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Ruth C. Plymale
Ouachita Baptist University, plymaler@obu.edu

Michael J. Grove
The Pennsylvania State University

Diana Cox-Foster
The Pennsylvania State University

Nancy Ostiguy
The Pennsylvania State University

Kelli Hoover
The Pennsylvania State University

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Plant-mediated alteration of the peritrophic matrix and baculovirus infection in lepidopteran larvae

Ruth Plymale[a], Michael J. Grove[b], Diana Cox-Foster[b], Nancy Ostiguy[b], Kelli Hoover[b,*]

[a]*Department of Entomology, Cornell University, Ithaca, NY 14853, USA*
[b]*Department of Entomology, The Pennsylvania State University, University Park, PA 16802, USA*

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Abstract

The peritrophic matrix (PM) lines the midgut of most insects, providing protection to the midgut epithelial cells while permitting passage of nutrients and water. Herein, we provide evidence that plant-mediated alteration of the PM contributes to the well-documented inhibition of fatal infection by *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) of *Heliothis virescens* F. larvae fed cotton foliage. We examined the impact of the PM on pathogenesis using a viral construct expressing a reporter gene (AcMNPV-hsp70/lacZ) orally inoculated into larvae with either intact PMs or PMs disrupted by *Trichoplusia ni* granulovirus occlusion bodies containing enhancin, known to degrade insect intestinal mucin. Larvae possessing disrupted PMs displayed infection foci (lacZ signaling) earlier than those with intact PMs. We then examined PMs from larvae fed artificial diet or plant foliage using electron microscopy; foliage-fed larvae had significantly thicker PMs than diet-fed larvae. Moreover, mean PM width was inversely related to both the proportion of larvae with lacZ signaling at 18 h post-inoculation and the final percentage mortality from virus. Thus, feeding on foliage altered PM structure, and these foliage-mediated changes reduced baculoviral efficacy. These data indicate that the PM is an important factor determining the success of an ingested pathogen in foliage-fed lepidopteran larvae.

Keywords: Tritrophic interactions; *Autographa californica* nucleopolyhedrovirus; Midgut physiology; *Heliothis virescens*; *Trichoplusia ni* granulovirus

1. Introduction

Ingested foliage impacts the performance of pathogens against larval lepidopterans, particularly with respect to a family of arthropod-specific viruses, the Baculoviridae (reviewed in Duffey et al., 1995; Cory and Hoover, 2006). Tritrophic interactions of baculoviruses, host plants, and herbivores have been studied extensively because of the potential of these pathogens as highly selective biological control agents (Szewczyk et al., 2006). In many instances, consumption of host plant foliage increases larval resistance to baculoviruses; although, in some cases plants enhance the performance of insect pathogens (reviewed in Cory and Hoover, 2006). In particular, *Heliothis virescens* larvae fed cotton foliage are less susceptible to mortal infection by *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) than larvae fed iceberg lettuce or artificial diet (Hoover et al., 2000). Here, we describe how plant-mediated changes to the peritrophic matrix (PM) influence *H. virescens* larval susceptibility to AcMNPV. To our knowledge, this is the first study to compare the effects of plant foliage relative to artificial diet on PM structure.

Baculoviruses are double-stranded DNA viruses which comprise two genera—the *Granuloviruses* (GVs) and the *Nucleopolyhedroviruses* (NPVs) (Theilmann et al., 2005). These viruses are orally infectious, gaining access to insect hosts by ingestion of proteinaceous occlusion bodies (OBs) on the surface of plant foliage. In lepidopteran hosts, the OBs dissolve in the larva's alkaline midgut fluids, releasing occlusion-derived virions (ODV) (Pritchett et al., 1982). ODV pass through the PM, initiating infections of the midgut columnar epithelial cells (Granados and Lawler, 1981; reviewed in Volkman, 1997; Bonning, 2005). Infection of a midgut epithelial cell is termed a primary infection. Within the nucleus of the midgut cell, viral

*Corresponding author. Tel.: +1 814 863 6369.
E-mail address: kxh25@psu.edu (K. Hoover).

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replication produces budded virus (BV), a second viral phenotype, which buds from the midgut cells to spread infection throughout the insect body (reviewed in Bonning, 2005). Progeny BV move out of the midgut via tracheolar cells servicing the midgut (Engelhard et al., 1994). The infection of tracheal epidermal cells and other tissues outside of the midgut is termed a systemic infection. In *H. virescens*, movement of virus from the midgut into the tracheal system is essential for NPV success; this is because infected midgut cells may be sloughed, removing viral inoculum from the midgut (Washburn et al., 1995). Conversely, *H. virescens* larvae do not appear to slough tracheal infections (Washburn et al., 1995) and, thus, a single tracheolar infection will ultimately kill the host, as most tissues will eventually become infected (Vail and Vail, 1987).

Since baculoviruses are orally infectious, the larval lepidopteran midgut is uniquely positioned to influence the early stages of infection, both through interactions between the virions and ingested foliage (Felton and Duffey, 1990) and through direct contact between the virions and the midgut environment (Granados and Lawler, 1981; Keddie et al., 1989). Within the midgut, one of the first barriers to establishing an infection is the PM (Derksen and Granados, 1988). The semipermeable PM lines the midgut, surrounding the food bolus (reviewed in Terra, 2001). It is composed of a mesh of chitin microfibrils overlaid with proteoglycans, proteins, and glycoproteins (reviewed in Lehane, 1997). Originally, the PM was thought to protect the midgut epithelium from physical abrasion by ingested plant material (Sudha and Muthu, 1988), but it has also been shown to shield the midgut epithelium from microbial and chemical threats. Due to its semipermeable nature, the PM acts as an ultrafilter, decreasing microbial access to the midgut epithelium (reviewed in Lehane, 1997; Wang and Granados, 1998; Barbehenn, 2001; Mitsuhashi et al., 2007). In its role as an ultrafilter, the PM separates most tannins and other large allelochemicals from the midgut epithelium, while allowing passage of smaller phenolics, nutrients and digestive enzymes (reviewed in Lehane, 1997; Barbehenn, 2001). The PM is also able to adsorb ingested chemicals (reviewed in Lehane, 1997; Barbehenn, 2001) and may serve as a functional antioxidant, protecting midgut epithelial cells from significant oxidative damage (Summers and Felton, 1996; Barbehenn and Stannard, 2004).

Whereas the PM affects the interaction between ingested material and the midgut epithelium (Barbehenn and Martin, 1992; Pimenta et al., 1997; Wang and Granados, 1998; Villalon et al., 2003), the PM itself can be influenced by ingested material (Derksen and Granados, 1988; Shao et al., 2001; Mitsuhashi et al., 2007). For example, consumption of maize plants producing an insect-inducible protease was shown to significantly disrupt PM structure (Pechan et al., 2002), decreasing the ultra-filtration ability of the PM (Mohan et al., 2006). Here, we provide further evidence of the ability of host plants to influence PM structure and describe how these changes impact baculoviral pathogenesis and efficacy in larval *H. virescens*.

2. Materials and methods

2.1. Insects

H. virescens eggs were acquired from the North Carolina State University Insectary, from two colonies, one established in 1997 and the other established in 2002 (Raleigh, NC). Larvae from the 2002 colony were used when larvae from the 1997 colony were no longer available. Larvae were reared through the third instar on semisynthetic diet (Southland Products Inc., Lake Village, AR) at 25 °C and a 16L:8D photoperiod, unless otherwise indicated.

2.2. Plants

Cotton, *Gossypium hirsutum* cv. Acala SJ2, seeds were donated by the California Planting Cotton Seed Distributors (Bakersfield, CA). Plants were grown in a growth chamber under a day:night temperature regime of 29:24 °C and a 16L:8D photoperiod to the six to eight leaf stage. Then, the upper two or three leaves were harvested for use in experiments, unless otherwise indicated. Oakleaf lettuce, *Lactuca sativa* L. cv. Oakleaf, and tobacco, *Nicotiana tabacum* L. cv. K-326, were grown under the same conditions described above. Oakleaf lettuce was grown for 3 weeks, at which time all the leaves were harvested for use in experiments. Tobacco was grown to the four-leaf stage and the upper two fully expanded leaves were harvested for use in experiments. Iceberg lettuce was purchased from a local grocery store; the outer three leaves were discarded and the inner leaves used in experiments after thoroughly washing the foliage.

2.3. Virus production

The virus used in this study was AcMNPV-hsp70/lacZ, derived from the E2 parental strain and containing the *Escherichia coli* lacZ reporter gene driven by the *Drosophila* hsp70 promoter (provided by Loy Volkman, University of California, Berkeley, CA; Engelhard et al., 1994). OBs were harvested from virus-killed *H. virescens* cadavers and partially purified using deionized water as described previously (Hoover et al., 1995), quantified using a hemacytometer, and stored at 4 °C in sterile, neutrally buoyant 60% glycerol with 0.002% sodium azide (Engelhard et al., 1994).

Trichoplusia ni GV (TnGV; provided by Ping Wang, Cornell University Experiment Station, Geneva, NY), was amplified in *T. ni* larvae (Wang et al., 1994). OBs 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).

2.4. Bioassay method

Late third instar *H. virescens* larvae displaying head capsule slippage were collected and held at 4 °C for up to 3 days. This procedure was not found to alter subsequent larval growth or susceptibility to baculovirus (data not shown). These larvae were then transferred to 28 °C and checked frequently for molting. Newly molted fourth instars (designated as 4^0s) were placed individually in 30 mL plastic cups and fed on artificial diet or foliage (Hoover et al., 2000), as specified for each experiment, for 8 h (designated as 4^8s). 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, as indicated by frass production. Larvae that had produced frass during the 8 h feeding period were then microinoculated *per os* with a 1 μL aliquot of AcMNPV-hsp70/lacZ OBs as described in Washburn et al. (1995). After inoculation, larvae were returned to their individual cups and fed their pre-inoculation food of artificial diet or foliage. Controls consisted of an additional group of larvae treated in the same way but not inoculated. Inoculated and control larvae were maintained at 28 °C and checked daily for mortality or pupation. New foliage was added as needed. Larvae that died within 3 days after inoculation were removed from the experiment as handling deaths (mortality by virus does not occur in fourth instar *H. virescens* prior to day 4; data not shown). All larvae were transferred to artificial diet after molting to the fifth instar (5^0) 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; Hoover et al., 2000).

2.5. Influence of foliage on AcMNPV pathogenesis

Newly molted fourth instar *H. virescens* were placed in 30 mL clear plastic cups containing artificial diet or fresh cotton foliage. These larvae were allowed to feed for 8 h, then microinoculated *per os* with OBs as described above; each larva received approximately 95 OBs of AcMNPV-hsp70/lacZ in a 1 μL aliquot. Thirty to 50 larvae per treatment were inoculated and monitored for mortality as internal controls. Analysis of variance for categorical data (proc CATMOD) was used to calculate the effect of food ingested on larval mortality using SAS (v. 9.1.3) (Zar, 1999).

At various times post-inoculation, 15–30 larvae were dissected as whole-mounts. The time points ranged from 12 to 41.5 h post-inoculation (hpi) among all experiments; a subset of time points was sampled within each experiment. 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 ethanol and the dorsal cuticle was slit from posterior to anterior. Whole-mounts were processed for lacZ expression as described previously (Engelhard et al., 1994). Whole-mounts were fixed in 2% paraformaldehyde in cytoskeletal extraction buffer (CEB) for 18 h at 4 °C and rinsed with CEB. The food bolus was removed, and then larvae were incubated in a 0.8 mg/mL X-gal solution for 12–24 h at room temperature in the dark.

Whole-mounts were examined and scored using a Nikon dissecting microscope (6.7–80 ×); 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 likewise categorized depending upon the locations of infection foci. 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 only midgut infections by the total number of larvae dissected. Likewise, the proportion of larvae with systemic infections was calculated by combining the number of larvae displaying midgut plus tracheal infections with those possessing tracheal only infections; the sum was divided by the total number of larvae dissected. Analysis of variance for categorical data (proc CATMOD) was used to determine the effects of food ingested and hours post-inoculation on viral pathogenesis (Zar, 1999).

2.6. Electron microscopy of the PM

Discs of artificial diet, cotton foliage, iceberg lettuce, oakleaf lettuce and tobacco foliage were prepared; artificial diet discs measured 4 mm in diameter and 1.5 mm high, while foliage discs measured 10 mm in diameter. Half of the artificial diet and cotton foliage discs were treated with 1×10^9 TnGV OBs in a 1 μL-aliquot of sterile ultrapure water. Intact TnGV OBs were used as a source of enhancin and were equivalent to purified enhancin because *H. virescens* larvae are not susceptible to TnGV. This was verified in a preliminary bioassay of *H. virescens* larvae with no observed viral deaths from TnGV at the high dosages used here. The iceberg lettuce, oakleaf lettuce and tobacco foliage discs remained untreated. Untreated and TnGV-treated food discs were placed individually in a 24-well plate, with wells containing 2% agar (4 mm thick) to prevent food discs from dehydrating or sticking to the plate.

Newly molted fourth instar *H. virescens* larvae were added individually to each well and allowed to feed for 8 h; larvae 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 0.1 M phosphate buffer, pH 7.4) (Dykstra, 1992). A sub-sample of midguts from two to four larvae per treatment group was further processed for

electron microscopy. Selected midguts were rinsed three times for 5 min each at room temperature (0.1 M cacodylate buffer, pH 7.4). Samples were secondarily fixed in 1% osmium tetroxide for 1 h, rinsed three times in cacodylate buffer for 5 min each, and dehydrated through an ethanol series (Hunter, 1984).

Following ethanol dehydration, midgut samples from three to four larvae were freeze fractured in liquid nitrogen for scanning electron microscopy (SEM). The sample fragments underwent critical point drying (Baltec CPD-030 Critical Point Dryer, Techno Trade, Manchester, NH), and were sputter-coated with 10 nm of gold–palladium (Baltec SCD-050 Sputter Coater, Techno Trade, Manchester, NH) (Dykstra, 1992; Echlin, 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).

Following osmium fixation, midguts from two to three larvae were cut into thirds and the middle third of each midgut was used for transmission electron microscopy (TEM). These midgut sections were dehydrated through an ethanol series, further dehydrated in acetone, then embedded in eponate resin and cut into 70 nm sections (Dykstra, 1992). Sections were stained with uranyl acetate and lead citrate. Between 2 and 11 prepared sections were viewed per larva, 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). At least 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. Data were analyzed using the general linear model (proc GLM) to determine the effects of TnGV consumption and food on PM width (SAS v. 9.1.3) (Zar, 1999).

2.7. Effects of PM and host plant on AcMNPV pathogenesis

To examine the influence of PM degradation and ingested foliage on early viral pathogenesis, newly molted fourth instar *H. virescens* larvae were transferred individually to a 24-well plate containing an untreated disc of artificial diet or cotton foliage or a disc treated with with 2×10^7 TnGV OBs and allowed to feed for 8 h as described above. Larvae that had consumed the entire food disc were then microinoculated *per os* with approximately 26 OBs of AcMNPV-hsp70/lacZ in a 1 μL aliquot as described above. We used a lower viral dose in this experiment than in the studies described above to ensure that the full augmentative effect of enhancin-containing TnGV OBs on larval mortality could be observed. Following inoculation, larvae were fed untreated artificial diet or foliage. Inoculated larvae were dissected as whole-mounts from 3 to 18 hpi, with 21–30 larvae dissected per time point. Thirty uninoculated larvae were dissected at 3 and 18 hpi as controls for false positive lacZ signaling. Dissected larvae were processed for lacZ signaling and scored as described above. Thirty larvae per treatment were inoculated and monitored for mortality as internal controls.

Eighteen hpi was selected as the final sampling point in this experiment because we have observed that once the source of enhancin is removed, it no longer influences viral performance, probably because passage of gut contents requires only a few hours in this insect (data not shown). Preliminary pathogenesis sampling beyond 18 hpi did not yield substantial new information concerning the influence of PM on viral pathogenesis. This is not surprising because *H. virescens* larvae possess a Type II PM, which is produced as a sleeve from a single region of the anterior midgut and moves posteriorly with the food bolus (Ryerse et al., 1992); although the specific rate of production for *H. virescens* larval PM is unknown, Type II PM production rates of 3–7 mm/h have been reported for dipterans at 30 °C (Waterhouse, 1954; Becker, 1978). Further, the relationship between lacZ signaling at 18 hpi and effects on viral performance was statistically evaluated (see Section 3).

To study the effect of different host plants on larval infection, newly molted fourth instar *H. virescens* larvae were fed artificial diet, cotton foliage, iceberg lettuce, oakleaf lettuce or tobacco foliage for 8 h, then microinoculated *per os* with approximately 50 OBs of AcMNPV-hsp70/lacZ in a 1 μL aliquot as described above. Following inoculation, larvae were fed artificial diet or foliage and dissected as whole-mounts at 18 hpi; 23–29 larvae were dissected per treatment. Ten uninoculated larvae per treatment were dissected as controls for false positive lacZ signaling. Dissected larvae were processed for lacZ signaling and scored as described above. Thirty larvae per treatment were inoculated and monitored for mortality as internal controls.

Analysis of variance for categorical data (proc CATMOD) was used to determine the effects of food ingested and TnGV OBs consumption on larval mortality and the influences of food, TnGV (enhancin), and hours post-inoculation on viral pathogenesis. A general linear model (proc GLM) was used to assess the effects of TnGV consumption and food on PM width. Regression analysis (proc REG) was used to determine the relationships between PM width and the larval infection parameters of lacZ signaling at 18 hpi, percentage mortality, and time to death. Correlation (proc CORR) analysis was performed to characterize the relationship between PM width and time to pupation. Regression analysis (proc REG) was used to examine the relationship between lacZ signaling at 18 hpi and larval mortality (Zar, 1999). All analyses were performed using SAS (v. 9.1.3, Cary, NC).

3. Results

3.1. Influence of foliage on AcMNPV pathogenesis

We began by comparing viral pathogenesis in *H. virescens* larvae fed artificial diet to that of larvae fed cotton foliage, using a construct of AcMNPV expressing lacZ (AcMNPV-hsp70/lacZ). Two time-course experiments were conducted; the first included time points at 18 and 41.5 hpi and the second covered time points between 12 and 20 hpi in 2 h increments. Ingestion of cotton foliage significantly influenced the percentage of lacZ positive larvae (Fig. 1) and markedly reduced the proportion of insects with evidence of infection. At any given time point, two to four times more artificial diet-fed larvae were lacZ positive, as compared to cotton-fed larvae. The proportion of larvae with lacZ foci increased in both groups of larvae as time post-inoculation increased. Artificial diet-fed larvae experienced significantly higher mortality from virus than larvae fed cotton foliage (Fig. 1).

While ingested cotton foliage significantly reduced the percentage of larvae with evidence of infection, foliage did not significantly impact the spread of the virus beyond the midgut (i.e., from primary midgut cell infections to systemic infection of the tracheolar cells), as compared to artificial diet. The progression of infection from primary to systemic foci, described as the ratio between the percentage of systemically infected larvae and the percentage of larvae with primary infections, was not significantly influenced by food ingested (Fig. 2).

3.2. Electron microscopy of the PM

Because mortality by virus in cotton-fed larvae was related to reduced primary midgut infections and not to the spread of infection out of the midgut, we examined PM structure as a potential barrier to virions in establishing primary midgut infections. Scanning electron micrographs (SEM) of PMs from larvae after 8 h of feeding on artificial diet or cotton foliage did not reveal any obvious differences in surface characteristics of the PM between treatment groups (Fig. 3A and C, respectively). These images did confirm that the *H. virescens* larval PM is comprised of at least two layers (Ryerse et al., 1992). The surface structure of the PM in artificial diet-fed larvae was strongly affected by ingestion of TnGV OBs, which contain the metalloprotease enhancin. Consumption of TnGV-treated diet resulted in a significantly thinned and more porous PM than that seen in larvae fed untreated artificial diet (Fig. 3A and B, insets). The PM of larvae fed artificial diet was robust, had a textured surface that was quilted in appearance, and was easily distinguished from the midgut epithelium. In contrast, the PM of larvae fed artificial diet + TnGV was extremely thin, to the point of complete degradation in some cases and had a highly porous surface, making it difficult to distinguish from the midgut epithelium. On the contrary, ingested TnGV seemed to have a minor influence on the surface appearance of the PM in cotton-fed larvae. The PMs of larvae fed either cotton foliage or

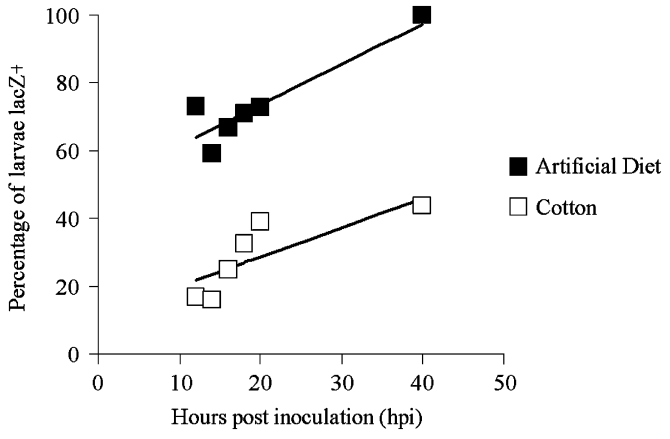


Fig. 1. Time course of the percentage of *H. virescens* larvae signaling lacZ. Food ingested prior to viral inoculation influenced percentage of larvae signaling lacZ over time ($\chi^2 = 69.6$, d.f. = 1, $p < 0.0001$). Final larval mortality was also significantly influenced by food ingested (percentage mortality ± 1 S.E.: artificial diet = 88.7 ± 9.2%, cotton = 69.7 ± 6.1%, $\chi^2 = 14.9$, d.f. = 1, $p = 0.0001$). There were 18–27 inoculated larvae dissected at each time point; each larva was inoculated as a 4^8 *per os* with 95 occlusion bodies.

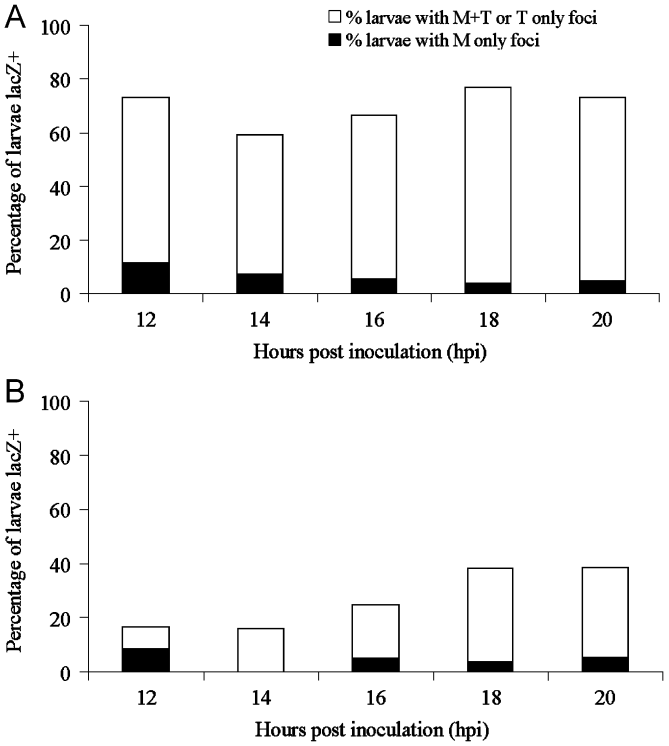


Fig. 2. Time course of the percentage of *H. virescens* larvae signaling lacZ. Larvae were fed artificial diet (A) or cotton foliage (B) for 8 h prior to inoculation with 95 OBs AcMNPV-hsp70/lacZ. The progression of infection from primary (M only) to systemic (M + T or T only) foci, represented by the relationship between the percentage of systemically infected larvae and the percentage of larvae with primary infections, was not significantly affected by food ingested ($\chi^2 = 1.75$, d.f. = 1, $p = 0.1863$). This experiment was performed one time, with 18–27 inoculated larvae dissected at each time point.

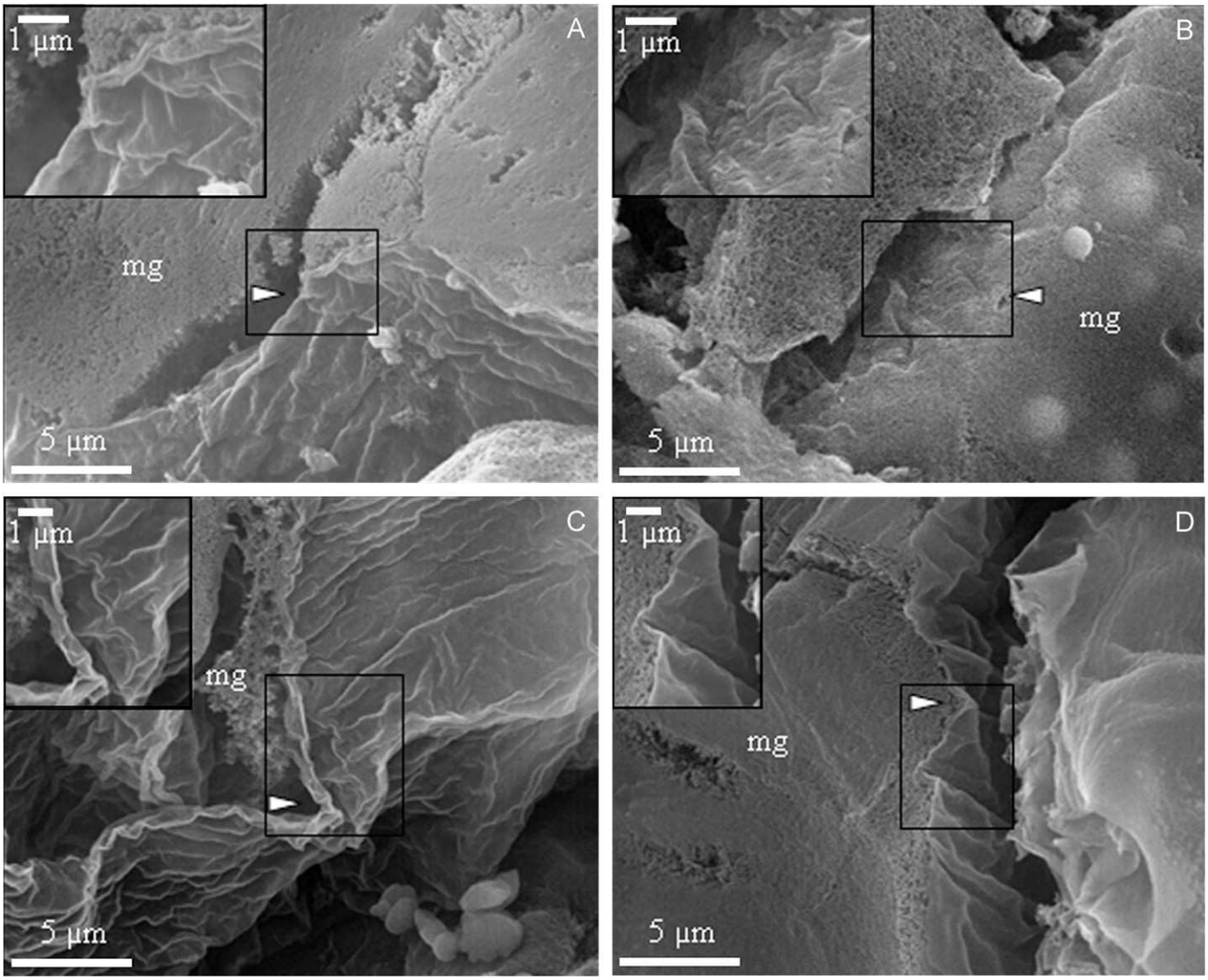


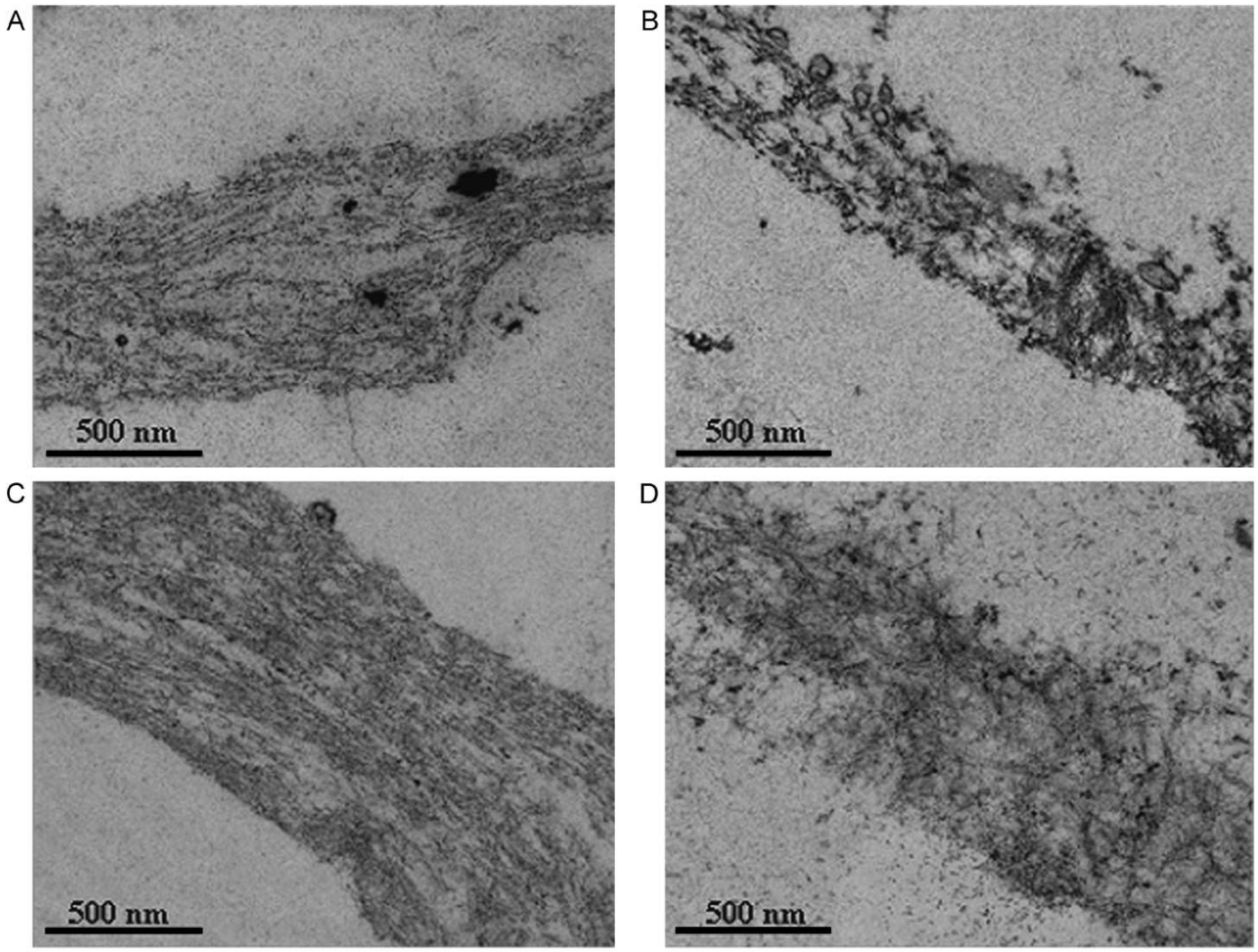
Fig. 3. Scanning electron micrographs of the PM from *H. virescens* larvae, revealing the influence of food and ingested TnGV OBs on PM structure. A = artificial diet-fed, B = artificial diet + TnGV-fed, C = cotton-fed, D = cotton + TnGV-fed. Midgut epithelium = mg; white arrow points to the PM. Magnification of large pictures = 5000 ×; scale bars = 5 μm. Insets in the upper right corner of each image are 15,000 × with 1 μm scale bars; the enlarged region shown in the insets is delineated by the box in the 5000 × images. The PM surface was visibly degraded by ingested TnGV OBs in artificial diet-fed larvae. SEM images were taken from the midguts of three to four larvae per treatment.

TnGV-treated cotton foliage appeared, in the SEM images, to be of similar, robust surface quality (Fig. 3B and D, respectively).

However, transmission electron micrographs (TEM) revealed a potential reason for the differences we observed in viral performance among treatments. TEMs were taken from the medial midguts of two larvae per treatment, with 2–11 cross-sections viewed per larva, to augment observations made from the SEM images. While the SEM images showed a similar PM surface texture in artificial diet and cotton-fed larvae (Fig. 3A and C, respectively), measurements taken from the TEM images indicated a two-fold thicker PM in larvae fed cotton foliage than in larvae fed artificial diet (Fig. 4C and A, respectively). The TEM images also provided quantitative information on the effect of TnGV OBs on PM integrity. Larvae fed TnGV-treated artificial diet showed a 4.5-fold reduction in PM width with a substantial decrease in apparent PM organization, as compared to larvae fed untreated artificial diet (Fig. 4B and A, respectively). Also, consumption of TnGV (enhancin) produced a 1.4-fold reduction in PM width and disrupted PM structure in cotton-fed *H. virescens* larvae (Fig. 4D and C, respectively).

3.3. Effect of PM and host plant on AcMNPV pathogenesis

Given the ability of TnGV OBs containing enhancin to disrupt PM structure, the effects of TnGV OBs on larval mortality and *in vivo* pathogenesis of AcMNPV-hsp70/lacZ were examined. An initial experiment evaluating the



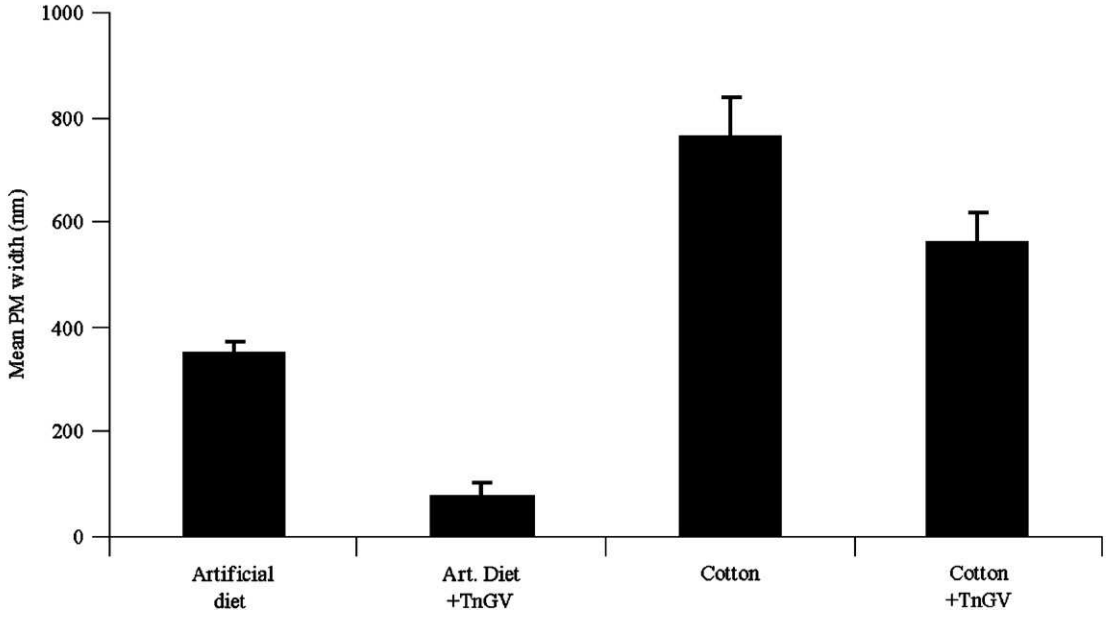


Fig. 4. Effect of food and TnGV OBs on PM width of *H. virescens* larvae. Transmission electron micrographs of the medial midgut of *H. virescens* larvae are shown at the top of the figure. A = artificial diet-fed, B = artificial diet + TnGV-fed, C = cotton-fed, D = cotton + TnGV-fed. Note the dramatic disorganization of the PM in TnGV-treated larvae. Magnification = 20,000 ×; scale bars = 500 nm. Mean measured PM widths are shown at the bottom of the figure. The PM of cotton-fed larvae was two-fold thicker than that of artificial diet-fed larvae ($F = 111$, d.f. = 1, $p < 0.0001$). Ingestion of TnGV OBs significantly decreased PM width by 4.5-fold in larvae fed artificial diet and 1.4-fold in larvae fed cotton foliage ($F = 34.6$, d.f. = 1, $p < 0.0001$). Two larvae were used for TEM imaging per treatment, with 2–11 cross-sections viewed per larva; error bars represent 1 S.E.

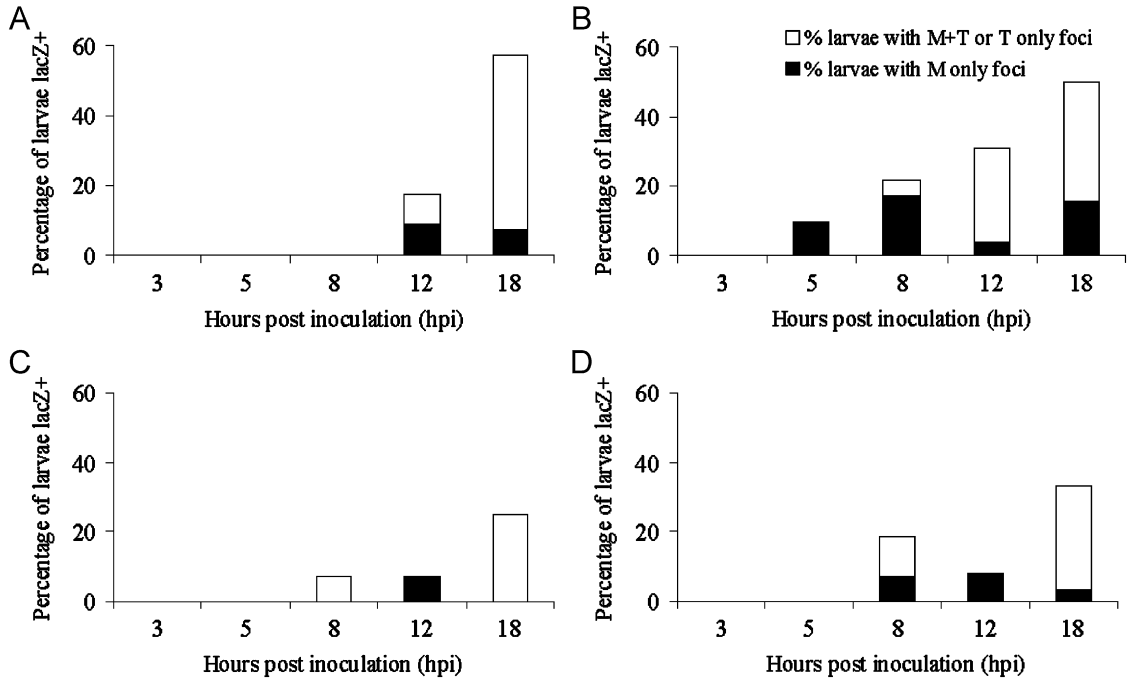


Fig. 5. Time course of the percentage of *H. virescens* larvae with primary or systemic infection foci post-inoculation. Larvae were fed artificial diet (A), artificial diet treated with TnGV OBs (B), cotton foliage (C), or cotton foliage treated with TnGV OBs (D) for 8 h post-molt to the fourth instar prior to oral inoculation with 26 occlusion bodies per larva. Viral spread from primary (M only) to systemic (M + T or T only) foci was not influenced by food ingested prior to inoculation or by TnGV (food $\chi^2 = 0.09$, d.f. = 1, $p = 0.7621$; TnGV $\chi^2 = 0.38$, d.f. = 1, $p = 0.5392$).

influence of TnGV on larval mortality demonstrated that TnGV OBs did not significantly influence mortality of artificial diet-fed larvae (artificial diet = 67.9%, artificial diet + TnGV = 72%), but doubled the mortality of cotton-fed larvae (cotton = 35.7%, cotton + TnGV = 73.1%). The dramatic increase in mortality observed in cotton-fed larvae with PMs disrupted by the enhancin in TnGV compared to cotton-fed larvae with intact PMs, indicates that the PM impeded viral infection in cotton-fed insects. Subsequent experiments suggested that disruption of the PM by enhancin in TnGV OBs also influenced the progression of virus infection, although this effect was not statistically significant (Fig. 5). In artificial diet-fed larvae, consumption of TnGV OBs reduced the time to first lacZ signaling from 12 to 5 hpi (Fig. 5). While the overall effect of TnGV on artificial diet-fed larvae was not statistically significant, there was a significant interaction between TnGV and hours post-inoculation in artificial diet-fed larvae ($\chi^2 = 5.85$, d.f. = 1, $p = 0.0155$), suggesting that the constantly forming PM may be an impediment to viral infection. In cotton-fed larvae, consumption of enhancin-containing TnGV OBs did not influence the timing of lacZ signaling but did increase the proportion of larvae signaling at 8 hpi by 2.5-fold (Fig. 5). Further, both primary and systemic infection foci were observed in TnGV-treated larvae at 8 hpi, while only primary foci were found in untreated cotton-fed larvae at this time point, suggesting a more rapid establishment of systemic infection foci in TnGV-treated larvae (Fig. 5). These results also suggest that the PM negatively impacts AcMNPV infection in *H. virescens* larvae.

Given that the PM of cotton-fed *H. virescens* larvae negatively influenced AcMNPV infection, the role of the PM in larvae fed various host plants was investigated to ask if this effect was specific to cotton foliage or generalizable to other *H. virescens* host plants. Similar to the effects of cotton foliage, larvae ingesting oakleaf lettuce or tobacco foliage prior to viral inoculation experienced a significant reduction in mortality as compared to larvae fed artificial diet. Mortality of oakleaf lettuce-fed larvae was reduced an average of $24 \pm 16.4\%$ compared with artificial diet-fed larvae ($\chi^2 = 16.98$, d.f. = 1, $p < 0.0001$); mortality of larvae fed tobacco foliage was reduced an average of $22.2 \pm 1.7\%$ compared with artificial diet-fed larvae ($\chi^2 = 10.21$, d.f. = 1, $p = 0.0014$).

Given the general plant effects on *H. virescens* viral mortality, transmission electron micrographs of medial midguts from larvae fed artificial diet, cotton foliage, oakleaf lettuce, iceberg lettuce or tobacco foliage were compared for differences in PM width and structure (Fig. 6). Three larvae were used for TEM imaging per treatment, with eight cross-sections viewed per larva. PM width was significantly influenced by food ingested; the PMs of larvae fed cotton, oakleaf lettuce or iceberg lettuce foliage were significantly thicker than that of artificial diet-fed larvae (Fig. 6). Interestingly, the PM of tobacco-fed larvae was the same width as that of artificial diet-fed larvae, in apparent contrast with the reduction in viral

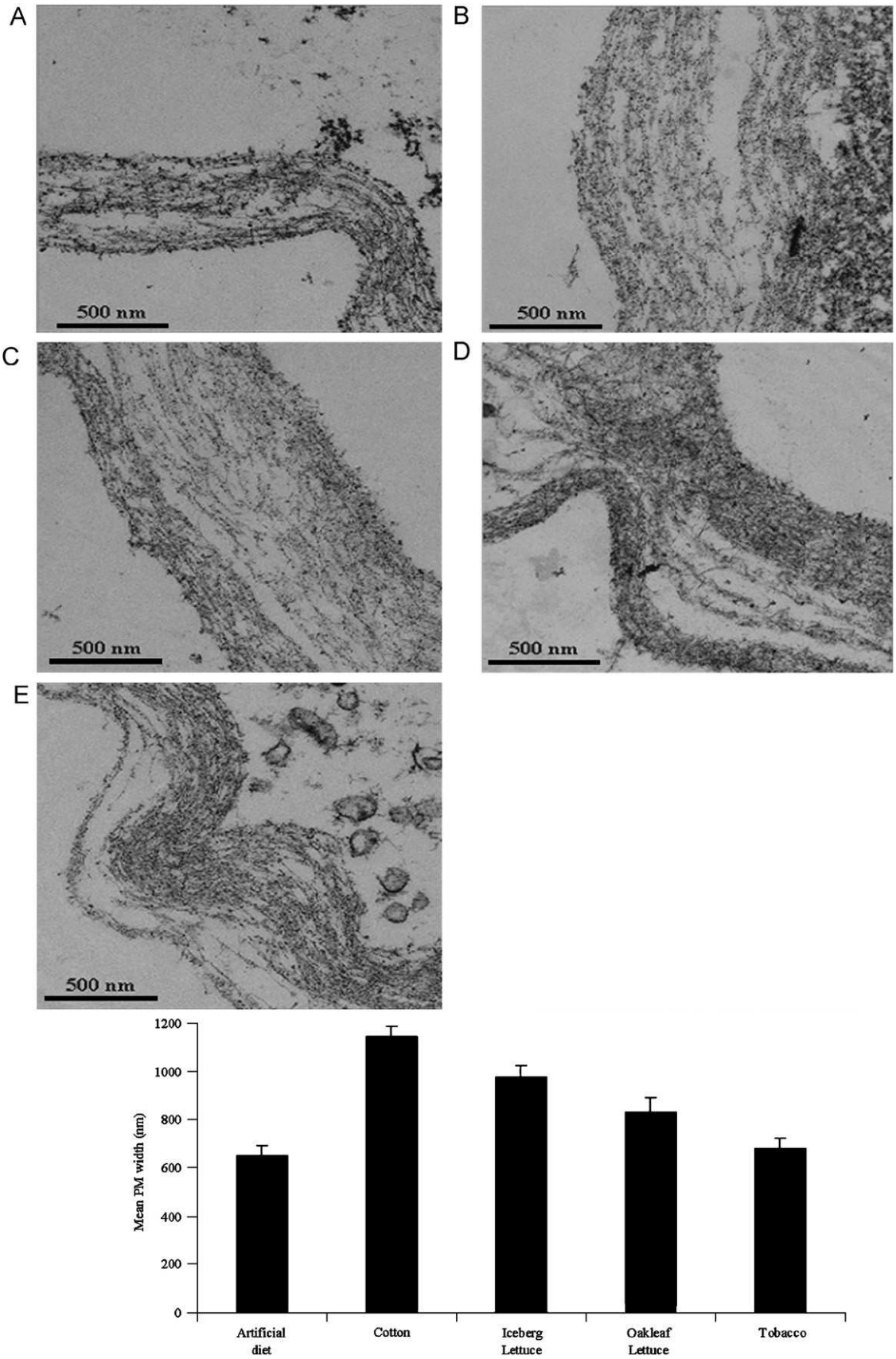


Fig. 6. Effect of food on PM width of *H. virescens* larvae. Transmission electron micrographs of the medial midgut from *H. virescens* larvae fed artificial diet (A), cotton foliage (B), iceberg lettuce (C), oakleaf lettuce (D) or tobacco (E) are shown at the top of the figure. Magnification = 20,000 ×; scale bars = 500 nm. The PMs of plant-fed larvae have multiple layers, compared to the PM of artificial diet-fed larvae. Mean measured PM widths are shown at the bottom of the figure. PM width was significantly influenced by food ingested for the first 8 h of the fourth instar ($F = 21.2$, d.f. = 4, $p < 0.0001$); foliage-fed larvae had thicker PMs than larvae fed artificial diet. Three larvae were used for TEM imaging per treatment, with eight cross-sections viewed per larva; error bars represent 1 S.E.

mortality seen in tobacco-fed larvae. Also notable in the TEM images are the distinct multiple layers of the PM from plant-fed larvae, as compared to fewer layers in the PM from artificial diet-fed larvae (Fig. 6).

The mean PM thickness for each treatment group measured from the TEM images was plotted against the percentage infection at 18 hpi and percentage mortality of that group (Fig. 7). The treatment groups represented the sum of our data, with each group being defined by the *H. virescens* larval colony and food ingested; the groups were 1997 larvae fed artificial diet, 97 larvae fed artificial diet + TnGV, 97 larvae fed cotton foliage, 97 larvae fed cotton foliage + TnGV, 2002 larvae fed artificial diet, 02 larvae fed cotton foliage, 02 larvae fed iceberg lettuce, 02 larvae fed oakleaf lettuce and 02 larvae fed tobacco. Mean PM width was predictive of the percentage of virally infected larvae at 18 hpi; as PM width increased, the percentage of larvae signaling lacZ decreased (Fig. 7). PM width was also inversely related to viral mortality (Fig. 7); the thicker the PM, the lower the mortality by virus. Further, the percentage of virally infected larvae at 18 hpi was positively related to larval mortality.

In addition to influencing larval infection by virus, PM width also influenced larval development. Both time to death of infected larvae and time to pupation of uninfected control larvae were positively related to PM width (Fig. 8). Infected larvae possessing thicker PMs took longer to die than larvae possessing thinner PMs. Larvae that survived to pupation required a longer time to pupate, the thicker their PM.

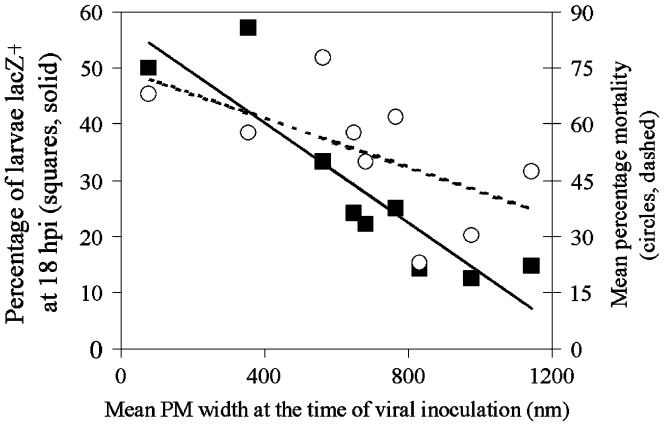


Fig. 7. Host plant-mediated relationships between the mean PM width of *H. virescens* larvae at the time of viral inoculation, the percentage of larvae found to be lacZ positive at 18 hpi, and the corresponding mean percentage larval mortality from AcMNPV-hsp70/lacZ. Larvae were fed foliage or artificial diet for 8 h prior to virus challenge. As PM width increased, the percentage of infected larvae observed at 18 hpi decreased (% infected larvae at 18 hpi = 58.0−0.04 × (mean PM width); $R^2 = 0.80$; $F = 27.8$, $p = 0.0012$). Similarly, PM width and percentage of larval mortality by virus were inversely related (mean percentage mortality = 74.6−0.03 × (mean PM width); $R^2 = 0.36$; $F = 3.99$, $p = 0.0860$). The percentage of larvae infected at 18 hpi was determined from 18 to 27 inoculated larvae dissected for each treatment, and the percentage larval mortality was determined from 30 to 50 larvae per treatment.

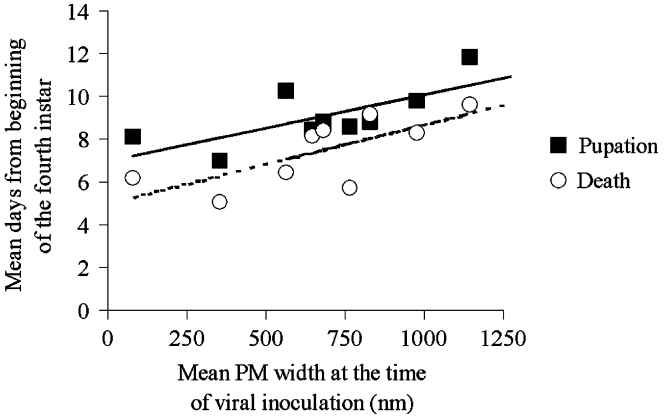


Fig. 8. Relationships between the mean PM width of *H. virescens* larvae and mean time (days) to death or pupation from the beginning of the fourth instar (4^0) of virally inoculated or control larvae. Note that the greater the PM width, the longer the time to death or pupation (mean days to death = 4.97 + 0.004 × (mean PM width), $R^2 = 0.53$, $F = 7.80$, $p = 0.0268$; mean days to pupation = 6.97 + 0.003 × (mean PM width), $R^2 = 0.51$, $F = 7.30$, $p = 0.0360$). Time to death was determined from 30 to 50 larvae per treatment and time to pupation from 20 to 30 larvae per treatment.

4. Discussion

The host plant consumed prior to challenge with baculoviruses has been shown to modify susceptibility of lepidopteran larvae to disease in numerous systems (reviewed in Cory and Hoover, 2006). In this study, we demonstrated that host plant foliage decreased performance of a viral pathogen by altering PM structure, thereby reducing the ability of the virus to establish infections in the midgut. Plant-fed larvae generally possessed a thicker PM than artificial diet-fed larvae, and this thickened PM was correlated with a reduction in infections at 18 h post-inoculation and a reduction in larval mortality. Thus, our data indicate that the PM is a key factor in resistance of lepidopteran larvae to baculoviruses when insects are fed on their natural host plants.

The concept that the PM is an impediment to viral infection in plant-fed larvae was reinforced when we observed increased mortality in TnGV-fed larvae with enhancin-degraded PMs. Feeding larvae with TnGV OBs containing the metalloprotease enhancin, which degrades the PM by digesting invertebrate intestinal mucin (Lepore et al., 1996; Wang and Granados, 1997), produced PMs that were highly disrupted and significantly thinner than those of controls. When TnGV-treated larvae were inoculated with baculovirus, we observed higher viral mortality in TnGV-treated plant-fed insects, suggesting that the PM hinders baculoviral infection in plant-fed larvae. While we did not directly measure PM permeability in this study, previous work has shown that exposing the PM to enhancin significantly increases its permeability to AcMNPV OBs (Peng et al., 1999). Thus, we suggest that reduction of PM permeability to virions is one mechanism

whereby ingested plant material reduces viral infection and mortality.

Interestingly, despite earlier onset of initial midgut infections in artificial diet-fed larvae treated with TnGV, mortality by baculovirus was unchanged by feeding TnGV OBs to these larvae. This finding is in agreement with a previous report (Washburn et al., 1995) which concluded that the PM is not a barrier to baculoviruses in artificial diet-fed *H. virescens*. Our results suggest the potential for physiological differences between diet- and plant-fed larvae and emphasize the importance of including natural host plants in studies of insect responses to ingested pathogens (reviewed in Duffey et al., 1995).

In this study, larval *H. virescens* fed cotton foliage possessed a thicker PM and experienced significantly fewer midgut infections and lower mortality than corresponding larvae fed artificial diet. These findings suggest that the altered PM found in plant-fed *H. virescens* larvae protects these larvae from baculoviral infection. An important function of the PM is protection of the midgut from abrasion by ingested materials (Sudha and Muthu, 1988). In addition to physically shielding the midgut, the PM chemically protects the midgut, binding toxic compounds and effectively removing them from the midgut (Bernays and Chamberlain, 1980; reviewed in Lehane, 1997; reviewed in Barbehenn, 2001). The PM has also been shown to protect the midgut against ingested bacterial (Granados et al., 2001; Hayakawa et al., 2004; Martin, 2004) and viral pathogens (Shapiro et al., 1987; Wang and Granados, 1998; reviewed in Monobrullah, 2003; Guo et al., 2007). Given these protective functions, the thicker PM observed in plant-fed larvae would seem to be advantageous. However, although there are undoubtedly protective benefits to the more robust PM observed in plant-fed *H. virescens* larvae, there may also be costs associated with forming this thicker structure. The removal of the PM has been shown to increase the rate of digestion (Villalon et al., 2003), suggesting that a thicker PM may reduce the efficiency of the digestive process. This potential decrease in digestive rate in plant-fed larvae may manifest as a decreased growth rate in plant-fed larvae. Although the PM is not the only factor determining larval growth rate, we did observe slower growth rates in larvae possessing thicker PMs. Additionally, forming a thicker PM may simply be more costly, requiring more nutrients and energy to construct than a thinner PM. In summary, our findings support a potential trade off between the protective and digestive functions of the PM.

While having the ability to change PM structure with the type of food ingested may be advantageous for phytophagous lepidopteran larvae, the mechanism by which ingested foliage influences PM structure is unknown. Studies on PM formation are difficult to perform. Whereas aspects of PM formation in blood-feeding insects are relatively well characterized (Ramos et al., 1994; Shao et al., 2001, 2005; Devenport et al., 2004, 2005; Kato et al., 2006), the regulation of PM formation in phytophagous insects is less well understood. We propose three potential mechanisms whereby ingested foliage could alter PM structure. First, ingested foliage may increase PM width and influence PM permeability by increasing synthesis of PM proteins and/or proteoglycans. Proteoglycans are a primary structural component of the PM, and have been hypothesized to influence PM integrity and permeability (reviewed in Lehane, 1997). PM proteins are secreted and bind to the PM after the chitin and proteoglycan framework is assembled. Ingested foliage may be able to influence the amount and/or composition of the PM proteins secreted. Second, ingested foliage may manipulate the structure of previously formed PM, possibly through increasing cross-linking among PM proteins or between PM proteins and the chitin framework. We observed both quantitative and qualitative changes to the PM when larvae were fed cotton foliage. Quantitatively, we observed a thicker PM in cotton-fed larvae than in artificial diet-fed larvae. Qualitatively, we observed a differential response to TnGV treatment in diet and cotton-fed larvae. The PM of diet-fed larvae was almost completely degraded when larvae were fed enhancin-containing TnGV OBs. In contrast, the PM of cotton-fed larvae was affected, but the level of degradation was less, suggesting qualitative changes in PM structure. Third, the PM may be thickened through compaction if the PM is synthesized at the same rate regardless of food ingested if the foliage food bolus is moving more slowly than the artificial diet food bolus. This latter hypothesis of PM thickening is unlikely, based on previous findings that PM passage rates are independent of food consumption (reviewed in Peters, 1992). Our results also suggest that PM formation is independent of food passage rates, since we observed similar food passage rates in *H. virescens* larvae fed artificial diet and those fed iceberg lettuce (data not shown), but the PM of iceberg lettuce-fed larvae is 1.5-fold thicker than the PM of artificial diet-fed larvae. Thus, PM width is likely independent of food passage rates. One or more of these hypothetical mechanisms may be responsible for the observed changes to the PM; consequently, interactions between ingested foliage and the PM warrant further investigation.

Though we have shown that the PM of plant-fed larvae influences viral infection, the PM is likely to be one of many factors affecting pathogen success. Our data suggest a more complex interaction, given that larvae fed tobacco foliage had significantly reduced viral mortality, as compared to those fed artificial diet, but the mean PM width observed in tobacco-fed larvae was similar to that of diet-fed larvae. This result could indicate qualitative differences between the two PMs, or may signify that host plants can influence viral efficacy in other ways. Other effects of host plants on viral infections include plant influences on virion integrity and midgut cell sloughing. Foliar phenolics may interact with viral OBs, reducing their dissolution and release of infective virions (Felton and Duffey, 1990; Hoover et al., 1998). Ingested plant material

may also increase the rate of sloughing of primary midgut infections before the virus becomes established and spreads systemically (Hoover et al., 2000). In Hoover et al. (2000), a greater number of infection foci were observed at 24 hpi in cotton-fed *H. virescens* larvae co-inoculated with AcMNPV and 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 (Washburn et al., 1998; Dougherty et al., 2006). However, the authors did not rule out alternative explanations. M2R may have multiple modes of action in the midgut. In addition to blocking midgut cell sloughing, M2R was shown to inhibit chitin synthetase *in vitro*, preventing PM formation (Bartnickigarcia et al., 1994; Wang and Granados, 2000), and can inhibit plant peroxidases and polyphenol oxidases assayed *in vitro* (Hoover, unpublished data). Thus, results observed by Hoover et al. (2000) could be explained by an M2R-mediated disruption of the PM or inhibition of plant-derived oxidative enzymes rather than (or in addition to) decreased rates of midgut cell sloughing.

In conclusion, we have shown that ingested plant material alters the structure of the PM in larval *H. virescens*, such that the PM of plant-fed larvae was more robust than that of artificial diet-fed larvae. Further, we have demonstrated that the thickened PM found in plant-fed larvae influenced viral pathogenesis, reducing the number of midgut infections and ultimately decreasing larval mortality, compared with artificial diet-fed larvae. Finally, 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 the interactions between insect tissues, ingested plant foliage and viral inoculum.

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