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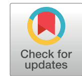
Acquisition of lethal infection, hypermobility and modified climbing behavior in nucleopolyhedrovirus infected larvae of *Anticarsia gemmatalis*



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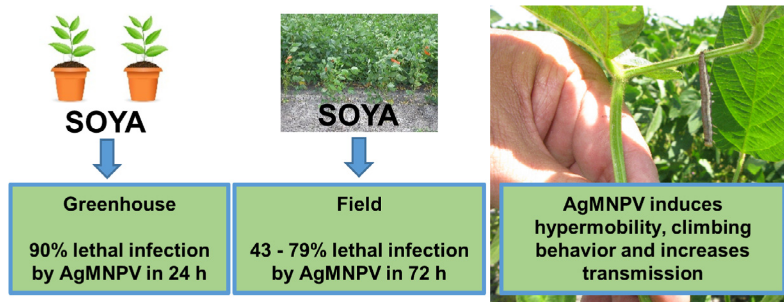
GRAPHICAL ABSTRACT



ABSTRACT

The Anticarsia gemmatalis multiple nucleopolyhedrovirus (AgMNPV) is the basis for viral insecticides in several countries in Latin America. The rate of acquisition of infection and the transmission of the pathogen from infected to healthy conspecifics both influence the efficacy of virus insecticides. By comparing the leaf area consumed by larvae with their stage-specific susceptibility to infection, we estimated that fourth instars were approximately twice as likely to acquire infection as second or third instars. Greenhouse trials indicated that 63% of third instars became infected within 1 h of exposure to soya plants treated with 1.5×10^7 occlusion bodies (OB)/m^2 obtained from mixtures of local AgMNPV isolates, as did 90% of larvae sampled at 24 h. Acquisition of lethal infection was slower in field trials performed in the soya-growing Huastecas region of Mexico. Virus-induced mortality varied from 43 to 79% in natural infestations of larvae sampled at 72 h post-application depending on the dose of unformulated OBs applied with wetting agent, although the majority of larvae had already acquired a lethal infection at 24 h post-application. Spray application volume (200 or 400 l/ha) did not significantly affect the prevalence of infection at 1 h post-application. Infected fifth instars moved twice as far and died significantly higher on plants than control larvae. Overall, 22% of third instars that subsequently foraged on these plants acquired a lethal infection. We conclude that most insects acquire infection within 24 h of virus application and that OBs in feces and regurgitate of infected larvae contribute significantly to pathogen transmission.

1. Introduction

The Anticarsia gemmatalis multiple nucleopolyhedrovirus (AgMNPV, *Alphabaculovirus*; *Baculoviridae*) has been the basis for the most widely used viral biopesticide in the world (de Castro Oliveira et al., 2006). This virus has been used to control the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) in Brazil, Paraguay, Uruguay and Mexico (Haase et al., 2015). A large-

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scale extension program under the control of EMBRAPA in Brazil resulted in the adoption of AgMNPV-based insecticides over an area that grew from one million hectares in 1990–91 to up to two million hectares in the 2003–04 production cycle, representing approximately 10% of the country's soya production (Moscardi et al., 2011). Use of AgMNPV-based insecticides in Brazil has subsequently declined with the increasing use of insect growth regulators and transgenic crops (Sosa-Gómez, 2017).

In Mexico a Brazilian isolate of AgMNPV was introduced in 1999 and used in a series of field trials in the major soya-growing region of Huastecas in Tamaulipas State, under the auspices of the INIFAP (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias), a federal government research organization (Avila-Valdez and Rodríguez-del-Bosque, 2008). Beginning in 2005 growers in the Huastecas region were encouraged to adopt a system of integrated pest management involving virus-based control of *A. gemmatalis* infestations. The virus was produced locally by infecting larvae on soya crops and collecting and processing infected cadavers. The use of the virus was subsequently employed on ~15,000 ha of soya in this region (Williams et al., 2013).

The acquisition of a lethal nucleopolyhedrovirus infection occurs when a larva consumes a sufficient number of OBs to result in larval death. The time taken to acquire a lethal infection is a key aspect that can determine the efficacy of a virus-based insecticide for three reasons. First, OBs are rapidly inactivated by solar ultraviolet radiation or certain phytochemicals following their application so that the insecticidal activity of OBs on leaf surfaces can fall markedly over the course of 24 h (Cory and Evans, 2007). Second, the susceptibility of larvae to infection decreases as larvae develop both within instar (intrastadial developmental resistance) and from one instar to the next (Hoover et al., 2002; McNeil et al., 2010). Third, speed of kill is an aspect of virus phenotype that influences the feeding damage that an infected larva can cause prior to death (Simón et al., 2008; Wilson et al., 2000). These factors individually or in combination act to reduce the efficacy of virus based insecticides as crop protection agents as the interval between application of OBs to the crop and acquisition of infection by the pest increases.

Acquisition of a lethal infection in a pest population depends on multiple factors related to the innate susceptibility of larvae, the stage structure of the pest population, the quantity and distribution of OBs applied, pest feeding behavior and crop architecture, among others (Lacey et al., 2015; Hajek and Shapiro, 2018). Preliminary field trails performed in the Huastecas region in 2015 suggested that acquisition of infection was rapid in *A. gemmatalis* larvae such that the majority of larvae acquired a lethal infection within 24 h of application of the virus. During these preliminary trials it was also clear that infected larvae climbed to the upper parts of the plant prior to death, a virus-induced modification of behavior that is likely to improve virus transmission (Young et al., 1987; Young and Yearian, 1989). In order to quantify the speed of acquisition of lethal infection we performed greenhouse and field trials using virus doses established as effective for *A. gemmatalis* control of this pest in Brazil (Moscardi and Sosa-Gómez, 2007). We also quantified virus-induced hypermobility and climbing behavior in *A. gemmatalis* and demonstrated that infected larvae release biologically significant quantities of inoculum prior to death; a previously unrecognized route of transmission in this species.

2. Materials and methods

2.1. Insect colony, virus and soya plants

A laboratory colony of *A. gemmatalis* was started using apparently healthy larvae collected in 2013 and 2014 from soya crops at the INIFAP-Las Huastecas field station (22°33′59″ N, 98°09′58″ W) in Tamaulipas State, Mexico. The colony was maintained in the INECOL insectary in Xalapa, Veracruz State, at 26 ± 2 °C, 60–80% humidity and 14 h:10 h L:D photoperiod. Larvae were reared individually on diet (Greene et al., 1976) and adults were provided with continuous access to 10% honey solution. Eggs were treated with 0.5% sodium hypochlorite for 5 mins and rinsed in distilled water prior to eclosion.

The virus sample used in this study was obtained from a mixture of AgMNPV isolates originating from 30 diseased larvae collected from soya crops in the INIFAP-Las Huastecas field station in 2013 and 2014. This mixture, named AgMNPV-30 wt, had higher pathogenicity, measured in terms of concentration-mortality metrics, than any of its component genotypes (Del-Angel et al., 2018). The virus sample was amplified in fourth instars using the diet incorporation technique (Lacey and Kaya, 2007). Virus-killed insects were homogenized in sterile water and the homogenate was filtered through a fine metal gauze (80 µm pore size) to remove insect debris. The resulting OB suspension was quantified using a Neubauer cell counting chamber (Hawksley, UK) and stored at 4 °C for up to one month prior to use in greenhouse and field experiments. The fidelity of the amplification was confirmed by comparison of HindIII restriction endonuclease profiles of genomic DNA extracted from amplified and inoculum OBs, following the methods described elsewhere (Del-Angel et al., 2018).

Soya var. Huasteca 400 (Maldonado-Moreno et al., 2010) was grown from seed in 30 cm tall plastic bags filled with 4 kg damp soil in an experimental greenhouse in the INECOL installations in Xalapa. No fertilizers or pesticides were applied.

2.2. Leaf feeding and infection risk

To determine the foliar area consumed by each instar of *A. gemmatalis* and therefore the likelihood of consuming a lethal dose of OBs, a group of 20 s instars (4–6 h after molting) were individually placed on 20 mature soya leaves, the area of which had been previously measured using Easy Leaf Area automated digital image analysis software (Easlon and Bloom, 2014). Larvae were prevented from escaping by enclosing each leaf in a 9 cm diameter plastic Petri dish with a piece of wet paper towel over a 24 h period. After this, each larva was removed and the leaf area measured again to determine the leaf area consumed by each insect. The procedure was performed on three occasions using different batches of insects. The entire procedure was repeated for third and fourth instars. Leaf area values were not normally distributed and were subjected to Kruskal-Wallis test and pairwise comparisons by Tukey (Nemenyi) test using the PMCMR package (Pohlert, 2014) in RStudio v. 0.98.501 (RStudio Inc., Boston). The instar specific likelihood of infection was calculated as follows. The number of OBs/cm^2 (a) required to infect 50% of insects was calculated using published LD_{50} values (b) from larvae that consumed OBs on the surface of leaf disks (Boucias et al., 1980), or semi-synthetic diet (Moscardi, 1983), over a 24 h period, divided by leaf area consumed over a 24 h period (c), i.e. $a = b/c$, assuming a uniform distribution of OBs on leaf surfaces. The relative risk of infection for third (a_3) and fourth (a_4) instars was then calculated relative to that of the second instar (a_2), i.e., a_2/a_3 or a_2/a_4, respectively (Goulson et al., 1995). It was not possible to determine the dose-mortality response for the AgMNPV-30 wt mixture as the *A. gemmatalis* colony was lost during the study and could not be replaced.

2.3. Acquisition of lethal infection: greenhouse experiments

To estimate the rate of acquisition of infection over a 72 h period in third instars, groups of 10 soya plants (average height 51 ± 1.4 cm) were arranged in a 1 m^2 square and sprayed with a suspension of 1.5×10^7 OBs (equivalent to 1.5×10^{11} OB/ha) in a volume of 40 ml of water with 0.1% (vol/vol) Tween 80 as a wetting agent. Control plants were treated identically with a 0.1% Tween 80 solution without OBs. After 30 mins, when plants had dried completely, plants were placed together in a 1 m × 60 cm × 60 cm cage constructed from PVC tubes and nylon gauze, and a group of 150 larvae in the third instar was placed on to plants. At intervals of 6, 12, 24, 48 and 72 h a random sample of 24 larvae were collected from plants placed individually in

the wells of a 24-well tissue culture plate with diet and reared until pupation or death. Deaths from polyhedrosis disease were confirmed by examining Geimsa-stained smears of larval tissues to detect the presence of OBs. Five replicates of the experiment were performed at different time points.

The entire experiment was subsequently performed following identical procedures except that larvae were sampled at 1, 3 and 6 h after being placed on treated and control plants. For both experiments the prevalence of infection over time was analyzed by fitting generalized linear mixed-effects models with a binomial error structure and multiple comparisons of means by Tukey contrasts in RStudio.

2.4. Acquisition of lethal infection: field experiments

In October 2016, a flowering soya crop (var. Huasteca 400, average height 56.2 ± 1.1 cm) in the early stages of pod production was selected in the fields surrounding the INIFAP – Las Huastecas experimental station. The presence of a suitable infestation of *A. gemmatalis* larvae on the crop was assured by sampling prior to field trials. Sampling was performed by briskly shaking the stems of soya plants over a white sheet of plastic placed on the ground to obtain a sample of larvae present on 1 m^2 of foliage (Herzog and Todd, 1980). The plastic sheets were treated with 0.5% sodium hypochlorite solution between treatments to avoid cross-contamination. No applications of virus or synthetic insecticides had previously been made to the crop.

2.4.1. Effect of dose on acquisition of infection

Each experimental unit consisted of a pair of adjacent rows of 40 m in length in the center of the soya crop with 4 rows of plants between selected pairs of rows. One of the following three treatments was randomly assigned to each of four pairs of rows: (i) 2×10^8 OB equivalent to 5.0×10^{10} OB/ha (low dose), (ii) 6×10^8 OB equivalent to 1.5×10^{11} OB/ha (high dose), (iii) water control. Treatments were applied at 8.00 hrs using a manual sprayer (Pro-Sprayer, RL Flo-Master, Lowell, MI). The application volume was 1.6 l for each pair of rows (6.4 l/treatment), equivalent to 400 l/ha, which was within the typical range of volumes (200–600 l/ha) for insecticide applications to this crop (Avila-Valdez and Rodríguez-del-Bosque, 2004). Virus treatments and control were applied with 0.1% Tween 80 as wetting agent. A data logger (Hobo, Onset Computer Corp., Bourne, MA) was placed 30 cm above the ground at the center of the experimental plot to collect temperature and humidity data.

At intervals of 3, 24, 48 and 72 h post-application a total of 35 larvae were collected from three randomly selected points in each pair of rows by shaking plants over a plastic sheet. Sampling points were selected by taking a random number, between 1 and 40 steps, in either direction along the row. Larvae were placed individually in a 30 ml plastic cup with a cube of diet, placed in an insulated box and taken to the laboratory where they were reared at 23 ± 1 °C until death from polyhedrosis disease or pupation. Virus-induced deaths were confirmed by microscopic observation of OBs in Geimsa-stained smears of larval tissues.

Mixed-effects models with a binomial error structure were fitted to virus-induced mortality results with OB dose and sample time as fixed factors using the Ime4 package (Bates et al., 2017) and the glmer function in RStudio. Simultaneous tests for general linear hypotheses and multiple comparisons of means by Tukey contrasts were conducted to establish differences between treatments groups using the multcomp package (Hothorn et al., 2017).

2.4.2. Effect of application volume on acquisition of infection

To determine whether rapid acquisition of lethal infection could be improved by increasing the volume of application and thereby the coverage of crop foliage, eighteen pairs of rows, 20 m in length with 4 rows separation were selected and marked with stakes. Six pairs of rows were randomly assigned to one of the following three treatments: (i) 1×10^8 OB applied in a volume of 400 ml, (ii) 1×10^8 OB applied in a volume of 800 ml, (iii) water control of 800 ml. All treatments were applied with 0.1% Tween 80 as wetting agent. At 1 h after treatment larvae were collected from three randomly selected points within each pair of rows (35 larvae in total), individually placed in cups with diet and transported to the laboratory for rearing until death from polyhedrosis disease or pupation, as described in the previous experiment. The prevalence of virus-induced mortality was compared between high and low volume treatments by fitting a generalized linear model with a binomial error structure specified in RStudio.

2.5. Virus-induced modification of larval behavior

As field observations indicated that AgMNPV infection could induce modifications in larval climbing activity, with implications for virus transmission, we decided to quantify this behavior. For this, fourth instars were inoculated with 1×10^8 OBs/ml of sucrose and food coloring solution using the droplet feeding technique (Hughes and Wood, 1981). This concentration was estimated to result in 90% lethal infection. A total of 30 larvae that consumed the inoculum in 10 mins were individually transferred to the wells of a 24 well tissue culture plate with diet and incubated for 4 days at 25 ± 1 °C in a laboratory incubator. Following this, each infected larva, now in the early fifth instar, was released on the lowest leaves of a soya plant 10 cm above the soil surface. Each plant (average height 51.0 ± 1.4 cm) was covered with a cage 1 m × 60 cm × 60 cm in the greenhouse. Larval feeding on leaves was observed on all plants in both infected and control treatments. The height of larvae on plants was recorded at 8 h intervals over a 48 h period. The minimum vertical distance moved was calculated from the height measurement at each time point from the height measurement at the previous time point. Control larvae were treated identically but were not inoculated with OBs.

To determine whether foraging of infected larvae had contaminated plants with significant quantities of OBs, the leaf on which each larva had died was removed and discarded immediately following the death of the larva. Larvae that had not died but were moribund and had stopped moving by the 48 h observation time were treated identically. Plants were then placed in five groups of five plants within cages 1 m × 60 cm × 60 cm and 50 larvae in the third instar were released on to the central portion of experimental plants for a 24 h period. After this, 24 larvae from each group of plants were selected at random and reared individually in tissue culture plates with diet until death or pupation. The prevalence of polyhedrosis disease was confirmed by observation of OBs in Geimsa-stained smears of larval tissues.

Distance moved over time was normalized by square-root transformation and analyzed by fitting a linear mixed-effects model. The suitability of the model was determined by examining the AIC value and the distribution of fitted values and standardized residuals. The heights of larvae on plants at death or moribund state (infected larvae) or at the end of the 48 h observation period (control larvae) were normally distributed and were compared by *t*-test.

3. Results

3.1. Leaf feeding and infection risk

The leaf area consumed by larvae over a 24 h period increased significantly with instar (Fig. 1), from 0.48 ± 0.05 cm^2 in the second instar, 2.03 ± 0.17 cm^2 in the third instar and 7.55 ± 0.43 cm^2 in the fourth instar (Kruskal-Wallis $H = 133.5$, $d.f. = 2$, $P < 0.001$). The reported LD_{50} of these instars on surface contaminated leaf disks over 24 h also increased from 103 OBs in the second instar, to 432 and 682 OBs in the third and fourth instar, respectively (Boucias et al., 1980). Therefore to infect 50% of the population assuming a uniform distribution of OBs on foliage (Goulson et al., 1995), this would require a density of 216, 213 and 90 OB/cm^2 consumed in 24 h, meaning that the

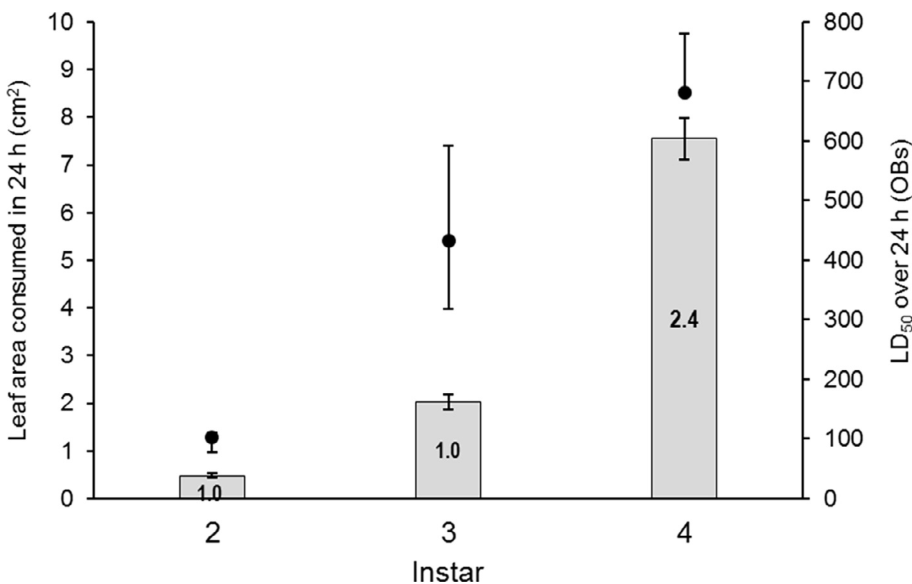

Fig. 1. Mean leaf area consumed in 24 h period (gray columns) and LD_{50} values (black points) for surface-contaminated leaf disks reported by Boucias et al. (1980) for *Anticarsia gemmatalis* second, third and fourth instars. Numerical values within columns indicate risk of lethal infection relative to that of the second instar (see text for details). Error bars indicate SE (columns) or 95% confidence interval of mean (points). For clarity only half the error bar may be shown.

second and third instars had a similar risk of lethal infection, compared to a 2.4-fold greater risk of infection in the fourth instar. These estimates were almost identical when the LD_{50} values published by Boucias et al. (1980) were replaced with estimates reported by Moscardi (1983). Using a diet surface contamination procedure, the LD_{50} values were estimated at 9.3, 28 and 70 OBs (Moscardi, 1983), equivalent to densities of 19, 14 and 9 OBs/cm^2 for second, third and fourth instars, respectively. These values would represent a relative risk of infection of 1.4 (third instar) and 2.1 (fourth instar) compared to that of second instars based on LD_{50} values reported by Moscardi (1983).

3.2. Acquisition of infection: greenhouse experiments

Application of 1.5×10^7 OB/m^2 to plants (equivalent to 1.5×10^{11} OB/ha) resulted in a high prevalence of infection that increased significantly over time (GLM: $b = 1.69$, $z = 6.09$, $d.f. = 19$, $P < 0.05$). The prevalence of virus-induced mortality ranged from 82 to 84% in larvae sampled at 6 and 12 h and reared in the laboratory on diet until death, to 97% in larvae sampled at 48 and 72 h and reared in the laboratory until death (Fig. 2A). None of the larvae collected from control plants died from polyhedrosis disease. These findings indicated that the dose of OBs applied resulted in a high prevalence of infection and that ~ 90% of third instars acquired a lethal infection in less than 24 h.

The previous experiment was performed again but samples were taken at intervals of 1, 3 and 6 h followed by laboratory rearing on diet to determine the prevalence of lethal infection (Fig. 2B). The prevalence of infection increased significantly over time from 63% (range of SE: 58–67%) in larvae sampled at 1 h, to 77% (range of SE: 71–81%) in larvae sampled at 6 h (GLM: $b = 0.51$, $z = 2.70$, $d.f. = 11$, $P < 0.05$). None of the larvae collected from control plants died from polyhedrosis disease during laboratory rearing.

3.3. Acquisition of infection: field experiments

The weather during the field experiments was warm and sunny (average temperature 29.1 °C, min. 22.8 °C, max. 39.6 °C). Relative humidity averaged 82% (min. 41%, max. 100%) but no rainfall occurred during this period. Larval sampling revealed the presence of an infestation by *A. gemmatalis* larvae of less than 1.5 cm in length at an average density of 19.1 ± 0.6 larvae/m^2 (average of 30 samples), which is above the action threshold of 10 larvae/m^2 for larvae of less than 1.5 cm in length (Avila-Valdez and Rodríguez-del-Bosque, 2004). Low numbers of *Trichoplusia ni* (Hübner), *Spodoptera eridania* (Stoll) and *Chrysodeixis includens* (Walker) were also present on the soya crop but were readily distinguished from *A. gemmatalis* and these species were not sampled.

3.3.1. Effect of dose on acquisition of infection

The prevalence of infection in larvae collected from field plots (Fig. 2C) increased from 10 to 43% in samples taken at 3–72 h post-application in the low dose treatment (equivalent to 5×10^{10} OB/ha) and 22–79% in samples taken from the high dose treatment (equivalent to 1.5×10^{11} OB/ha) over the same period. The prevalence of infection increased significantly with dose and over time (GLM: $b = 0.55$, $z = 2.96$, $d.f. = 23$, $P < 0.05$). The interaction of dose * time was not significant. (Fig. 2C).

No lethal virus infections were observed in control larvae. Of the 1680 larvae collected from field plots, parasitoids emerged from 22 (1.3%) larvae during laboratory rearing and one larva died from fungal infection; these insects were not included in the analysis.

3.3.2. Effect of application volume on acquisition of infection

To determine whether volume of application could improve the speed of acquisition of infection a second experiment was performed. The prevalence of infection in larvae collected at 1 h post-application did not differ significantly when OBs were applied in 400 ml (7.6% lethal infection, range of SE: 5.5–10.4%) or 800 ml (10% lethal infection, range of SE: 8.1–12.2%), which were equivalent to 200 or 400 l/ha (GLM: $b = -2.19$, $z = -9.552$, $d.f. = 10$, $P = 0.39$). None of the control larvae died from lethal polyhedrosis disease. Of the 630 larvae collected from field plots, parasitoids emerged from just three larvae during laboratory rearing and four larvae died from fungal infection; these insects were not included in the analysis

3.4. Virus-induced modification of larval behavior

Following 48 h of observation, control larvae in the fifth instar were measured at an average height of 24 ± 1.6 cm which was almost exactly halfway up the experimental plant (Fig. 3A). In contrast, infected fifth instars died (n = 20) or entered a moribund state and stopped moving (n = 10) at an average height of 35.3 ± 2.5 cm, significantly higher than the controls ($t = 3.83$, $d.f. = 58$, $P < 0.001$). Infected larvae demonstrated high mobility on soya plants and many were observed to move more than 30 cm during the 48 h period, whereas control larvae showed a greater tendency to move and feed within the central portion of plants (Fig. 3B). The distance moved by larvae during

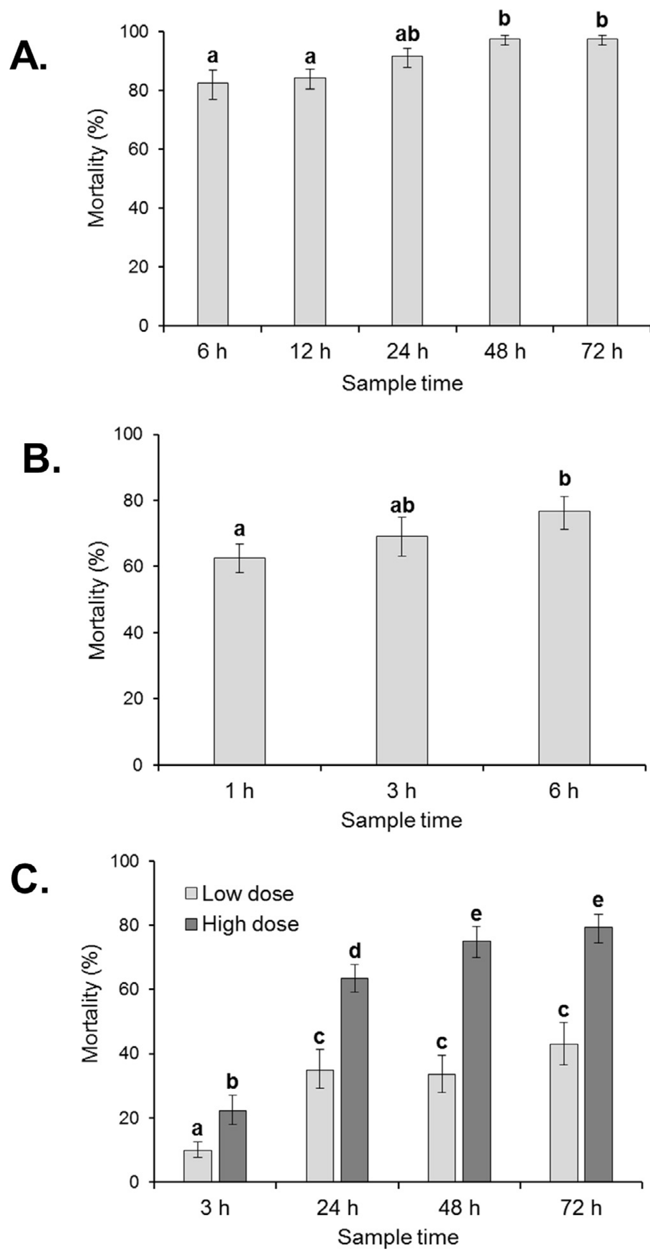

Fig. 2. Mean prevalence of AgMNPV-induced mortality of *Anticarsia gemmatalis* larvae sampled from greenhouse-grown soya plants at intervals of (A.) 6–72 h or (B.) 1–6 h following exposure to OB-treated plants (1.5×10^7 OBs, equivalent to 1.5×10^{11} OB/ha). (C.) Results of field trial involving low (5×10^{10} OB/ha) and high (1.5×10^{11} OB/ha) dose OB treatments and sampling of larvae at 3–72 h post-application. Error bars indicate SE. Columns headed by different letters differ significantly (Mixed-effects models with binomial error structure, Tukey $P < 0.05$).

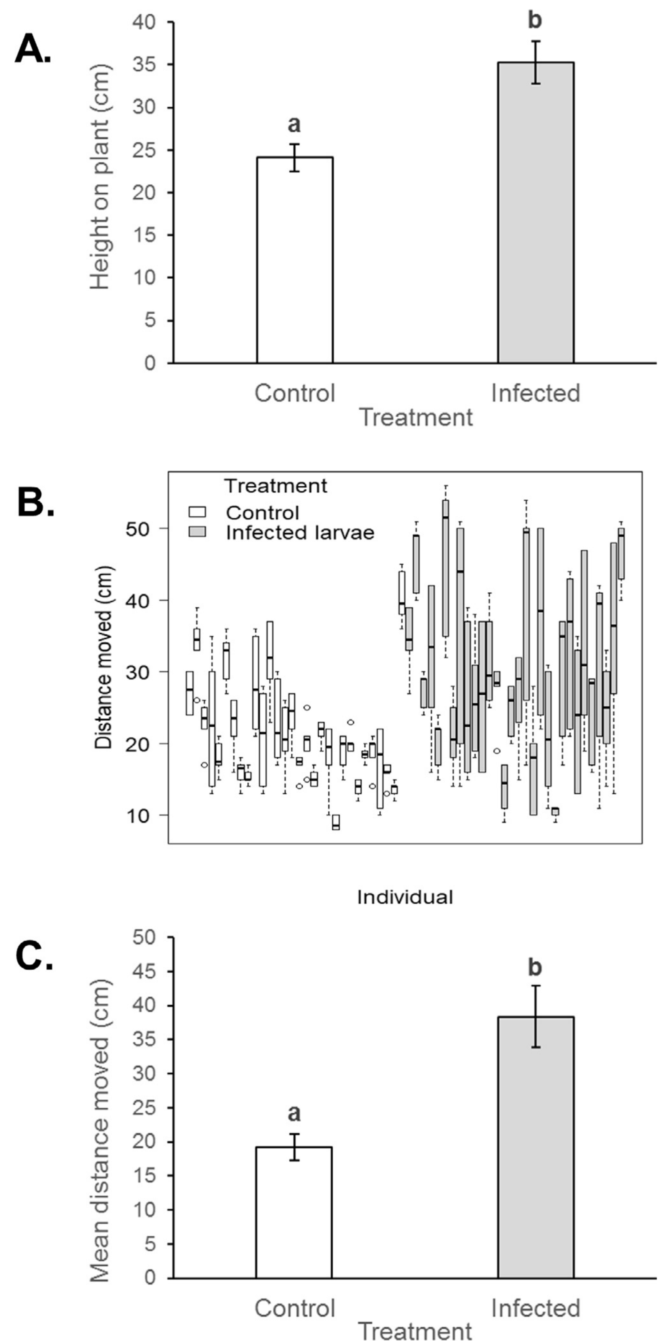

Fig. 3. (A.) Mean height of *Anticarsia gemmatalis* fifth instars on soya plants at death or moribund state (AgMNPV-infected larvae), or at end of 48 h observation period (control larvae). (B.) Box and whisker plot of vertical distances moved by 30 infected and 30 control larvae at observation intervals of 8 h over the 48 h experimental period. Each box and whisker indicates the movements of a single larva. Horizontal black line indicates median, boxes indicate interquartile range. (C.) Mean of the total distance moved by larvae during the 48 h experimental period. Error bars in A and C indicate SE. Columns headed by different values indicate significant differences (A) *t*-test, (C) Tukey, $P < 0.05$.

the experiment decreased over time ($F = 53.73$, $d.f. = 1, 298$, $P < 0.001$). Infected larvae moved approximately twice the distance moved by control larvae (Fig. 3C; $F = 14.08$, $d.f. = 1, 58$, $P < 0.001$). Of the third instar larvae released onto plants that had been exposed to the movement of infected larvae, 22% (range of SE: 19–26%) subsequently developed lethal polyhedrosis disease during individualized laboratory rearing. No virus disease was observed in control larvae.

4. Discussion

Experiments performed in this study revealed differences in the time required for *A. gemmatalis* larvae to consume a lethal quantity of OBs when feeding on soya plants under greenhouse and open field conditions. In addition, measurements of larval movement on soya plants revealed virus-induced hypermobility prior to death, with implications for the transmission and dispersal of this pathogen. Greenhouse experiments revealed that a significant proportion of *A. gemmatalis* third instars acquired lethal infection within one hour of exposure to OB treated plants. Sampling over longer periods revealed that additional larvae acquired lethal infection up to 24 h post-exposure, although after

this time the increase in the prevalence of lethal infection was minimal (Fig. 2A).

The susceptibility of larvae to virus infection usually decreases markedly as insects develop (Briese, 1986). Developmental resistance likely involves three main factors: (i) a stage-related increase in the thickness and reduction in the porosity of the peritrophic matrix through which virions pass to reach midgut epithelial cells (Levy et al., 2012; Wang and Granados, 2000), (ii) an increase in the rate of sloughing of infected midgut cells in later, compared to earlier instars (Kirkpatrick et al., 1998), which reduces the period during which the virus can replicate and produce budded virions that establish a systemic infection (McNeil et al., 2010) and (iii) a decreasing surface area:-volume ratio in the gut of larvae, which means that in order to reach midgut cells, virus particles must pass through an increasing volume of food bolus as the larvae increase in size (Hochberg, 1991). Following the reasoning used previously (Goulson et al., 1995), we assumed that ingestion of leaf tissue would be proportional to ingestion of OB inoculum across different instars, as long as OBs were uniformly distributed on leaf surfaces. Following this approach it was estimated that the relative likelihood of lethal infection in second and third instars would be similar, whereas fourth instars would have a 2.4-fold higher probability of infection (Fig. 1). Similar estimates were obtained using numerically different LD_{50} values calculated from leaf disk and diet surface contamination assays performed using slightly different methodologies (Boucias et al., 1980; Moscardi, 1983). These findings support the use of the virus to target larval stages of up to 1.5 cm in length which correspond to first to fourth instars (Moscardi, 1983; Sosa-Gómez, 2017). Although it was not possible to determine the dose-mortality response for the AgMNPV-30 wt mixture of isolates in all three instars of *A. gemmatalis* as our insect colony was lost, it is likely that the LD_{50} values would have differed from those published using different strains of the virus and another colony of this insect. Nonetheless, the fact that two different published studies provided similar estimates of the relative risks of infection for different instars suggests that the relationship between instar-specific susceptibility to infection and feeding behavior is not particularly sensitive to insect colony or virus strain-specific dose-mortality characteristics.

The acquisition of infection was slower in field conditions than observed in larvae feeding on caged greenhouse plants. This may be due to: (i) a more heterogeneous age structure in field sampled larvae compared to the uniform use of third instars in greenhouse trails, (ii) greater heterogeneity in the application of OBs due to the complexity of the plant canopy under field conditions and, (iii) rapid UV-inactivation of OBs applied in the field, especially on the uppermost leaves of the crop, compared to a greatly reduced exposure to UV radiation in the greenhouse experiments (Lasa et al., 2007). That said, by 72 h after the start of the first field trial ~80% of larvae had acquired a lethal infection and died during subsequent laboratory rearing, which is very similar to the prevalence of polyhedrosis disease observed in field trials performed in Brazil (Moscardi, 1989), Mexico (Avila and Rodríguez-del-Bosque, 2005; Avila-Valdez and Rodríguez-del-Bosque, 2008) and the United States (Fuxa and Richter, 1999). The remaining ~20% of larvae likely comprised a combination of larvae that were to large to be highly susceptible to infection (fifth and sixth instars) or larvae that did not consume OB-contaminated foliage during the sampling period.

Increasing the volume of spray application from 200 to 400 l/ha did not significantly increase the acquisition of lethal infection over a one hour period in the second field trial. Previous studies in Brazil reported no significant differences in the prevalence of infection when OBs were applied to soya crops in volumes of 100–300 l/ha, whereas the prevalence of infection was significantly reduced at 50 l/ha, presumably due to poor coverage of foliar surfaces (Silva and Moscardi, 2002). The relationship between spray volume, droplet size and crop coverage is complex and is sensitive to environmental conditions during spraying (Matthews, 2000). To avoid excessive droplet evaporation during application and rapid inactivation by solar UV radiation, we applied treatments early in the morning (08.00 hrs) when air temperatures were cooler and wind currents were not noticeable (Hunter-Fujita et al., 1998).

As AgMNPV-infected cadavers do not liquefy due to an absence of viral encoded degradative enzymes, chitinase and cathepsin (Ishimwe et al., 2015), the insect generally remains intact on the upper leaves and stems of the plant. The cadavers break down slowly and releases OBs over a period of several weeks, thereby increasing the period for which transmission to conspecifics can occur (Young and Yearian, 1989). The absence of rapid cadaver liquefaction is also likely to facilitate virus dispersal through the action of invertebrate predators (Fuxa et al., 1993; Young and Yearian, 1987) and birds (Entwistle et al., 1993; Reilly and Hajek, 2012), which can release large quantities of viable OBs in their feces, far from the site at which the infected cadaver was consumed.

Greenhouse observations on larval behavior on plants revealed that AgMNPV infected larvae showed virus-induced hypermobility and climbing behavior, also known as tree-top disease. Climbing behavior is a phototactic response that is frequently observed in baculovirus infected larvae shortly prior to death (Goulson, 1997; van Houte et al., 2014a). This is a virus-induced behavior, the molecular basis for which is currently being investigated (Ros et al., 2015; Zhang et al., 2018). The *egt* gene has been implicated in climbing behavior in the case of the gypsy moth (*Lymantria dispar*) nucleopolyhedrovirus (Hoover et al., 2011). Larvae that die high on the host plant are more likely to transmit the pathogen to conspecifics feeding on the same or adjacent plants following the release of OBs through liquefaction of the insect tissues. Rainfall can then wash the OBs over much of the lower foliage thereby facilitating transmission (D'Amico and Elkinton, 1995; Goulson, 1997).

In contrast, baculovirus-induced host hypermobility appears to involve a viral protein tyrosine phosphatase (*ptp*), although this may differ between group I and group II alphabaculoviruses (Katsuma et al., 2012; van Houte et al., 2014b). Also, the phosphatase activity of the enzyme may not be necessary to elicit hypermobility in all nucleopolyhedroviruses, so that different mechanisms may be responsible for these behaviors in different baculovirus pathosystems (Katsuma, 2015). The fact that both control and infected larvae were in the mid-fifth instar when height on plant measurements were taken (although control larvae were slightly larger than infected insects), would appear to rule out pre-molting climbing behavior, which has been observed in some species of lepidopteran larvae during studies on baculovirus manipulation of host behavior (Ros et al., 2015).

The fact that 22% of third instars acquired a lethal infection after feeding on plants that had previously been exposed to infected *A. gemmatalis* larvae, suggests that significant quantities of OBs had been released from the infected larvae during the 48 h period of the mobility experiment, as each moribund larva or larval cadaver and the leaf on which it died, was immediately removed (< 8h) from the experimental plant. Our findings are in line with previous studies on other lepidopteran nucleopolyhedroviruses in which between 13 and 50% of healthy larvae that consumed plants, over which infected conspecifics had moved and fed, themselves acquired polyhedrosis disease from OBs released in the feces and regurgitate of infected larvae prior to death (Vasconcelos, 1996; Rebolledo et al., 2015).

Previous studies performed using moribund larvae of *A. gemmatalis* placed on soya plants resulted in a prevalence of transmission that averaged 20.6% when the infected cadaver was removed from the plant within 24 h of death and leaves were subjected to laboratory bioassay on second instars, or between 6.7 and 20.8% when larvae were released and allowed to forage on contaminated plants (Young and Yearian, 1989). These authors attributed transmission to the presence of the infected cadaver, but it seems more likely that OBs were released over the foliage while the original infected insect was still alive and capable of defecation and regurgitation. These findings also suggest that significant quantities of OB inoculum may remain of the host plant even when the larval cadavers are removed by bird or invertebrate predators

immediately following death; a concept that requires further experimentation.

We conclude that the efficacy of AgMNPV as a viral insecticide is largely determined by acquisition of the infection during the first 24 h of feeding on OB-treated plants. Analysis of the relationship between leaf area consumed and susceptibility to infection supports the concept of targeting virus applications against early instars (second, third and fourth instars) that have a similar risk of acquiring a lethal infection. AgMNPV infection results in virus-induced climbing behavior and hypermobility involving the release of significant quantities of OBs that were subsequently transmitted to healthy third instars. These findings should contribute to the efficient use of this virus as a biological insecticide for control of *A. gemmatalis* larvae on soya crops in Mexico and elsewhere.

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