# In vivo apoptosis induction and reduction of infectivity by an Autographa californica multiple nucleopolyhedrovirus p35− recombinant in hemocytes from the velvet bean caterpillar Anticarsia gemmatalis (Hübner) (Lepidoptera: Noctuidae)

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Abstract

Baculoviruses have long been shown to regulate apoptosis in cultured insect cells. Recently, this phenomenon was also reported to occur in vivo, reinforcing the importance of apoptosis in insect immunity against viruses. The vP35del virus, an Autographa californica multiple nucleopolyhedrovirus (AcMNPV) recombinant, was previously shown to induce apoptosis in Anticarsia gemmatalis cultured cells. In order to verify the AcMNPV interaction with hemocytes, apoptosis induction in vivo and its effects upon infectivity, we studied the course of intrahemocoelic infection of recombinant viruses (vHSGFP and vHSGFP/P35del) in A. gemmatalis larvae. Insect development and mortality were monitored and infection progress was followed by light, fluorescence and electron microscopy. For all doses tested, vHSGFP/P35del caused lower mortality than vHSGFP. Mortality of 95% occurred with a dose of 4×106 PFUs of vHSGFP, which was reduced to 60% for vHSGFP/P35del. GFP expression was first observed at 3 h p.i. for the two viruses, increasing for vHSGFP (40% at 120 h p.i.) and decreasing for vHSGFP/P35del (0% at 120 h p.i.). The virus vHSGFP/P35del induced apoptosis in hemocytes, with some budded virus being produced, and fragmented cells were observed between 24 and 72 h p.i. The recombinant vHSGFP induced typical wild-type cytopathic effects, with low production of occluded viruses until 120 h p.i. Plasmatocytes and granular hemocytes type 1 were the hemocytes most susceptible to both viruses. For these experimental conditions, we concluded that A. gemmatalis is a semi-permissive host to AcMNPV; moreover, apoptosis reduces AcMNPV infectivity and the p35 gene is essential for blocking apoptosis in this system.

Keywords: Apoptosis; Baculoviridae; GFP; Hemocytes; Hemolymph; Lepidoptera; Ultrastructure

## 1. Introduction

The family Baculoviridae consists of enveloped, doublestranded DNA viruses which are pathogenic toward arthropods, especially lepidoptera larvae. Baculovirus species are distributed between two genera, Granulovirus (GV) and Nu cleopolyhedrovirus (NPV).

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the type-species of the Nucleopolyhedrovirus genus and the best-known species among baculoviruses [3].

Infection specificity and consequent safety in relation to non-target organisms are characteristics that make these viruses agents of great potential for integrated pest management [2,24]. Baculoviruses are also useful as heterologous gene expression vectors, since their genome supports large DNA inserts at non-essential gene loci. Foreign proteins can be successfully expressed upon infection of a permissive insect cell line [18,26].

Apoptosis is a phenomenon of cellular self-destruction that can be triggered by diverse stimuli, and viral infections are among them. By activating an apoptotic response, the host cell can abort viral infection, avoiding its spread to the whole organism. For survival and progeny production, viruses had to circumvent this response during evolution. One strategy for achieving such circumvention is the acquisition of antiapoptotic genes [21,35].

Baculoviruses are known to possess at least two types of antiapoptotic genes: p35 and iap. The gene p35 encodes a broad-spectrum caspase inhibitor, described in five baculovirus species up to date [10,13,14,19,28]. Another way of apoptosis inhibition by P35, still not completely understood, is related to reactive oxygen species (ROS) neutralization, and prevention of cytochrome C liberation by the mitochondria [29]. The IAPs (inhibitors of apoptosis proteins), which were first described in Cydia pomonella granulovirus (CpGV), are also found in organisms ranging from yeasts to humans. IAPs are metalloproteins that inhibit apoptosis at upstream pathways before caspase activation or by interacting directly with these enzymes [9].

AcMNPV possesses p35 and iap genes; however, until now, antiapoptotic function was observed only for the P35 protein. Apoptosis induction by p35 mutant (p35−) AcMNPV viruses is best characterized for Spodoptera frugiperda and its derived cell line, SF-21, with a reduction in viral infectivity and progeny production [7,9]. In other cases, despite presenting intact antiapoptotic genes, AcMNPV induces apoptosis and displays reduced infectivity and propagation in some insect cell lines [5,27,41] and in Spodoptera litura larvae [40].

Despite the existence of a great deal of information related to mutant baculovirus induction of apoptosis in vitro, only recently it was reported in vivo [6,40]. These studies have shown that apoptosis is an important antiviral response in insects which appear to lack acquired immunity, and that the strategies to counteract cell death are one of the determinants of baculovirus host-range [7]. Investigations in this field, especially those focusing on in vivo assays, are important for shedding new light on insect immune strategies against pathogens and for emphasizing the role of apoptosis during the evolution of immunity in these organisms.

In a previous study [33], it was reported that the virus vP35del, derived from AcMNPV, which has a deletion in the p35 gene, induced massive apoptosis in a cell line derived from A. gemmatalis (UFL-AG-286) [30]. Apoptosis occurred between 9 and 16 h p.i., with a total lack of progeny production [33]. In contrast, a mutant (vApAg) derived from A. gemmatalis multiple nucleopolyhedrovirus (AgMNPV), induced apoptosis in a delayed manner, between 24 and 48 h, with some progeny production [4,33]. Recently, it was observed that vApAg induces apoptosis in A. gemmatalis larval hemocytes in a manner very similar to that of apoptosis induction in UFL-AG-286 cells (Silveira et al., submitted).

In this work, we investigated apoptosis induction in vivo by an AcMNPV p35− virus in A. gemmatalis larval hemocytes and its effects on infectivity. Recombinant viruses containing the egfp gene were inoculated intrahemocoelically into 4th instar larvae. Insect development and mortality were monitored, infection progress in hemocytes was followed by light and transmission electron microscopy, and infected cells were counted under fluorescence microscopy.

## 2. Materials and methods

### 2.1. Insects and viruses

A. gemmatalis 1st instar larvae, obtained from Embrapa (Brazil), were reared on an artificial diet [17] at 24–27 ◦C, with a 12:12 h dark/light regime. The molts were monitored, and 4th instar larvae (between 12 and 24 h after molt) were used for all experiments.

The recombinants vHSGFP and vHSGFP/P35del were propagated in BTI-Tn-5B1-4 (Tn-5B) cells [15] and maintained in TC-100 medium (GIBCO-BRL Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum at 27 ◦C. The viruses vHSGFP and vHSGFP/P35del are derived from the AcMNPV L1 strain and have the gene egfp under control of the Drosophila melanogaster constitutive promoter hsp 70 at a site adjacent to the polyhedrin gene [6]. Additionally, vHSGFP/P35del has the antiapoptotic gene p35 deleted [8]. The inocula were titered by the TCID50 method following the protocol described by O'Reilly et al. [26].

### 2.2. Bioassays

To observe the effects of viruses on insect mortality and development, larvae were injected with 20 µl of viral inoculum directly into the hemocoel by using an insulin microsyringe. Three different concentrations of inoculum were used (2×106, 2×107, 2×108

PFU/ml) for each virus, and twenty larvae were inoculated for each one. Each larva was reared separately in a plastic cup. Mortality and development were reported every day for 25 days. Larvae were considered dead if they did not react to mechanical stimulation. Deaths attributed to injection trauma were not considered. Controls were obtained by the inoculation of an equal volume of TC-100 medium and by no inoculation.

### 2.3. Infected cell countings

To verify the percentage of infected cells during the time of infection and the structural alterations promoted by viruses in hemocytes, insects were injected with 20 µl of viral inoculum at the highest concentration (2×108 PFU/ml), as described. Hemolymph samples were collected in anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citric acid, pH 4.5) [23], observed directly in a fluorescence microscopy under the blue excitation filter, and by DIC (differential interferential contrast) in an Axiophot Zeiss microscope. For each time after infection (3, 24, 48, 72, 96 and 120 h), at least seven hundred cells were counted in two subsamples obtained from a mix of hemolymph collected from ten insects.

### 2.4. Transmission electron microscopy

To observe the ultrastructural alterations induced in hemocytes by viral infection, hemolymph samples were collected from insects injected with 20 µl of viral inoculum (2 × 108 PFU/ml) after 12, 24, 48, 72, 96 and 120 h p.i. Samples were fixed for 30 min (2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) with 5% sucrose), centrifuged at 750 g for 5 min, the pellet washed in the same buffer, post-fixed (1% osmium tetroxide, 0.8% potassium ferricyanide in the same buffer), contrasted in block with 0.5% uranyl acetate, dehydrated in acetone, and embedded in Spurr's resin. The ultrathin sections were contrasted with uranyl acetate/lead citrate and observed in a TEM JEOL 100C and JEOL 1011 at 80 kV. 2.5. Hemocyte identification A. gemmatalis larval hemocyte-types were identified by structural and ultrastructural characteristics described previously [32,34].

## 3. Results

### 3.1. Bioassays

Increasing doses of vHSGFP resulted in increasing larval mortality, decreasing larval pupation, and decreasing adult emergence (Table 1, Fig. 1). The dose of 4 × 104 PFU/larva resulted in 21% death, while 79% of individuals formed pupae and 53% of these emerged as moths (Fig. 1A). For 4 × 105 PFU/larva, larval mortality was around 53%, and 47% of pupae were formed, but none of them emerged as adults (Fig. 1B). For 4 × 106 PFU/larva, 95% of individuals died. One pupa was formed (5%), but it did not emerge as an adult (Fig. 1C). For vHSGFP/P35del, the same tendency was observed, but in comparison to the previous system, the viral effects were milder for each dose (Table 1, Fig. 2). No larvae injected with 4 × 104 PFU died. All of them became pupa, and 70% emerged as adults (Fig. 2A). For 4 × 105 PFU/larva, larval mortality was around 22% and 78% of individuals formed pupa; however only one (5%) reached adulthood (Fig. 2B). For 4×106 PFU/larva, 60% of individuals died, 40% became pupa, but none emerged (Fig. 2C).

For the vHSGFP highest dose, some liquefaction of dead larvae was observed, but for the majority of them and for lower doses, there was melanization of cadavers (not shown). Some pupae were abnormal for larvae inoculated with either vHSGFP (4 × 104 and 4 × 105 PFU/larva) or vHSGFP/P35del (all doses), especially for the latter. Despite molting inhibition, the larva body hypertrophied in such a way that the normal proportions between the body and the head were lost. Later, the body decreased in size, melanized and hardened. Prolegs and head persisted (not shown). None of the abnormal pupae emerged.

Table 1
Percentage of dead A. gemmatalis larvae, non-viable pupae and moths in a bioassay of different doses of vHSGFP and vHSGFP/P35del

| Dose (PFU) | Virus | Dead larvae (%)[a] | Non-viable pupae (%) | Moths (%) |
|---|---|---|---|---|
| $4 \times 10^4$ | vHSGFP | 21 | 37 | 42 |
| | vHSGFP/P35del | 0 | 30 | 70 |
| $4 \times 10^5$ | vHSGFP | 53 | 47 | 0 |
| | vHSGFP/P35del | 22 | 73 | 5 |
| $4 \times 10^6$ | vHSGFP | 95 | 5 | 0 |
| | vHSGFP/P35del | 60 | 40 | 0 |

[a] n = 20 for each treatment. Larvae were injected with 20 µl of viral inoculums directly into the hemocoel using an insulin microsyringe and were observed for 25 days. Deaths attributed to injection trauma were not considered. Deleterious effects were milder for vHSGFP/P35del than for vHSGFP for all doses tested

### 3.2. Hemocyte identification

Six hemocyte types were recognized in A. gemmatalis larvae based on structural and ultrastructural characteristics previously described [32,34]. They included prohemocytes (pr), plasmatocytes (pl), granular hemocytes type 1 (gh1), granular hemocytes type 2 (gh2), oenocytoids (oe), and spherulocytes (sph).

### 3.3. DIC, fluorescence microscopy and cell counting

Fluorescence occurred preferentially for gh1 and pl at all times post-infection studied. These cells were promptly recognized because of their characteristic shapes. Gh1 are round, rich in thin surface projections (filopodia) and spread symmetrically, maintaining a circular shape. Pl are somewhat elongated or fusiform, and spread maintaining this asymmetrical shape.

For both systems, GFP expression was observed from 3 h p.i., (around 3%) (Fig. 3). The number of fluorescent cells remained similar for both viruses until 48 h p.i. (8–12%). After 48 h p.i., the increasing tendency continued for vHSGFP, which reached 40% of fluorescencent cells at 120 h p.i. For vHSGFP/P35del, a plateau (9% of fluorescent cells) was established between 24 and 72 h p.i. Then, the number of fluorescent cells decreased, reaching values around 0.01% after 120 h p.i. (Fig. 3).

For vHSGFP/P35del, apoptotic cells, recognized because of their fragmentation into apoptotic bodies, were eventually found at 12 h p.i., more frequently at 24 (Figs. 4A–4D) and 48 h p.i. (not shown), and then became rare after 96 h p.i. (not shown). After 48 h p.i., there was some polyhedra assembly for vHSGFP (Figs. 4E, 4F), which did not occur for vHSGFP/P35del (not shown). After 96 h p.i., the number of gh2 slightly increased for both systems. These cells were intact and had an extremely low rate of fluorescence (Figs. 4G, 4H). Infected sph were also occasionally found (not shown).

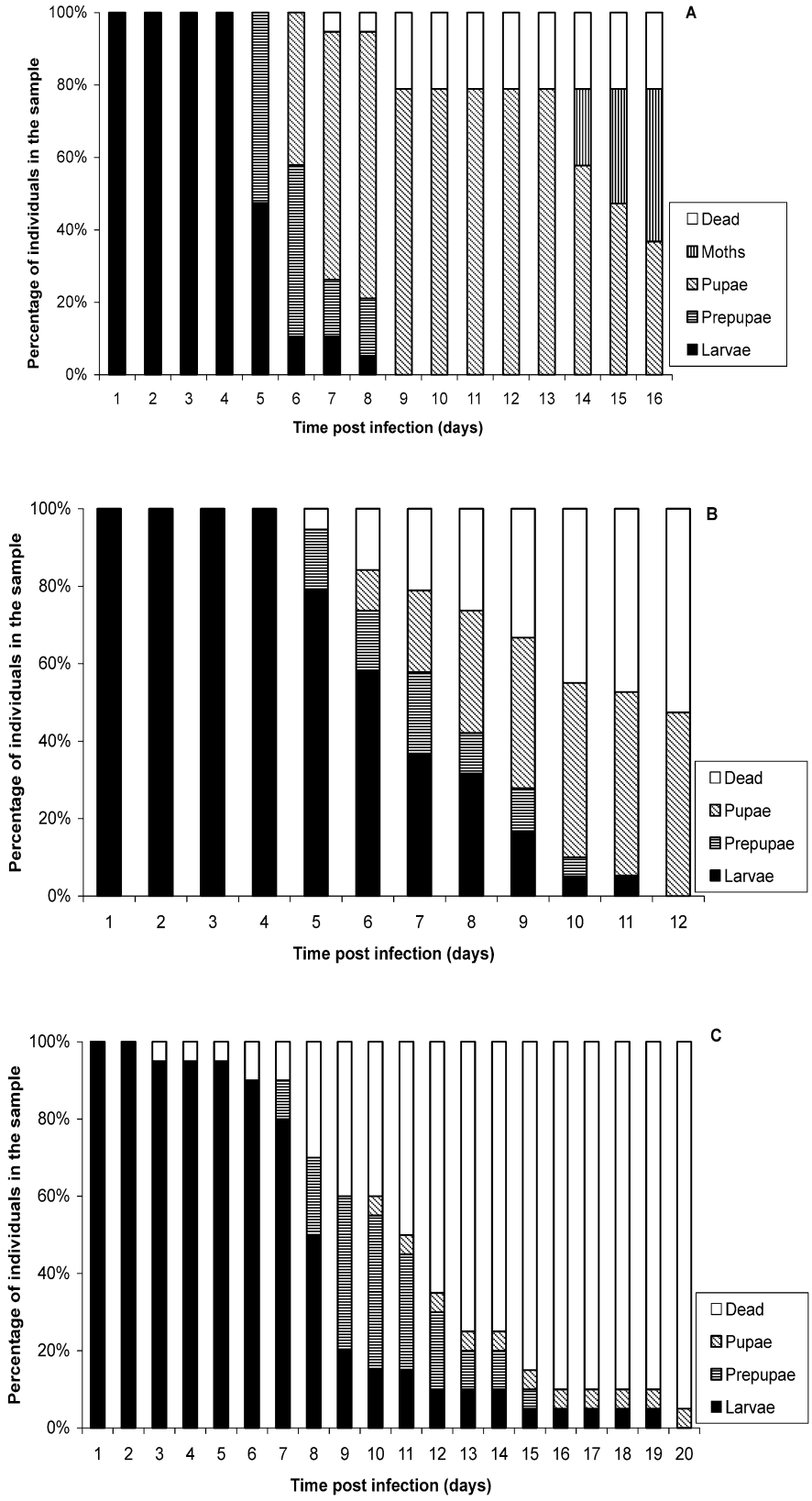


Fig. 1. Bioassay composition over time of infection for larvae infected with different doses of vHSGFP. (A) $4 \times 10^4$; (B) $4 \times 10^5$; (C) $4 \times 10^6$ PFU. Larvae were injected with 20 µl of viral inoculum directly into the hemocoel using an insulin microsyringe. Observations were made for 25 days. Data are reported until the last day in which an event occurred (death, prepupae or pupae formation, adult emergence). $n = 20$ for each treatment. Higher doses resulted in higher mortality and reduced numbers of pupae and moths.

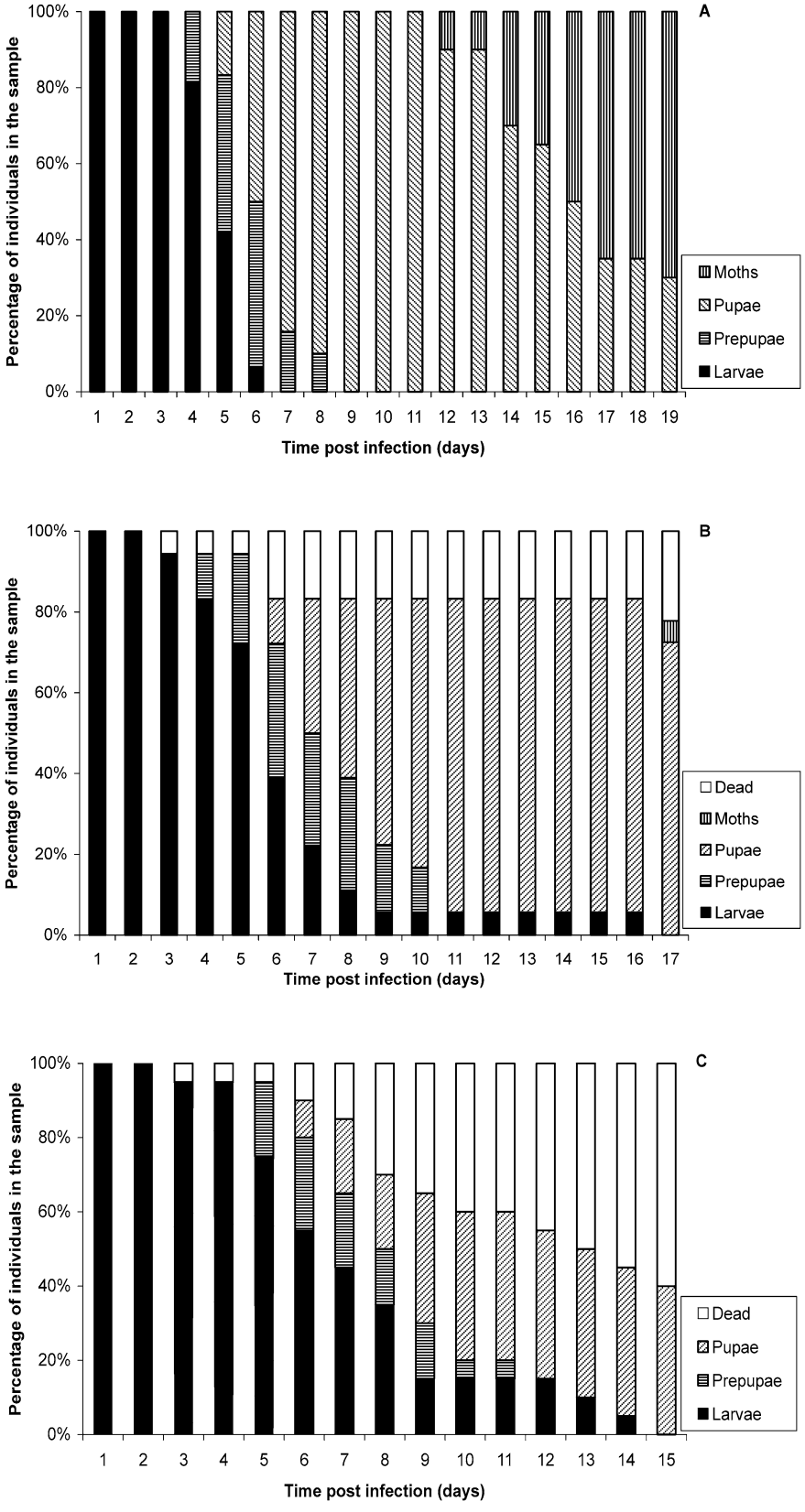


Fig. 2. Bioassay composition over time of infection for larvae infected with different doses of vHSGFP/P35del. (A) $4 \times 10^4$;. (B) $4 \times 10^5$; (C) $4 \times 10^6$ PFU. Larvae were injected with 20 µl of viral inoculum directly into the hemocoel using an insulin microsyringe. Observations were made for 25 days. Data are reported until the last day in which an event occurred (death, prepupae or pupae formation, adult emergence). $n = 20$ for each treatment. Higher doses resulted in higher mortality and reduced numbers of pupae and moths.

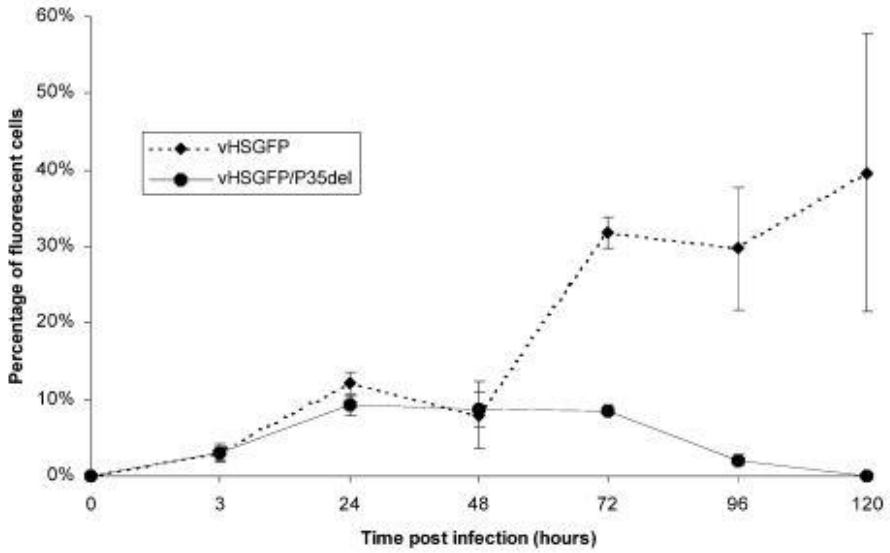


Fig. 3. Percentage of egfp-expressing cells over time of infection with vHSGFP and vHSGFP/P35del. Larvae were injected with 4 × 106 PFU of virus, cohorts of ten insects were sacrificed for hemolymph collection and at least seven-hundred cells were counted in a DIC/fluorescence microscope at each time. Values are plotted as means ± SD.

### 3.4. Transmission electron microscopy

At 12 h p.i., the major part of the hemocytes were intact for both systems. At that time of infection, for vHSGFP, rare cells, especially pl and gh1, presented sign of viral morphogenesis such as nuclear hypertrophy, nucleocapsid assembly, envelopment, nuclear envelope folding with budded virus protrusion through the cytoplasm, and, eventually, fibrillar aggregate accumulation (Fig. 5A). Higher numbers of hemocytes displayed the events described above after 24 h p.i. (Figs. 5B–5D). After 48 h p.i., some polyhedra assembly was visualized (Figs. 5E–5F), but until late in infection (72–120 h p.i.) the number of cells presenting polyhedra was very low. At 72 and 120 h p.i., fibrillar aggregates were larger and nuclear envelope folds were more extensive, sometimes forming organized arrays of tubes throughout the cytoplasm (Fig. 5C). Pr, pl, gh1, oe and eventually sph were shown to be susceptible to vHSGFP.

For vHSGFP/P35del, after 12 h p.i., some nuclear hypertrophy and nuclear envelope folding also occurred, but, in some cases, these events were associated with surface blebbing, chromatin condensation and fragmentation into apoptotic bodies. These apoptotic events became more frequent at 24 and 48 h p.i. (Figs. 6A–6F). Many apoptotic cells presented segregation of organelles in some of the apoptotic bodies formed (Figs. 6E, 6F). Despite chromatin condensation, apoptotic body formation for sph was not observed (Fig. 6C).

Exocytosis of gh1 unstructured granule contents was observed in association with filopodia emission directed to an apoptotic oe (Fig. 6D). Some nucleocapsid assembly and envelopment were also found, despite cell death (Fig. 6B). Apoptosis and replication events decreased after 96 h p.i., and there was no polyhedra assembling. Pl, gh1, pr, oe and, eventually, sph were shown to be susceptible to vHSGFP/P35del, especially the first two cell types, as was the case for vHSGFP.

Necrosis of infected cells and phagocytic activity of pl and gh1 were observed for both systems (Figs. 7A–7F). Gh1 were shown to emit thin long projections which could be directed to small cell fragments, forming multiple phagosomes simultaneously (Figs. 7A–7B), or which could be directed to entire cells or cell fragments of large dimensions (Fig. 7C). Pl were shown to engulf entire cells more frequently than gh1 (Fig. 7E). 4. Discussion In this work, we have shown that AcMNPV lacking the p35 gene causes apoptosis in A. gemmatalis hemocytes. However, the intensity and speed of death were different from that induced in a cell line derived from A. gemmatalis. In hemocytes, apoptosis occurred between 24 and 72 h p.i., at low frequency, with the occurrence of some nucleocapsids assembling and enveloping. For UFL-AG-286 cells, apoptosis was massive and rapid (until 16 h), without progeny production [33]. One possible reason for this discrepancy could be the difference between the two cellular models as concerns sus ceptibility to AcMNPV, despite UFL-AG-286 cells being derived from the same organism, suggesting that A. gemmatalis has tissue-specific susceptibility to AcMNPV. UFL-AG-286 cells are permissive to AcMNPV [31]. However, we have demonstrated that between 24 and 48 h p.i., only 8% of hemocytes were infected by vHSGFP and vHSGFP/P35del, and after 120 h p.i. vHSGFP reached only 40% of infection in hemocytes, which indicates a low rate of infection in these cells. A similar rate of infection was found for the same recombinant in S. frugiperda hemocytes [6].

Another explanation for the low occurrence of apoptosis could be the turnover of hemocytes by cell division in circulation and in hemopoetic organs, added to putative factors that can neutralize virions, which does not occur in vitro. However, vApAg, which is derived from AgMNPV, has been shown to induce more intense apoptosis in A. gemmatalis hemocytes between 24 and 72 h p.i., under very similar experimental conditions (Silveira et al., submitted). This reinforces the hypothesis that less intense apoptosis induction by vHSGFP/P35del is related to the reduced susceptibility of A. gemmatalis hemocytes to AcMNPV.

A. gemmatalis larvae have been shown to be semipermissive or permissive to high doses of AcMNPV by intrahemocoelic infection, since 95% of larval mortality was obtained with the injection of 4 × 106 PFUs. In Trichoplusia ni, this virus induces 95% death with an

injection of less than 1 PFU [6], and AgMNPV causes 50% death in A. gemmatalis with 0.1 PFU (Silveira et al., submitted).

The absence of the p35 gene further reduced the infectivity level, with maximum larval mortality of 60% obtained with a 10 times higher dose than that used for vHSGFP with similar mortality (53%). This probably can be related to a drop in BV production and a consequent reduction in systemic virus spread. For other systems, apoptosis can more drastically reduce infectivity in vivo. In S. frugiperda larvae, 1000-fold more BVs of AcMNPV p35− and 25-fold more PIBs (polyhedral inclusion bodies) are required to reach an LC50 in comparison to the wild-type or revertant viruses [11,12].

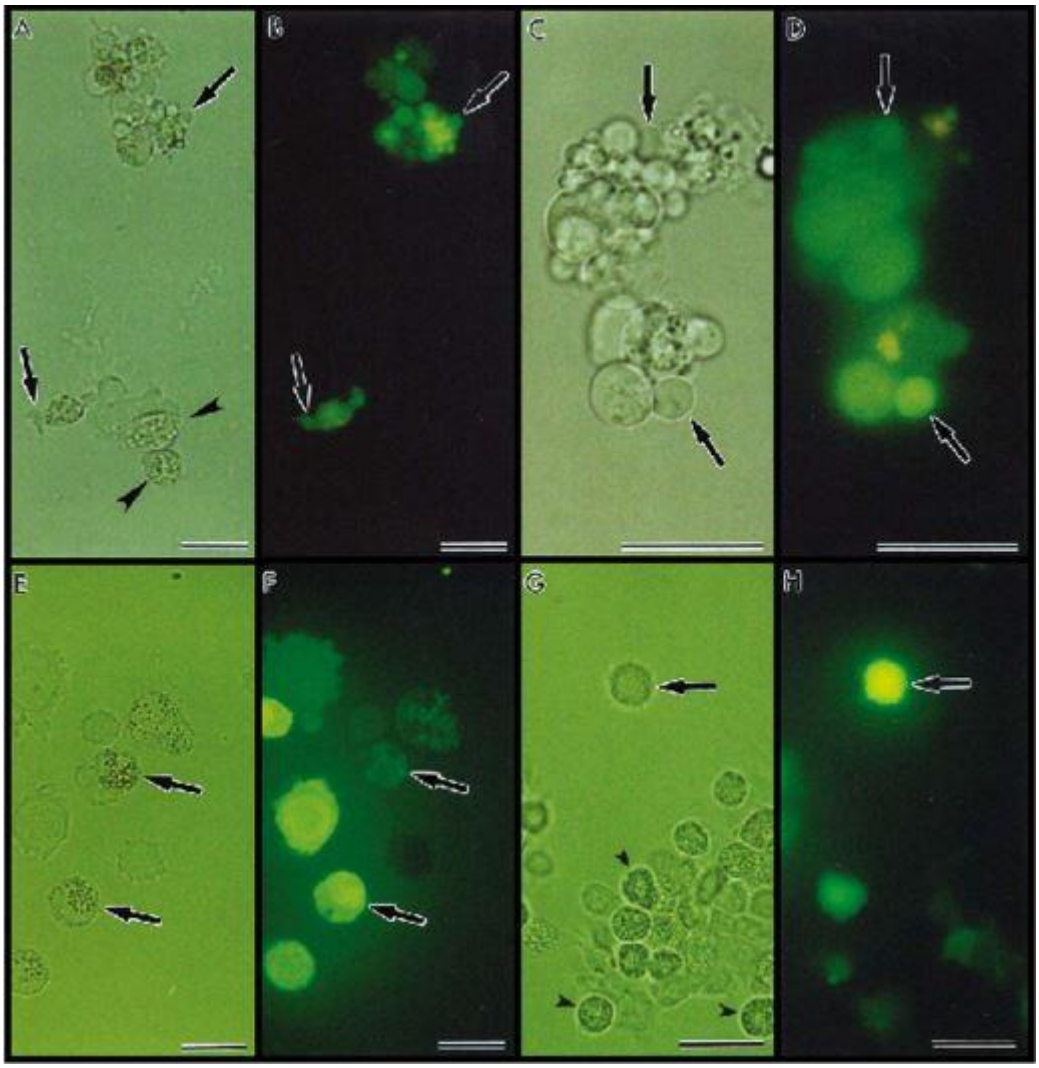


Fig. 4. DIC and fluorescence (blue excitation filter) microscopy micrographs of hemocytes from A. gemmatalis larvae infected intrahemocoelically with 4 × 106 PFU of vHSGFP/P35del (A–D) and vHSGFP (E–H). Hemolymph was collected in anticoagulant buffer and a sample was taken for observation and photography. (A–D) 24 h p.i. Arrows indicate apoptotic bodies, arrowheads indicate gh2. (E–F) 120 h p.i. Arrows indicate cells presenting polyhedra. (G–H) 120 h p.i. Arrow indicate gh1, small arrowheads indicate gh2. Bars represent 20 μm.

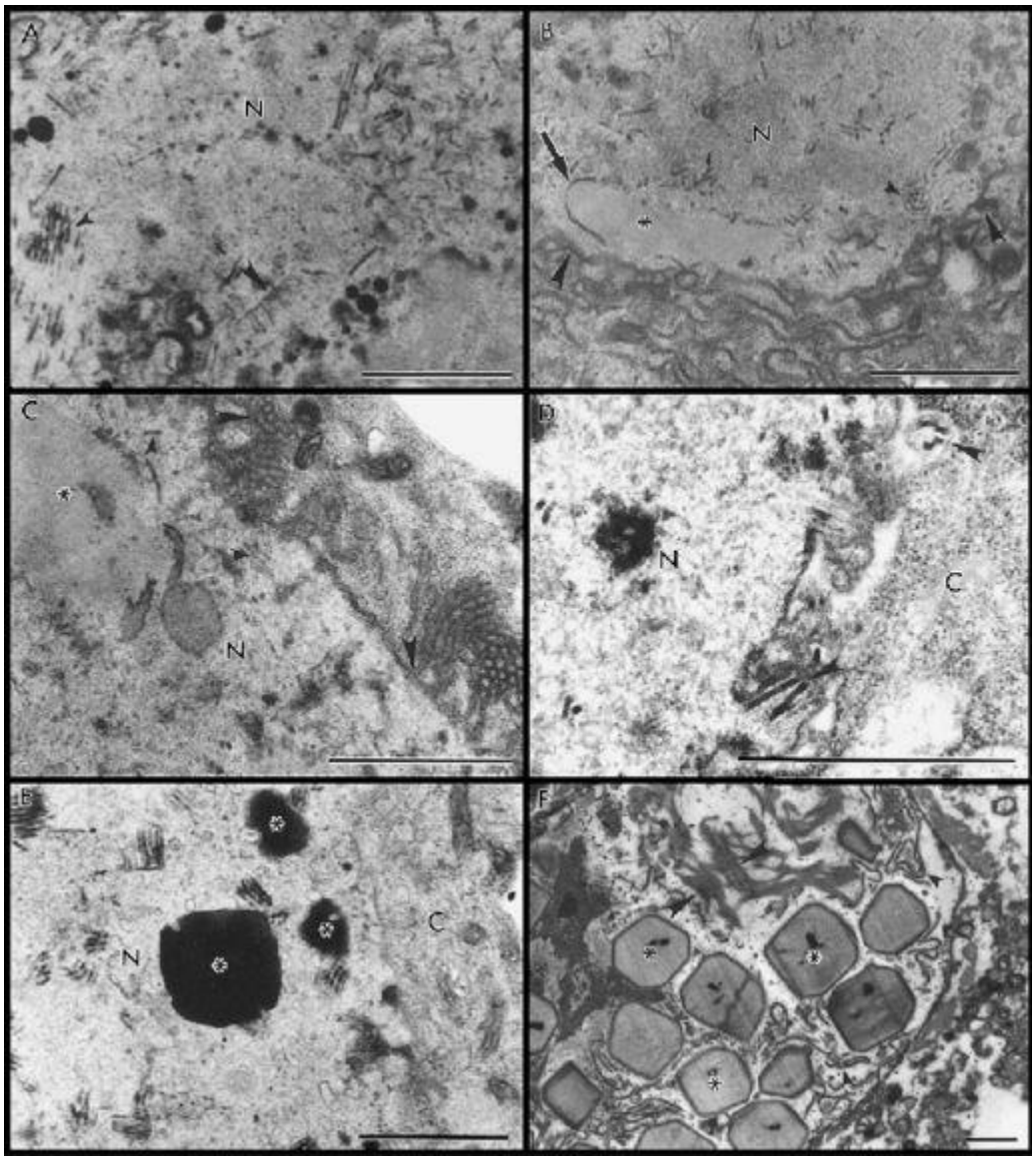


Fig. 5. TEM micrographs of A. gemmatalis hemocytes infected by vHSGFP. (A) 12 h p.i. A putative plasmatocyte, presenting nucleocapsids assembling and initial enveloping (large arrowhead). Note some groups of long immature nucleocapsids (small arrowhead). N—nucleus. (B) 24 h p.i. A putative plasmatocyte presenting nucleocapsids assembling and enveloping (small arrowhead), a nuclear fibrillar aggregate (*) associated with a membranous profile (arrow) and nuclear envelope folding (large arrowheads). N—nucleus. (C) 72 h p.i. Putative granular hemocyte type 1 presenting a peculiar profile of the nuclear envelope that protrudes as organized tubes throughout the cytoplasm (large arrowheads). N—nucleus; (*) fibrillar aggregate; small arrowheads–nucleocapsids. (D) 72 h p.i. Formation of transport vesicles containing nucleocapsids (arrowheads). N—nucleus; C—cytoplasm. (E) 120 h p.i. A putative plasmatocyte, presenting polyhedra assembly (*). N—nucleus; C—cytoplasm. (F) 120 h p.i. A necrotic cell with the nucleus filled with polyhedra (*), fibrillar aggregates (large arrowheads), and membranous profiles (small arrowheads). Bars represent 1.5 μm.

In addition to mortality, another deleterious effect attributed to viral infection, mainly caused by vHSGFP/P35del, was the production of abnormal pupae. These were derived from larvae that had survived infection and attempted to form pupae despite incomplete molting. This probably derives from successful expression of the enzyme EGT (ecdisteroyd UDP-glucosyltransferase), which inhibits molting by inactivating the ecdysone hormone [25]. This anomalous phenotype had been previously reported in S. frugiperda larvae infected by

vHSGFP/P35del, which presented decreasing tissue fluorescence during the time of infection [8]. This effect suggests a milder infection that did not result in larval death, but that was sufficient to impair normal development.

Insect hemocytes are a complex of cell types that circulate in hemolymph. They are involved in defense responses such as wound repair, phagocytosis, nodulation, encapsu lation, coagulation, synthesis and secretion of immunological factors [22]. Hemolymph has been shown to contribute to baculovirus dissemination in permissive hosts [1,16,39]. However, hemocyte resistance, apoptosis or an effective cellular immune response against infection, like melanization of tracheal foci, may restrict the replication of NPVs in specific virus–host combinations [6,8,36–38,40].

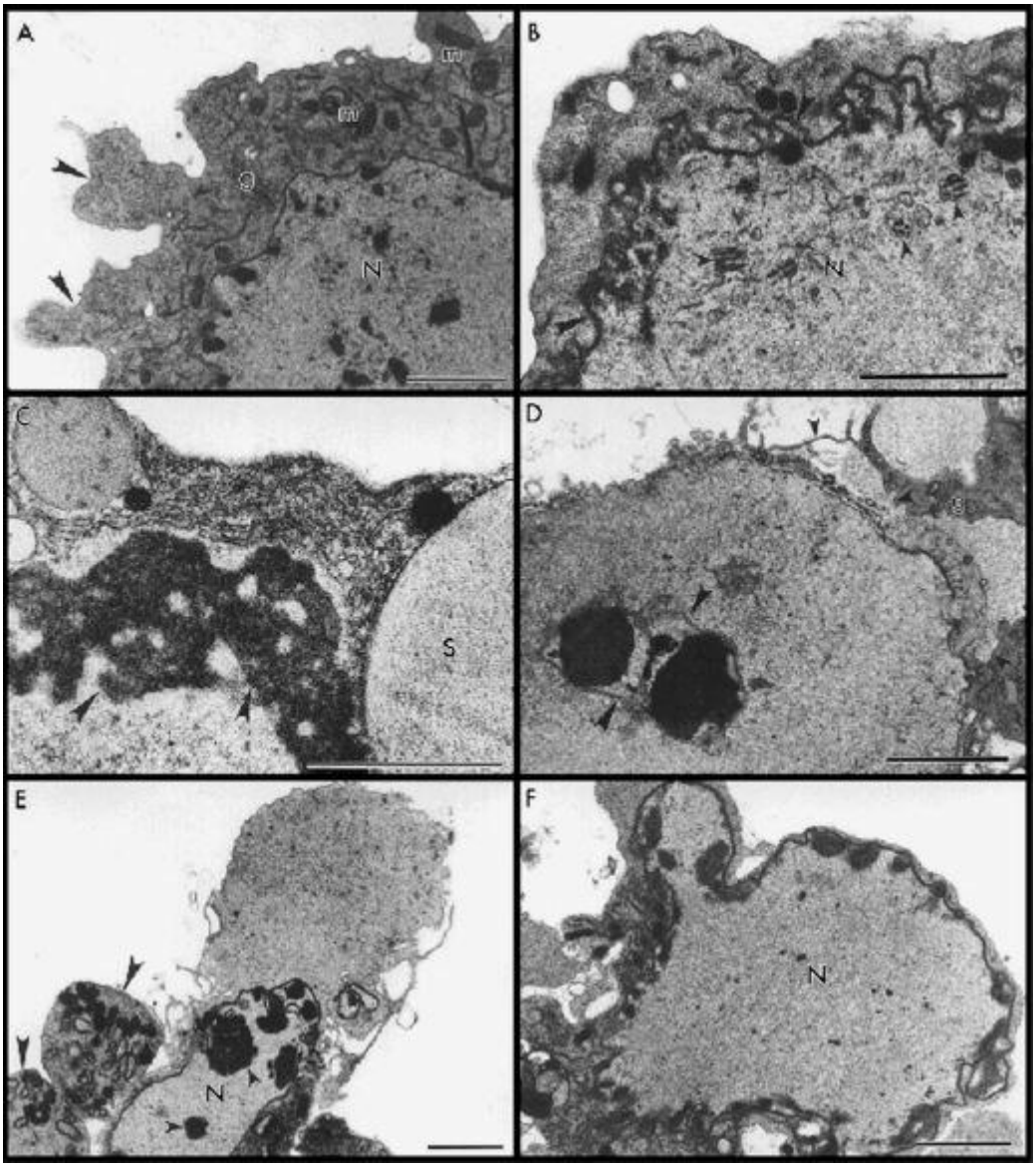


Fig. 6. TEM micrographs of A. gemmatalis hemocytes after 24 h p.i. (A, C–D) and 48 h p.i. (B, E–F) by vHSGFP/P35del. (A) A putative granular hemocyte type 1 presenting surface blebbing (arrowheads). N—nucleus, m—mitochondria, g—Golgi complex. (B) A plasmatocyte presenting nucleocapsids enveloping (small arrowheads) and some convolution of the nuclear envelope (large arrowheads). N—nucleus. (C) A

spherulocyte presenting chromatin condensation (arrowheads). S—spherule. (D) An oenocitoyd presenting chromatin condensation. Large arrowheads indicate the nucleus. A gh1 (g) liberated the contents of an unstructured granule (small arrowheads) over the oe surface. (E) A putative apoptotic plasmatocyte. The nucleus (N) displays some patches of condensed chromatin (small arrowheads). Organelles are segregated in some apoptotic bodies (large arrowheads). (F) A putative apoptotic plasmatocyte. N—nucleus. Bars represent 2 µm.

We have found that pr, pl, gh1 and oe from A. gemmatalis presented signs of infection, but sph and gh2 appeared to be more resistant to AcMNPV infection. Gh2 have also shown some resistance to AgMNPV and vApAg ([32], Silveira et al., submitted). The resistance of sph to AcMNPV may be one of the factors that limits the permissiveness of A. gemmatalis to AcMNPV. Kislev et al. [20] showed NPV replication events in hemocytes from Spodoptera litorallis after oral infection, or intrahemocoelic injection of polyhedra and free ODVs. For the three modes of infection, Pl were shown to be the major cell type for NPV replication. Cells classified as adipohemocytes at that time, which presented clear characteristics of sph, also did not become infected in those experiments.

Phagocytic activity of pl and gh1 was frequently observed against infected cells and apoptotic bodies, which has been observed before for insects infected with AgMNPV and vA-pAg ([32], Silveira et al., submitted). Pl and gh1 are reported to be the only Lepidoptera larval hemocytes capable of adhering to foreign surfaces, which is important for phagocytosis, nodulation and encapsulation [22].

A recurrent event found for A. gemmatalis hemocytes infected by baculoviruses is the necrosis of infected cells, which generally presented mature virogenic stroma. Together, phagocytosis and necrosis of infected hemocytes are clues to a putative recognition of these virus-infected cells as altered self, and the triggering of cytotoxic responses against them, in addition to apoptosis [32]. To date, apoptosis is the best-described antiviral response in insects [7]. Apoptosis reduces baculovirus replication in vitro, and AcMNPV mutants lacking the p35 gene have reduced infectivity in S. frugiperda larvae when compared to the wild-type virus [9]. More recently, apoptosis in vivo induced by baculovirus infection was demonstrated. Apoptosis was shown to be correlated with reduced viral propagation of AcMNPV in a non-susceptible host, S. litura [40] and with reduced infectivity of vHSGFP/P35del in S. frugiperda [8].

In the present work, we have shown that an AcMNPV p35− virus induces apoptosis in vivo in larval hemocytes from a semi-permissive host upon intrahemocoelic injection, with some reduction in infectivity. This corroborates the current belief that apoptosis is an important element in insect immunity against viral infections. Other defense weapons demonstrated here were phagocytosis and necrosis of infected cells, which requires rigorous

investigation for a better understanding of antiviral immunity in insects, as well as differential susceptibility of the different hemocyte types to baculovirus infection.

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