Research Article

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A novel use of *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) as inoculative agent of baculoviruses

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Abstract

Background: Alphabaculoviruses are Lepidoptera-specific virulent pathogens that infect numerous pests, including the *Spodoptera* complex. Due to their low environmental persistence, the traditional use of Alphabaculoviruses as bioinsecticides consist in high-rate spray applications with repeated treatments. Several abiotic and biotic factors can foster its dispersion, promoting their persistence in the agroecosystem. Amongst biotic factors, predatory arthropods can disperse the viruses by excretion after preying on infected individuals. Therefore, this study focused on promoting predator's ingestion of nucleopolyhedrovirus (NPV)-treated diets, and the later exposition of the insect host to leaf surfaces contaminated with predator excreta. The virus–host–predator system studied was *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV), *Spodoptera littoralis* (Boisduval) and *Nesidiocoris tenuis* (Reuter). The infective potential of *N. tenuis* feces and the retention time of SpliNPV were assessed under laboratory conditions after feeding on treated diets (sucrose solution and *Ephestia kuehniella* eggs).

Results: Mortality of *S. littoralis* larvae was lower via *N. tenuis* excretion than in positive control (spray application) in the first infection cycle, together with a delay in host death. In the second infection cycle, both SpliNPV-treated diets triggered 100% mortality. Both diets allowed the transmission of SpliNPV, with a faster excretion via sucrose solution compared to *E. kuehniella* eggs. SpliNPV remained in *N. tenuis* digestive tract and was viable after excretion at least for 9 days for both diets.

Conclusions: This study demonstrated the potential of the predator *N. tenuis* as inoculative agent of baculoviruses, representing a new alternative that, along with inundative applications, might contribute to improve pest management strategies.

Keywords: *Alphabaculovirus*; bioinsecticides; microbiological control; predatory mirids; *Spodoptera littoralis*

1 INTRODUCTION

Alphabaculovirus, one of the four genera within the *Baculoviridae* family, include Lepidoptera-specific virulent pathogens that infect many species of agricultural pests.[1–3] Morphologically, alphabaculovirus particles belong to the nucleopolyhedrovirus (NPV) type because their virions are embedded in crystalline occlusion bodies (OBs) shaped by the virus-encoded polyhedrin protein. Most commonly, infection of a susceptible host starts following ingestion of OB-contaminated food substrate. OBs are then solubilized in the alkaline gut lumen, releasing their virions, which enter the midgut epithelial cells and undergo a first round of replication. The newly produced virions are then transmitted to other host tissues within the hemocoel, where they replicate in secondary and subsequent rounds until virtually all host cells are infected. OBs are formed in the late phases of replication and released upon host death.[4,5] Outside their host, NPVs persist inactive as mature OBs until subsequent infections in the larval stages of new hosts occur.[6–8] However, their persistence outside the host on plant surfaces is negatively influenced by abiotic factors such as solar ultraviolet (UV) light, alkaline pH, and high temperatures.[9] Mostly, lepidopteran-specific baculoviruses from the genus *Alphabaculovirus* (NPV) and *Betabaculovirus* (granulovirus type, GV) have been commercially developed so far,[10] and because of their restricted host range, are considered very safe insecticides.[6,8]

The use of baculoviruses as bioinsecticides consist in spray applications at high concentrations (rates for field use vary between 0.5–5 $\times$ 10^{12} OBs per hectare) with repeated treatments due to their low environmental persistence.[10] The commercial production of baculovirus OBs currently uses *in vivo* procedures that require rearing and harvesting large numbers of host

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larvae.[11] As a result, the production of baculovirus-based insecticides at low cost and large quantities represents a challenge for a commercial-scale up manufacturing.[12–14]

Baculoviruses have the potential to infect, multiply, spread (both horizontally and vertically) and persist in populations that constantly fluctuate.[6,15] When using baculoviruses in huge amounts with a curative strategy, the virus reduces pest population levels, but its reproductive success decreases because transmission to new host is a fundamental process in disease ecology; in the end, this reduces the persistence of the virus because of the speed of the death of the host.[10,16,17] When the pest levels are low, the use of a preventive strategy following inoculative releases of baculoviruses could increase the persistence in the agroecosystem. This strategy, which uses much lower virus loads, has proven successful in a few instances. However, it has not been explored much in agricultural settings as a preventive strategy to reduce the number of curative applications.

After the release of OBs from infected corpses to the environment, different abiotic and biotic factors can contribute to the dispersion of the virus, promoting the access to new hosts and favoring its persistence.[18,19] Amongst biotic factors, several studies reported that predatory arthropods can disperse the viruses by excretion of viable OBs after preying on baculovirus-infected larvae of different lepidopteran pests.[16,19–23] Unfortunately, the relevance of predatory arthropods as biotic agents in the dissemination of baculoviruses under field conditions depends on several factors, such as the coincidence of infected prey and predators, the acceptance of infected prey as food, or the interactive behavior of predator and prey in relation to virus acquisition.[24,25] In addition, the interaction necessary with the infected prey/host to acquire the virus requires a certain pest level in the crop. In short, it is a random process, unpredictable and consequently, not useful for pest control. Nevertheless, we hypothesize that these drawbacks could be overcome with man-guided dissemination of predator species that are routinely used for other target pests, if previously fed with baculoviruses. In Integrated Pest Management programs the term 'integrated' should not only involve the use of compatible tactics but also explore their possible synergies.[26] In this sense, the release of predators in biological control tactics with entomopathogenic OBs in their digestive tract would allow the combination of macrobiological and microbiological strategies in one single step, and therefore reduce the crop inputs, since the total amount of baculovirus used would be reduced and the predators should be used in any case to control other pests.

In order to improve the management of baculovirus under inoculative strategies, this study focused on promoting predator's ingestion of OBs via virus-treated diets, without mediation of infected-prey, and subsequently exposing the insect host to leaf surfaces contaminated with predator feces. The pathogen–host system studied was *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV) and the host where it was first isolated, *S. littoralis* (Lepidoptera: Noctuidae). The predator species selected was *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae), mainly used in Spain in tomato glasshouse crops for the biological control of whiteflies and the tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae).[27–29]

2 MATERIALS AND METHODS

2.1 Insects and baculovirus

Insects rearing and bioassays were conducted in the Universidad Politécnica de Madrid (UPM), Spain. The controlled environmental conditions inside climatic chambers were 25 ± 2 °C, 65 ± 10% relative humidity (RH), and 16 h:8 h (light/dark) photoperiod.

The laboratory rearing of *S. littoralis* (Boisduval) (Lepidoptera: Noctuidae) was started in 2018 and was collected on *Medicago sativa* L. in Los Palacios (Sevilla, Spain). Larvae were mass-reared in plastic boxes (30 cm × 20 cm × 10 cm) on an artificial diet described for the rearing of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae).[30] Once pupation occurred, pupae were transferred to ventilated methacrylate cages (40 cm × 30 cm × 30 cm). Emerged female and male adults were fed with a 30% (*v/v*) honey solution. Zig-zag folded filter paper was placed inside for oviposition. For bioassays, < 24 h-old eggs were collected to obtain larvae of uniform age.

Nesidiocoris tenuis was purchased from Insectaria® (Logroño, Spain). The predator was reared following a procedure previously described.[31] *Nesidiocoris tenuis* adults were fed *ad libitum* with a diet based on *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs provided by Agrobío® (Almería, Spain), and fresh green beans (*Phaseolus vulgaris* L.) as a source of water and sugar, as well as oviposition substrate. The SpliNPV isolate was amplified in *S. littoralis*, OBs were extracted and purified from NPV-killed insects, and OB concentration was determined at Universidad Pública de Navarra (Spain), using an improved hemocytometer (Hawkley Ltd, Lancing, UK).

2.2 Infection of L_1 *S. littoralis* larvae with SpliNPV via *N. tenuis* excretion

A baculovirus concentration of 1.40×10^8 OBs mL^{-1}, corresponding to the 95% lethal concentration (LC_{95}) × 20 against *S. littoralis* L_2 larvae was used.[32] Two different diets treated with SpliNPV were offered to *N. tenuis* to evaluate the acquisition and excretion of the baculovirus: (a) SUC diet, a 0.5 mol L^{-1} sucrose (Azucarera, Madrid, Spain) water solution cotton[29]; (b) EPHES diet, consisting in defrosted eggs of *E. kuehniella* (EPHEScontrol; Agrobío, Almería, Spain).

To prepare SUC diet, Eppendorf tubes were filled with 1 mL each of 1.40×10^8 OBs mL^{-1} SpliNPV suspension ($LC_{95} \times 20$) and sealed with cotton. For EPHES diet, *E. kuehniella* eggs were treated for 5 min by immersion in a 1.40×10^8 OBs mL^{-1} SpliNPV suspension ($LC_{95} \times 20$), in proportion 1:1 *w/v* (1 g:1 mL), and then placed on filter paper disks (Filter-Lab, Barcelona, Spain) to absorb the excess of moisture. Both SpliNPV-treated diets were offered *ad libitum* in feeding cages to < 72 h-old *N. tenuis* females and males separately, together with a fresh green bean pod as a source of water.

Thus, the sequential procedure of four steps was as follows: (step 1) predator OBs acquisition: OBs ingestion by *N. tenuis* adults (< 72 h-old) on SpliNPV-treated diets during 48 h; (step 2) predator excretion: eight adults (4 ♀, 4 ♂) were placed in each experimental unit consisting of a plastic cage (2 cm height, 9 cm Ø) with a tomato leaflet (*Solanum lycopersicum* L.) on a 1 cm-thick layer of agar-agar/distilled water (1.5% *w/v*) during 24 h to promote direct excretion; (step 3) pest larvae exposure to predator's excreta: after removing *N. tenuis* adults, 15 L_1 larvae of *S. littoralis* were placed on each tomato leaflet, where they fed on for 72 h; (step 4) individualization of pest larvae for mortality assessment: *S. littoralis* larvae were individually placed in blisters with 28 alveoli (Arapack, Zaragoza, Spain) previously half-filled with artificial diet. Larval mortality was daily evaluated until the surviving individuals reached the prepupal stage. In addition, to detect the presence of OBs in the predator's droppings, the drops excreted by five females and five males (step 2) per treatment were collected in 50 µL of distilled water. The presence of OBs was checked by counting a sample (20 µL) from each replicate

using a Neubauer chamber (Reichert Scientific Instruments, Buffalo, NY, USA) under the optical microscope (400× magnification lens; Leica, Microsystems GmbH, Wetzlar, Germany).

These were the criteria followed for the length of the earlier described steps: (1) a short period of 48 h in the step of OBs ingestion by predators, considering that in a commercial scenario the constant adding of the baculovirus to predator's diet would not be economically feasible and, before a sale order is placed, it is realistic to implement baculovirus supply for this interval; (2) an excretion time of 24 h because predators move in search of their prey and do not stay long in the same place, and (3) a time of 72 h for pest feeding on leaflets to favor the exposition to predator's excreta, and to avoid horizontal transmission through the corpses of other larvae before isolation in step 4.

The treatments assessed were: a negative control (tomato leaflets with excretion of *N. tenuis* previously fed on untreated laboratory-breeding diet of *E. kuehniella* eggs and fresh green beans); SUC and EPHES (tomato leaflets with predator's excreta previously fed on the SpliNPV-treated diets described earlier), and a positive control (tomato leaflets sprayed until the point of run-off with $LC_{95} \times 20$ SpliNPV suspension). Seven replicates per treatment were performed.

Additionally, a second infection cycle was developed with the dead larvae obtained in SUC and EPHES treatments. All corpses of infected larvae of each treatment were pooled in Falcon tubes with 20 mL of distilled water and ten sterile glass beads. The corpses were disintegrated at 2500 rpm in a vortex (Genie2; Scientific Industries, New York, USA). The suspension obtained was evenly distributed with a cotton swab on the tomato leaflets. Three treatments were considered: negative control-2 (water spraying), SUC-2 and EPHES-2. After air-drying the tomato leaflets, steps 3 and 4 described earlier for the first cycle of infection were repeated (15 L_1 *S. littoralis* larvae per replicate, seven replicates per treatment), assessing daily mortality once larvae were transferred to blisters and until survivors reached the prepupal stage.

An estimation of the number of OBs in both pools of dead larvae (first and second infection cycles) was performed by means of an optical microscope (400×) and a Neubauer camera (Reichert Scientific Instruments, Buffalo, NY, USA). A total of five samples by diet treatment and cycle were analyzed.

2.3 Assessment of *N. tenuis* retention of SpliNPV

Retention capacity was defined as the number of days that the predator excreted infective OBs after finishing baculovirus ingestion. To assess this point, the sequential procedure previously described was performed until 15 days after predator's ingestion of baculovirus-treated diets ($LC_{95} \times 20$ of SpliNPV in SUC and EPHES diets). Once the feeding period (48 h) finished, *N. tenuis* adults of T0 (retention time = 0 days) were immediately placed on tomato leaflets for excretion (24 h), while the rest (T3, T6, T9, T12 and T15) were transferred to cages with BV-free diet (*E. kuehniella* eggs and green bean pod) and sequentially placed (after 3, 6, 9, 12 and 15 days respectively) on tomato leaflets for excretion (24 h).

Once the excretion period finished, the mirids were removed from the experimental units and *S. littoralis* larvae (15 L_1 per leaflet) were introduced. Larvae fed on leaflets for 72 h, and then were individually transferred to alveoli of blisters half-filled with artificial diet. A negative control (tomato leaflets with excretion of *N. tenuis* previously fed on BV-free *E. kuehniella* eggs and fresh green beans) was assessed for each retention time. Five replicates per treatment (diet × retention time) were performed. The mortality of *S. littoralis* larvae was daily assessed until pupation.

2.4 Statistical analysis

Data of the cumulative mortality (end of evaluation) of *S. littoralis* larvae (first and second infection cycles) and estimation of OBs in predator's dropping were analyzed with one-way analysis of variance (ANOVA) to determine statistically significant differences among the different treatments (Bonferroni test). The data of estimation of OBs recovered from corpses (first and second infection cycles), and cumulative mortality at different retention times were analyzed using two-way ANOVA to determine the main effects of the principal factors (diet and cycle of infection, diet and retention time) as well as their interaction. If differences were observed in the interaction, pairwise multiple comparisons were run thereafter with the Bonferroni test. Prior to analysis, the data were checked for homoscedasticity and normal distribution. The level of statistical significance was $P \leq 0.05$.

Daily mortality data of the virus host were subjected to Kaplan–Meier survival analysis and median time to death (MTD) values were calculated. Multiple pairwise procedures to compare survival curves among treatments (different diets and retention times) were subjected [Log-Rank (Mantel–Cox) test, $P \leq 0.05$]. The analyses were performed with SPSS Statistics Software Package for Windows version 24.0.0.0.[33]

3 RESULTS

3.1 Infection of L_1 *S. littoralis* larvae with SpliMPV via *N. tenuis* excretion

The exposure of *S. littoralis* larvae to the baculovirus via *N. tenuis* excretion (first infection cycle) caused an increase of mortality. The percentages of accumulated mortality until prepupal stage (day 17 of evaluation) showed significant differences between SUC and EPHES treatments (81.23 ± 4.48% and 50.47 ± 3.62%, respectively), both mean values significantly higher than the negative control (4.75 ± 1.90%) but lower than positive control (100% of mortality) ($F_{3,27} = 187.45$; $P < 0.001$) (Fig. 1(a)). All analyzed droppings of *N. tenuis* excreta that have been fed on treated diets contained OBs in lower numbers than in the initial baculovirus concentration supplied. Besides, no significant differences were found between SUC treatment ($2.11 \times 10^6 \pm 1.08 \times 10^5$) and EPHES treatment ($2.40 \times 10^6 \pm 2.65 \times 10^5$) ($U = 55.00$, $P = 0.705$). In the negative control, no OBs were observed. On the contrary, after the replication of the virus in the host (second - cycle of infection) a 100% mortality of exposed *S. littoralis* larvae was recorded in both diets, whereas for negative control was 1.02 ± 0.90% (Fig. 1(b)).

The survival curves in the first cycle were also significantly different between both diets ($\chi^2 = 230.556$, df = 2, $P < 0.001$). In SUC treatment, the lethal effect of the virus in the host was faster than in EPHES treatment, and MTD values were 7.00 ± 0.62 and 15.00 ± 2.75 days, respectively. The reduction of survival in the positive control could not be calculated, as more than half of the larvae died on the tomato leaflets before they could be transferred, indicating much faster infection rates. In the same way, in the second cycle of infection, mortality in SUC-2 and EPHES-2 also occurred in the early larval instars.

Regarding the number of OBs present in each pool of dead larvae, a significantly lower number was observed in the second cycle of infection ($F_{1,16} = 457.02$, $P \leq 0.001$). In contrast, there were no significant differences between the SUC and EPHES

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treatments ($F_{1,16} = 2.09$, $P = 0.167$). Similarly, no interaction between the main factors was observed ($F_{1,16} = 0.56$, $P = 0.464$) (Table 1). No OBs were detected in any negative control of both infection cycles.

3.2 *Nesidiocoris tenuis* retention of SpliNPV

Mortality of *S. littoralis* larvae was affected by both factors considered, diet ($F_{2,72} = 91.22$, $P \leq 0.001$) and retention time ($F_{5,72} = 22.27$, $P \leq 0.001$). In addition, a significant interaction (diet × time) was also observed ($F_{10,72} = 7.23$, $P \leq 0.001$) and data were analyzed by pairwise multiple comparisons (Fig. 2).

The results revealed that SpliNPV remained in *N. tenuis* digestive tract and was viable after excretion at least for 9 days (T9) after the cessation of feeding on treated diets (Fig. 2, uppercase letters). No statistical differences were found between host mortality in SUC and EPHES treatments when the evaluation was extended until pupal stage, but in both cases the cumulative mortality was much higher than that of the control until T9 evaluation (Fig. 2, lowercase letters). There was a significant reduction of *S. littoralis* mortality in SUC treatment between T6 and T9, while in the case of EPHES diet, it was observed a delay in this significant decrease, which occurred from T9 to T12.

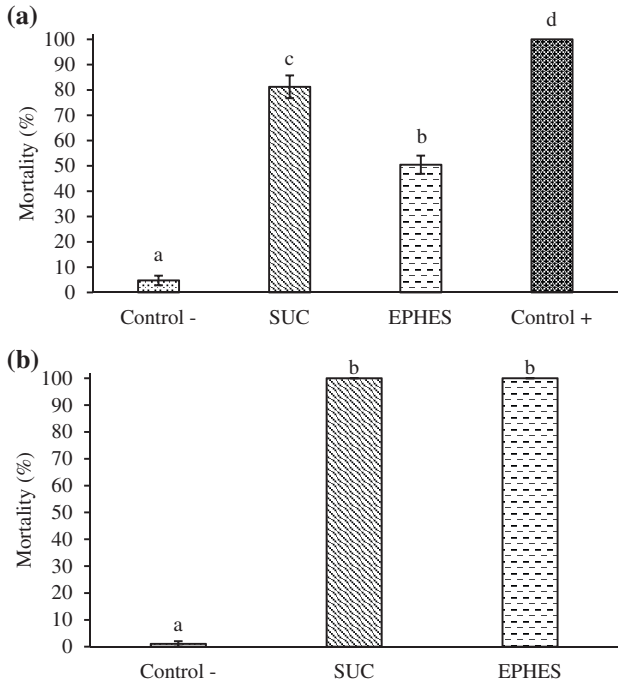

Figure 1. Cumulative mortality of *Spodoptera littoralis* larvae exposed to *Nesidiocoris tenuis* excreta [SUC: 0.5 mol L^{-1} sucrose solution + SpliNPV ($LC_{95} \times 20$). EPHES: *Ephestia kuehniella* eggs + SpliNPV ($LC_{95} \times 20$)]. (a) First infection cycle; (b) second infection cycle (no positive control performed in this cycle). Data are the mean (± standard error) of seven replicates/treatment ($n = 15$). One-way ANOVA. Different letters indicate significant differences ($P \leq 0.05$).

The values of the MTD of SUC and EPHES treatments in each retention time were evaluated and the comparison of the survival curves are shown in Table 2. For the T0 treatment, the MTD value was again much shorter when the predator ingested the virus in the sucrose solution but, in the rest of the retention times considered, the values were similar or equal for both diets. In addition, the survival curves of T0 and T3 were significantly different. As expected, MTD values increased as retention time did so, particularly in sucrose treatment.

Finally, a longer duration of the surviving individual larval stage was observed in SUC and EPHES treatments compared with the negative control. Figure 3 shows the cumulative pupation along time for each retention time; for T0, a clear delay was observed in surviving larvae (Fig. 3(a)); in the case of the retention times T3, T6 and T9, despite the progressive decrease of mortality, a delay in pupation was also detected, more visible in EPHES treatment (Fig. 3(b)–(d)); in T12 and T15, the difference amongst accumulated pupation curves of SpliNPV treatments and control almost disappeared (Fig. 3(e),(f)).

4 DISCUSSION

Baculoviruses are important control agents of relevant pests of Lepidoptera, whose management has become difficult due to pesticide resistance and the pressure to reduce pesticide residues.[10] They are highly specific and compatible with beneficial arthropods and vertebrates.[6,12] However, as biological agents, the inundative spray of baculoviruses implies considerable production inputs and high costs. An early inoculative application with a seasonal colonization approach has scarcely been explored.[6]

This study is the first evidence to demonstrate baculovirus OBs dispersal by *N. tenuis*, triggering infection in the lepidopteran hosts of the pathogen even in absence of direct interaction. In addition, the results support the fact that OBs are released by the predator in a more inoculative way than a baculovirus spray application. The levels of cumulative mortality reached were

Table 1. Estimation of occlusion bodies (OBs) recovered from the corpses of *Spodoptera littoralis* larvae after two cycles of infection

	Diet[†]		
Infection cycle (IC)	SUC[‡]	EPHES[§]	Mean
First	7.86×10^6 (2.20×10^5)	7.34×10^6 (3.88×10^5)	**7.60×10^6 (2.27×10^5) A**
Second	2.62×10^6 (1.56×10^5)	2.46×10^6 (4.98×10^4)	**2.54×10^6 (8.20×10^4) B**
Mean	**5.24×10^6 (8.83×10^5) a**	**4.90×10^6 (8.35×10^5) a**	

Note: Two-way ANOVA. Data are the mean (standard error) of five replicates/treatment ($n = 15$). Different lowercase letters within the same row or different uppercase letters within the same column indicate significant differences due to diet factor or to infection cycle respectively ($P \leq 0.05$). Bold vales indicate ($P \leq 0.05$).
[†] Diet offered to *Nesidiocoris tenuis* adults for 48 h.
[‡] SUC: 0.5 mol L^{-1} sucrose solution + *Spodoptera littoralis* multiple nucleopolyhedrovirus (SpliNPV) [95% lethal concentration (LC_{95}) × 20].
[§] EPHES: *Ephestia kuehniella* eggs + SpliNPV ($LC_{95} \times 20$).

lower via predator excretion than in the positive control (the same amount of OBs sprayed uniformly), suggesting that *S. littoralis* larvae ingested more OBs in the latter treatment. This was probably due to a lower concentration in the predator droppings and the less uniform distribution and release of the virus via predator excretion compared to the spray application. Along with the lower cumulative mortality, a delay in host death was also detected in comparison with the positive control. Some *S. littoralis* larvae died in the prepupal stage with a higher size than those in the negative control, which probably allowed them to produce a larger amount of OBs. Han *et al.*[34] reported that the *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) ecdysteroid UDP–glucosyltransferase (*egt*) gene product can modulate the host by prolonging the larval development in *S. exigua* before its death, and as consequence facilitates tree-top disease and a higher yield of viral OBs. Velasco *et al.*[14] showed that in the *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV)–*S. frugiperda* pathosystem, in addition to the increase in virus yield, OB maturation and pathogenicity also increased with the time after infection. In this way, the high levels of virulence obtained in the second cycle of infection proved the efficient replication of SpliNPV in the host via *N. tenuis* excretion. This trade-off phenomenon is also explained by Velasco *et al.*,[14] high inoculum concentration will infect a large fraction of the larvae, but also will shorten larval survival time and reduce the overall harvest of OBs.

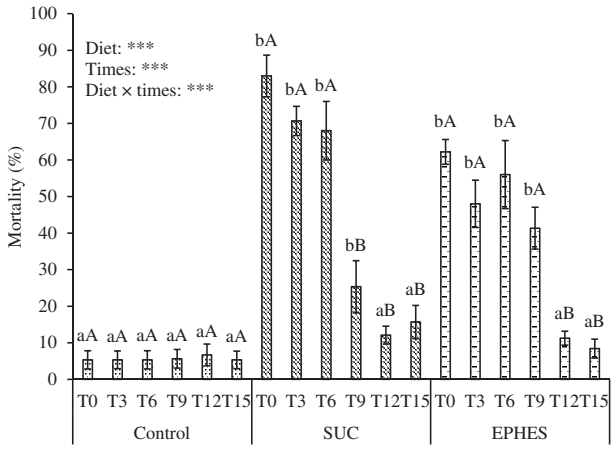

Figure 2. Cumulative mortality of *Spodoptera littoralis* larvae exposed 72 h to *Nesidiocoris tenuis* excreta after different retention times. T = time between the cessation of feeding on treated diets and the start of excretion on tomato leaflets (0, 3, 6, 9, 12 and 15 days). Diet treatments offered to *Nesidiocoris tenuis* adults for 48 h: Control: negative control without SpliNPV; SUC: 0.5 mol L^{-1} sucrose solution + SpliNPV ($LC_{95} \times 20$); and EPHES: *Ephestia kuehniella* eggs + SpliNPV ($LC_{95} \times 20$). Data are the means ± standard error of five replicates/treatment ($n = 15$). Two-way ANOVA analysis performed with diet and retention time as factors. Significant differences represented by asterisks (***$P \leq 0.001$). Multiple pairwise comparisons for each factor were performed with Bonferroni test ($P \leq 0.05$): different lowercase letters and different uppercase letters indicate differences due to the diet factor and due to retention time, respectively.

The acquisition of SpliNPV through treated diets did not affect the temporary retention of the baculovirus by *N. tenuis* compared to that observed in different predator species previously fed with infected prey. Abbas and Boucias[35] reported that *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) excreted infective OBs for 4 days when fed on *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV)-infected larvae of *A. gemmatalis* (Hübner) (Lepidoptera: Noctuidae) during 24 h. Also, Young and Yearian[36] documented a 10-day retention time of infective OBs in the feces of *Nabis roseipennis* Reuter (Hemiptera: Nabidae) when fed on larvae infected with AgMNPV. As baculovirus persistence on the surface of plants is scarce and OBs need to be ingested by new hosts before degrading,[6] the lifespan of OBs excretion while the predator is moving in the plant could be relevant for virus persistence. During passage through the digestive tract, OBs avoid damage from exposure to sunlight UV, which is the most degrading environmental factor to their viability.[16] In addition, predators while searching for prey, can deposit the viral OBs in plant microhabitats which are more protected from sunlight than in the case of a spray application.

Both types of diets used to supply the OBs to the predator allowed the horizontal transmission of SpliNPV despite their differences in terms of efficacy, probably due to the distinct state of matter (liquid and solid). Besides, sucrose could act as a phagostimulant for *N. tenuis*.[29] Nevertheless, the piercing-sucking mouthparts of *N. tenuis*, could represent a handicap for the acquisition of the baculovirus via *E. kuehniella* eggs, which presumably would be mostly retained in the chorion.[37] Taking the earlier mentioned into account, significant differences were observed in the initial virulence and in the retention pattern between both SpliNPV-treated diets. Apparently, the OB excretion was faster when they were ingested in a sucrose solution than via *E. kuehniella* eggs, initially fostering a higher ingestion of the virus by *S. littoralis* larvae.

Table 2. MTD (median time to death) values of *Spodoptera littoralis* larvae and comparison of survival curves between diets along the time

Retention time	MTD days (95% confidence limits) SUC[†]	EPHES[‡]	Comparison of survival curves
T0	9 (5.33–12.66)	21 (19.58–22.42)	$\chi^2 = 17.299$, **$P \leq 0.001$**
T3	21 (20.46–21.54)	22 (21.15–22.85)	$\chi^2 = 15.811$, **$P \leq 0.001$**
T6	23 (22.18–23.81)	23 (20.89–25.10)	$\chi^2 = 0.245$, $P = 0.621$
T9	26 (24.90–27.10)	25 (23.75–26.25)	$\chi^2 = 0.185$, $P = 0.667$

Note: T = retention time: 0, 3, 6 and 9 indicate the number of days between cessation of feeding on treated diet and excretion on leaflet (MTD values for T12 and T15 could not be calculated by SPSS software due to the low number of mortality events occurring in these retention times). Pairwise comparisons: Log Rank test ($P \leq 0.05$). Bold vales indicate ($P \leq 0.05$).
[†] SUC: 0.5 mol L^{-1} sucrose solution + *Spodoptera littoralis* multiple nucleopolyhedrovirus (SpliMNPV) [95% lethal concentration (LC_{95}) × 20].
[‡] EPHES: *Ephestia kuehniella* eggs + SpliMNPV ($LC_{95} \times 20$).

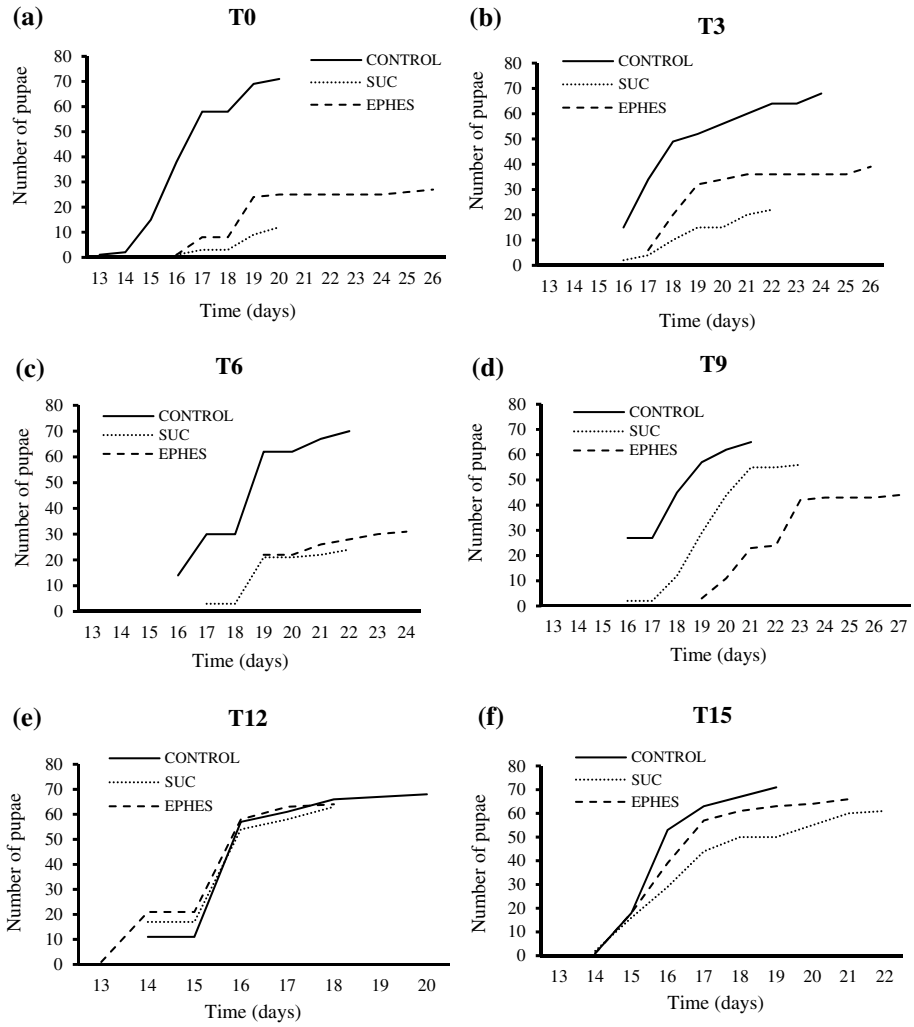

Figure 3. Comparative pupation of surviving larvae of *Spodoptera littoralis* exposed to *Nesidiocoris tenuis* excreta [SUC: 0.5 mol L^{-1} sucrose solution + SpliNPV (LC_{95} × 20). EPHES: *Ephestia kuehniella* eggs + SpliNPV (LC_{95} × 20)], after different retention times (T0, T3, T6, T9, T12 and T15). T = time between the cessation of feeding on treated diets and the start of excretion on tomato leaflets.

In addition, the delay observed in the pupation of survivors suggests a sublethal infection. The low virulence of baculoviruses is associated with covert infection.[38] Cabodevilla *et al.*[39] reported sublethal infections by SeMNPV in field populations of *S. exigua* with significant increases in larval developmental time, among other symptoms. The authors conclude that sublethal infections can persist between generations and reduce host fitness in terms of development and reproductive capacity. *Spodoptera* larvae that ingest OBs but do not die could emerge as covertly infected adults.

Nesidiocoris tenuis is commonly released in horticultural crops of southern Spain, and its target prey, whiteflies and *T. absoluta*[27–29] coexist in the crops with the susceptible baculoviruses hosts *S. littoralis* and also *S. exigua*.[40] This mirid is a generalist predator that can contribute to the control of lepidopteran pests, preying on eggs and early larvae,[28,41] so the interaction with uninfected and baculovirus-infected prey (such as *Spodoptera* sp. larvae) may be likely under a field scenario. Therefore, considering its mobility and the possibility of acquiring the baculovirus through treated diets (e.g., in a biofactory), the introduction of the virus inoculum in the crop by this means represents a remarkable chance.

Another question to be discussed is the number of predators used per leaflet in our study. In field releases, biocontrol companies recommend an inoculative rate of 0.5–1.5 individuals of *N. tenuis* per m^2 and up to 5 individuals per m^2 if pest attack is intense, being the initial release of the predator in groups of at least 25 and at most 100 individuals per 'releasing point'.[42,43] In our work, a higher density of eight adults per leaflet excreting during 24 h was evaluated. Although it is difficult to transpose the laboratory conditions performed to a field scenario, baculovirus excretion on leaves and stems close to the 'releasing points' of *N. tenuis* could reach similar levels of inoculum, serving as potential infective OB reservoirs.

5 CONCLUSION

The application of SpliNPV via predator excretion can facilitate the horizontal transmission between the host larvae in a more persistent manner, because the appearance of infected larvae is slower but also more continuous and extended in time than in the case of an inundative strategy (e.g., the positive control of this study) implemented in curative control cases. Hence, the introduction of the nucleopolyhedrovirus in association with *N. tenuis*, if it

proves to be efficient, would entail a more sustainable type of application, with savings in water and spraying costs, particularly indicated in a preventive control strategy. The laboratory results of this work are promising but require more research and their validation in field conditions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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