescens*, differences of only a few hours in age post-molt at the time of viral challenge results in dramatic differences in susceptibility to AcNPV (Washburn et al. 1995).

In conclusion, we present here the first evidence of intraspecific differences in susceptibility to baculoviral infection among non-selected lab colonies of *H. virescens*. The less inbred AGR and NCSU02 colonies were significantly less susceptible to AcNPV infection than the NCSU97 colony; the mechanism regulating this decreased susceptibility appears to be restricted to the midgut environment and may be to the result of a number of midgut differences, including different rates of midgut cell sloughing and/or PM structure. Our results demonstrate that insect source may be a significant variable in experimental outcomes and we stress the importance of being familiar with the background of colony insects used in experiments.
References


Bacillus thuringiensis toxins among unselected strains of Plutella xylostella.

Applied and Environmental Microbiology 67: 4610-4613.


Figure 6.1. Influence of colony source and cotton foliage on mortality of *H. virescens* larvae inoculated with occlusion bodies. Mean percentage mortalities of *H. virescens* larvae from three colonies fed artificial diet or cotton foliage, then orally inoculated with AcNPV-hsp70/lacZ occlusions. Error bars represent one standard error of the mean (for experiments with error bars, N = 2 or 3 replicates, with 28-30 larvae/replicate). Mortality was significantly influenced by *H. virescens* colony; NCSU97 larvae were more likely to die than AGR or NCSU02 larvae ($\chi^2 = 9.32$, df = 2, $p = 0.0095$). Overall, food ingested prior to inoculation did not significantly influence mortality ($\chi^2 = 3.61$, df = 1, $p = 0.0573$), but there was a significant interaction between colony and food ($\chi^2 = 8.04$, df = 2, $p = 0.0179$). Larvae from the NCSU97 colony fed artificial diet were more likely to die from oral viral inoculation than those fed cotton foliage.
foliage. In contrast, food did not differentially influence mortality of AGR or NCSU02 larvae.
Figure 6.2. Influence of colony source and cotton foliage on mortality of H. virescens larvae injected with budded virus. Mean percentage mortalities of H. virescens larvae from three colonies fed artificial diet or cotton foliage, then injected with AcNPV-hsp70/lacZ budded virus. Error bars represent one standard error of the mean (for experiments with error bars, N = 4 replicates, with 28-30 larvae/replicate). Larval mortality was not significantly influenced by colony or food ($\chi^2 = 3.40$, df = 2, $p = 0.1826$; $\chi^2 = 3.19$, df = 1, $p = 0.0741$, respectively).
Chapter 7. Conclusion

Interactions between insect herbivores and the pathogens that infect them can be modulated by host plants. Ingestion of foliage simultaneously with baculovirus exposure has been shown to reduce viral efficacy in several larval lepidopterans (reviewed in Duffey et al. 1995; Cory and Hoover 2006). *Heliothis virescens* F. larvae fed cotton (*Gossypium hirsutum* L.) foliage are less susceptible to mortal infection by *Autographa californica* multiple nucleopolyhedrovirus (AcNPV) received *per os* than larvae fed iceberg lettuce or artificial diet (Hoover et al. 1998a). Although it has been suggested that this cotton-mediated inhibition of virus disease involves multiple mechanisms, the specific mechanisms underlying inhibition of disease were not fully described (Hoover et al. 1998a; Hoover et al. 2000). This dissertation provides the first detailed study of how ingested cotton foliage influences AcNPV pathogenesis by decreasing primary midgut infections and ultimately reducing *H. virescens* larval mortality. Moreover, these results provide the first evidence showing that peritrophic matrix structure is significantly influenced by food consumed and describe a protective role for the peritrophic matrix in baculoviral infection.

The primary objective of this dissertation was to characterize the influence of ingested cotton foliage on pathogenesis of *Autographa californica* nucleopolyhedrovirus (AcNPV) in *H. virescens* larvae. Chapter 2 details the differences in pathogenesis observed in fourth instar *H. virescens* larvae fed artificial diet or cotton foliage. Current hypotheses of foliar interference with baculoviral infection may be divided into two groups of biological outcomes as (1) prevention of primary midgut infections and/or (2) decreased establishment of systemic infections. Primary infections may be decreased if
fewer virions are released from occlusions, or if those released are unable to successfully interact with midgut cells (Felton and Duffey 1990a; Feldman et al. 1999) (Fig 1 A-D). In addition to influencing the establishment of primary infections, ingested foliage may also increase midgut cell sloughing, potentially decreasing establishment of systemic infections (Hoover et al. 2000) (Fig 1 E-F). As reported in Chapter 2, the proportion of artificial diet-fed larvae that were virally-infected throughout the early time points (12-20 hours post-inoculation) was consistently two to four-fold greater than the proportion of virally-infected cotton-fed larvae, suggesting that consumption of cotton foliage interferes with the ability of the virus to establish midgut infections. In contrast, the proportions of artificial diet and cotton-fed larvae possessing systemic viral infections were similar, indicating that ingested cotton foliage did not influence the spread of infection beyond the midgut. Taken together, these data suggest that cotton foliage inhibits mortality from baculovirus either through direct interactions with virions and/or by altering midgut physiology, thereby reducing the ability of the virus to establish infections in the midgut, not by increasing sloughing of infected midgut cells (Hoover et al. 2000).

In this study, larvae were fed artificial diet or cotton foliage, and then orally inoculated with virus. While this technique allows for detailed study of viral pathogenesis, it does not resemble the natural method of exposure, which is consumption of viral occlusions on host plant foliage. The deposition and subsequent consumption of OBs on host plant foliage allows interactions between virus, plant and insect to take place outside of the larval gut. In the case of cotton, viral occlusions may be negatively affected by high pH foliar exudate (Elleman and Entwistle 1985), larval saliva and/or
larval regurgitant. Specifically, hydrogen peroxide, produced at the feeding site by salivary glucose oxidase (Eichenseer et al. 1999; Peiffer and Felton 2005), may increase foliar redox cycling, perhaps resulting in binding of phenolics to occlusion bodies (Felton and Duffey 1990b) and/or oxidation of the polyhedrin protein.

In addition to the experiments reported in Chapter 2, other experiments were performed in which ingested cotton foliage did not reduce larval mortality. The plants used in these experiments were grown under different light sources and had different growth rates and foliar characteristics. Foliar peroxidase values varied substantially among different growth environments, and, although they were not measured, it is likely that other foliar compounds varied as well. These results reflect the influence of variation in host plant quality as a component of variation in larval susceptibility to infection.

The experiments described in Chapter 3 were designed to ask whether ingested cotton foliage influenced the PM of *H. virescens* larvae and, if so, whether these alterations influence viral pathogenesis. Although multiple protective functions have been reported for the PM, how ingested foliage influences PM physical structure has not been previously documented. Food-mediated changes to the PM of *H. virescens* larvae may provide larvae some protection from viral infection (Fig 1 C-D). As described in chapter 3, the PM of cotton-fed *H. virescens* larvae was found to be twice as thick as the PM of diet-fed larvae, based on TEM images of the PM, and was more resistant to tearing. In addition to protective roles, the PM also functions in digestion, compartmentalizing digestive enzymes and separating undigested from digested food. Although I suspect that the thickened PM observed in cotton-fed larvae does not
adversely affect digestion, the influence of a PM thickening on digestion could be determined in future studies using the Waldbauer coefficients of digestibility (Waldbauer 1968).

The thickened, tougher PM observed in cotton-fed larvae may be formed primarily to protect larval midgut cells from damage by ingested foliage (Summers and Felton 1996; reviewed in Barbehenn 2001; Barbehenn and Stannard 2004). There are a number of ways whereby ingested foliage may influence PM structure. For example, ingested foliage may stimulate increased synthesis of PM components, leading to a thicker PM. Also, ingested foliage may alter the structure of previously-formed PM through cross-linking between foliar and PM components. For instance, oxidized phenolics, lipid peroxides, free radicals and/or reactive oxygen species generated in the midgut during phenolic redox cycling and/or tannins found in ingested foliage may cross-link PM proteins. Further, a decreased rate of food movement in cotton-fed larvae, compared with larvae fed artificial diet, may result in physical accumulation (thickening) of the PM if the PM is produced at a constant, food-independent rate. Finally, ingested foliar phenolics may inhibit serine proteases (Jedinak et al. 2006), potentially reducing degradation of the PM, compared with artificial diet-fed larvae.

While protecting the *H. virescens* larval midgut from damage by ingested plant material, a thickened PM may secondarily protect the larva from ingested pathogens. As described in Chapter 3, resistance of cotton-fed *H. virescens* larvae to baculovirus was reduced when the PM was disrupted by the metalloprotease enhancin prior to AcNPV inoculation, suggesting that the PM of cotton-fed larvae is a major factor in baculoviral resistance. The influence of the PM on viral efficacy was further characterized through a
pathogenesis study. Disruption of the PM by enhancin resulted in earlier infection in artificial diet-fed larvae, suggesting that disruption of the putative PM barrier allowed virions immediate access to susceptible midgut cells. Treatment with enhancin also increased the proportion of virally-infected cotton-fed larvae at eight hours post-inoculation. Despite significantly increasing midgut infections, disruption of the PM by enhancin did not influence the spread of infection out of the midgut in larvae fed artificial diet or cotton foliage. Among all treatments, the proportion of $lacZ$ positive larvae at 18 hours post-inoculation was negatively related to PM width. Together, these findings provide compelling evidence that plant-mediated changes in PM structure influence the success of baculoviral infection in $H.\virescens$ larvae and call attention to the PM as a potentially important pest control target.

The observations that ingestion of cotton foliage alters PM structure and reduces midgut viral infections show possible mechanisms of host plant influence on pathogens that infect through the midgut. Foliage-mediated changes to the PM may influence efficacy of other ingested pathogens and understanding the interactions between ingested foliage and insect pathogens is likely to become more important as pathogens experience increasingly widespread use as biocontrol agents. $Bacillus\ thuringiensis$ crystal toxins bind to the PM (Bravo et al. 1992); although the amount of binding has not been correlated with toxin potency, PM disruption by chitinase or enhancin significantly increases toxin efficacy (Morris 1976; Granados et al. 2001). The increased width and assumed reduced permeability of the PM in cotton foliage-fed insects may contribute to the reduction in $Bt$ efficacy observed in cotton-fed $Helicoverpa\ zea$ (Ali et al. 2004).
While the results described in chapters 2 and 3 strongly suggest that the PM influences baculoviral infection, it is important to remember that the PM is only one of many factors determining pathogen success. Plant-mediated inhibition of disease is likely multifaceted and highly interactive. In addition to altering PM structure, ingested foliage may influence virion integrity. Foliar phenolics may interact with viral OBs, reducing OB dissolution and/or infectivity (Felton and Duffey 1990a; Feldman et al. 1999). If ingested foliage reduces OB dissolution, the concentration of PM-degrading enhancing factors available may also be decreased, if these factors are located within the occlusion body. The decreased degradation of a PM thickened by ingested plant materials could result in a formidable barrier to the fewer ODV that may be released if OB dissolution is lessened. It is also possible that hydrogen peroxide, which is highly diffusible, and other reactive products generated by redox cycling could damage midgut cell receptors preventing virions from binding and fusing to initiate midgut infections. Thus, further work is needed to clarify the interactions between insect tissues, ingested plant foliage and viral inoculum.

The impact of other *H. virescens* food plants on viral mortality of developmentally-matched fourth instar was investigated, as described in chapter 4. The influence of selected host plants on *H. virescens* susceptibility to baculoviral infection has been reported previously (Ali et al. 1998; Hoover et al. 1998b); however, researchers did not control for developmental age within the instar, which could have been a confounding factor in the relative susceptibility to virus in these studies. Ingestion of iceberg lettuce prior to inoculation did not significantly influence larval mortality compared with consumption of artificial diet (Hoover et al. 1998b). In contrast, cotton,
tobacco and oakleaf lettuce foliage significantly reduced susceptibility of *H. virescens* larvae when ingested prior to viral inoculation, compared with artificial diet. Cotton and tobacco foliage contain relatively high levels of phenolics, oakleaf lettuce moderate levels and iceberg lettuce low levels (Bi et al. 1997; Hoover et al. 1998b; DuPont et al. 2000). We speculate that the physiological response of larvae to these and other foliar compounds may alter their susceptibility to baculoviral infection (Ojala et al. 2005; Lee et al. 2006). The ability to influence viral efficacy appears to be widespread among *H. virescens* host plants (Ali et al. 1998), and ingested foliage has recently been reported to affect efficacy of *Bt* to several lepidopteran larvae (Ali et al. 2004).

The ecological ramifications of decreased viral efficacy in cotton-fed *H. virescens* on host plant selection are significant. *H. virescens* is highly polyphagous and is a pest of cotton, soybean and tobacco in the United States (Fitt 1989). While herbivores are generally believed to select host plants which optimize their fitness, fitness may be determined by a range of factors, as seen in *H. virescens* (reviewed in Dicke 2000). Larval development time of *H. virescens* fed soybean foliage is shorter than that of larvae fed tobacco or cotton foliage, and *H. virescens* pupal weight is higher in larvae fed soybean or tobacco foliage than larvae fed cotton foliage (Laster et al. 1982; but see Ali et al. 1998). Further, *H. virescens* larvae feeding on cotton squares have a significantly longer larval development time and significantly lower pupal weight than larvae feeding on cotton foliage (Ali et al. 2003). Maternal food stress has been shown to decrease egg provisioning and the ability of progeny to handle food stress (Gould 1988; reviewed in Hunter 2002). Thus, increased *H. virescens* pupal weight may manifest at the population level as higher quality progeny.
Influences on insect growth are not the only avenue by which host plants can affect herbivore fitness. Baculovirus OBs are quickly inactivated on cotton foliage by UV irradiation and dew that collects on the surface of foliage; in contrast, inactivation on soybean foliage by UV irradiation is slower, and soybean dew does not adversely affect OB persistence (tobacco foliage was not tested) (Young and Yearian 1974; McLeod et al. 1977). Thus, *H. virescens* larvae may be more likely to encounter infectious OBs on soybean foliage. While the susceptibility of larvae fed cotton, soybean or tobacco foliage was similar, significantly fewer progeny viral occlusions were produced in cotton-fed larvae than soybean-fed larvae, resulting in a significantly lower total viral activity in cotton foliage-fed larvae (tobacco-fed larvae were not tested) (Ali et al. 2002). Moreover, the susceptibility of larvae fed cotton squares was only slightly less than that of larvae fed cotton foliage, but larvae fed squares produced a smaller number of less infectious progeny viral occlusions than larvae fed cotton foliage (Ali et al. 1998; Ali et al. 2002).

Thus, there may be trade-offs with respect to maximizing fitness for *H. virescens* among host plants. Since larvae may grow better on soybean and tobacco than cotton foliage but the larvae are equally susceptible to virus on foliage from these host plants, natural selection could favor a shift toward preference for soybean or tobacco. However, because viral persistence is lower on cotton than soybean, larvae may encounter a lower effective viral dose on cotton, receiving better protection from infection on cotton. Further, because fewer occlusions are produced in larvae fed cotton foliage than larvae fed soybean foliage (Ali et al. 2002), feeding on cotton may result in a smaller viral reservoir available to infect subsequent generations of *H. virescens*, providing
population-level protection from virus in cotton-fed insects. A similar scenario can be envisioned if *H. virescens* were constrained to the cotton field. *H. virescens* pupal mass is higher in insects fed cotton foliage than those fed cotton squares (Ali et al. 2003).

Further, while the susceptibility to viral infection larvae fed foliage or squares is similar, the number and infectivity of viral occlusions produced is lower in insects fed squares (Ali et al. 2002).

Whereas host plant quality with respect to maximizing growth is probably a more consistent selective force than exposure to orally infectious pathogens, it is possible that *H. virescens* may evolve a preference for soybean or tobacco over cotton foliage and/or cotton foliage over cotton squares. If, however, pressure from viral pathogens was increased by repeated use of a viral insecticide, host plant selection may shift to cotton squares. Nevertheless, host plant selection may be influenced by mortality factors other than pathogens; parasitism rate of *H. virescens* and *H. zea* by *Cardiochiles nigriceps* is lower in cotton than tobacco (Neunzig 1969), perhaps also driving a shift in host plant selection toward cotton.

Although baculoviruses are not widely used as bioinsecticides in the United States at this time, their use as a part of integrated pest management (IPM) strategies may increase as the problems of slow speed of kill and high cost of production are resolved (Szewczyk et al. 2006). Thus, it is important to assess how baculoviral efficacy is influenced by other components of IPM. Systemic acquired resistance (SAR) is a long-lasting response typically induced in reaction to pathogen infection (reviewed in Durrant and Dong 2004). Activation of SAR by the synthetic plant elicitor benzo-(1, 2, 3)-thiadiazole-7-carboxthioic acid S-methyl ester (BTH) provides protection against an array
of bacterial, fungal and viral plant pathogens; consequently, BTH and other SAR activators may be used with increasing frequency in IPM systems (Friedrich et al. 1996; reviewed in Vallad and Goodman 2004). Induction of SAR in cotton foliage by BTH application significantly increased foliar peroxidase activity and total phenolics, compared with untreated plants, but did not affect the concentration of catecholic phenolics, as reported in Chapter 5. Efficacy of baculoviral pathogens to lepidopteran larvae may be influenced by foliar phenolics (Felton et al. 1987; Felton and Duffey 1990b; but see Ali et al. 1999); in particular, catecholic phenolics have been shown to decrease baculoviral infectivity in vitro. Thus, since a change in catecholic phenolics was not observed following BTH application, consumption of SAR-induced foliage was not expected to influence H. virescens larval mortality. While ingestion of foliage from BTH-treated plants reduced larval mortality compared with ingestion of foliage from untreated plants, the difference was not significant. Therefore, activators of SAR, including BTH, and baculoviruses are likely to be compatible components of an IPM system.

The effect of intraspecific variation in H. virescens on AcNPV virulence is described in Chapter 6. Although the pathogenicity of baculoviral strains is known to vary within a given insect host species (Hodgson et al. 2001), variability in susceptibility to baculoviruses among different insect strains has not been described, except in cases where selection for resistance to NPV has been described (Piubelli et al. 2006). As reported in Chapter 6, larvae from the less-inbred strains displayed significantly lower mortality than larvae from the more-inbred strain, when fed artificial diet, suggesting that the less-inbred strains possess a level of inherent resistance to baculoviral infection. In
addition, ingestion of cotton foliage reduced viral mortality only in larvae from the more-inbred strain. These differences in virus-insect colony-plant interactions appear to be related to differences in midgut physiology. Inoculation with budded virus, which bypasses the midgut, eliminated colony and plant influences on host response to virus infection. Decreased susceptibility of larvae from the less-inbred strains could be the result of differences in rates of midgut cell sloughing, differences in peritrophic matrix structure and permeability to virions, differences in the molecular structure of midgut cell receptors, or subtle differences in midgut pH. Further, these results demonstrate that insect source may be a significant variable in experimental outcomes; therefore, it is important to be familiar with the background of insects used in experiments.

In conclusion, the work described in this dissertation will contribute to the fields of insect pathology and biological control, and may have implications for host plant usage and selection by polyphagous insect herbivores. The findings in this dissertation suggest that feeding on plants that favor production of a thicker PM may not only protect the insect from some secondary plant compounds, but also from orally infectious pathogens. The balance of selective forces between those providing protection from pathogens and those maximizing fitness by permitting faster growth rates and higher fecundity will likely tip in the direction of the stronger selective force.

The pathogenesis results described in Chapter 2 verify that baculoviral infection proceeds from the midgut into the tracheal system (Engelhard et al. 1994). Further, the results reported in Chapter 3 provide direct evidence of food-mediated structural changes to the PM and confirm the PM as a protective barrier against ingested pathogens. Although the PM appears to be a barrier to baculoviral infection in *H. virescens*, it likely
acts in concert with other mechanisms to provide protection from ingested pathogens. Future research in this area should attempt to qualify whether ingested foliage reduces OB dissolution and/or infectivity \textit{in vivo} and how important the PM barrier is in blocking ODV.

With respect to biological control, the results produced by this dissertation suggest that baculoviruses could be more widely used in IPM systems, since baculoviral efficacy is not compromised by induction of SAR. The findings reported herein suggest that efficacy of baculoviruses and other ingested pathogens could be greatly improved if a compound was added to degrade the PM. The PM has many protective functions; its disruption would leave insects highly susceptible to naturally-occurring and/or applied pathogens, even those of low virulence. Consequently, the PM is likely to become an important pest control target (Kramer and Muthukrishnan 1997; Hayakawa et al. 2000).
References


Figure 7.1. Possible mechanisms of host plant influence on baculoviral infection. A, C and E represent scenarios in artificial diet-fed larvae; B, D and F plant-fed larvae. A and B show potential affects on OB dissolution; C and D potential affects on the PM; E and F potential affects on midgut cell sloughing.
Appendix A: *Heliothis virescens* colony protocols

Environmental chamber settings:
23-25°C (80°F), 65% RH, 14 hours light:10 hours dark.

Important times:
- Eggs hatch ~2 days after sterilization
- Larvae develop into pupae within 3 weeks
- Adults emerge from pupae within 5-9 days
- Adults begin to lay eggs 2-3 days after emerging

Supplies and suppliers:

Table A.1. Diet ingredients listed with protocol

<table>
<thead>
<tr>
<th>Product Number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>cups</td>
<td>5226413 Xpedx, $300 min order to avoid shipping</td>
</tr>
<tr>
<td>lids</td>
<td>5101707 Xpedx</td>
</tr>
<tr>
<td>Cheesecloth</td>
<td>21910-130 VWR</td>
</tr>
<tr>
<td>Fabric</td>
<td>batiste Wal-Mart</td>
</tr>
<tr>
<td>Sucrose</td>
<td>902978 MP Biomedical</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>194738 MP Biomedical</td>
</tr>
</tbody>
</table>

Notes:
- Observe all sanitation practices listed on the following page. Failure to do so may result in colony collapse.
- Record all colony work in the red colony notebook, not the black experiment notebook.
- Do not use abbreviations or shorthand (aside from initials) in the notebook. Other people may not be familiar with what the notations stand for.
- Use neat handwriting. Others will need to read your entries.
- Write dates as [day] [month] [year], eg. 27 Apr 2005
- Give a written reason in the notebook for any procedural change.
- Record the lot number of diet and any chemical used
- Fold under tape corners!
- Use milliQ water for all solutions
- Wash dishes following the protocol above the sink. Do not use soap unless specifically instructed to do so.
**Heliothis Daily Work List**

_gid Set up 3 trays of neonates from eggs sterilized two days prior
- Ethanol 533 hood before and after use
- Freeze tub containing remaining neonates.
- Wash tub of previously frozen neonates
- Place trays in Hv growth chamber, in filing cabinets. Write harvest date and location of trays on calendar [P C8 (# trays)].
- Submerge empty 2 gallon plastic bags in 10% bleach ice chest. Make sure tops are open.

_gid Feed moths
  - Once moths have emerged, change sponge vials daily, using only autoclaved vials and sterile 16% sucrose + 0.01% sodium chloride
    - Wash sponge vials removed from buckets
    - Wet dried sponges with distilled water (washed the previous day) and place in dried vials
    - Wrap and autoclave assembled, dried sponge vials
    - Wash stoppers
    - Put away dry stoppers and autoclaved sponge vials

_gid Change cloths on buckets.
- Put fabric (not cheesecloth) on buckets once moths have begun to lay eggs.
  - Use sanitized bowls to transport cloths.
- Autoclave cheesecloth and fabric as needed, making sure there is at least enough for the next day.
Sterilize eggs

- Put away dry fabric pieces from previous day
- Completely rinse eggs from fabric and place fabric pieces on 532 “rack” to dry
- Do not save cheesecloth after you have removed eggs; throw it away in the autoclave bag.
- Make sure there is enough egg bleach for the next day.
- Ethanol out hood before removing filter from funnel
- Cut dried, weighed eggs in half. Put half in 533 15°C incubator, half in 532 28°C incubator, with most recent tub on the bottom. Leave the egg tub(s) on the counter if the incubator is being chilled for an experiment.

Harvest pupae, sterilize, dry, count, weigh and set up in buckets

- Make and autoclave sucrose/sodium chloride, being sure there is at least enough for the next day.
- Put now empty trays in 10% bleach ice chest.

Make diet

- Make sure there is enough prepared diet for the next day; if Friday, enough for the weekend.
- Wash diet dishes
- Autoclave lab coat after making diet.

Weekly:

- Autoclave and/or wash lab coats
- Bleach walls and floor of *Heliothis* growth chamber
Sanitation Guidelines

10% bleach and 70% ethanol are used for sanitation throughout the lab. Bleach should be applied first, to thoroughly cover the area, then wiped off. Ethanol is applied second because it evaporates quickly, leaving a clean, dry surface.

If it seems prudent to sterilize something not mentioned here, ask questions. More often than not, it will be a very good idea ☺. Also, be sure to write down everything you do in the lab notebook. If there is a contamination problem, good notes will help us trace the source. The data recorded in the lab notebook are also important sources of methods, enabling us duplicate experiments.

If you see any “odd” insects or notice any behaviors out of the ordinary in any stage of development, please save the insects and tell Ruth. If a colony cup looks odd to you, containing a sick or malformed insect or fungus, do not open the cup!

Personal:

Wash hands with germicidal/antimicrobial soap for 15 seconds; wash all parts of hands, up to elbows. Hands should be washed when you first arrive at the lab, after using the bathroom, after eating, before going into the hood and before beginning any other sensitive task. Hands should also be washed when you are leaving the lab at the end of the day.

Put up long hair.

Cover skin/clothes with lab coat when performing any colony task, unless you are in the hood. A newly-autoclaved coat with cuffs is required when making diet. Any coat, newly autoclaved or previously worn, may be used for other tasks. Lab coats should be autoclaved weekly.

Wear a mask when entering the growth chamber. The hairnet is optional.

Wear a mask and hairnet when making diet.

Wear a mask when cleaning frozen moth buckets.

Gloves:

Gloves should be worn for every colony task.

Ethanol gloves when you put them on, out of the box. Ethanol gloves if you have touched a contaminated surface, including your face, personal items, lab handles, tools, etc (pretty much anything you didn’t clean).

Do not touch common use areas (doorknobs, computer, phone) with gloves on.

Remove gloves when leaving the room. When removed, gloves may be initialed and left on the counter, or be discarded. Gloves should not be kept in clothing pockets—they will accumulate microorganisms. If you are carrying something from one room to another, remove one glove and touch the doorknob with your bare hand.

If your gloves seem or look dirty, do not hesitate to discard and replace them.
Hood:
You should only use the 533 hood. There are hoods in 528, but they are not available for insect colony work.
Never go into the hood without gloves.
If you have not recently washed your hands, use Purel water-free wash up to your elbows before entering the hood.
Wear short sleeves in the hood, or roll up long sleeves.
Clean all hood surfaces before using the hood and after finishing a task. When cleaning the hood, use lots of ethanol. Ethanol sterilizes on contact, so the hood surface must be thoroughly covered.
Ethanol items before placing them in the hood, including gloved hands and containers/tools.
Clean facemarks off of the front of the hood with ethanol when finished.
Fan on hood should never be turned off.

Lab:
Always bleach and ethanol counters before beginning and after finishing a task.
All lab equipment must be sanitized before use, either autoclaved or cleaned with bleach and ethanol. Anything that can be autoclaved should be, including metal and glass items, paintbrushes and the ceramic Buchner funnel. Two layers of foil should be used to cover items without a lid for autoclaving. Autoclaves are located in 529 and 438. If there is no other option, you may bleach and ethanol a clean metal or glass item to sanitize, but that is a last resort. The ceramic Buchner funnel may be autoclaved or bleached and ethanoled before use. All plastic objects should be bleached and ethanoled before use.
Perform morning, afternoon and Friday bleaching. Move UV light each evening to shine on the computer, a counter in 532 or 533, or the injector. Preference is given to areas in which virus has been manipulated.
Mop floor and walls of Heliothis growth chamber with 10% bleach weekly.
Autoclave lab coats weekly.
Place 30-well trays and 2 gallon plastic bags flat in bleach ice chest; that which is not submerged is not sterilized.
Be responsible for your own dishes. Wash dirty dishes after finishing a task. Put away dry dishes or wrap and autoclave.
Do not leave materials on the lab counter overnight without labeling them, unless they belong there. Return pens and markers to the appropriate container after you finish using them.
Diet, insects and gloves must be discarded in the autoclave bag.
The autoclave bag can only be filled 1/2 to 2/3 full, then must be removed from the stand, twisted shut, taped with autoclave tape and autoclaved. Bags that are more full will not fit in the autoclave.
Do not open 2 gallon bags containing diet trays in the cold room. Bring bags down to the lab before opening them.
Sliding glass cabinet doors should remain closed unless you are currently removing items from a cabinet.
The 10% bleach ice chest is used for the sanitation of large plastic items, particularly trays and 2 gallon bags. If you notice the bleach is low, make more and add it to the chest. The chest should remain approximately 4 inches from the -80°C freezer to allow the freezer to vent.
Schedules

Eggs harvested: day 1
  ↓ 2 days
Neonates hatched and started: day 3
  ↓ 21 days
Pupae harvested: day 24
  ↓ 7 days
Adults emerge: day 31
  ↓ 3 days
Egg laying begins: day 34
  ↓ 2 days
Egg laying peaks: day 36

Plan 5 weeks ahead from egg to egg

Neonates hatched and started: day 1
  ↓ 21 days
Pupae harvested: day 22
  ↓ 7 days
Adults emerge: day 29
  ↓ 5 days
Egg laying peaks: day 34
  ↓ 2 days
Neonates hatched and started: day 36
  ↓ 5-8 days
PM>4: day 41-44

Plan 7 weeks ahead from egg or neonate to PM>4
Making Squirted Diet

1. Wash hands. Put on newly-autoclaved lab coat with cuffs, mask and hairnet. Put on gloves and ethanol them. Bleach and ethanol entire counter on “fridge side”, liberally applying solutions. Wipe counter off with paper towels between applications. If necessary, you can use the “door side” counter, but this is not preferred, because of air disturbance caused by opening the door. Initial and remove gloves, and place on counter.

2. Gather the following. To sanitize, bleach and ethanol all but the blender base and counter liner. Allow sanitized items to air dry.
   - Waring blender, vented lid and blender base
     Assemble blender if needed, lining up “peg” with notches.
     Blender does not need to be disassembled to sanitize. Verify that blender nut is tightened using blue handled channel locks before sanitation.
   - Rubbermaid condiment squirter and lid
   - Short clear tubing
   - Short-necked plastic funnel
   - 12-18” strip VersiDry counter liner

3. Using a sterile graduated cylinder, measure 2790mL milliQ water (930mL X 3) into two autoclaved 2L beakers (1395 each) or 3 1L beakers (930 each). Boil by microwaving for 35 minutes.

4. Place blender base on counter next to mQ water jug. Place sanitized blender on base.
   Place counter liner next to base, with plastic side down. Place sanitized condiment squirter and lid, tubing, short-necked plastic funnel and blender lid on liner.

5. Gather, per 3L batch of diet, 12 trays. Remove gloves before leaving the room. Go next door (533) and gather 2 1/2 to 3 sleeves of 1 oz portion cups. Replace gloves.

6. Get bag of tobacco budworm dry diet mix from 532 freezer or 558 walk-in freezer. The outer bag should have an orange line at the top. If it does not, add one using an orange Sharpie. Make sure it is tobacco budworm diet, not gypsy moth diet.

7. Center bubble on uncovered scale and brush scale off with paintbrush.

8. Weigh out dry diet mix into three white weigh boats, 162 g per boat. Record exact weight of mix in the notebook. Set boats aside. If you open a new bag, ethanol a new shot glass and allow to dry before putting in bag as a scoop.

9. Close bags, leaving them as you found them. If you got the bag from the 558 freezer, leave it in the 532 freezer. There should always be one bag in the 532 freezer.
10. Lay the trays out on the counter, turning “longways” to fit two deep. Fill 12 30-well trays with cups. Save remaining cups to be returned to 533 when you are finished.

11. Make sure the water is boiling when you take it out of the microwave. If it is not, put it in for more time.

12. Pour boiling water into blender. Add dry mix. Fasten on vented lid and blend on low for 30-35 seconds. You may lightly hold the blender handle if you like. Work quickly; the diet will not properly mix if the water has cooled.

13. When making the last batch of diet for the day, heat additional milliQ water (approximately 1500mL) in the same beaker(s) in microwave for 17 minutes. This water is for cleaning the blender.

14. Using plastic funnel, pour diet into condiment squirter. Only one squirter should be used per person. Diet will be hot!!! You may wear autoclave gloves when holding blender and squirter. Replace lid on blender after pouring to keep diet warm. Be careful not to overfill squirter.

15. Firmly place clear tubing on spout of squirter lid.

16. Holding the squirter vertically, squirt diet into cups, filling cups ~8-10 mL. If you are standing above the trays, this will be to or slightly above the dividing ledge in the tray. Be aware that underfilling will not provide the insects with enough to eat, while overfilling of cups will result in fewer trays produced. Be careful when squirting the first few cups, that you do not overfill them. The diet often comes out very fast at the beginning. If you severely overfill a cup, you can pour some of the diet into another.

17. Use your gloved hand to scrape remaining diet out of the blender into the funnel.

18. Once all diet has been squirted, stack trays, turning each alternately horizontal and vertical. Place more recently squirted trays on the top, since they are the least dry.

19. Go into 533 and sanitize hood. Leaving hood open, place trays of diet inside, stacked 4 high. Place an empty tray on top, turned alternately, to keep top diet tray from drying out. Cover the hood with vinyl cover and turn on the UV light. Record the time on the hood white board. The diet should be in the hood 30 to 45 minutes.

20. Pour boiling “wash” water into blender and blend for 30 seconds on low.

21. You do not need to wash the blender if you will be immediately making another batch of the same type of diet.
22. Record diet making in the red colony notebook, including:
   mix received and opened dates
   amount of mix used
   amounts of water used for each step
   microwaving time
   number of trays produced
   UV time
   fate of trays (see #22).

23. Any diet that will not be used immediately may be bagged (2 trays per 2 gallon bag)
    labeled with tape (fold under tape corner) using an ethanol-resistant VWR marker
    (Southland diet, the date, your initials) and placed in the gray plastic \( Hv \) diet box
    in the cold room on the bottom right until use.

24. Wash diet-making equipment, using brushes. Rinse 3 times with tap water and 3
    times with distilled. Add 2 additional tap water rinses if soap is used, to remove
    soap residue. After the other dishes are washed, take apart the blender using
    channel locks and wash it. Take the lid apart and wash each part and the gasket.
    No diet pieces or residue should remain on the blender, blade or lid.

25. Clean off any diet spilled on blender base and counter using a sponge or razor blade.
    Put away blender base. Bleach and ethanol counter.

26. Wrap lab coat worn for autoclaving. Label with [size] and “cuff” and add autoclave
    tape. Autoclave or combine with other wrapped items.
Setting up neonates

1. Place sterilized eggs in 28°C 532 incubator to hatch. If 28°C chamber is not available or is cooler than 28°C, place on 532 counter. The eggs will hatch in about 2 days. Eggs darken immediately before they hatch. Do not discard darkened eggs, unless it has been 4 days or more since they have been sterilized and they have not hatched.

2. Unless otherwise instructed, set up 3 trays each day.

3. Wash hands. Gather paintbrush and neonates from 532 and diet from cold room. Use the oldest diet first. Do not remove diet from bags in the cold room; bring bags down to the lab before opening.

4. Go into 533 before putting on gloves; ethanol your gloves.

5. Sterilize 533 hood by spraying a liberal amount of ethanol on all surfaces and wiping with a Kimwipe. Move the ice cream cups if needed to facilitate cleaning.

6. Remove diet from bags and place in hood. The bag should not enter the hood. Wait for moisture inside cups to evaporate, if necessary. Larvae can drown even in small drops of water. If the trays are wet, spread them in a single layer on the bottom of the hood to speed drying.

7. Place a new, dry Kimwipe on right hood floor. Sterilize and place in hood: sleeve of clear plastic lids, tub of neonates and autoclaved paintbrush. Put neonate tub on top of Kimwipe.

8. Open the neonate tub and place lid on Kimwipe. Make sure paintbrush bristles are dry by dabbing on Kimwipe. Place two neonates on a plastic lid and close the lid onto a cup. When you are finished with a tray, you may leave it in the hood or set it outside of the hood, on the floor or counter.

9. When finished with all of the trays, label one cup in each tray using tape with folded corner and an ethanol-resistant VWR marker. Label trays with setup date and pupal harvest date, 3 weeks later. Any additional information should be recorded in the red colony notebook, not on the tape label. Do not bag the trays once the neonates have been added: cups will mold.

10. When you are finished with the neonate tub, close the lid. Wrap the Kimwipe around the tub and thoroughly ethanol the Kimwipe. Freeze the tub in the 533 freezer. Remove and wash previously frozen neonate tubs. There should only be one neonate tub in the freezer at a given time!

11. Remove all other items that you have used from the hood and wipe all hood surfaces down with ethanol, including the outside of the glass, to remove face smudges.
12. Place all trays in 23°C growth chamber (C8, 531A), in a cabinet wherever there is space, stacking as needed. Record the pupal harvest date and location of the insects on the calendar, using the following notation. In the date corresponding to three weeks from today, write P-C8 (3). This indicates that pupae should be harvested on this date, and that 3 trays are in the C8 chamber.

13. Record in the red colony notebook the number of trays set up, the egg date on the neonate tub, the date the diet was made and who made it, the location of the trays, and the anticipated pupation date. Be sure to record “set up on bench” if you did not use the hood.

14. If you used bagged diet from the cold room, place used bags in the 10% bleach ice chest in 532. Remove any tape labels from the bags and make sure they are open and that the bleach enters the bag.

15. After a minimum one hour soak, rinse all surfaces of each bag with 3 tap water rinses and 3 distilled water rinses.

16. Hang rinsed bags on drying rack, making sure corners are open and not folded over.
Harvesting pupae

To ensure colony mixing, pupae will be harvested one time every five days. For your convenience, pupae have been divided into five day groups by color on the C8 chamber calendar. All pupae for each color group should be collected together, at the end of the five day period. If needed, pupae may be harvested over two days, then combined and sterilized. You may hold a portion of pupae at $15^\circ C$ overnight.

The total number of trays to be harvested at a given time can be figured out by adding the number of trays set up on each day. The number of trays set up each day and the location of the trays is recorded on the calendar. All trays are kept in the $Hv$ C8 growth chamber.

Pupae should be handled gently. They are not invincible and damage may kill them, reducing the number of moths and decreasing egg production.

1. Pupae should develop within 3 weeks (12 days) of setting up neonates.

2. Wash hands and put on lab coat. Put on gloves; ethanol your gloves. Bleach and ethanol a white plastic bowl or clear box to put pupae in. Begin collecting pupae when bowl/box is dry.

3. Pupae may be harvested in 532, on the “door” side, or in 533. Bleach and ethanol entire counter, liberally applying solutions. Wipe counter off with paper towels between applications.

4. Sort through cups, saving tape labels from each tray. Harvestable pupae are uniformly brown and not deformed. Write the following headings in the lab notebook or on scrap paper: live larva, dead larva, partially-formed/diseased pupa, dead pupae and live but not used. As you sort, set aside cups that contain harvestable pupae. For all other cups, mark them down under the appropriate heading and discard cups. Do not open the cups unless they contain harvestable pupae. If you see a particularly odd insect, bag it and save in the 533 fridge to show Ruth.

5. For cups with harvestable pupae: open cups and locate pupae. Gently squeeze each pupa and look for posterior rotation, which indicates life. The rotation may be slight or dramatic. All pupae should be harvested into the same container, to enable mixing of pupae from different dates. Try to clean the live pupa of larval cuticle, diet or frass before putting it in the bowl.
   - If there is only one pupa in the cup:
     - If it rotates, transfer it to the harvest container.
     - If it does not rotate, record as “dead pupa” and pitch.
   - If there are two or more pupae, check each for rotation. If one pupa from a cup does not rotate, neither may be used for the colony.
     - If all of the pupae rotate, transfer all to the harvest container.
     - If neither rotate, record as “dead pupa” and pitch.
     - If one rotates and the other does not
- Pitch the one that does not and record as “dead pupa”
- Pitch the one that does and record as “live but not used”. These pupae should be included in the count of “live pupae”, but should not be included in the buckets.

6. Make sure that pupae are clean of larval cuticle, diet and frass before you sanitize them. You may wash them with distilled water if you wish.

7. Sanitize pupae by soaking for 10 minutes in 12.5% formaldehyde. Formaldehyde is extremely carcinogenic and should be used only in the hood!! Measure 80mL (2 parts) of mQ water into a graduated cylinder. Take the cylinder and the pupae into the hood, and add 40mL (1 part) 37.5% formaldehyde (found in the flammable cabinet). Pour the solution onto the pupae and allow to sit for 10 minutes, swirling a couple of times to ensure good coverage. Using the lid of the bowl or box, pour the formaldehyde off of the pupae into the Hv formaldehyde waste container in the hood. Be careful not to use the gypsy moth formaldehyde (see step 21 if no bottle).

8. Take pupae to sink and rinse 5 times with milliQ water. The first three rinses should last one minute and pupae should be swirled in water for the entire minute. The last two rinses should consist of submerging the pupae and then draining the water. After last rinse, gently "pour" pupae onto paper towels to dry.

9. When dry, check the bubble on a scale and weigh pupae in tared weigh boat. Record the weight of the pupae and the number of pupae in the red Hv colony notebook. Gently divide pupae into equal sized groups and place them in 2 or more sterilized (bleached then ethanoled) 5 gallon buckets, depending on the number of pupae. You should have 100-200 pupae per bucket. If possible, you should set up at least two buckets, in case something happens to one.

10. Cover buckets with a single layer of 18"x18" sterile (autoclaved) cheesecloth, securing with a rubber band. Precut cheesecloth squares may be in the 533 Hv diet supplies cabinet. A square removed from the foil packet will need to be cut in half to form two useable squares. If there are no autoclaved precut squares, see step 22. Using tape with folded corner and an ethanol-resistant VWR marker, label buckets with date that pupae were harvested and sterilized and the number of pupae.

11. Insert bleached and ethanoled #2 stoppers into bucket feeding ports. Stoppers may be found in the 533 Hv colony supplies cabinet.

12. Record details of harvest in the red colony notebook, including:
   - the date the larvae were set up and initials of the person who set them up
   - the number of trays harvested from each date
   - the number of live pupae harvested
     - including live pupae that will not be used for the colony
the number of larvae living but not pupated
the number of partially-developed pupae
the number of dead pupae
the state of diet pupae were harvested from (dry, wet, gummy, etc.)
details of the sterilization method, including product number and lot number of the formaldehyde
the number of buckets used and the number of pupae per bucket
the total number of pupae
the total weight of pupae

13. Inform Ruth of any dramatic changes noticed while harvesting, including increased mold, increased number of live larvae or decreased number of live pupae, and changes in diet appearance or consistency.

14. Place buckets in 23°C C8 growth chamber.

15. Show any dead or abnormal insects to Ruth.

16. Clean counter and wash dishes. If lab coat worn is dirty, place in “to be washed” container. Otherwise, wrap and autoclave.

17. Submerge empty trays right side up in the 10% bleach ice chest in 532.

18. After a minimum one hour soak, rinse trays with 3 tap water rinses and 3 distilled water rinses.

19. Place trays on drying rack or dish rack to dry, with cups facing down. Trays may be stacked if they do not totally overlap.

20. Enter information from notebook in pupae worksheets of “Hv colony progress.xls” on 532 computer desktop.

21. If there is no “Hv formaldehyde” waste bottle, you must create one. Get a sizable empty waste bottle and a red waste label (from the blue “Chemical Waste Logbook”). Fill out the waste label as below and attach to the bottle. Label the bottle “Hv waste formaldehyde”

<table>
<thead>
<tr>
<th>Name</th>
<th>Kelli Hoover</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rm. &amp; bldg.</td>
<td>532 ASI</td>
</tr>
<tr>
<td>Container Contents:</td>
<td></td>
</tr>
<tr>
<td>Formaldehyde 12.4% 50-00-0</td>
<td></td>
</tr>
<tr>
<td>Methanol 4% 67-56-1</td>
<td></td>
</tr>
<tr>
<td>Water 83.6% 7732-18-5</td>
<td></td>
</tr>
</tbody>
</table>

22. If there are no autoclaved precut cheesecloth squares, get the cardboard cheesecloth template from the right of the Hv colony supplies cabinet in 533. Wrap the
template with cheesecloth, so that the cloth is in the same orientation as the template. Cut cloth at both ends of the cardboard. Do not cut sides of the cloth. Fold cloths individually, wrap in foil, label “Hv cheesecloth” and autoclave.
Moth care: making sucrose/sodium chloride

1. Wash your hands. Put on gloves; ethanol your gloves.

2. Bleach and ethanol the counter near the scale, using a liberal amount of each solution and wiping with a paper towel between applications.

3. Center bubble of uncovered scale and dust the scale off with paintbrush.

4. Make 1.5L of 16% sucrose + 0.01% sodium chloride
   - Weigh 240 g sucrose using a clean, autoclaved spatula and white weigh boat. The sucrose is in the walk-in cooler in a short bucket, with no lid.
   - Weigh 0.15g sodium chloride using a clean, autoclaved spatula and weigh paper. The sodium chloride is in the “less hazardous chemicals”. Do not use the one that says “ok for bacteria”.

5. Record the actual amounts and the lot numbers of sucrose and sodium chloride weighed in the red colony notebook.

6. Place a stir bar in an autoclaved 2L beaker and add the sucrose and sodium chloride.

7. Using an autoclaved graduated cylinder, measure and add 1.5L milliQ water.

8. Stir until dissolved.

9. Pour sucrose into 3 autoclaved 500mL bottles, taking care not to spill. Spills must be rinsed with water so that a sticky residue does not remain. Screw the lids on, then loosen slightly.

10. Using a tape label with folded corner, label each bottle with “16% sucrose + 0.01% sodium chloride” [date] [your initials]. Place a small piece of autoclave tape on the label.

11. Place a large piece of autoclave tape, with folded-under corner, on the lid of each bottle.

12. Place the bottles in a Pyrex baking dish and autoclave using the liquid cycle set for a 20 minute exposure.

13. When the autoclave is finished, immediately remove bottles. Check lids to see that they are loose, but do not remove them. Place bottles in the 533 refrigerator, on the “body” second shelf.

14. The sucrose/sodium chloride bottle currently in use should be kept on the right hand side of the top door shelf of the 533 refrigerator.
15. Use all older bottles before opening a new bottle of sucrose/sodium chloride.

Moth care: feeding moths

Moths should emerge 5-9 days after pupal harvest. Check buckets daily and note the date of moth emergence in the red colony notebook. After the moths have begun to emerge, move them near or under the hanging light bulb.

1. Begin to feed a bucket as soon as the first moth emerges.

2. Wash your hands and put on gloves; ethanol your gloves.

3. To make a feeder vial:
   - Get an autoclaved sponge vial from the vial box. Remove foil “lids” and place in tub in foil cabinet. Using a transfer pipet, drip 5-6 full pipets of sterile sucrose to the sponge. The sponge should soak up the sucrose as it is added. If the sucrose runs off the sponge, add it more slowly. The sponge should be saturated and there should be a 3-5mm of sucrose at the bottom of the vial. There should be a small puddle of sucrose at the bottom of the vial, but no sucrose should leak when the vial is held horizontally. Take filled vials to the 23°C growth chamber. Screw the vial into a lid glued to a bucket.

4. Replace existing vials with new vials every day.

5. Do not throw away old vials; they can be reused. Remove used sponges and wash, being sure to wash off any eggs laid on the sponge. Wash the vials. When the vials are dry, wet sponges with distilled water and squeeze out. Insert damp sponges into vials and set vials on the counter next to the dish drainer in 532. When sponges are dry and there is no condensation in the vials, wrap with foil and autoclave.

6. Record in the red colony notebook that you replaced the vials, the dates on the buckets, the date the sucrose/sodium chloride was made and who made it.

7. Never use sponge vials that have not been autoclaved.

8. Return autoclaved sponge vials to the box.
Moth care: harvesting eggs

Females should begin laying eggs 2-3 days after emergence. Once they begin to lay, the cheese cloth top should be changed to a tighter-weave fabric. The fabric must be replaced daily or larvae hatch, feed on eggs and create a general mess. You must wear a dust mask, lab coat and gloves at a minimum when changing the fabric. You may wear a hairnet if you wish.

1. Wash your hands. Put on a lab coat. Put on gloves; ethanol your gloves. Bleach and ethanol 4 plastic bowls (two for egg sterilization) and hang on drying rack to dry. Be careful not to drip ethanol on clean dishes. Remove one glove to open door and go to 533.

2. Get autoclaved fabric piece (s) (not cheesecloth) from 533 cabinet. Place the fabric in a sanitized plastic bowl. Get dust mask from 533 cabinet. Take bowl containing fabric to the growth chamber, along with sinkers, replacement feeder vials, and second plastic bowl.

3. Place the bucket on a table.

4. Hang eight sinkers on old cloth, at opposite corners and folds.

5. Holding the bucket against you (be careful of the vials), gently remove rubber band, beginning from the opposite side.

6. Lay new cloth on top of old, making sure it is centered.

7. Move sinkers to new cloth, adding additional sinkers if desired. If you wish, place two sinkers on the back of the fabric you are planning to remove. These weights help this fabric not to collapse in, trapping moths.

8. Gently pull old cloth out from underneath new, being careful not to trap moths or release them into the room. Trapped moths will appear as bumps or darker shadows under the cloth. Moths accidentally released can be reinserted into buckets through feeder ports. If the moths escape and fly away, squirt with ethanol and smush.

9. Re-center new cloth if necessary. Holding the bucket against you, replace rubber band, beginning at opposite side of bucket. Stretch rubber band before placing on bucket to decrease cloth movement. Remove sinkers. Pull on cloth until taut, being careful not to dislodge rubber band. After putting on the rubber band, lift up the sides of fabric to see if there are any moths on the outside of the bucket.

10. Place bucket under light bulb, at least a foot from the larval cabinet.
11. Eggs should be harvested from a bucket until the moths stop laying, then the bucket should be frozen in the 558 walk-in freezer.
Moth care: sterilizing eggs

** Work quickly. Bleach removes the outer eggshell and eggs will drown if they are submerged for a long period. Cloths must be sterilized individually and must not be left in the bleach solution longer than one minute. Eggs from all cloths should be filtered into a common filter.

1. Make a weak bleach solution (~0.1% sodium hypochlorite, 84 mL 6% bleach plus 4916 mL MQ water) or get from “Save for Hv” bleach container in the right cabinet under the sink. Make sure there is enough bleach for the next day.

2. Wash your hands and put on gloves; ethanol your gloves.

3. Bleach and ethanol three white plastic bowls (or use bowls previously sanitized), large ceramic Buchner funnel and two small clear neonate tubs and lids.

4. Screw clamp (found in drawer under MQ water) onto distilled water faucet and place funnel into clamp. Place autoclaved coffee filter (533 cabinet) in funnel.

5. Remove a single egg-laden fabric from the bowl and open it up. Fold in quarters with eggs to the outside. Lay fabric in the bottom of a white plastic bowl and cover with weak bleach solution. Push fabric down to make sure it is all wet. Allow to soak for 1 minute, swirling if desired.

6. Remove the cloth from bowl and pour bleach solution and detached eggs into the funnel.

7. Open the fabric to half of the total size, with eggs outside. Lay the fabric over one gloved hand, with the bottom of the fabric in the white plastic bowl. Rinse the eggs down into the white plastic bowl with cool tap water, gently sweeping or brushing the eggs off of the cloth with your fingers. Do not squeeze the eggs. When finished with that half, turn the fabric over and do the other half.

8. Stop when bowl is nearly full of water. Rake your fingers right and left through the top of the water to break the surface tension. Your fingers should be submerged to the knuckle. Then, using your finger as a spoon, swirl the water in the bowl and set the bowl aside. The viable eggs will sink and gather in the middle.

9. Continue rinsing the cloth into a new bowl, if needed.

10. When water in the swirled bowl has stopped moving, pour off some water from the bowl, being careful not to disturb the eggs on the bottom of the bowl. You may pour out floating eggs. This is fine; those eggs will not hatch. Pour the remaining viable eggs into the coffee filter.
11. When you think all eggs have been removed from a fabric, open the fabric fully and rinse under fast water, until you are sure no eggs remain. Wring out fabric and hang damp fabric over metal grid. Pitch cheesecloth once eggs have been removed.

12. Pour the remaining water and viable eggs into filter, swirling water as needed to get eggs stuck to the bowl.

13. Rinse bowl with a small volume of cool tap water to collect any remaining eggs. Pour rinse into filter.

14. When all the fabrics have been rinsed, rinse eggs down from sides of filter using a mQ water squirt bottle. All of the eggs should accumulate at the bottom of the filter. Eggs should be evenly distributed through the bottom of the filter, not accumulated to one side.

15. Using ethanoled “general use” forceps (in the forceps drawer or dish drainer), remove all threads and hatched larvae that are in the filter. We do not want them weighed with the eggs.

16. Removing gloves before opening the door, wipe out 533 hood with ethanol. Return to 532, retrieve wet filter, take it to 533 and place it on hood “floor” to dry.

17. After the filter is dry, take it to 532 and weigh the filter and eggs on the enclosed scale. Fold over the sides of the filter to ensure that they do not touch the sides of the scale. If the filter touches the scale anywhere but the weigh plate, you will not get an accurate reading. Record the following in the red colony notebook; record the first three and in the file “Hv colony progress.xls” on 532 computer desktop:
- the weight of the filter plus eggs
- the number of buckets eggs were harvested from
- the date(s) on the buckets
- a summary of the sterilization process, something like “soaked in 0.1% sodium hypochlorite for 1 minute.”

18. Ethanol a pair of scissors. Using these scissors, cut off the sides of the filter. Cut/tear the remaining piece in half, being careful not to damage the eggs. Place each half of the eggs in separate sterilized small clear plastic neonate tubs.

19. Retrieve 3 ice cream cups with Hv Southland diet from the 532 refrigerator, bottom of the door or left crisper. Tear pieces of filter with eggs from one or both halves. Place one piece, containing ~150 eggs (~0.13g of eggs), in each ice cream cup, with the paper down.

20. Cover each clear plastic tub with a lid, making sure it is firmly on. Using tape with a folded corner and an ethanol-resistant VWR marker, label each tub with the date.
sterilized and your initials. Label ice cream cups directly on the lid with “Hv97 eggs”, the date and your initials

21. Place one tub containing eggs in the 532 28°C incubator, placing the new tub under older tubs. If incubator is unavailable, place the tub on 532 counter. The eggs should hatch in 2-3 days. Place the other tub in the 533 15°C incubator (display reads 18°C). This will delay hatching approximately 3 weeks. Place the ice cream cups in the 23°C C8 chamber, making sure the tops are covered by a tray to decrease condensation.

22. Wipe out the 533 hood with ethanol. “Wash” all bowls and the funnel and put away the clamp.

23. Put away equipment used, once dry.
Cleaning buckets

1. Retrieve frozen buckets from 558 ASI walk-in freezer and bring to 532 or 533.

2. Wash hands. Put on lab coat and mask. You may wear a hairnet if you wish. Put on gloves and ethanol them.

3. Bleach and ethanol counter.


5. Place a piece of freezer paper on the counter, with shiny side down.

6. Gently pour contents of bucket onto paper. Inspect bucket, making sure all moths and pupae are out. Use forceps to dislodge insects if necessary.

7. Separate moths, pupae and pupal casings. Pupae are hard and cannot be squished. Pupal casings are empty and easily squished. Discard pupal casings.

8. Record the date on the bucket(s) harvested, the number of buckets harvested from and the number of moths and unemerged pupae in notebook. Discard all insects.

9. Spray inside of bucket with bleach and wipe up, removing scales. Continue bleaching and wiping until bucket is white and dry.

10. Remove lab coat and place in the “to be washed” can.

11. Wash buckets in dishwasher, on “plastic” setting, using cycle A1. Do not forget to add a level spoon of detergent.


13. Record in the file “Hv colony progress.xls” on 532 computer desktop

- under the “moth emergence” tab:
  - the date on the bucket(s) harvested, the number of buckets harvested from and the number of moths and unemerged pupae

- under the “egg harvest” tab:
  - the number of moths
Appendix B: Occlusion Body Purification Method for *Autographa californica* nucleopolyhedrovirus

Materials, all should be sterile:
Virus PBS, recipe from N. Hayes-Plazolles per liter,
- 8.00g NaCl (136.8 mM)
- 0.20g KH$_2$PO$_4$ (1.47 mM)
- 1.15g Na$_2$HPO$_4$ (8.09 mM)
- 0.20g KCl (2.68 mM)
pH to 7.00, autoclave and chill.
Plastic 15 and 50 mL screw-capped conical tubes
Hand homogenizer
Large metal spatula
25, 10, 5 mL disposable pipets
Drummond variable speed pipetaid
60% glycerol
1.5 mL Eppendorf tubes
1% sodium azide
Tube racks for 50 and 15 mL conical tubes and 1.5 mL Eppendorf tubes
Ethanol-resistant marker
600-1000 mL beaker
1000 mL Erlenmeyer flask
gloves

1. Prior to purification, freeze cadavers of virus-killed larvae at 4°C in 50mL tubes on the day that they die, before much melanization occurs. Try not to get much frass or diet in with the cadavers. Cadavers may be stored at 4°C for a few days to months. Cadavers may be stored at -80°C for months to years.

2. On the day before purification, remove cadavers from freezer and place in the refrigerator to defrost. Cadavers may also be defrosted at room temperature the day of purification.

3. On the day of purification, chill centrifuge capable of going 2000g and rotor to fit 50 and 15 mL conical tubes to 4°C.

4. Collect materials listed above and take to the room with the sterile hood.
5. With gloved hands, clean out the sterile hood and place the following items inside, wiping each with ethanol:
   - 50 mL tube rack
   - 50 mL tubes, 9
   - Virus PBS
   - 10 and 25 mL pipets,
   - Drummond pipetaid
   - Hand homogenizer
   - Large metal spatula
   - Cadavers
   - Ethanol-resistant marker
   - 600-1000 mL beaker
   - 1000 mL Erlenmeyer flask

6. Uncover the beaker and set to the side. Using the spatula, transfer a few cadavers to the hand homogenizer. Pipet virus PBS into the homogenizer and homogenize.

7. Transfer homogenate to 50 mL tube.

8. Add more PBS to the homogenizer, if there are still cadavers, and continue homogenizing. Repeat until all cadavers have been homogenized.

9. The homogenate from ~30 cadavers should be placed in each 50 mL tube. Add virus PBS to bring the total volume to 30 mL (one mL PBS/cadaver) and invert to mix.

10. Centrifuge at 45g for 5 minutes in chilled centrifuge, to pellet insect fragments and frass.

11. Gently pour or pipet supernatant into clean 50 mL tube(s). Inspect supernatant for cuticle fragments.

12. If no cuticle fragments are found, skip to step 13. If fragments are found, centrifuge at 100g for 5 minutes in chilled centrifuge. Gently pour or pipet supernatant into clean 50 mL tube(s).

13. Centrifuge at 3000g for 10 minutes to pellet occlusion bodies.

14. Pour off supernatant. Do not pipet, you will disturb the “fat” layer.

15. Add 5mL PBS to pellet and resuspend by pipetting. Add 25 mL PBS for a total of 30 mL (or total volume equivalent to number of cadavers). Centrifuge at 3000g for 10 minutes, then pour off supernatant.

16. Repeat step 15 two times.
17. Add 3 mL 60% glycerol to pellet (one mL per 10 cadavers) and resuspend (pipetting, vortexing).

18. Aliquot 1 mL into 1.5 mL Eppendorf tubes. Add 2 μL 1% sodium azide to each 1 mL of virus.

19. Label tubes with name of virus (AcNPV), host insect species, date and “60% glycerol + 0.02% sodium azide”.

20. Refrigerate tubes for immediate use or freeze at -80°C for long term storage.
Appendix C: Counting NPV Occlusion Bodies on a Hemacytometer

Materials:
- 2-20 μl pipetman
- other pipetman
- pipet tips
- 60% glycerol
- sterile Eppendorf tubes
- tube rack
- AcNPV stock
- ethanol-proof marker
- hemacytometer and cover slip (Hausser Scientific)
- waste beaker for tips
- lens paper
- water
- 10% bleach
- hand counter

Procedure:

1. Clean out the sterile hood with ethanol.

2. Wipe each item below with ethanol and place in the hood:
   - pipetmen
   - pipet tips
   - beaker of sterile Eppendorf tubes
   - tube rack
   - marker
   - glycerol

3. Remove four tubes and place in the rack. Recover the beaker with foil and remove from hood.

4. Close lids of all the tubes. On two, write “G” for glycerol; on one, write 1E–2; on one, write 1E–3.

5. Pour 60% glycerol into the G tubes, without touching the tubes to the lip of the bottle. Reclose the 60% glycerol bottle and remove from the hood.

6. Pipet 990 μl of 60% glycerol into the 1E–2 tube and the 1E–3 tube.

7. Vortex the AcNPV stock for 5 to 10 seconds, on vortexer speed 3. Wipe with ethanol and place in the hood in the tube rack.
8. Pipet 10 μl of the virus stock into the 1E–2 tube. Vortex mixture for 5 to 10 seconds, on vortexer speed 3, then wipe the tube with ethanol and return to the hood.

9. Pipet 100 μl of the 1E–2 mixture into the 1E–3 tube.

10. Retrieve the hemacytometer. Squirt it with ultrapure milliQ water. Shake off the excess water and then dab with lens paper to dry. Do not wipe; you may scratch the grids. Squirt the coverslip with water and dab or wipe with lens paper.

11. Place the hemacytometer in the hood. Place the cover slip over the hemacytometer, making sure it is centered over the middle.

12. Vortex 1E–3 mixture for 5 to 10 seconds at speed 3, then wipe the tube with ethanol and return to hood. Slowly pipet 10-12 μl of the 1E–3 mixture into each side of the hemacytometer, placing the pipet tip into the groove on each side.

13. Remove the hemacytometer to the BX-60 scope. Turn the scope on. View the hemacytometer with the 40X objective. Make sure the phase ring is on phase two.

14. Move the hemacytometer slide so that the central hemacytometer square, the one with three lines marking the smaller squares, is in the center of view. (Microsoft would only allow two lines, but three are there.)

15. Move the slide so that small square one is in the field of view.

16. Turn the fine focus knob toward you, putting the slide out of focus. Continue until you are just past being able to see the grid.

17. Slowly scroll the fine focus knob away from you, clicking the hand counter with each polyhedron you see. Each polyhedron will be roughly square, with rounded corners. As you focus in and out, the polyhedra will appear bright white with black centers. Do not count circles or irregularly shaped objects. Polyhedra may be different sizes, so keep
that in mind. You may see some very large square crystals, considerably larger than other polyhedra. Do not count these; they are not virus.

18. Continue until you have counted all of small square 1.

19. Scroll over and count small square 2. Continue until you have done all 13 small squares within one grid.

20. Record the number of polyhedra counted for all 13 small squares in the notebook.


22. Move the slide until the second grid of the hemacytometer is visible. Count small squares 1-13.

23. Record the number of polyhedra counted for all 13 small squares in the notebook.

24. Gently remove the hemacytometer from the microscope. Set the hemacytometer on the counter and gently remove the coverslip.

25. Holding the coverslip over the autoclave bag, squirt off with 10% bleach, then milliQ water. Gently wipe or dab the coverslip with lens paper and place it in the hood, on tube rack.

26. Holding the hemacytometer slide over the autoclave bag, squirt off with 10% bleach, then milliQ water. Gently dab the slide with lens paper and place in the hood.

27. Repeat steps 11-25 a minimum of two additional times, for 3 hemacytometer counts total. One count consists two grids from a single hemacytometer slide. If you want to be more confident, you may count up to 5 hemacytometers.

28. Calculate the number of polyhedra in the stock:
   a) Average the number of polyhedra per 13 small squares
   b) Divide the average the number of polyhedra per 13 small squares by 13, giving the average the number of polyhedra per 1 small square.
   c) Multiply the average polyhedra per small square by 25, giving the average number of polyhedra per square millimeter.
   d) Using the following equation, calculate the number of polyhedra per microliter in the virus stock:
      \[
      \text{Number of polyhedra /}\mu\text{l} = \frac{\text{number polyhedra per square millimeter}}{\text{dilution factor}} \times 10
      \]
      Here the dilution factor is 1000 (1E-3).

29. Record all calculations in the notebook. Record the number of polyhedra per microliter on the tube containing the virus stock.
Appendix D: Processing of Whole Infected Larvae for \textit{lacZ} Visualization


** Wear gloves at all times. Both paraformaldehyde fixative and X-gal are highly toxic.

1. Surface sterilize larvae in 70\% ethanol and dry. Place larvae on ice pack, if desired.

2. Dissect larvae on paraffin-filled dissecting dish, with dorsal side up. Open cuticle, securing with stainless steel pins and minutens to avoid accidentally precipitating the x-gal. You may dissect under cytoskeletal extraction buffer (CEB) if desired.

3. Flood wholemount dissections with 2\% paraformaldehyde fixative (fix) and store at 4\°C for 12-18 hours.

4. Remove fixative and rinse dissections with at least 2, preferably 3, washes of CEB.

5. Flood dissections with X-gal staining solution prepared within the last 5 days and store in the dark at room temperature 8-12 hours or overnight. Dissections may be stored on a shaker or left on the counter.

6. Remove X-gal and rinse dissections with 2-3 washes of CEB.

7. Flood dissection with fix and place at 4\°C for long-term storage.

Detailed procedures for solution preparation and dissection follow.
Chemical List

Bleach
sodium hypochloride, NaOCl, CAS number: 7681-52-9

DMSO
dimethyl sulfoxide, C₂H₆SO, CAS number: 67-68-5

ethanol
C₂H₅OH, CAS number: 64-17-5

EGTA
ethylenebis(oxyethylenenitrilo)tetraacetic acid, C₁₄H₂₄N₂O₁₀,
CAS number: 67-42-5

magnesium acetate
Mg(OAc)₂, C₄H₆O₄Mg·4 H₂O, CAS number: 16674-78-5

magnesium chloride
MgCl₂, CAS number: 7786-30-3

paraformaldehyde
(CH₂O)ₙ, CAS number: 30525-89-4

PIPES
piperazine-1,4-bis(2-ethanesulfonic acid), C₈H₁₈N₂O₆S₂, CAS number: 5625-37-6

potassium chloride
KC₁, CAS number: 7447-40-7

potassium ferricyanide
K₃Fe(CN)₆, CAS number: 13746-66-2

potassium ferrocyanide
K₄Fe(CN)₆·3H₂O, CAS number: 14459-95-1

sodium hydroxide
NaOH, CAS number: 1310-73-2

potassium hydroxide
KOH, CAS number: 1310-58-3

sucrose
D(+) Sucrose, C₁₂H₂₂O₁₁, CAS number: 57-50-1

ultrapure milliQ water

X-gal
5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, C₁₄H₁₅BrClNO₆, CAS number: 7240-90-6
Cytoskeletal Extraction Buffer (CEB)

CEB may be made from “scratch”, if a large amount is needed, or from stock solutions, if a small amount is needed. Both methods are detailed here.

CEB is:
10mM PIPES
60mM sucrose
100mM potassium chloride
5 mM magnesium acetate
1 mM EGTA
in ultrapure milliQ water

CEB should be brought to pH 6.8 using NaOH or KOH.
CEB should be filter-sterilized before use. Alternatively, it may be autoclaved if it is removed from the autoclave as soon as the cycle is finished. If the CEB is left in the autoclave, the sucrose will begin to caramelize.
CEB should be stored at 4°C.

Chemical Characteristics:
PIPES, Piperazine-1,4-bis(2-ethanesulfonic acid), C$_{8}$H$_{18}$N$_{2}$O$_{6}$S$_{2}$
   CAS number: 5625-37-6
   Molecular weight: 302.36 g/mol

Sucrose, D(+) Sucrose, C$_{12}$H$_{22}$O$_{11}$
   CAS number: 57-50-1
   Molecular weight: 342.30 g/mol

Potassium chloride, KCl
   CAS number: 7447-40-7
   Molecular weight: 74.55 g/mol

Magnesium acetate, Mg(OAc)$_{2}$, C$_{4}$H$_{6}$O$_{4}$Mg·4 H$_{2}$O
   CAS number: 16674-78-5
   Molecular weight: 214.45 g/mol

EGTA, Ethylenebis(oxyethylenenitrito)tetraaceticacid, C$_{14}$H$_{24}$N$_{2}$O$_{10}$
   CAS number: 67-42-5
   Molecular weight: 380.35 g/mol

Water, H$_{2}$O
   CAS number: 7732-18-5
   Molecular weight: 18.01 g/mol
### Making CEB from “Scratch”

Per one liter, dissolve in 1L ultrapure milliQ water:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molar Concentration</th>
<th>Mass required (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIPES</td>
<td>10 mmol/L</td>
<td>3.024 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>60 mmol/L</td>
<td>20.538 g</td>
</tr>
<tr>
<td>KCl</td>
<td>100 mmol/L</td>
<td>7.455 g</td>
</tr>
<tr>
<td>Mg(OAc)₂</td>
<td>5 mmol/L</td>
<td>1.073 g</td>
</tr>
<tr>
<td>EGTA</td>
<td>1 mmol/L</td>
<td>0.38 g</td>
</tr>
</tbody>
</table>

Per five liters:

15.118 g PIPES
102.69 g sucrose
37.275 g potassium chloride
5.369 g magnesium acetate
1.902 g EGTA
Making CEB from Stock Solutions

Per one liter:

100 mL 0.1M PIPES
To make 100 mL 0.1M PIPES


60 mL 1M sucrose
To make 100 mL 1M sucrose:


100 mL 1M potassium chloride
To make 100 mL 1M potassium chloride:


50 mL 0.1M magnesium acetate
To make 100 mL 0.1M magnesium acetate:


10 mL 0.1M EGTA
To make 100 mL 0.1M EGTA:


Making 2% Paraformaldehyde Fixative

Per 500 mL:
10 g paraformaldehyde, CAS number 30525-89-4
250 mL ultrapure milliQ water
NaOH or KOH
250 mL sterile CEB

Chemical Characteristics:
Paraformaldehyde, K₄Fe(CN)₆·3H₂O
   CAS number: 14459-95-1
   Molecular weight: 422.39 g/mol

Water, H₂O
   CAS number: 7732-18-5
   Molecular weight: 18.01 g/mol

1. In the sterile hood, aliquot 250 mL CEB to beaker or flask.
2. Weigh out paraformaldehyde in the fume hood.
3. Transfer paraformaldehyde to a beaker and add milliQ water.
4. Place beaker on stir plate. Stir and apply low heat. Do not heat about 60°C.
5. Add NaOH or KOH dropwise until the paraformaldehyde dissolves. If the fix is going to be used for anything concerning actin, use KOH instead of NaOH because the sodium depolymerizes actin.
6. Calibrate the pH meter to 7.0 using the standard. Be sure to rinse between solutions.
7. Add 250 mL CEB to paraformaldehyde solution. Adjust the pH of the mixture to 6.8.
8. Pour paraformaldehyde fixative into 500 mL glass bottle and cap. Store at 4°C.

Table D.1. Waste label for 2% paraformaldehyde fixative:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% concentration</th>
<th>CAS number</th>
</tr>
</thead>
<tbody>
<tr>
<td>paraformaldehyde</td>
<td>2%</td>
<td>30525-89-4</td>
</tr>
<tr>
<td>PIPES</td>
<td>0.15%</td>
<td>5625-37-6</td>
</tr>
<tr>
<td>sucrose</td>
<td>1.03%</td>
<td>57-50-1</td>
</tr>
<tr>
<td>KCl</td>
<td>0.37%</td>
<td>7447-40-7</td>
</tr>
<tr>
<td>Mg(OAc)₂</td>
<td>0.05%</td>
<td>16674-78-5</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.02%</td>
<td>67-42-5</td>
</tr>
<tr>
<td>water</td>
<td>96.4%</td>
<td>7732-18-5</td>
</tr>
</tbody>
</table>
**Making Enhancement Buffer (EB)**

EB is:
- 5 mM potassium ferrocyanide
- 5 mM potassium ferricyanide
- 2 mM magnesium chloride

**Chemical Characteristics:**
- Potassium ferrocyanide, $K_4Fe(CN)_6 \cdot 3H_2O$
  - CAS number: 14459-95-1
  - Molecular weight: 422.39 g/mol

- Potassium ferricyanide, $K_3Fe(CN)_6$
  - CAS number: 13746-66-2
  - Molecular weight: 329.26 g/mol

- Magnesium Chloride, $MgCl_2$
  - CAS number: 7786-30-3
  - Molecular weight: 95.211 g/mol

per 100 mL, dissolve in 100 mL ultrapure milliQ water:
- 0.217 g potassium ferrocyanide

<table>
<thead>
<tr>
<th>5 mM $K_4Fe(CN)_6 \cdot 3H_2O$</th>
<th>1 mol</th>
<th>1 L</th>
<th>433.39 g $K_4Fe(CN)_6 \cdot 3H_2O$</th>
<th>100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 L</td>
<td>1000 mmol</td>
<td>1000 mL</td>
<td>1 mol $K_4Fe(CN)_6 \cdot 3H_2O$</td>
<td>=0.217 g $K_4Fe(CN)_6 \cdot 3H_2O$</td>
</tr>
</tbody>
</table>

- 0.165 g potassium ferricyanide

<table>
<thead>
<tr>
<th>5 mM $K_3Fe(CN)_6$</th>
<th>1 mol</th>
<th>1 L</th>
<th>329.26 g $K_3Fe(CN)_6$</th>
<th>100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 L</td>
<td>1000 mmol</td>
<td>1000 mL</td>
<td>1 mol $K_3Fe(CN)_6$</td>
<td>=0.165 g $K_3Fe(CN)_6$</td>
</tr>
</tbody>
</table>

- 0.019 g magnesium chloride

<table>
<thead>
<tr>
<th>2 mM $MgCl_2$</th>
<th>1 mol</th>
<th>1 L</th>
<th>95.21 g $MgCl_2$</th>
<th>100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 L</td>
<td>1000 mmol</td>
<td>1000 mL</td>
<td>1 mol $MgCl_2$</td>
<td>=0.019 g $MgCl_2$</td>
</tr>
</tbody>
</table>

EB is photosensitive and should be stored in a foil-covered bottle at 4°C until use.
Table D.2. Waste label for enhancement buffer:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% concentration</th>
<th>CAS number</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_4$Fe(CN)$_6$·3H$_2$O</td>
<td>0.217%</td>
<td>14459-95-1</td>
</tr>
<tr>
<td>K$_3$Fe(CN)$_6$</td>
<td>0.165%</td>
<td>13746-66-2</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>0.019%</td>
<td>7786-30-3</td>
</tr>
<tr>
<td>water</td>
<td>99.6%</td>
<td>7732-18-5</td>
</tr>
</tbody>
</table>
Making X-gal Staining Solution

Notes:
X-gal is toxic!
X-gal reacts with metals to form an insoluble blue precipitate. If processing
 wholemounts, use only stainless steel pins. Do not handle dissected items with
 metal forceps.
X-gal is photosensitive. Wrap containers in aluminum foil or otherwise exclude light.
X-gal staining solution must be made within five days of being used.

X-gal staining solution is 1.6 mg/mL X-gal. Find the amount of each ingredient needed
per total volume on the next page.

**When making X-gal staining solution, completely dissolve the X-gal powder in DMSO
before adding the EB. If the EB is added too quickly, the X-gal will not go into
solution.

X-gal staining solution is:
0.8 mg/mL X-gal
4% DMSO
Enhancement buffer

Chemical Characteristics:
X-gal, 5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, C_{14}H_{15}BrClNO_{6}
   CAS number:  7240-90-6
   Molecular weight: 408.63 g/mol

DMSO, dimethyl sulfoxide, C_{2}H_{6}SO
   CAS number: 67-68-5
   Molecular weight: 78.13 g/mol

Table D.3. Waste label for X-gal staining solution:
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% concentration</th>
<th>CAS number</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-gal</td>
<td>0.08%</td>
<td>7240-90-6</td>
</tr>
<tr>
<td>DMSO</td>
<td>4%</td>
<td>67-68-5</td>
</tr>
<tr>
<td>K_{4}Fe(CN)<em>{6} 3H</em>{2}O</td>
<td>0.208%</td>
<td>14459-95-1</td>
</tr>
<tr>
<td>K_{2}Fe(CN)_{6}</td>
<td>0.158%</td>
<td>13746-66-2</td>
</tr>
<tr>
<td>MgCl_{2}</td>
<td>0.018%</td>
<td>7786-30-3</td>
</tr>
<tr>
<td>Total Volume Required (mL)</td>
<td>X-gal (mg)</td>
<td>DMSO (mL)</td>
</tr>
<tr>
<td>---------------------------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>0.2</td>
</tr>
<tr>
<td>7.5</td>
<td>6</td>
<td>0.3</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>0.4</td>
</tr>
<tr>
<td>15</td>
<td>12</td>
<td>0.6</td>
</tr>
<tr>
<td>20</td>
<td>16</td>
<td>0.8</td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>1.0</td>
</tr>
<tr>
<td>30</td>
<td>24</td>
<td>1.2</td>
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<td>35</td>
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<td>1.2</td>
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</tr>
<tr>
<td>100</td>
<td>80</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Table D.4. X-gal staining solution
Cuticle Dissections of Larvae to Produce Wholemounts
Virus infected insects. Be clean!

1. Cut a piece of lab soaker for your dissection area. Place the following items on the lab soaker.

- 2% paraformaldehyde fixative, chilled, from 533 fridge or cold room
- plastic transfer pipet, labeled “fix”
- dissecting scissors and forceps for dissection and minuten manipulation
- stainless steel pins, size 0
- stainless steel minutens, 0.20 mm
- 70% ethanol
- ice pack
- dissecting tray
- plastic Ziploc box, small square size
- tape labels, a long detailed label and a short label with time, treatment and dish number

2. Put on gloves.

3. Retrieve insects to be dissected from the 532 28°C incubator. Get all the insects from one row at a time, usually 3-5 insects, leaving the rest of the tray in the incubator. The times on the insects selected should encompass 10-15 minutes.

4. Open the cups and put the larvae in the ethanol, leaving them any time from 30 seconds until they stop moving. Transfer larvae to the ice pack.

5. Place the appropriate short label, that matching the hours post inoculation, diet and treatment of the larvae, on the outside of the plastic box. Place the corresponding long, detailed label on the box lid. Transfer the stickers from the first and last larvae in the group to the “empty” end of the long label.

6. Transfer larvae from the ice pack to the dissecting dish.
- Position insects in a circle, “head to tail”.

7. Pin a larva directly behind the head capsule. Stick a pin in the fleshy “hump” near the posterior end of the larva. Stretch the insect, but do not tear it, and push the posterior pin into the paraffin. The heads of both pins should be angled away from the insect. Repeat with other larvae. At this point, you may flood the dissecting dish with CEB if desired. Dissecting under liquid may make the process easier.

8. Make a small transverse (horizontal) cut near the posterior pin. Stick the blade of the scissors in this cut and cut up the insect to the head, making sure not to cut the gut. Pointing the tip of the scissors up slightly will reduce the chances of cutting the gut.
9. With “dissection” forceps, grasp the cut cuticle on one side of a larva and gently pull away from the gut. With “minuten” forceps, grasp a minuten. Firmly press the minuten through the cuticle and into the paraffin. Minutens should be placed so that the entire gut of the insect, including foregut and hindgut, is visible.

10. With forceps, grasp the Malpighian tubules above and below the gut and gently pull off of the gut to the posterior. Be careful not to damage gut.

11. Drip-drop 2% paraformaldehyde fixative (fix) directly onto the dissected larva. Do not squirt; the speed of the liquid may damage the gut. It is fine if fix runs off of the dissections, but make sure each is well covered. You may use up to 12 mL of fix per dish, but can use less.

If you previously flooded the dissecting dish with CEB, dissect all of the larvae, and pour the CEB into the autoclave bag. You may then apply the fix, making sure it is not diluted by excess CEB.

If you are a slow dissector, apply fix to each dissection as you finish it, to prevent them drying or melanizing. If you a fast dissector, you can dissect all of the insects and then apply the fix all at once.

12. Put the lid on the Ziploc box. Place the Ziploc box in fridge, making sure to put all the boxes from each time point together.

13. Using 10% bleach and 70% ethanol, sanitize everything you used, including chair arms, squirt bottles, ice packs, the microscope, forceps and scissors. Pay particular attention to cleaning the scissor blades, “unhooking” if necessary to get better access.
Disecting Midgut and Removing Food Bolus

Virus infected insects. Be clean!

1. Cut a piece of lab soaker for your dissection area (or re-use existing piece). Retrieve scissors and forceps.

2. Put on gloves.

3. Retrieve fixed dissections from the fridge.

4. Remove the dissecting tray from the Ziploc box. Wipe the bottom of the tray if fix has spilled.

5. Make small transverse cut at the back of the midgut, or gently poke the gut with scissors to create a small hole. Stick the blade of the scissors in this cut or hole and cut slowly but steadily up the gut. Cut slowly, carefully following the previous cut to reduce jagged edges. Make sure not to tear the gut, and that no pieces of gut tissue are lost.

6. Grasp the food bolus and gently but firmly pull it out of the gut. Get as much food out as you can, but do not tear the gut. Place what you remove in the autoclave bag or a designated shot glass, to be autoclaved. The food bolus contains virus!

7. Place the dissecting tray back in the Ziploc box and take it to the chemical waste area to remove fix and add X-gal.

Note: the food bolus is removed to prevent direct interactions of cotton foliage and foliar surface microorganisms with X-gal staining solution.
Removing Fix and Adding X-gal

Virus infected insects. Be clean!

1. Put on gloves. Retrieve Ziploc boxes from the person removing the food bolus.

2. Remove the lid from the Ziploc box. If you are processing several boxes, make sure that you place the lids so that you know which lid goes with each box.

3. Using a transfer pipet labeled “waste,” remove fix from the dissections. Be gentle, but get as much as you can—pipetting gently around the edges of each dissection. Do not pipet directly off of the dissection or touch the dissection; you may damage the dissection. Tip the dissection dish to the side and gently press on the paraffin. Remove any additional fix. Repeat until all fix is removed. Squirt fix into fix waste (paraformaldehyde waste, not formaldehyde waste).

4. Using a transfer pipet labeled “CEB,” gently drip CEB onto the dissections. Drip along the body, moving from one end to the other and particularly aiming to flush out the gut and rinse away any remaining food. Do not squirt the CEB, as dissections are fragile after fixing. Make sure the dissections are thoroughly covered—you may use up to 12 mL of CEB per dissecting dish.

5. Using the “waste” transfer pipet, gently remove CEB from the paraffin and around the edges of each dissection. Tip the dissection dish to the side and gently press on the paraffin. Remove any additional CEB. Repeat until all CEB is removed. Squirt the CEB into the fix waste container.

6. Repeat steps 4 and 5 two additional times, making sure to gently remove as much liquid as you can after each CEB wash.

7. Using a transfer pipet labeled “X-gal”, gently drip X-gal onto the dissections. Drip along the body, moving from one end to the other. Do not squirt the X-gal, as dissections are fragile after fixing. Make sure the dissections are thoroughly covered—you may use up to 12 mL of X-gal per dissecting dish.

8. Put the lid on the Ziploc box, making sure it is the correct lid.

9. Place the Ziploc box inside a cardboard box or cover with foil to exclude light. Label the outside of the cardboard box or foil with tape to indicate which Ziploc boxes are inside. Place box on shaker, set at a 40-60 rpm, or on the counter.
Removing X-Gal
Virus infected insects. Be clean!

1. Put on gloves.

2. Get the Ziploc box from the shaker or counter and take to chemical waste area.

3. Remove the lid from the Ziploc box. If you are processing several boxes, make sure that you place the lids so that you know which lid goes with each box.

4. Using a transfer pipet labeled “waste,” remove the X-gal from the dissection dish. Be gentle, but get as much as you can, pipetting gently around the edges of the body. Do not pipet directly off of the dissection or touch the dissection; you may damage the dissection. Tip the dissection dish to the side and gently press on the paraffin. Remove any additional X-gal. Repeat until all X-gal is removed. Squirt X-gal into X-gal waste.

5. Using a transfer pipet labeled “CEB,” gently drip CEB onto the dissections. Drip along the body, moving from one end to the other. Do not squirt the CEB, as dissections are fragile after fixing. Make sure the dissections are thoroughly covered—you may use up to 12 mL of CEB per dissecting dish.

6. Using the “waste” transfer pipet, gently remove CEB from the paraffin and around the edges of each dissection. Tip the dissection dish to the side and gently press on the paraffin. Remove any additional CEB. Repeat until all CEB is removed. Squirt the CEB into the X-gal waste container.

7. Repeat steps 5 and 6, making sure to gently remove as much liquid as you can.

8. Using a transfer pipet labeled “fix,” gently drip fix onto dissections. Drip along the body, moving from one end to the other. Do not squirt the fix, as dissections are fragile after fixing. Make sure the dissections are thoroughly covered—you may use up to 12 mL of fix per dissecting dish.

9. Place the lid on the Ziploc box and stack with other boxes from the same timepoint on a cafeteria tray. Label the tray with the timepoint, diet and treatment of dishes it contains. Carefully carry the tray to the walk-in cold room and place on a shelf.
Appendix E: Purification of GV Occlusion Bodies from *Trichoplusia ni*

Larvae

(can be used for other species also)

Materials needed for procedure:
- Polytron
- Sterilized beaker or flask (200-400mL)
- Sterilized cheese cloth (6 in²)
- Sterile 50mL conical screw cap centrifuge tubes (disposable)
- 2% SDS sterilized by filtering or autoclaving
- assorted sterile pipets
- sterilized water, ice cold
- sterilized funnel
- sterilized centrifuge tubes (high speed, need to get from Cui lab)
- ice in bucket
- sterilized forceps
- 1M cysteine
- 10 mg/mL gentamycin sulfate

Critical Parameters:
1) It is important to keep the OB solution cold. This will help prevent melanization.

2) Check the low speed centrifugation pellet for OBs with a 40x objective (phase contrast). If there are a large number of OBs, dilute the pellet with 25mLs of water and repellet the debris.

3) While washing the OBs to remove excess SDS (steps 8-14), it is important to thoroughly resuspend the OBs.

Procedure

1. All purification steps should be done steriley in a laminar flow hood. All work should be done on ice and as quickly as possible. The sterile water should be ice cold.

2. Transfer cadavers to clean 50mL tube, taking care not to move any black (melanized) larvae. Resuspend the larvae in ice cold water in a volume equal to 1 mL per larva. Add cysteine for a final concentration of 10 mM to inhibit melanization (Homogenize the larvae using the Polytron).

3. Using a sterile funnel, filter the homogenate through 4 layers of sterile cheesecloth into a sterile flask on ice.
4. Rinse the blender with a few mLs of sterile water and pass through funnel with cheesecloth. Using a pipette squeeze any remaining liquid from the cheese cloth.

5. Add an equal volume of 2% SDS to the filtered homogenate. The final concentration of SDS should be 1%. Incubate the mixture at room temperature for 30 minutes.

6. Divide the homogenate into 50mL conical tubes and centrifuge the suspension in the GLC tabletop centrifuge at 1500rpm for 10 minutes. This step will remove large host debris.

7. The virus will be in the supernatant. Divide it equally into as many high speed tubes as needed. Also, resuspend the low speed pellets in 10mLs of water and check under the 40X objective for the presence of OBs. If needed, resuspend in a greater volume and repellet to recover additional OBs.

8. To pellet the OBs, centrifuge the supernatant in the Sorvall RC5 superspeed at 10,000rpm for 10 minutes at 4°C.

9. Discard the supernatant into a waste flask and check for the presence of OBs. Repellet the supernatant if necessary.

10. Resuspend the pellets in the original volume of sterile water (1mL per larva) using a pipette. Place the resuspended pellet into new high speed tubes, reducing the number of centrifuge tubes if possible.

11. Centrifuge at 10,000rpm for 10 minutes at 4°C.

12. Wash the pellets with water at least 2 additional times (steps 10 and 11). This is important to prevent melanization and remove excess SDS.

14. After the last wash, resuspend OB pellets in water and transfer into a sterile 3 dram vial (3.7mL). Keep the suspension highly concentration. For example, resuspend pellets from 100 larvae in 5 mL of water, and pellets from 200 larvae in 10mL. This should give an approximate concentration of 2 x 10^{12} OBs/mL. Sonicate OBs suspension twice for 20 seconds each time and then vortex.

15. Add gentamycin sulfate. Final concentration should be 100μg/mL.
Appendix F: Foliar Peroxidase Extraction and Assay

To extract soluble peroxidases:

1. Select leaves and cut from plant. Keep leaves on ice when not handling. Cut off petioles.

2. Weigh 0.1 to 0.3 g of leaf tissue (I usually shoot for close to 0.3g, but don’t go over). If this is a single large piece of leaf, cut into smaller pieces. Place leaf tissue into plastic centrifuge tube.

3. Add to each tissue sample 640 µL 10% Triton-X 100 (solubilizes hydrophobic regions such as vacuoles) (1 mL Triton-X 100 plus 9 mL pH 7 0.1 M potassium phosphate buffer) and 2 mL 7% PVPP (binds phenolics) (7 g of PVPP in 100 mL of pH 7 0.1 M potassium phosphate buffer). The Triton-X 100 and PVPP solutions are collectively called the grinding buffer or the extraction buffer.

4. Homogenize leaf tissue with the Polytron tissue homogenizer until there are no big pieces. Make sure that no leaf pieces are stuck to the bottom of the Polytron when you are finished with each tube and rinse with water between tubes.

5. Centrifuge at 5000 g for 15 minutes at 4°C (7500 g for 15 minutes). Remove the supernatant immediately (the pellet will get mixed back in if the supernatant is not removed) and place in a labeled centrifuge or Eppendorf tube. Keep these tubes on ice until they are assayed.

Peroxidase substrate solution:

5 mM guiacol and 35 µM hydrogen peroxide in pH 7, 0.1 M potassium phosphate buffer. To make, put 12 µL guiacol and 4 µL 30% hydrogen peroxide in 20 mL buffer.

Assay for cuvettes:

1. Add 1 mL of substrate solution to a 1.5 mL disposable cuvette.

2. Add 10-50µL of leaf extract. Start with 10 µL and increase as needed. The aliquot of leaf extract you add will depend on the activity—you want to get a linear change in absorbance reading for 30 seconds.

3. Mix the cuvette quickly by inverting with parafilm covering. Do not shake the cuvette; bubbles will give an inaccurate absorbance reading.

4. Read the change in absorbance at 470 nm for 30 seconds, with readings every 5 seconds. Make sure you record the units that the spec is reading in; some specs give the results in ΔAbs/sec. Peroxidase activity is reported as the change in absorbance at 470 nm (ΔOD_{470}) per gram leaf tissue per minute with 1 unit = 0.001 Δ Abs/g/min.
Assay for microplates:
1. Add 5-9 µL leaf extract to multiple wells, avoiding “border” wells.

2. Using a multi-channel pipetter, add substrate to a total of 200 µL.

3. Quickly place the plate in the microplate reader and mix.

4. Read the change in absorbance at 470 nm for 30 seconds, with readings every 5 seconds. The aliquot of leaf extract you add will depend on the activity—you want to get a linear change in absorbance reading for 30 seconds. Make sure you record the units that the plate reader is reading in. Peroxidase activity is reported as the change in absorbance at 470 nm (ΔOD470) per gram leaf tissue per minute with 1 unit = 0.001 Δ Abs/g/min.

Formula for calculating activity:
\[
\text{ΔOD/g leaf/min} = \frac{\text{ΔOD}_{470}/ \text{min}}{\text{g foliage / aliquot}} \times \frac{\text{mL reaction mixture}}{}
\]
RUTH PLYMALE CURRICULUM VITA

**Education**

PhD, Entomology, August 2006  
Pennsylvania State University, State College Pennsylvania  
Dissertation: Influence of ingested cotton foliage on baculoviral infection in *Heliothis virescens*

BS, Botany, May 2000, *summa cum laude*  
University of Arkansas, Fayetteville Arkansas

**Honors and Awards**

Travel award for international meeting, Microbial Control Division of the Society for Invertebrate Pathology, 2005

Phi Kappa Phi Honor Society, University of Arkansas, 1998

Golden Key National Honor Society, University of Arkansas, 1998

Barry M. Goldwater scholarship, University of Arkansas, 1997

**Presentations**

“The peritrophic matrix hinders baculoviral infection in cotton-fed *Heliothis virescens*,” poster at Entomological Society of America meeting, December 2005, Ft. Lauderdale, FL

“Impact of the peritrophic matrix on baculoviral pathogenesis in a tritrophic system,” paper at XXXVIIIth Annual Meeting of the Society for Invertebrate Pathology, August 2005, Anchorage, AK

“The peritrophic matrix hinders baculoviral infection of *Heliothis virescens* (Lepidoptera: Noctuidae) larvae fed cotton foliage,” paper at Entomological Society of America meeting, November 2004, Salt Lake City, UT

“Inhibition of Baculoviral Disease by Ingested Cotton Foliage is Influenced by Host Strain,” poster at Pennsylvania State University Graduate Exhibition, March 2003

“Inhibition of Baculoviral Disease by Ingested Cotton Foliage,” poster at XXXVth Annual Meeting of the Society for Invertebrate Pathology, August 2002, Igassu Falls, Brazil

**Professional Affiliations**

Entomological Society of America, member 2004-2006

Society for Invertebrate Pathology, member 2002-2006