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Properties of nucleopolyhedrovirus occlusion bodies from living and virus-killed larvae of *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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A B S T R A C T

Previous studies report that the pathogenicity of nucleopolyhedrovirus occlusion bodies (OBs) is reduced if OBs are collected from living infected insects compared to virus-killed insects. We compared the production and characteristics of SfMNPV OBs collected from living and virus-killed fourth instars of *Spodoptera frugiperda* at 3-4 days, 5 days and 6-7 days post-inoculation. Cumulative virus-induced mortality increased from 10.5% at 3 days to 99.6% at 7 days post-inoculation. The production of OBs/mg larval weight increased significantly between the first and second samples, but did not increase thereafter. The total number of OBs produced in living and virus-killed larvae increased at each sample time. The size (cross-sectional area) of OBs increased significantly during the experiment, probably due to OB maturation. The median prevalence of immature OBs decreased over time from 25-44% in the first samples to 9% in the final sample. OB pathogenicity was compared in second instars that consumed a discriminating concentration of inoculum (2 x 10^4 OB/ml). Virus-induced mortality increased markedly in larvae that consumed OBs collected later (6-7 days) compared to OBs from earlier samples, but was similar for OBs from living or virus-killed insects at each time point. We conclude that the pathogenicity of OB samples was sensitive to the prevalence of immature OBs rather than the viral genome content of OBs, which did not differ over sample times or in OBs from living or virus-killed insects. These findings can inform decisions on the production of biological insecticides and laboratory studies on the insecticidal properties of OBs.

Keywords: SfMNPV; OB production; OB size; OB maturation; pathogenicity; genome copies; biological insecticide; Fall armyworm

1. Introduction

The efficient production of baculoviruses is critical to their commercial development as the basis for biological insecticides (Lacey et al., 2015). Advances in cell culture techniques have stimulated interest in the production of baculovirus insecticides in bioreactors, although this technology faces a variety of technical constraints related to the yield, production costs and stability of virus strains in cell culture (Claus et al., 2012; Reid et al., 2016). As a result, current methods for the production of virus occlusion bodies (OBs) require the inoculation, rearing and harvesting of large numbers of host larvae (Grzywacz and Moore 2017). The quantity and insecticidal activity of OBs produced *in vivo* depend on a range of variables, including genetic factors such as host and virus strains, environmental factors such as temperature and numerous operational factors such as inoculum dose, inoculation method, insect stage, diet, rearing densities and harvesting of infected individuals (Hunter-Fujita et al., 1998; Shapiro 1986). Optimization of these aspects of OB production are necessary as OB production is the most costly and time-consuming aspect of virus-based insecticide manufacturing (Grzywacz et al., 2014).

A few studies have reported that harvesting of nucleopolyhedrovirus (*Baculoviridae: Alphabaculovirus*) OBs prior to death of the host results in OBs with reduced insecticidal activity, usually measured in terms of dose-mortality metrics. Compared to OBs from virus-killed insects, OBs from living infected larvae were two-fold less pathogenic for Spodoptera litura nucleopolyhedrovirus (SpliNPV) (Takatsuka et al., 2007), or seven-fold less pathogenic for Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV) (Shapiro and Bell 1981) or Helicoverpa zea nucleopolyhedrovirus (HzNPV) (Ignoffo and Shapiro 1978), whereas no significant difference was observed in the pathogenicity of Spodoptera exigua multiple nucleopolyhedrovirus OBs from living or dead larvae (Smits and Vlak 1988). The production of OBs with reduced insecticidal activity in living larvae is an issue of interest as a fraction of the infected insects are often harvested prior to death, in order to minimize loss of OBs due to post-mortem liquefaction of larval corpses (Cherry et al., 1997; Grzywacz et al., 1998). The reasons that OBs from living larvae are less pathogenic than OBs from virus-killed insects is unclear but may be related to the reduced size or virion content of OBs from living infected larvae (Behle 2018; Takatsuka et al., 2007).

The Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) has been used as the basis for biological insecticide preparations against larvae of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Barrera et al., 2011; Behle and Popham 2012; Cruz et al., 1997; García-Banderas et al., 2020; Haase et al., 2015) that has spread across Africa, Asia and Oceania (Tay et al., 2022). In the present study we compared the

physical and biological characteristics of OBs collected from living and virus-killed larvae of *S. frugiperda* at different intervals post-inoculation, with the aim of identifying changes in OB characteristics during the development of polyhedrosis disease. These findings are likely to inform decisions on the timing of harvesting of virus infected insects during commercial OB production and laboratory studies on the insecticidal properties of nucleopolyhedrovirus OBs.

2. Methods

2.1. Insects and virus

The laboratory colony of *S. frugiperda* was started in 2016 from larvae collected in maize fields close to Xalapa, Veracruz, Mexico. Larvae were reared at 26 ± 1 °C on a semi-synthetic diet based on soybean flour, wheatgerm, yeast, agar, and vitamins (Hunter-Fujita et al., 1998). For oviposition, groups of adults were placed in paper bags with continuous access to 10% sucrose solution on a cotton pad.

A Nicaraguan wild-type isolate of SfMNPV (Simón et al., 2011) was amplified by inoculating *S. frugiperda* fourth instars with a suspension of 1 x 10^8 OBs in 10% sucrose and 0.1% food coloring solution (McCormick, Mexico City) using the droplet feeding method (Hughes et al., 1986). Larvae that consumed the suspension within 10 min were reared individually at 27 ± 0.5 °C until death and then homogenized in sterile water and filtered through a stainless steel mesh (80 μm pore size) to remove debris. The resulting OB suspension was counted in triplicate using a Neubauer chamber under a phase contrast microscope and stored at 4 °C prior to use in experiments.

All experimental procedures described in this study were performed under controlled laboratory conditions at 25 ± 1 °C, 70 ± 10% relative humidity and 14:10 h L:D photoperiod.

2.2. Inoculation and sampling of infected insects

Third instars of *S. frugiperda* were starved overnight and those that had molted to the fourth instar were selected and inoculated with a suspension of 2.1 x 10^8 OBs/ml using the droplet feeding method. This concentration was expected to infect 99% of experimental insects based on the results of preliminary studies, in order to minimize the presence of uninfected larvae in samples of experimental insects. Control larvae were inoculated with sucrose solution and food coloring without OBs. Groups of 24 inoculated larvae were placed in one of three 24-well tissue culture plates containing a piece of diet and were incubated in darkness at 25 ± 0.5 °C in a laboratory incubator (Heratherm, Thermo Scientific, USA). Each plate was then assigned at random to one of three sampling regimes

in which samples were taken at: (i) 3 and 4 days post-inoculation, (ii) 5 days post-inoculation, (iii) 6 and 7 days post-inoculation. At each sample time, larvae were classified as living or dead (unresponsive to the touch of a toothpick), counted, transferred to 1.5 ml microcentrifuge tubes (one tube for living larvae, another for virus-killed larvae), weighed on a precision balance (Explorer EX124, Ohaus, USA) and frozen at -20 °C. It is important to note that all tissue culture plates were checked on each day of the experiment and virus-killed larvae that had died prior to the assigned sample time were removed and discarded, so that samples taken at 3-4 days, 5 days or 6-7 days post-inoculation comprised only larvae that died (or were alive) on those sample dates and did not include larvae that died at earlier times (Fig. 1). This procedure was employed to avoid including insects that died at early times in samples taken at later sample times. The daily observation of larvae also allowed dead larvae to be collected prior to liquefaction. The procedure was performed on ten occasions using ten different batches of insects that represented ten independent experimental replicates. Sample sizes varied depending on the number of virus-killed or living infected insects at each time point (see Results section 3.1).

To produce OB suspensions all the larvae from each replicate were thawed and transferred to a glass homogenizer. Each microcentrifuge tube was washed with 300 µl of water to remove any insect remains and these washings were transferred to the homogenizer. Larvae were homogenized in 1.5 ml MilliQ water and the resulting suspension was filtered through a stainless steel mesh (80 µm pore size) to remove debris. Each OB suspension was then centrifuged at 2800 *g* for 15 mins and the resulting pellet was resuspended in 1 ml MilliQ water. To quantify OBs, each sample was diluted in Milli-Q water and triplicate samples were counted in a Neubauer counting chamber at x400 magnification. The OB suspensions were then stored at -20 °C until required.

2.3. Determination of OB size and maturity

For OB size measurements, 100 µl of OB suspension from each sample was pooled among all ten replicates to produce 1 ml of OB suspension that was adjusted to a concentration of 5 x 10^7 OBs/ml in MilliQ water. Each suspension represented a pooled sample of between 38 and 125 larvae (see Results section 3.1). Each suspension was mixed thoroughly on a vortex mixer and 20 µl droplets were pipetted on to an aluminum stub and allowed to dry. Each sample was then coated with gold-palladium in a Quorum Q150R sputter-coater and observed at x10,000 magnification in a FEI Quanta 250 FEG scanning electron microscope (SEM) at an accelerating voltage of 10 kV. Images of OBs (~100 OBs/image) were taken at 5-7 different points within each sample at a fixed resolution of 1536 x 1103 pixels, 71 dpi. The cross-sectional area of an average (±SE) of 438 ± 73 OBs from living and dead larvae was then estimated with reference to the 20 µm scale bar using

the ImageJ program (https://imagej.net) as described previously (Ramírez-Arias et al., 2019). The prevalence of mature and immature OBs was calculated by counting each type of OB in 3 - 5 photomicrograph images according to the presence or absence of the polyhedron envelope. Mature OBs have a smooth sealed exterior surface whereas immature OBs have an obvious pitted and irregular surface and lack the polyhedron envelope (Sajjan and Hinchigeri 2016).

2.4. Quantification of viral genomes

Samples of 2 x 10^8 OBs in 300 µl sterile water were obtained for all treatments and replicates. Virions were released from the OBs by adding 100 µl of 0.5 M Na_2CO_3 and 100 µl of 10% SDS for 10 min at 55 °C. The pH was adjusted by addition of 60 µl 0.1 M HCl. Debris was pelleted by centrifugation at 4200 *g* for 5 min and was discarded, whereas the virion-containing supernatant was incubated with 12.5 µl proteinase K (20 mg/ml) at 55 °C for one hour. Each sample was then treated with 20 µl RNase solution, followed by 200 µl 96% ethanol and cell lysis solution (PureLink Genomic DNA kit, Invitrogen, Waltham, MA). The sample was then loaded on to a spin column and centrifuged at 10,000 *g*, washed twice and DNA was eluted in 50 µl of elution buffer following the manufacturer's protocol. The concentration of DNA in each sample was then determined by calculating the average reading from triplicate samples in a spectrophotometer at 260 nm wavelength (BioSpec-Nano, Shimadzu, Japan).

Quantitative PCR (qPCR) based on SYBR fluorescence was performed in 96-well plates in a Mx3005P qPCR System (Stratagene, La Jolla, USA). The primers were designed based on the polyhedrin gene sequence and had been used in a previous qPCR study (Ramírez-Arías et al., 2019). The forward primer (5'-GAACCTTCACTCTGAGTACACGCAC) and reverse primer (5'-AGACGATGGGTTTGTAGAAGTTCTCC) amplified an 82 bp fragment of the SfMNPV polyhedrin gene. Amplifications were performed in a volume of 10 µl, comprising 5 µl of iQ SYBR Green Supermix (Bio-Rad), 3.6 µl of sterile Milli-Q water, 0.2 µl of each of the primers (forward and reverse, 10 mM) and 1 µl of a 1/1000 dilution of DNA (between 5 x 10^{-4} and 5 x 10^{-5} ng). Three reactions for each sample with a negative control were included in each analysis and the standard curve (range 5 x 10^{-3} ng to 5 x 10^{-7} ng viral DNA) was performed in triplicate to determine the efficiency of each reaction. The qPCR protocol consisted of an initial denaturation step at 95 °C for 3 min followed by 45 amplification cycles of 95 °C for 15 s and 60 °C for 30 s and, to determine the melting curve, a cycle of 30 s with increments of 0.5 °C between 60 °C and 95 °C. Data acquisition and processing was performed using MxPro software (Stratagene, La Jolla, USA).

2.5. Insecticidal activity of OBs

The mortality response of *S. frugiperda* larvae to a discriminating concentration of OBs was determined using pooled OB samples from the ten replicates of each treatment. For this, groups of 24 larvae were starved overnight as they molted to the second instar and, using the droplet feeding method, they were then inoculated with 2 x 10^4 OBs/ml that was previous determined to be the 50% lethal concentration (LC_{50}) in second instars from our laboratory colony (Ramírez-Arias et al., 2019). Control larvae consumed sucrose and food coloring solution without OBs. Insects that drank the suspension in 10 mins were individually transferred to the wells of a tissue culture plate with diet, maintained in darkness at 25 ± 0.5 °C and checked daily until death or pupation. The bioassay was performed on three occasions using different batches of insects.

2.6. Statistical analyses

The experiment was originally designed as a two-way ANOVA with infection status (living infected insects vs. virus-killed insects) and sample time as factors. However, only one larva was alive in the 6-7 day sample which resulted in an unbalanced design. Consequently, the remaining treatment combinations were treated as five separate groups and subjected to Kruskal-Wallis analysis followed by Dwass-Steel-Critchlow-Fligner (DSCF) pairwise comparisons (Hollander and Sethuraman 2015). The results of these analyses are shown as median values and the corresponding asymmetrical interquartile range (IQR). All analyses were performed using the R-based software Jamovi v.2.3.0 (Jamovi 2022). OB pathogenicity was determined by analyzing larvae mortality in bioassays by fitting a generalized linear model (GLM) with a binomial error distribution in GLIM 4 (Numerical Algorithms Group 1993). The SE values of binomially distributed mortality data are asymmetrical. There was no evidence of overdispersion in the mortality data.

3. Results

3.1. Larval mortality

Following inoculation of larvae, cumulative mortality due to lethal polyhedrosis disease increased from 10.5% (asymmetrical upper - lower range of SE: 1.1 - 1.2%) at 3 days post-inoculation to 99.6% (range of SE: 0.8 - 0.2%) at 7 days post-infection (Fig. 2A). A total of 15 larvae (2%) died from bacterial infection, confirmed by microscopic examination of Giemsa smears, which were discarded and were not considered in cumulative mortality calculations. Across all ten replicates, the number of sampled insects decreased over time

due to mortality at earlier time points (Fig. 2B). A total of 101 virus-killed larvae and 125 living infected larvae were collected and analyzed in the 3-4 day sample, representing an average (± SE) of 10.1 ± 1.1 virus-killed insects and 12.5 ± 0.9 living insects from each replicate. The 5 day sample consisted of 80 virus-killed larvae (average 8.0 ± 1.1 larvae/replicate) and 38 living larvae (average 3.8 ± 1.1 larvae/replicate). Finally, 44 virus-killed larvae (4.4 ± 0.8 larvae/replicate) and a single living larva comprised the sample taken at 6-7 days. The single living larva in the 6-7 day sample was removed from the study and is not considered further. No virus induced mortality was observed in control larvae that were discarded after 7 days post-inoculation.

3.2. OB production

The median weight of larvae in each sample varied significantly among groups (Kruskal-Wallis H = 34.9; d.f. = 4; P <0.001). Living larvae were significantly heavier than virus killed larvae at the same time point (Fig. 3A). The median weight of virus-killed larvae increased by almost four-fold during the experiment.

The production of OBs/mg of insect body weight differed significantly among experimental groups (H = 29.3; d.f. = 4; P <0.001). The number of OBs/mg larval weight in living infected insects was lower than measured in virus-killed insects, although this difference was only significant in the 3-4 day sample as increased variation was observed in the sample taken at 5 days post-inoculation (Fig. 3B). The difference between the OBs/mg values at 3-4 days and 5 days was borderline significant in living insects (P = 0.055). In virus-killed larvae OBs/mg values increased significantly between the first and second samples, but did not increase in the final sample taken at 6-7 days post-inoculation (Fig. 3B).

The total number of OBs produced in each virus-killed larva increased significantly at each sample time (H = 34.9; d.f. = 4; P <0.001), reflecting the growth of larvae during the infection period (Fig. 3C). The median number of OBs/larva increased from 9.3×10^7 OBs in larvae killed at 3-4 days post-inoculation to 7.2×10^8 OBs in larvae killed at 6-7 days post-inoculation. Interestingly, the number of OBs recovered from living infected larvae was similar to the numbers from insects that died from polyhedrosis disease at the same time point (Fig. 3C), suggesting that insect death was not solely dependent on total OB load.

3.3 Determination of OB size and maturity

The cross-sectional area of OBs varied from 0.111 μm^2 to a maximum of 6.226 μm^2 across a total of 2192 OBs measured. The cross-sectional area varied significantly among

the five experimental groups ($H = 264.9$; d.f. = 4; $P < 0.001$). The median cross-sectional area of OBs in virus-killed insects did not differ for samples taken at 3-4 days and 5 days post-inoculation, but increased significantly in OBs sampled at 6-7 days post-inoculation (Fig. 4A). The smallest OBs were collected from living larvae at 3-4 days post-inoculation but the size of these OBs increased significantly in the sample taken from living larvae at 5 days post-inoculation (Fig. 4A).

The prevalence of mature and immature OBs was determined by examination of scanning electron photomicrographs (Fig. 4B). The median prevalence of immature OBs collected from virus-killed larvae decreased steadily from 25.5% (IQR: 22.6 - 33.0) in the 3-4 day sample, to 8.9% (IQR: 2.4 - 12.3) in the sample collected at 6-7 days post-inoculation ($H = 39.2$; d.f. = 4; $P < 0.001$). The prevalence of immature OBs was markedly higher in living larvae collected at 3-4 days post-inoculation (44.2%, IQR: 36.1 - 55.1) compared to virus-killed insects at the same time point, but this difference decreased and was not significant in the sample taken at 5 days post-inoculation (Fig. 4B).

3.4. Quantification of viral genomes

The qPCR analysis of DNA extracted from samples of 2×10^8 OBs from each replicate resulted in amplification with a mean ±SD Cq value of 22.2 ± 1.7 across all samples and an efficiency of 105-106%, within the acceptable limits for qPCR studies (Taylor et al., 2019). The median copy number varied from 1.92×10^9 copies in the samples of OB from virus-killed insects collected at 5 days post-inoculation to 2.39×10^9 copies in the OB samples collected from virus-killed larvae collected at 3-4 days post-inoculation (Fig. 5). The qPCR measurements of copy number varied considerably within each time point but did not vary significantly among time points or between OB samples from living and virus-killed larvae ($H = 1.60$; d.f. = 4; $P = 0.806$). To confirm these findings the qPCR assay was repeated with similar non-significant variation among samples and time points ($H = 2.07$; d.f. = 4; $P = 0.724$; data not shown).

2.5. Insecticidal activity of OBs

The biological activity of OBs was determined by inoculating *S. frugiperda* second instars with 2×10^4 OB/ml previously estimated to result in 50% mortality (Fig. 6). Virus-induced mortality varied significantly among larvae inoculated with OBs from one of the five experimental groups (GLM: $\chi^2 = 73.48$; d.f. = 4; $P < 0.001$). Although all insects received the same concentration of inoculum, virus-induced mortality increased from ~3% in insects that consumed OBs from living and virus-killed insects collected at 3-4 days post-inoculation to 52% (range of SE: 6.0 - 5.9) in insects that consumed OBs collected from virus-killed at 6-7 days post-inoculation, with intermediate values (~16%) in the

samples from living and virus-killed insects taken at 5 days post-inoculation (Fig. 6). No virus-induced mortality was observed in control larvae.

4. Discussion

A systematic examination of the production and characteristics of OBs harvested from living and virus-killed larvae of *S. frugiperda* at three time points along the cumulative mortality curve resulted in findings that have applications in the production of biological insecticides and in the study of the insecticidal phenotype of nucleopolyhedrovirus OBs in the laboratory.

The study was conducted by inoculating fourth instars with a high concentration of inoculum (2.1 x 10^8 OBs/ml) to ensure that virtually all the experimental insects acquired a lethal infection. As a result, it was not necessary to analyze living larvae individually at each sample time to determine their infection status prior to the analysis of their OB load, which would have been very laborious given that we sampled a total of 164 living larvae distributed across the ten replicates of the study. In reality, in large-scale virus production facilities, the inoculum concentration has to be optimized to produce the highest number of highly insecticidal OBs from each batch of inoculated larvae. This usually requires a trade-off, as high inoculum concentration will infect a large fraction of the larvae, but very high inoculum concentration will shorten larval survival time and reduce the overall harvest of OBs (Behle 2018; Grzywacz and Moore 2017).

In the present study, both living and virus-killed larvae harvested at 3-4 days post-inoculation produced fewer OBs than larvae at later time points, whereas at 5 days and 6-7 days post-inoculation the production of OBs per larva increased significantly in virus-killed insects (Fig. 2C). Clearly, OB numbers at later time points reflect a combination of increased host resources due to larval growth during the systemic phase of the infection and the extended period for which the virus can replicate, compared to samples taken at earlier time points. The OB load per mg of larval weight also increased significantly over time (Fig. 2B). Studies with other nucleopolyhedroviruses have noted an increase in OB production per mg larval weight over time (Kumar et al., 2005; Takatsuka et al., 2007), and a 4-fold to 6-fold higher production of OBs per mg larval weight in virus killed larvae compared to living infected insects in the case of the *H. zea*/HzNPV pathosystem (Ignoffo and Shapiro 1978).

Scanning electron microscope examination revealed that OBs varied widely in size (range 0.111 - 6.226 μm^2 cross-sectional area). The size of OBs tended to increase at later sampling times and the prevalence of immature OBs decreased markedly. Indeed, the decrease in the prevalence of immature OBs over time was evident by simple visual

inspection of electron microscope images. Interestingly, samples of SeMNPV OBs from living larva also contained many small OBs (Smits 1987) and an increase in the size of SpliNPV OBs at successive sample times was also reported by Takatsuka et al. (2007). In the present study, the increase in OB size at later sample times was likely due to the OB maturation process in which immature OBs acquire a smooth sealed outer envelope of multilayered polyhedron envelope protein (PEP), in a process that is facilitated by a vermiform complex of P10 protein and electron dense spacers in the nucleus of the infected cell (Graves et al., 2019). Various functions have been assigned to the polyhedron envelope include preventing the loss of ODVs from the polyhedrin matrix, preventing the fusion of OBs during their development in the cell nucleus, and increasing the physical stability of OBs in the environment outside of the host (Gross et al., 1994; Sajjan and Hinchigeri 2016). As the polyhedron envelope is sensitive to sodium dodecyl sulfate (Lua et al., 2003), we avoided the use of this or other detergents during sample preparation, except when performing DNA extraction.

As OBs differed markedly in size and maturity, we suspected that they may differ in ODV content. To examine this, we determined the number of genomes occluded by OBs using qPCR targeted at the polyhedrin gene. All the genotypic variants present in the SfMNPV-NIC isolate are polyhedrin positive (Simón et al., 2004), so we were confident that amplification based on this gene would provide an accurate estimate of genome copies in each OB sample. Contrary to our supposition, the OB samples had a similar viral genome content, irrespective of the time point or status (living/dead) of the host. This suggests that there may be a threshold number of ODVs in the nucleus around which the OB forms and that the size of the OB that develops subsequently does not reflect the ODV content that it occludes. Previous statements that OB size is positively correlated with the number of ODVs that each OB occludes (Takatsuka et al., 2007) finds no support in the cited literature (Allaway 1983; Mazzone and McCarthy 1981). However, as we did not employ cell culture assay techniques or transmission electron microscopy, we cannot rule out the possibility that different OB samples had different numbers of ODVs, for example if the ratio of single nucleocapsid to multinucleocapsid ODVs changed over time but the number of genome copies remained stable at different sample times. This seems unlikely however, as the distribution of nucleocapsids among ODVs seems to be a characteristic of each nucleopolyhedrovirus strain (Fujimoto et al., 2017; Rohrmann 2014) and appears to be genetically determined as several genes influence the number of nucleocapsids packaged within ODVs (Beperet et al., 2013; Li et al., 2014; Yu et al., 2009), or the number of ODVs occluded within OBs (Li et al., 2014; Simón et al., 2008a; Yang et al., 2014). The identity of the host cell can also influence nucleocapsid aggregation within ODVs (Chaeychomsri et al., 2018; Xu et al., 2012).

OB samples were used to inoculate larvae at a standard discriminatory concentration (2×10^4 OB/ml) that was expected to result in 50% lethal infection in *S. frugiperda* second instars (Ramírez-Arias et al., 2019). The pathogenicity of OBs from each time point showed marked differences across sample times but did not differ significantly between OBs from living or virus-killed insects at a given time point (Fig. 6). It was previously suggested that differences in OB pathogenicity were due to variation in the number of ODVs occluded (Behle 2018). Our qPCR findings provided no evidence for this, whereas there was a closer correlation between OB pathogenicity and the prevalence of mature OBs in the inoculum. This suggests that OB maturation is associated with processes that influence key aspects of the primary infection process in the insect midgut. One possible mechanism is the incorporation of alkaline proteases into OBs during maturation. These proteases markedly facilitate OB degradation and ODV release in the alkaline conditions of the lepidopteran midgut (Mazzone and McCarthy 1981; Rorhmann 2019). An OB endogenous alkaline protease is also involved in the activation of P74 that forms part of the ODV *per os* infection factor (PIF) complex that is essential for virus entry into midgut epithelial cells (Peng et al., 2011). An additional candidate for maturation-mediated increases in OB pathogenicity could be an ortholog of the fusolin-like GP37 protein that is associated with OBs (Vialard et al., 1990), and that has chitin binding activity that may affect the integrity of the peritrophic matrix through which ODVs must pass to access midgut cells (Erlandson et al., 2019).

It would be interesting to verify these findings in other lepidopteran-nucleopolyhedrovirus pathosystems and begin to explore the reasons behind such large variation in OB pathogenicity over time. Although the genotypic variants present in the SfMNPV-NIC isolate differ in their individual pathogenicity and speed-of-kill characteristics (Simón et al., 2005, 2008b), the possibility that OBs from early time points comprise different proportions of genotypic variants with a different insecticidal phenotype than OBs produced at later times is considered to be highly unlikely. This is because the high inoculum concentration used would have ensured the transmission of most if not all variants to the experimental insects (Clavijo et al., 2010) and once infection has been achieved, the relative prevalence of variants within the host does not change significantly over the course of each infection (Simón et al., 2008b).

These findings have clear implications both for the commercial production and the study of alphabaculoviruses as the basis for biological insecticides. First, for mass production processes it is clear that OB harvesting should be delayed as long as possible before larval liquefication seriously reduces the final yield of OBs (Cherry et al., 1997; Grzywacz et al., 1998). If possible, OBs should not be harvested from living infected

larvae, but it is usually difficult to separate living insects for later harvesting for logistical reasons and the costs associated with increased handling of insects (Grzywacz and Moore, 2017). Second, in laboratories involved in the study and selection of alphabaculovirus isolates with high potential as biological insecticides it is clear that the procedures involving collecting OBs from virus-killed insects need to be standardized so that comparisons of OBs from different virus strains are performed at similar points on the cumulative mortality curve. This should ensure that the OB preparations under study have similar proportions of mature and immature OBs and therefore differences in OB potency will reflect the phenotype of the strains being compared rather than the time at which the OBs were harvested.

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CRediT authorship contribution statement

Esbeidy A. Velasco: Investigation, Methodology, Data Curation, Formal analysis, Writing - Original Draft. **Cindy S. Molina-Ruíz**: Investigation, Data Curation, Writing - Review & Editing. **Juan S. Gómez-Díaz**: Investigation, Resources. **Trevor Williams**: Conceptualization, Methodology, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Supervision, Visualization, Funding acquisition.

References

Allaway, G.P., 1983. Virus particle packaging in baculovirus and cytoplasmic polyhedrosis virus inclusion bodies. J. Invertebr. Pathol. 42 (3), 357-368.

Barrera, G., Simón, O., Villamizar, L., Williams, T., Caballero, P., 2011. Spodoptera frugiperda multiple nucleopolyhedrovirus as a potential biological insecticide: genetic and phenotypic comparison of field isolates from Colombia. Biol. Control 58, 113-120.

Behle, R.W., 2018. In vivo production of Agrotis ipsilon nucleopolyhedrovirus for quantity and quality. J. Econ. Entomol. 111, 101-107.

Behle, R.W., Popham, H.J., 2012. Laboratory and field evaluations of the efficacy of a fast-killing baculovirus isolate from *Spodoptera frugiperda*. J. Invertebr. Pathol. 109, 194–200.

Beperet, I., Barrera, G., Simón, O., Williams, T., López-Ferber, M., Gasmi, L., Herrero, S., Caballero, P., 2013. The sf32 unique gene of Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) is a non-essential gene that could be involved in nucleocapsid organization in occlusion-derived virions. PLoS One 8, e77683.

Chaeychomsri, S., Chaeychomsri, W., Siruntawineti, J., Ikeda, M., Kobayashi, M., 2018. Replication and occlusion body formation of Spodoptera exigua multicapsid nucleopolyhedrovirus in a homologous cell line. J. Adv. Agric. Technol. 5 (3), 236-244.

Cherry, A.J., Parnell, M.A., Grzywacz, D., Jones, K.A., 1997. The optimization of in vivo nuclear polyhedrosis virus production in *Spodoptera exempta* (Walker) and *Spodoptera exigua* (Hübner). J. Invertebr. Pathol. 70 (1), 50-58.

Claus, J.D., Gioria, V.V., Miceloud, G.A., Visnovsky, G., 2012. Production of insecticidal baculoviruses in insect cell cultures: potential and limitations. In: Soloneski, S. Larramendy, M. (Eds.), Insecticides – Basic and Other Applications. InTechOpen, London, pp. 127–152.

Clavijo, G., Williams, T., Muñoz, D., Caballero, P., López-Ferber, M., 2010. Mixed genotype transmission bodies and virions contribute to the maintenance of diversity in an insect virus. Proc. R. Soc. B: Biol. Sci. 277, 943-951.

Cruz, I., Figueiredo, M.L., Valicente, F.H., Oliveira, A.C., 1997. Application rate trials with a nuclear polyhedrosis virus to control *Spodoptera frugiperda* (Smith) on maize. An. Soc. Entomol. Brasil 26, 145-152.

Erlandson, M.A., Toprak, U., Hegedus, D.D., 2019. Role of the peritrophic matrix in insect-pathogen interactions. J. Insect Physiol. 117, 103894.

Fujimoto, S., Kokusho, R., Kakemizu, H., Izaku, T., Katsuma, S., Iwashita, Y., Kawasaki, H., Iwanaga, M., 2017. Characterization of a Bombyx mori nucleopolyhedrovirus variant isolated in Laos. J. Insect Biotechnol. Sericol. 86, 85-94.

García-Banderas, D., Tamayo-Mejía, F., Pineda, S., de la Rosa, J.I.F., Lasa, R., Chavarrieta-Yáñez, J.M., Gervasio-Rosas, E., Zamora-Avilés, N., Morales, S.I., Ramos-Ortiz, S., Valle, J., Martínez-Castillo, A.M., 2020. Biological characterization of two Spodoptera frugiperda nucleopolyhedrovirus isolates from Mexico and evaluation of one isolate in a small-scale field trial. Biol. Control 149, 104316.

Graves, L.P., Hughes, L.C., Irons, S.L., Possee, R.D., King, L.A., 2019. In cultured cells the baculovirus P10 protein forms two independent intracellular structures that play separate roles in occlusion body maturation and their release by nuclear disintegration. PLoS Pathog. 15 (6), e1007827.

Gross, C.H., Russell, R.L., Rohrmann, G.F., 1994. Orgyia pseudotsugata baculovirus p10 and polyhedron envelope protein genes: analysis of their relative expression levels and role in polyhedron structure. J. Gen. Virol. 75, 1115-1123.

Grzywacz, D., Moore, S., 2017. Production, formulation, and bioassay of baculoviruses for pest control. In: Lacey, L.A. (Ed.), Microbial Control of Insect and Mite Pests, Academic Press, San Diego, CA, pp. 109-124.

Grzywacz, D., Jones, K.A., Moawad, G., Cherry, A., 1998. The in vivo production of Spodoptera littoralis nuclear polyhedrosis virus. J. Virol. Meth. 71, 115-122.

Grzywacz, D., Moore, D., Rabindra, R.J., 2014. Mass production of entomopathogens in less industrialized countries. In: Morales-Ramos, J.A., Rojas, M.G., Shapiro-Ilan, D.I. (Eds.), Mass Production of Beneficial Organisms. Elsevier, Amsterdam, Netherlands, pp. 519-553.

Haase, S., Sciocco-Cap, A., Romanowski, V., 2015. Baculovirus insecticides in Latin America: historical overview, current status and future perspectives. Viruses 7, 2230–2267.

Hollander, M., Sethuraman, J., 2015. Nonparametric statistics: rank-based methods. In: Wright, J.D. (Ed.), International Encyclopedia of the Social and Behavioral Sciences, second ed. Elsevier, Oxford, UK, pp. 891–897.

Hughes, P.V., Van Beek, N.A.M., Wood, H.A., 1986. A modified droplet feeding method for rapid assay of *Bacillus thuringiensis* and baculoviruses in noctuid larvae. J. Invertebr. Pathol. 48 (2), 187-192.

Hunter-Fuijita, F.R., Entwistle, P.F., Evans, H.F., Crook, N.E., 1998. Insect Viruses and Pest Management. Wiley, Chichester, UK, pp. 620.

Ignoffo, C.M., Shapiro, M., 1978. Characteristics of baculovirus preparations processed from living and dead larvae. J. Econ. Entomol. 71 (2), 186-188.

Jamovi, 2022. Jamovi version 2.3.0. Available from: https://www.jamovi.org.

Kumar, C.M.S., Sathiah, N., Rabindra, R.J., 2005. Optimizing the time of harvest of nucleopolyhedrovirus infected *Spodoptera litura* (Fabricius) larvae under in vivo production systems. Curr. Sci. 88, 1682-1684.

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: back to the future. J. Invertebr. Pathol. 132, 1-41.

Li, S. N., Wang, J. Y., Yuan, M. J., Yang, K., 2014. Disruption of the baculovirus core gene *ac78* results in decreased production of multiple nucleocapsid-enveloped occlusion-derived virions and the failure of primary infection in vivo. Virus Res. 191, 70-82.

Lua, L.H., Nielsen, L.K., Reid, S., 2003. Sensitivity of Helicoverpa armigera nucleopolyhedrovirus polyhedra to sodium dodecyl sulfate. Biol. Control 26, 57-67.

Mazzone, H.M., McCarthy W.J., 1981. Gypsy moth nuclear polyhedrosis virus: biochemistry and biophysics. In: Doane, C.C., McManus, M.L. (Eds.), The Gypsy

Moth: Research Toward Integrated Pest Management, Technical Bulletin 1584, US Department of Agriculture, Washington, DC, pp. 487–495.

Numerical Algorithms Group, 1993. In: Francis, B., Green, M., Payne, C. (Eds.), The GLIM System: Release 4 Manual. Clarendon Press, Oxford, UK.

Peng, K., van Lent, J.W., Vlak, J.M., Hu, Z., van Oers, M.M., 2011. In situ cleavage of baculovirus occlusion-derived virus receptor binding protein P74 in the peroral infectivity complex. J. Virol. 85 (20), 10710-10718.

Ramírez-Arias, F.G., Lasa, R., Murillo, R., Navarro-de-la-Fuente, L., Mercado, G., Williams, T., 2019. Post-mortem incubation influences occlusion body production in nucleopolyhedrovirus-infected larvae of *Spodoptera frugiperda*. Biol. Control 135, 33-40.

Reid, S., Chan, L.C.L., Matindoost, L., Pushparajan, C., Visnovsky, G., 2016. Cell culture for production of insecticidal viruses. In: Glare, T., Moran-Diez, M. (Eds.), Microbial-Based Biopesticides. Methods in Molecular Biology, vol. 1477. Humana Press, New York, pp. 95-117. https://doi.org/10.1007/978-1-4939-6367-6_9

Rohrmann, G.F., 2014. Baculovirus nucleocapsid aggregation (MNPV vs SNPV): an evolutionary strategy, or a product of replication conditions? Virus Genes 49, 351–357.

Rohrmann, G.F., 2019. Baculovirus Molecular Biology [Internet]. 4th edition. National Center for Biotechnology Information (US), Bethesda, MD. Available from: https://www.ncbi.nlm.nih.gov/books/NBK543458/

Sajjan, D.B., Hinchigeri, S.B., 2016. Structural organization of baculovirus occlusion bodies and protective role of multilayered polyhedron envelope protein. Food Environ. Virol. 8, 86-100.

Shapiro, M., 1986. In vivo production of baculoviruses. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses, vol. II. CRC Press, Boca Raton, FL, pp. 31–62.

Shapiro, M., Bell, R.A., 1981. Biological activity of Lymantria dispar nucleopolyhedrosis virus from living and virus-killed larvae. Ann. Entomol. Soc. Am. 74, 27-28.

Simón, O., Williams, T., López-Ferber, M., Caballero, P., 2004. Genetic structure of a Spodoptera frugiperda nucleopolyhedrovirus population: high prevalence of deletion genotypes. Appl. Environ. Microbiol. 70, 5579-5588.

Simón, O., Williams, T., López-Ferber, M., Caballero, P., 2005. Functional importance of deletion mutant genotypes in an insect nucleopolyhedrovirus population. Appl. Environ. Microbiol. 71, 4254-4262.

Simón, O., Williams, T., Cabrera-Asensio, A., Ros, S., Gaya, A., Caballero, P., Possee, R.D., 2008a. Sf29 gene of Spodoptera frugiperda multiple nucleopolyhedrovirus is a viral factor that determines the number of virions in occlusion bodies. J. Virol. 82, 7897-7904.

Simón, O., Williams, T., López-Ferber, M., Taulemesse, J.M., Caballero, P., 2008b. Population genetic structure determines the speed of kill and occlusion body

prodction in Spodoptera frugiperda multiple nucleopolyhedrovirus. Biol. Control 44, 321-330.

Simón, O., Palma, L., Beperet, I., Muñoz, D., López-Ferber, M., Caballero, P., Williams, T., 2011. Sequence comparison between three geographically distinct Spodoptera frugiperda multiple nucleopolyhedrovirus isolates: detecting positively selected genes. J. Invertebr. Pathol. 107, 33-42.

Smits, P.H., 1987. Nuclear polyhedrosis virus as biological control agent of *Spodoptera exigua*. PhD thesis, Wageningen Agricultural University, Netherlands. 127 pp.

Smits, P.H., Vlak, J.M., 1988. Quantitative and qualitative aspects in the production of a nuclear polyhedrosis virus in *Spodoptera exigua* larvae. Ann. Appl. Biol. 112, 249–257.

Takatsuka, J., Okuno, S., Ishii, T., Nakai, M., Kunimi, Y., 2007. Productivity and quality of polyhedral occlusion bodies of a nucleopolyhedrovirus harvested from *Spodoptera litura* (Lepidoptera: Noctuidae) larvae. Appl. Entomol. Zool. 42 (1), 21-26.

Tay, W.T., Rane, R.V., Padovan, A., Walsh, T.K., Elfekih, S., Downes, S., Nam, K., d'Alençon, E., Zhang, J., Wu, Y. Nègre, N., Kriticos, D.J., Czepak, C., Otim, M.H., Gordon, K.H.J., 2022. Global population genomic signature of *Spodoptera frugiperda* (fall armyworm) supports complex introduction events across the Old World. Comm. Biol. 5, 297.

Taylor, S.C., Nadeau, K., Abbasi, M., Lachance, C., Nguyen, M., Fenrich, J., 2019. The ultimate qPCR experiment: producing publication quality, reproducible data the first time. Trends Biotechnol. 37 (7), 761-774.

Vialard, J.E., Yuen, L., Richardson, C.D., 1990. Identification and characterization of a baculovirus occlusion body glycoprotein which resembles spheroidin, an entomopoxvirus protein. J. Virol. 64, 5804–5811.

Xu, Y.P., Gu, L.Z., Lou, Y.H., Cheng, R.L., Xu, H.J., Wang, W.B., Zhang, C.X., 2012. A baculovirus isolated from wild silkworm encompasses the host ranges of Bombyx mori nucleopolyhedrosis virus and Autographa californica multiple nucleopolyhedrovirus in cultured cells. J. Gen. Virol. 93 (11), 2480-2489.

Yang, M., Wang, S., Yue, X.L., Li, L.L., 2014. Autographa californica multiple nucleopolyhedrovirus orf132 encodes a nucleocapsid-associated protein required for budded-virus and multiply enveloped occlusion-derived virus production. J. Virol. 88, 12586-12598.

Yu, I.L., Bray, D., Lin, Y.C., Lung, O., 2009. Autographa californica multiple nucleopolyhedrovirus ORF 23 null mutant produces occlusion-derived virions with fewer nucleocapsids. J. Gen. Virol. 90, 1499-1504.

Figure legends.

Fig. 1. Experimental design and sampling regime. Larvae of *Spodoptera frugiperda* were inoculated and incubated prior to sampling at 3-4 days, 5 days or 6-7 days post-inoculation. Larvae were classified as living or virus-killed at each sample time point. Occlusion bodies (OBs) were harvested and subjected to different analyses as indicated. A single living larva at 7 days post-inoculation was not included in the study.

Fig. 2. Larval mortality and larval sampling.(A) The cumulative mortality of larvae during the course of the experiment due to lethal polyhedrosis disease. Vertical bars indicate SE. (B) Numbers of living and virus-killed larvae sampled at each time point. Numbers within each column indicate total number of larvae collected of each type.

Fig. 3. Larval weight and production of occlusion bodies (OBs). (A) Median weight of living and virus-killed larvae at each sample time point. (B) Median logarithm of OB production/mg of larval weight. (C) Median logarithm of the number of OBs produced per larva. Vertical bars indicate the asymetrical interquartile range. Columns headed by identical letters did not differ significantly (Kruskal-Wallis/DSCF pairwise comparisons; $P > 0.05$).

Fig. 4. Size of OBs harvested from living and virus-killed insects at each sample time point. (A) Median cross-sectional area of OBs calculated from scanning electron photomicrographs. (B) Median percentage of immature OBs present in samples. Vertical bars indicate the asymetrical interquartile range. Columns headed by identical letters did not differ significantly (Kruskal-Wallis/DSCF pairwise comparisons; $P > 0.05$).

Fig. 5. Logarithm of SfMNPV genome copy number calculated by qPCR amplification of a *polh* gene fragment on DNA extracted from samples of 2×10^8 OBs from living insects (pale boxes) and virus-killed larvae (dark boxes) at each sample time point. Horizontal lines indicate medians, boxes indicate interquartile range, vertical bars indicate range, dots indicate individual data points from replicates within each range. P value indicates the result of a Kruskal-Wallis test.

Fig. 6. Mean mortality of *Spodoptera frugiperda* larvae inoculated with a discriminating concentration of 2×10^4 OBs/ml from living or virus-killed insects at each sample time point. The inoculum was expected to result in 50% larval mortality. Vertical bars indicate SE. Columns headed by identical letters did not differ significantly (GLM, $P > 0.05$).

Fig. 1

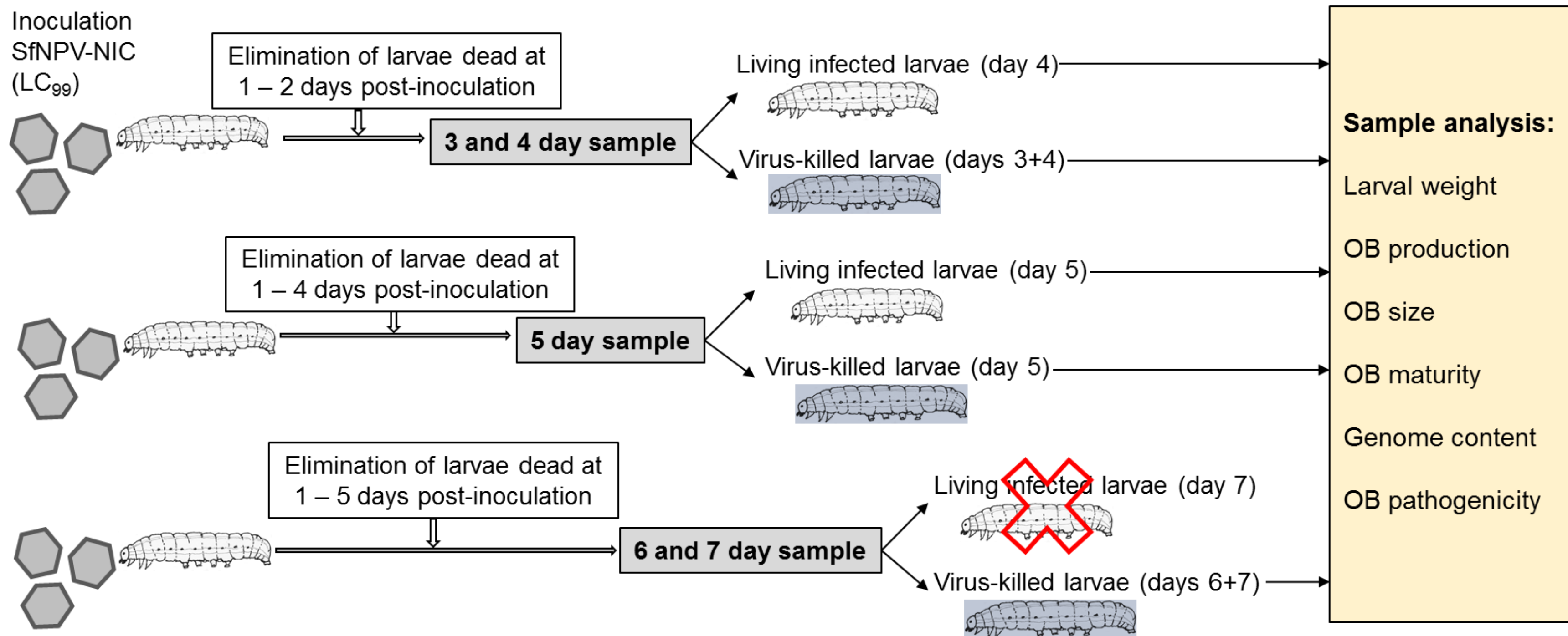


Fig. 2

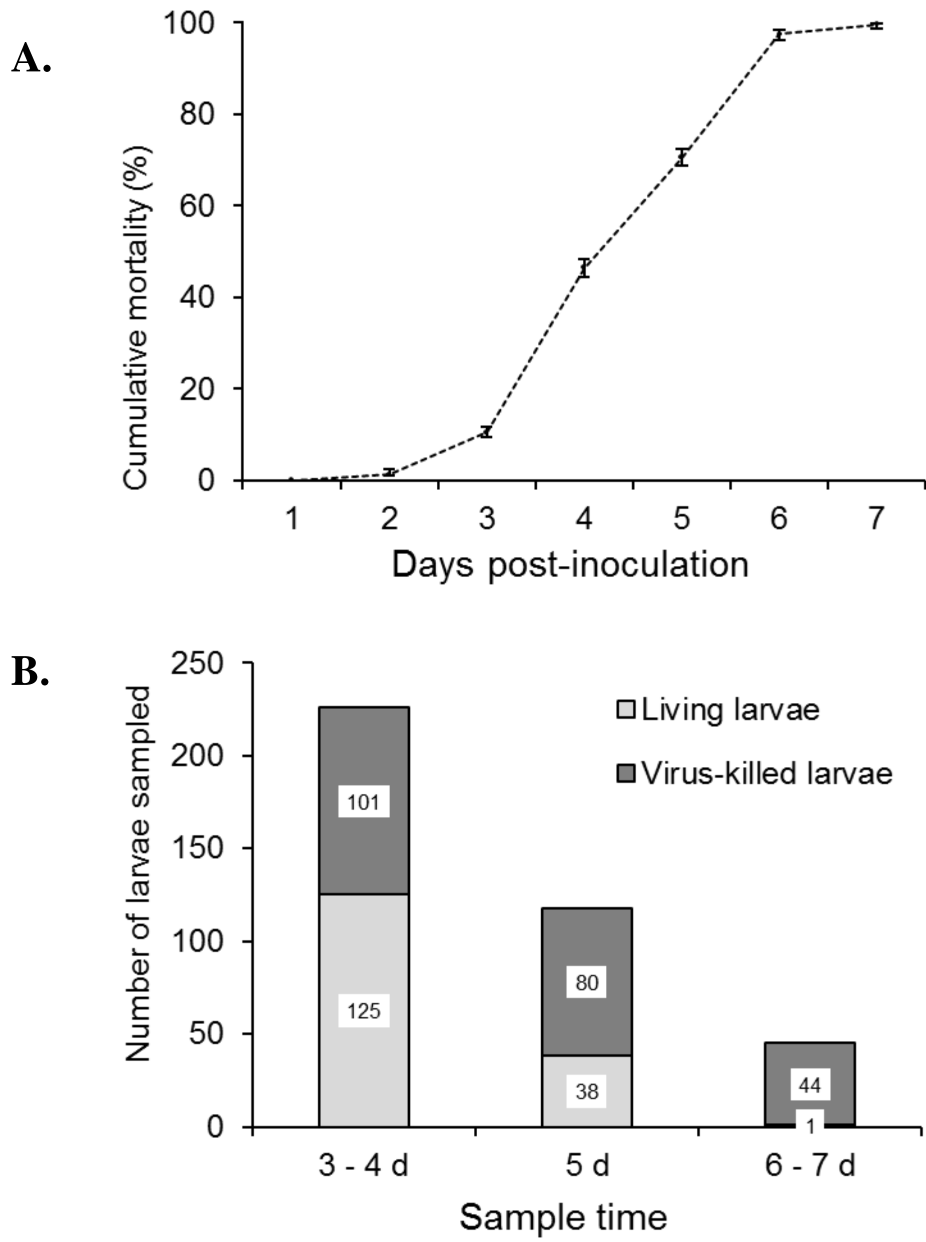
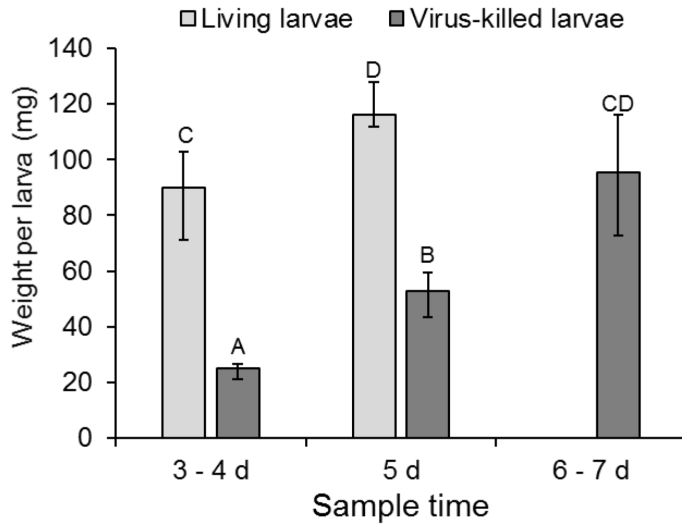


Fig. 3

A.



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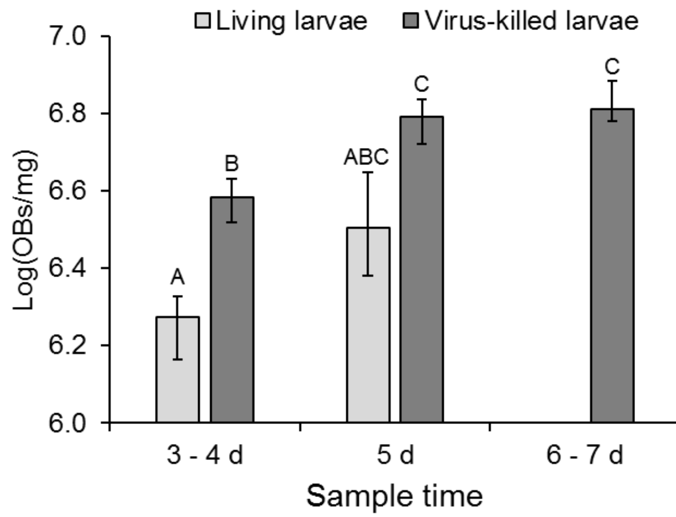

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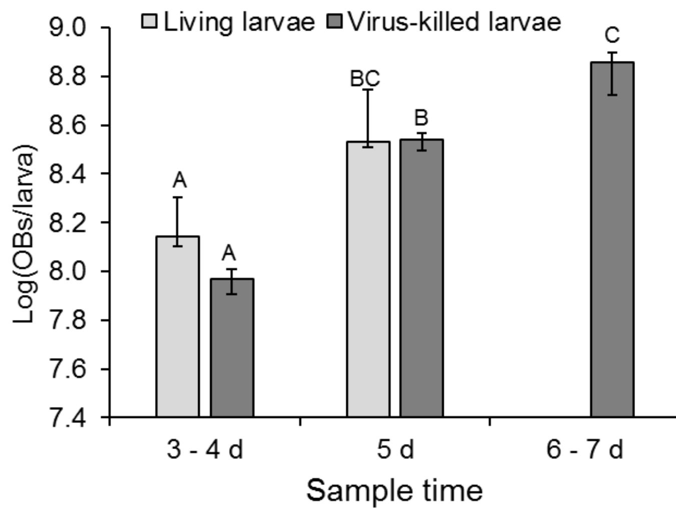

Fig. 4

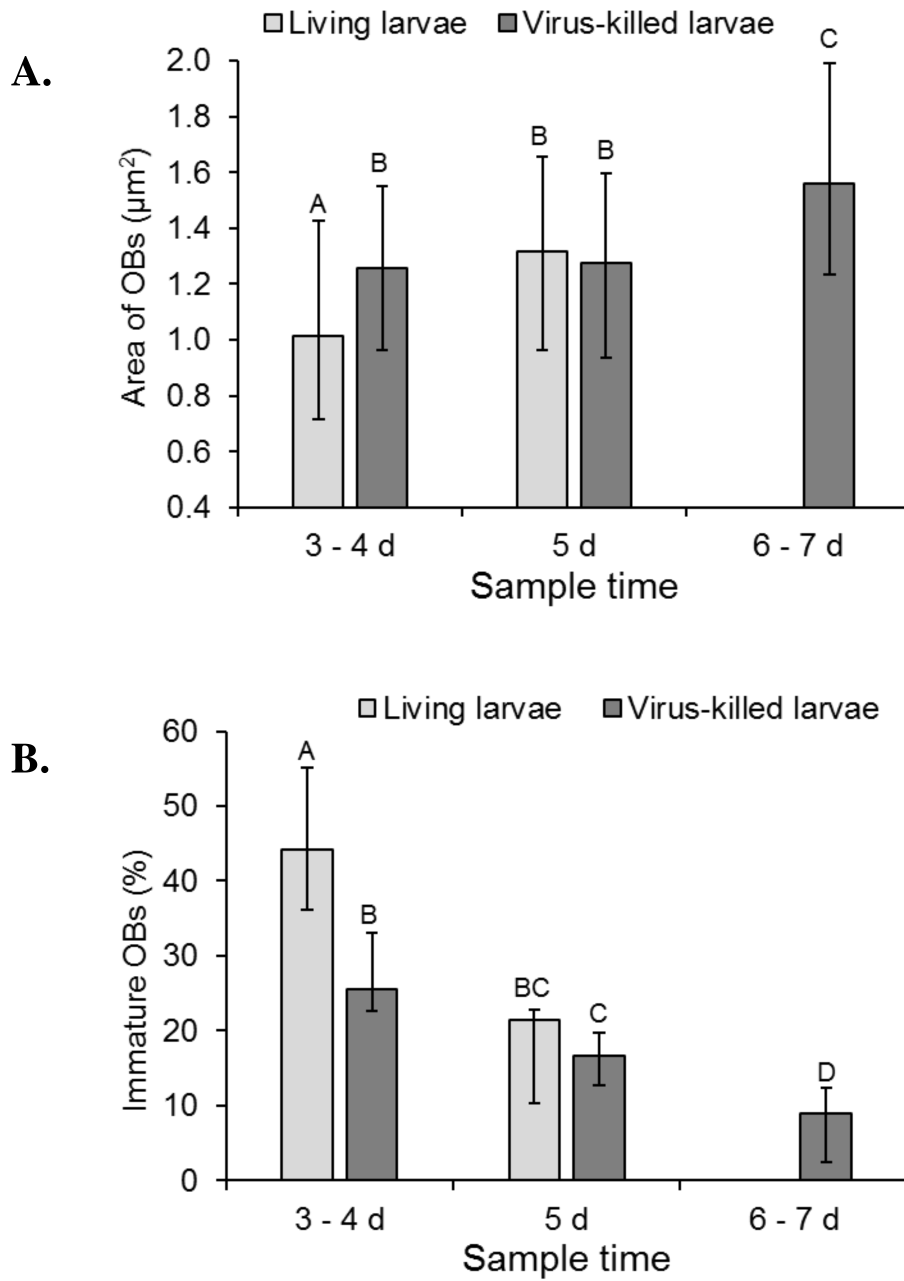

Fig. 5.

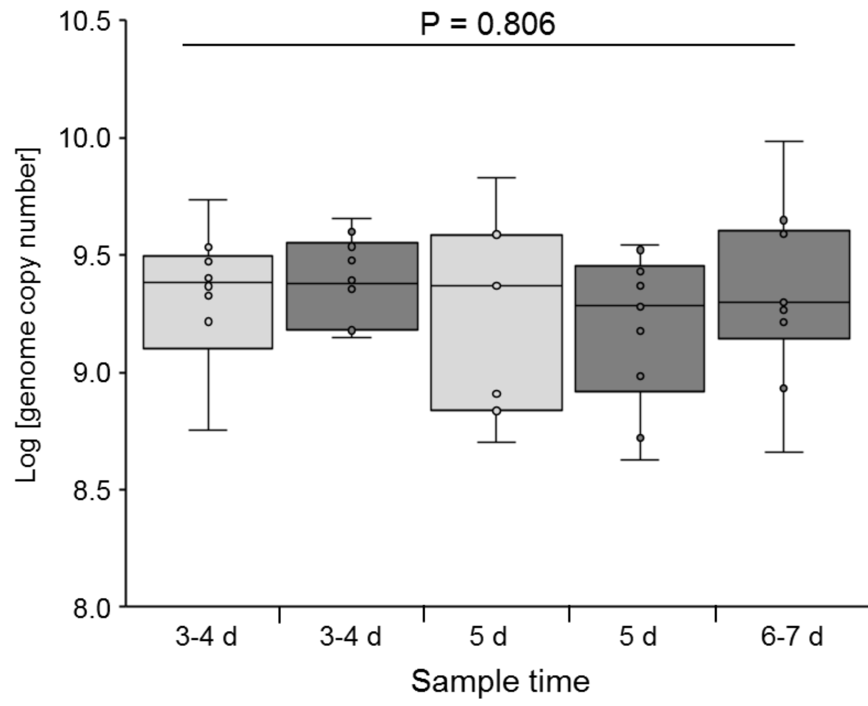

Fig. 6.