



Post-mortem incubation influences occlusion body production in nucleopolyhedrovirus-infected larvae of *Spodoptera frugiperda*

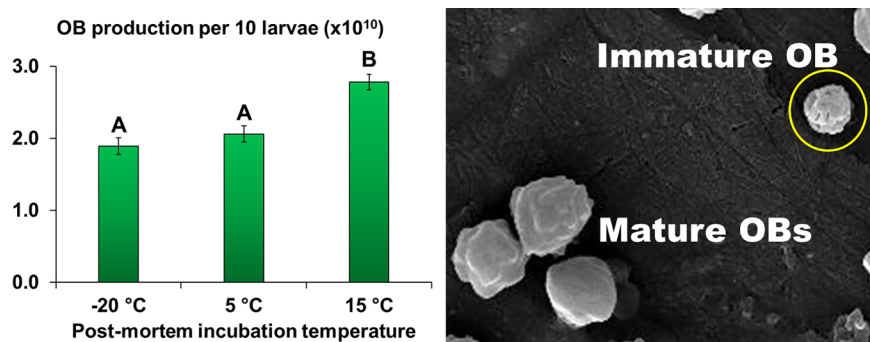
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GRAPHICAL ABSTRACT



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ABSTRACT

The inefficient production of nucleopolyhedrovirus occlusion bodies (OBs) can limit the commercialization of virus-based insecticides. The production of OBs in SfMNPV-infected *Spodoptera frugiperda* fourth instars was compared among groups of larvae that were frozen (−20 °C) immediately following death, or subjected to a 9-day period of post-mortem incubation at 5 °C or 15 °C. Incubation at 15 °C resulted in a ~40% increase in total OB production and OBs per mg larval weight, compared to the −20 °C and 5 °C treatments. OBs from the 5 °C treatment were ~10% smaller in cross sectional area and small OBs (< 1 μm²) were more abundant than in other treatments, possibly due to reduced post-mortem OB maturation in this treatment. SfMNPV genomic DNA in OB samples was 3.6-fold higher in the −20 °C treatment than the 5 °C treatment and 1.7-fold higher than the 15 °C treatment, possibly due to differences in the exposure of viral genomes to degradative enzymes. However, these differences did not affect the concentration-mortality relationship or speed of kill of OBs from the different treatments. The abundance of aerobic microbes increased from ~3 × 10⁷ CFU/ml in the −20 °C treatment, to approximately 2 × 10⁸ and 3 × 10⁸ CFU/ml in the 5 °C and 15 °C incubation treatments, respectively, similar to levels seen in other nucleopolyhedroviruses produced in insects. We conclude that post-mortem incubation at 15 °C likely involves continuing processes of virion occlusion and OB maturation that increase overall OB production without loss of insecticidal activity, although the value of this step in commercial virus insecticide production will depend on the cost of the incubation step and the value of the additional OBs produced.

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1. Introduction

Baculoviruses can form the basis of highly effective biological insecticides for control of insect pests of field and greenhouse crops and forests (Lacey, 2017). One such virus, the *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV), is being tested for control of the fall armyworm, *S. frugiperda* JE Smith (Lepidoptera: Noctuidae), a pest of maize, rice and sorghum in the US and Latin America (Williams et al., 1999; Barrera et al., 2011; Behle and Popham 2012; Haase et al., 2015). The use of the virus is also likely to attract attention as a potential biocontrol agent following the recent invasion of this insect across sub-Saharan Africa, India, China and elsewhere in Asia (FAO, 2019).

The production of large quantities of virus occlusion bodies (OBs) at low cost represents a challenge to the manufacturers of baculovirus-based insecticides (Shapiro, 1986; Grzywacz and Moore, 2017). This is because these viruses are currently produced by large-scale inoculation of lepidopteran larvae, although efforts continue to develop effective *in vitro* production systems (Reid et al., 2014).

Studies on the optimization of baculovirus *in vivo* production systems have focused on the selection of virus strains (Erlandson et al., 2007; Bernal et al., 2013; Opoku-Debrah et al., 2016) and suitable host species (Tompkins et al., 1981; Kelly and Entwistle 1988), the development of low-cost diets (Shapiro et al., 1981a; Elvira et al., 2010), inoculum delivery, dosage and larval rearing densities and conditions (Shapiro et al., 1981b; Cherry et al., 1997; Subramanian et al., 2006; Behle, 2017), hormonal enhancement of larval growth (Lasa et al., 2007; Liao et al., 2016), and harvesting and purification procedures that allow the collection of the greatest quantities of OBs before enzymatic liquefaction of virus-killed insects (Shapiro 1986; Grzywacz et al., 1998; Kumar et al., 2005). In general, virus production studies aim to achieve the highest yield of OBs in a short period of time, fidelity of amplification of the inoculum, high insecticidal activity of OBs and low levels of microbial contaminants that usually originate from the host insect microbiota (Tang et al., 2012). Where possible, these factors should be optimized at the same time as labor and the costs of materials and overheads are minimized (Shieh, 1989; Hunter-Fujita et al., 1998; Grzywacz et al., 2014).

One process that has been little explored is that of low temperature incubation. This involves the collection of moribund diseased larvae that are separated from their diet before death and incubated alone, or in groups, during the moribund stage and post-mortem period, to avoid the loss of OBs that often occurs following the liquefaction of larval tissues. Increased OB yields have been reported for the nucleopolyhedrovirus of *Spodoptera littoralis* (SpliNPV) following peri- and post-mortem incubation at temperatures between 4 and 24 °C (McKinley et al., 1989; Grzywacz et al., 1998). The incubation period involves low temperatures to reduce the proliferation of contaminant microbiota (Podgwaite et al., 1983; Grzywacz et al., 1997; Subramanian et al., 2006). However, the incubation period can range from 1 to 14 days, depending on the production system, which significantly extends the production cycle and increases the costs of production (Shapiro, 1986; Hunter-Fujita et al., 1998).

In the present study we asked whether there was any benefit to extended post-mortem incubation at low temperatures for the yield or the insecticidal characteristics of the resulting OBs. To examine these possibilities, we compared production of SfMNPV OBs in *S. frugiperda* larvae that were frozen immediately following death or were subjected to post-mortem incubation at low temperatures (4° or 15 °C). We also examined the influence of low temperature incubation on the insecticidal activity of OBs, OB size, the quantity of viral genomic DNA and the abundance of aerobic microbial contaminants.

2. Materials and methods

2.1. Insects and virus

A laboratory colony of *S. frugiperda* was reared on a semisynthetic diet based on soybean flour, wheatgerm, yeast, agar, and vitamins (Hunter-Fujita et al., 1998). The colony was started in 2016 using insects collected from maize fields within a 30 km radius of Xalapa, Veracruz, Mexico (19° 31' 50" N; 96° 54' 33" W).

A Nicaraguan wild-type isolate of SfMNPV (Simón et al., 2004) was amplified by allowing overnight-starved *S. frugiperda* fourth instars to drink 1×10^8 OBs in 10% sucrose and 0.1% food coloring solution (McCormick, Mexico City) over a 10 min period. Inoculated larvae were reared individually until death or pupation and then triturated in sterile water. The resulting OB suspension was counted in triplicate using a Neubauer chamber under a phase contrast microscope and stored at 4 °C for two weeks prior to use in experiments.

Unless stated otherwise, all insect rearing and virus production procedures described in this study were performed under controlled laboratory conditions at 25 ± 1 °C, $70 \pm 10\%$ relative humidity and 14:10 h L:D photoperiod.

2.2. Post-mortem incubation of virus-killed larvae

A group of 45 larvae, each between 30 and 45 mg in weight, were selected at 12–24 h after molting to the fourth instar. Each larva was individually offered a cube of diet ($4 \times 4 \times 4$ mm) that had been treated with 8×10^6 OBs, estimated to kill ~95% of inoculated insects. Larvae that consumed the diet in a 24 h period were individually transferred to clean 30 ml plastic cups with a piece of untreated diet and were maintained in darkness at 25 ± 0.5 °C in a laboratory incubator. Larvae were checked at 12 h intervals and as soon as they failed to respond to the touch of a toothpick larvae were considered to have died and were placed individually in a 1.5 ml centrifuge tube, weighed and randomly assigned to one of the following treatments: (i) immediate freezing at -20 °C, (ii) post-mortem incubation at 5 ± 1 °C in a dark bioclimatic chamber, (iii) post-mortem incubation at 15 ± 1 °C in a dark bioclimatic chamber. The post-mortem incubation period was 10 days, after which infected cadavers from the 5 and 15 °C treatments were frozen at -20 °C prior to processing. This procedure was performed on ten occasions with different batches of larvae to generate ten replicates of each treatment.

2.3. Quantification of OBs following post-mortem incubation

To avoid unusually large or small larvae unduly influencing the results, the distribution of all larval weights at death was examined (429 insects in total) and 40 individuals of less than 90 mg and seven individuals over 400 mg were identified as deviating from the normal distribution (Supplemental material, Fig. S1). These individuals were excluded from the experiment leaving 11–14 larvae (90.4–396.9 mg in weight) in each replicate of each treatment. A group of 10 of these larvae was then selected at random for OB extraction and the production of the OB stock suspension for each replicate.

To produce OB suspensions each larva was thawed in its centrifuge tube and transferred to a glass homogenizer. The plastic centrifuge tube was washed with 300 µl of 0.05% (v/v) Tween 80 to remove any insect remains and these washings were transferred to the homogenizer. Larvae were homogenized and the resulting suspension was filtered through a stainless steel mesh with an 80 µm pore size. This procedure was repeated for each of the ten larvae in each replicate. At the end of this process the homogenizer was washed with 4 ml of 0.05% Tween 80 that was also passed through the mesh filter. The resulting suspension was placed in a 15 ml sterile plastic centrifuge tube and adjusted to a total volume of 14 ml. The weight of the insect remains retained by the steel mesh was determined by drying in an oven at 50 °C for 24 h and

then weighed using an analytical balance (Explorer EX124, Ohaus, USA).

Each 14 ml volume of crude OB suspension was mixed on a vortex mixer, aliquoted into volumes of 1 ml in individual 1.5 ml sterile centrifuge tubes, half of which was frozen at -20°C for analyses of microbial contamination or DNA content, and the other half was stored at 5°C for OB counting and size measurements and for determination of lethal concentration metrics. For the analysis of OB size and insecticidal activity, pooled OB samples were prepared by mixing 500 μl of OB suspension from each of the 10 replicates of each treatment to produce 5 ml of pooled OB suspension for each treatment. All samples were thoroughly mixed using a vortex mixer at all stages. To quantify OBs, each OB sample was diluted in Milli-Q water and counted in triplicate in a Neubauer bacterial counting chamber.

2.4. OB size measurement

For OB size measurements the pooled OB suspension from each treatment was adjusted to a concentration of 5×10^7 OBs/ml, mixed on a vortex-mixer and 10 μl droplets were pipetted on to an aluminum stub and allowed to dry under a warm 40 W lamp for 15 min.

Each sample was then coated with gold-palladium in a Quorum Q150R sputter-coater and observed at $\times 10,000$ magnification in a FEI Quanta 250 FEG scanning electron microscope at an accelerating voltage of 10 kV. Images of OBs (~ 40 OBs/image) were taken at 18–25 different points within each sample at a fixed resolution of 1536×1103 pixels, 37 ppi. The cross-sectional area of each OB was then estimated with reference to the 20 μm scale bar using the ImageJ program (<https://imagej.net>).

2.5. Quantification of viral genomes

Samples of 2×10^8 OBs in 225 μl sterile water were obtained for all treatments and replicates. Virions were released from the OBs by adding 50 μl of 0.5 M Na_2CO_3 and 25 μl of 10% SDS for 5 min at 55°C . Debris was pelleted at 8000 rpm for 5 min and was discarded, whereas virion-containing supernatant was incubated with 12.5 μl proteinase K (20 mg/ml) at 55°C for one hour. Viral genomic DNA was then extracted with phenol and twice with a phenol-chloroform-isopropylalcohol and precipitated by addition of 3 M sodium acetate (pH 5.2) and ice-cold ethanol. This precipitate was centrifuged at 15,000 rpm for 15 min, washed with 70% ethanol and dissolved in 200 μl of Milli-Q water. The concentration of DNA in each sample was then determined by calculating the average reading from triplicate samples in a spectrophotometer (BioSpec-Nano, Shimadzu, Japan).

Quantitative PCR (qPCR) based on SYBR fluorescence was performed in a Mx3005P qPCR System real-time (Stratagene) in 96-well reaction plates. For viral load detection, specific primers were designed based on the polyhedrin gene sequence for SfMNPV-NIC genotype B (Simón et al., 2011), using Primer3 software (Untergasser et al., 2007). The forward primer Sfpolh1 (5'-GAACCTTCACTCTGAGTACACGCAC) and reverse primer Sfpolh2 (5'-AGACGATGGGTTTGTAGAAGTTCTCC) amplified an 82 bp region of the *polh* gene. Amplifications were performed in a final 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 DNA (0.301–3.786 ng). Three reactions with a negative control were included in each analysis and the standard curve (range 5×10^{-6} ng to 5 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).

2.6. Quantification of microbial contaminants

To quantify microbial contaminants in the OB suspension, samples from the five even-numbered replicates, representing half of the total number of replicates, were serially diluted in sterile Milli-Q water (10^{-3} – 10^{-7}) and 100 μl volumes of each dilution were spread over LB agar plates (LB agar, Bioxon) using sterile glass beads. Inoculated plates were incubated in darkness at $37 \pm 1^{\circ}\text{C}$ in a laboratory incubator for 24 h. The number of colony forming units (CFU) was counted and marked using a permanent marker in those dilutions that resulted in 30–300 CFU/plate. Plates where then incubated for a further 24 h period at $28 \pm 1^{\circ}\text{C}$ for the development of organisms that prefer cooler conditions. The number of additional CFU that appeared 24 h later was recorded.

2.7. Insecticidal activity of OBs

The concentration-mortality response of *S. frugiperda* larvae to OBs was determined using pooled OB samples from the ten replicates of each treatment, as described in the section on OB size measurements. For this, groups of 24 larvae were starved overnight as they molted to the second instar and were then allowed to drink from an aqueous suspension containing 10% sucrose, 0.1% food coloring and one of the following concentrations of OBs: 4.8×10^2 , 2.4×10^3 , 1.2×10^4 , 6.0×10^4 or 3.0×10^5 OBs/ml. This range of concentrations resulted in 10–90% mortality in previous studies with this virus (Simón et al., 2013). Insects that drank the suspension in 10 min were individually transferred to 30 ml plastic cups with diet, maintained in darkness at $25 \pm 0.5^{\circ}\text{C}$ and checked daily until death or pupation. The bioassay was performed on four occasions using different batches of insects.

To determine speed-of-kill responses, groups of 24 overnight-starved second instars were allowed to drink a suspension of 1×10^5 OBs/ml, estimated to kill $\sim 80\%$ of inoculated insects. Larvae that drank the suspension in 10 mins were individually incubated on diet in darkness at $25 \pm 0.5^{\circ}\text{C}$ and monitored at 8 h intervals until death or pupation. The experiment was performed on six occasions using different batches of insects.

2.8. Statistical analyses

Counts of OBs/group of ten larvae and OBs/mg of larval weight and CFU counts at 24 h and 48 h were log-transformed, whereas OB cross-sectional area values were normalized by square root transformation (\sqrt{x}) prior to one-way analysis of variance (ANOVA). The quantities of viral DNA in each qPCR reaction were subjected to one-way ANOVA without prior transformation. Treatment means were compared by Tukey test. The weights of tissue debris remaining following OB extraction and total DNA extracted from crude OB suspensions lacked homoscedasticity and were subjected to Kruskal-Wallis analysis followed by Dwass-Steel-Critchlow-Fligner (DSCF) pairwise comparisons (Hollander and Sethuraman, 2015). All ANOVAs and non-parametric tests were performed using the R-based program Jamovi v.0.9.1.12 (Jamovi, 2018). The frequencies of OBs of different size classes were subjected to contingency table analysis. Concentration-mortality results were subjected to logit regression with the Generalized Linear Interactive Modeling (GLIM 4) program with a binomial error distribution specified (Numerical Algorithms Group, 1993). Minor overdispersion in the mortality results was taken into account by scaling the error distribution where necessary (Crawley, 1993). Mean time to death values were estimated by Weibull survival analysis in GLIM 4.

3. Results

3.1. Quantification of OBs following post-mortem incubation

Inoculation of larvae with 8×10^6 OBs resulted in $> 90\%$ larval

mortality in all replicates. No mortality was observed in water-inoculated controls. The weight of groups of 10 randomly-selected virus-killed larvae that comprised each replicate varied between 2.1 and 2.4 g and did not differ significantly among groups assigned to each incubation treatment ($F = 0.987$, $df = 2, 27$; $P = 0.386$).

The production of OBs in each group of larvae differed significantly among the three post-mortem incubation regimes ($F = 15.5$; $df = 2, 27$; $P < 0.001$). OB production increased by 47% in the 15 °C treatment compared to the −20 °C treatment but was not significantly increased in the 5 °C treatment (Fig. 1A).

Similarly, the production of OBs per mg weight of larval tissues also differed significantly with post-mortem incubation regime ($F = 17.8$; $df = 2, 27$; $P < 0.001$), with the number of OBs/mg larval tissue over one-third higher in the 15 °C treatment compared to the other incubation treatments (Fig. 1B).

The tissue debris residue obtained by filtration after OB extraction also differed significantly among treatments ($F = 5.58$; $df = 2, 27$; $P = 0.009$). Median debris weight in the 15 °C treatment was approximately half that measured in the −20 °C treatment, with an intermediate value in the 5 °C treatment (Fig. 1C).

3.2. OB size measurement

The total number of OBs measured was 1029, 963 and 967 for the −20°, 5° and 15 °C treatments, respectively. Estimated cross-sectional area varied between 0.278 and 7.32 μm^2 . OBs extracted from larvae that experienced post-mortem incubation at 5 °C had a significantly lower cross-sectional area than OBs extracted from the other treatments ($F = 17.4$; $df = 2, 2964$; $P < 0.001$), but the difference was small, comprising a reduction of approximately 0.18 μm^2 or ~10% of the cross-sectional area (Fig. 2A). To investigate this effect, we examined the distribution of OB areas in each treatment (Fig. 2B). The prevalence of small OBs was significantly higher in the 5 °C treatment than the other treatments ($\chi^2 = 7.598$; $df = 3$; $P = 0.022$). Specifically, the number of OBs with an area of less than 1 μm^2 comprised 16.3% of the total OBs measured in the 5 °C treatment compared to 12.5 and 12.6% of the OBs measured in the −20 °C and 15 °C treatments, respectively. An alternative explanation, in which differences in mean OB size were due to a paucity of particularly large OBs in the 5 °C treatment, was not supported by the data, as OBs of over 4.0 μm^2 comprised less than 1.5% of measured OBs across all treatments and did not differ significantly in prevalence among treatments, with 9–15 OBs per treatment in total ($\chi^2 = 1.205$; $df = 3$; $P = 0.547$).

3.3. Quantification of viral genomes

The quantity of total DNA extracted from a crude suspension of 2×10^8 OBs varied between 30.1 and 378.6 ng/ μl . The quantity of total DNA, comprising host, virus and microbiota DNA, differed significantly among the three post-mortem incubation regimes, with decreasing total DNA extracted as post-mortem incubation temperature increased (Kruskal-Wallis $H = 23.7$; $df = 2$; $P < 0.001$) (Fig. 3A). The quantity of SfMNPV genomic DNA/ μl also differed significantly among the incubation regimes with amount of viral DNA in the −20 °C treatment approximately 3.6-fold higher than the 5 °C treatment and 1.7-fold higher than the 15 °C treatment ($F = 41.0$; $df = 2, 27$; $P < 0.001$) (Fig. 3B).

3.4. Quantification of microbial contaminants

The abundance of microorganisms differed significantly among the post-mortem treatments for microbes counted at 37 °C after 24 h ($F = 20.1$; $df = 2, 10$; $P < 0.001$), or when re-incubated at 28 °C for an additional 24 h period ($F = 22.1$, $df = 2, 10$, $P < 0.001$) (Fig. 4). Initial aerobic CFU counts on LB agar at 24 h were only slightly increased when re-incubated 48 h in all cases (Fig. 4). Mean CFU counts (\pm SE) in

pooled OB suspensions from larvae frozen at −20 °C varied between 3.3×10^7 and 3.7×10^7 CFU/ml following incubation at 37 and 28 °C, respectively, which were significantly lower than for OB suspensions from the 5 °C or 15 °C regimes, in which microbial contaminants were approximately six-fold and nine-fold more abundant, respectively (Fig. 4).

3.5. Insecticidal activity of OBs

The concentration-mortality responses of insects that consumed OBs from the different incubation treatments did not differ significantly ($F = 0.70$; $df = 2, 53$; $P = 0.50$). Similarly, the LC_{50} values and the corresponding 95% CI values overlapped broadly among the incubation

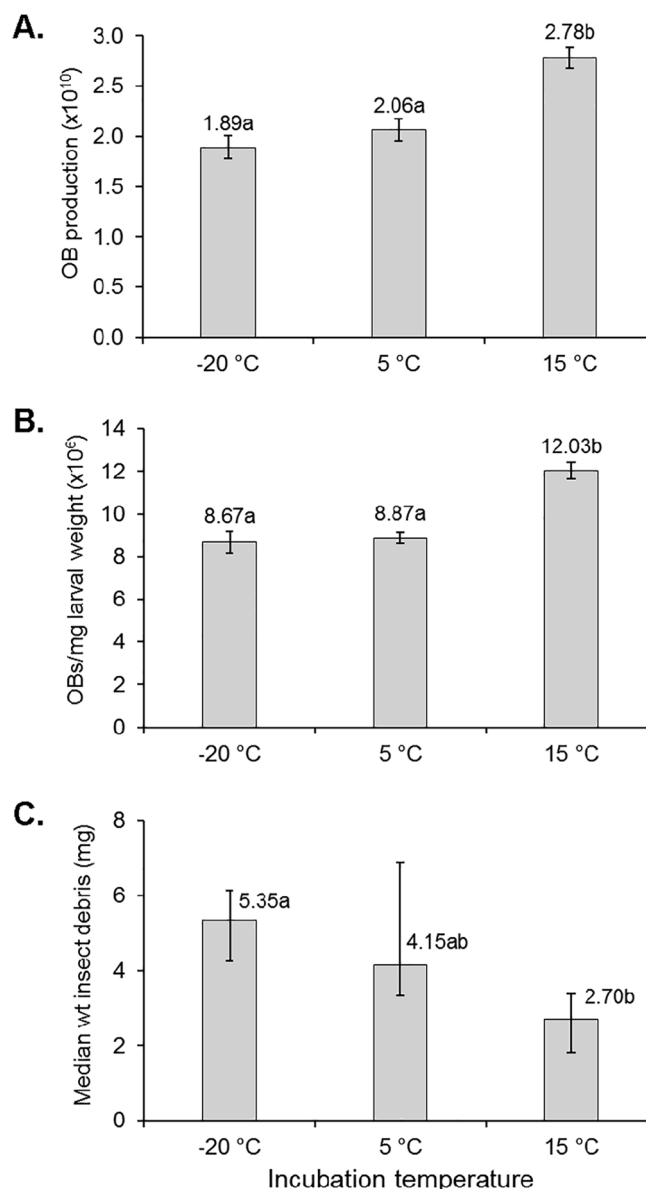


Fig. 1. Occlusion body (OB) production in larvae that were immediately frozen (−20 °C) following death or subjected to post-mortem incubation at 5 or 15 °C. (A) Mean OB production from each group (replicate) of 10 virus-killed larvae, (B) mean OB production per mg larval weight, (C) median weight of larval remains following filtration of larval homogenate in each group of 10 larvae. Vertical bars indicate SE (A and B) or interquartile range (C). Values above columns indicate means or medians. Values followed by identical letters did not differ significantly (Tukey, $P > 0.05$ for A and B, or DSCF pairwise comparisons for C).

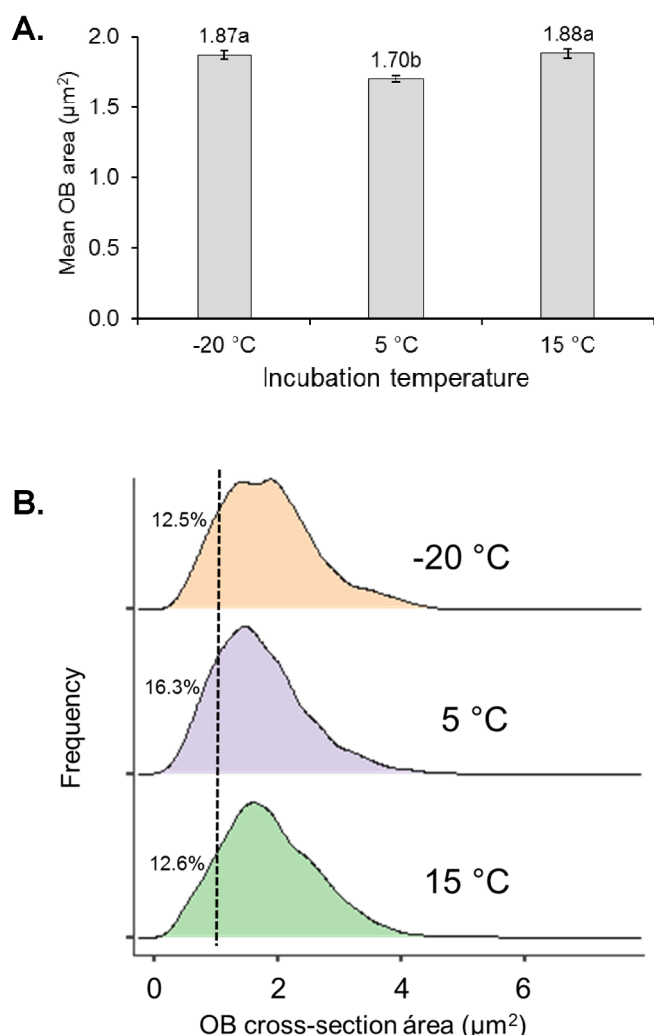


Fig. 2. Occlusion body (OB) measurements performed by scanning electron microscopy. (A) Mean OB cross-sectional area in each post-mortem treatment calculated by image analysis. (B) Frequency-area distribution of OBs from each treatment. Dotted vertical line indicates fraction of OBs of $< 1 \mu\text{m}^2$ with the corresponding percentage value. Vertical bars in (A) indicate SE. Mean values above columns in (A) followed by identical letters did not differ significantly (Tukey, $P > 0.05$).

regimes (Table 1).

Mean time to death values were based on larvae that succumbed to an LC_{80} concentration of inoculum that killed 77.8, 79.2 and 82.6% of experimental insects in the -20° , 5° and 15°C treatments respectively (Table 1). Mean time to death values did not differ significantly among incubation regimes and varied between 96.7 h and 99.7 h, depending on post-mortem incubation temperature.

4. Discussion

Post-mortem incubation at 15°C was demonstrated to result in a clear increase in OB production in SfMNPV-killed larvae. No such increase was observed in the 5°C incubation treatment, which was similar to the no incubation treatment involving immediate freezing (-20°C) after death. The biological activity of OBs was not adversely affected by post-mortem incubation, although several effects were detected on average OB size, DNA content and the abundance of microbial contaminants. As will become apparent, we believe these results reflect the outcome of two opposing post-mortem processes of: (i) continued ODV occlusion and OB maturation and (ii) the action of degradative enzymes

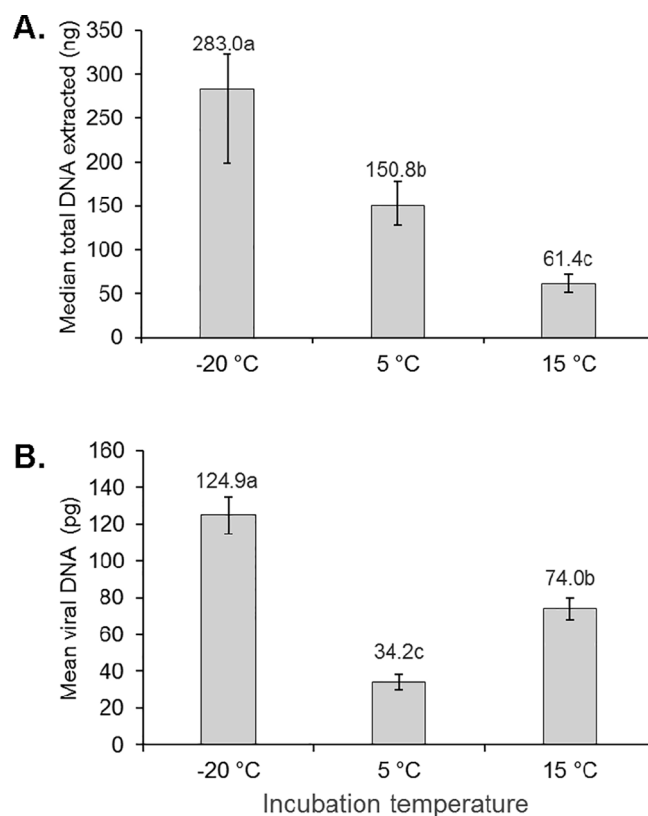


Fig. 3. Analysis of DNA extracted from occlusion body suspensions in each post-mortem treatment. (A) Median concentration of total DNA extracted measured by UV-spectroscopy. (B) Mean quantity of viral DNA detected by qPCR amplification using primers targeted at the *polh* gene of SfMNPV. Vertical bars indicate (A) interquartile range or (B) SE. Values above columns indicate median and mean values in A and B, respectively. Values followed by identical letters did not differ significantly ($P > 0.05$, DSCF pairwise comparisons for A; Tukey for B).

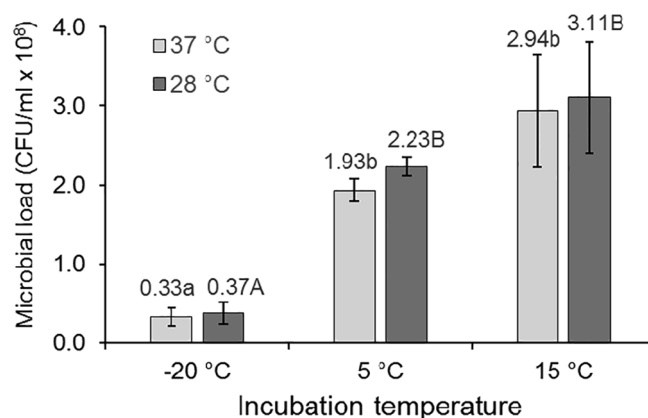


Fig. 4. Quantification of aerobic microbial contaminants in OB suspensions subjected to immediate freezing of larvae following death (-20°C) or post-mortem incubation at 5° or 15°C . Mean numbers of colony forming units were determined following 24 h incubation at 37°C and 24 h additional incubation at 28°C . Vertical bars indicate SE based on five replicate samples from each treatment. Mean values above columns followed by identical letters did not differ significantly for comparisons of lower case (37°C) and upper case (28°C) values (Tukey, $P > 0.05$).

from the host and associated microbiota.

The quantity of OBs per larva and per mg larval weight both increased by $\sim 40\%$ during post-mortem incubation at 15°C , suggesting that the processes involved in OB production, such as ODV occlusion

Table 1

Logit regression of concentration-mortality responses, 50% lethal concentrations (LC₅₀) and mean time to death of *Spodoptera frugiperda* second instars that consumed OB suspensions obtained from three different post-mortem incubation temperature treatments. Mean time to death values were estimated by Weibull analysis for larvae that consumed LC₈₀ inoculum.

Incubation temperature	Slope ± SE	Intercept ± SE	LC ₅₀ (95% CI) (OBs/ml × 10 ⁴)	Mean time to death (95% CI) (h)
–20 °C	1.050 ± 0.13	–10.42 ± 1.26	2.04 (1.42–2.97)	99.9 (97.4–102.4)
5 °C	0.792 ± 0.08	–8.11 ± 0.81	2.08 (1.98–4.05)	99.8 (97.4–102.3)
15 °C	0.840 ± 0.10	–8.58 ± 1.02	2.72 (1.81–4.14)	96.8 (94.5–99.2)

The error distribution of logit models was scaled to account for moderate overdispersion in the results (Crawley, 1993); scale parameter was 2.0 (–20 °C), 1.0 (5 °C) and 1.9 (15 °C).

and OB maturation, continued for a period after the death of the host insect. Presumably these processes were temperature dependent as no increase in OB production was observed in the 5 °C treatment. In previous studies, OB yields increased by 2.2–2.8-fold following 7–14 d of peri- and post-mortem incubation at 4–24 °C in *Spodoptera littoralis* larvae infected with their homologous nucleopolyhedrovirus (SpliNPV) (McKinley et al., 1989; Grzywacz et al., 1998). In contrast, the production of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) or *Spodoptera exempta* nucleopolyhedrovirus (SpexNPV) OBs did not increase significantly following 9 days of peri- and post-mortem incubation of the homologous host larvae at 14 or 26 °C (Cherry et al., 1997).

Insect remains retained during filtration of cadaver homogenate were significantly reduced in the 15 °C treatment, probably due to the activity of degradative enzymes from the host and microbial community, including virally-encoded enzymes such as chitinase and cathepsin that promote larval liquefaction and favor the release of OBs from the insect cadaver (Hawtin et al., 1997; Ishimwe et al., 2015), for subsequent dispersal and horizontal transmission (D'Amico et al., 2013). As the observed differences among the incubation treatments were very small, comprising just a few milligrams of insect remains in each group of ten infected larvae, the increased OB yield in the 15 °C treatment was unlikely to be due to enzymatic breakdown of tissues markedly improving OB extraction, although this process may have contributed to an improved efficiency of OB extraction to some degree.

Despite the increase in the total number of OBs produced in larvae incubated at 15 °C, OB size (cross-sectional area) in this treatment was almost identical to that of the –20 °C treatment, which we took as an indicator of a similar prevalence of OB maturation in these treatments, whereas when incubated at 5 °C, a significantly increased fraction of small OBs (< 1 μm²) was observed in the OB population. We suggest that this may have resulted from a modest production of OBs that did not mature over the 10-day incubation period due to the low temperature (5 °C) and which did not significantly affect the total OB production values in this treatment. In contrast, in the 15 °C treatment, OB maturation presumably continued to completion in the OBs that developed during the post-mortem incubation so that mean OB size was similar to that of the –20 °C treatment in which OB maturation was presumably halted completely.

OB maturation appears to be an important but incompletely understood process, in which the multilayered polyhedron envelope (PE), comprising phosphorylated PEP protein, in association with P10 and a carbohydrate component, forms a smooth sealed surface over the occlusion body (Van Lent et al., 1990; Sajjan and Hinchigeri 2016). The functions of the PE may include preventing the loss of ODVs from the OB matrix, preventing the fusion of developing OBs in the cell nucleus, improving the physical stability of OBs and protecting them from physical assaults in the environment outside of the host (Gross et al., 1994; Sajjan and Hinchigeri 2016). In the absence of the PE, viral OBs appear small and pitted, probably due to the loss of ODVs from the polyhedrin matrix (Li et al., 2015). Importantly, the use of 0.5% sodium dodecyl sulfate during OB extraction can result in the loss of the PE structure, especially in OBs grown in cell culture (Lua et al., 2003). However, in present study we used a very low concentration (0.05%) of

Tween 80 for OB extraction and used pure water for OB dilution and the large majority of OBs appeared undamaged during electron microscopic examination.

Analysis of the DNA extracted from OB suspensions revealed a decrease in total DNA, comprising host, viral and microbial DNAs, with increasing incubation temperature (Fig. 3A). We assume that this reflects the temperature dependent activity of host and microbial degradative enzymes during the incubation period. Quantification of viral DNA by qPCR targeted at the polyhedrin gene revealed a different pattern. The quantity of viral DNA was lowest in the 5 °C treatment, highest in the –20 °C treatment and intermediate in the 15 °C treatment. As many of the ODVs produced late in infection are not occluded (Chung et al., 1980; Kelly 1981; Chaeychomsri et al., 2018) and immature OBs contain fewer ODVs (Allaway 1983; Kumar et al., 2005), the reduced quantity of viral DNA measured in the 5 °C treatment may result from degradation of genomes within non-occluded ODVs and immature OBs that lacked the protective PE structure. In contrast, in the 15 °C treatment we suggest that loss of viral genomes was partially offset by continuing occlusion of ODVs and OB maturation during the incubation period, providing improved protection of viral genomes from enzymatic degradation, resulting in the intermediate levels of viral DNA observed in this treatment. Clearly, support for this hypothesis would require further study on the dynamics of OB development and maturation following the death of the host.

Despite differences among incubation treatments in OB size and genome content, the biological activity of OBs from different treatments did not vary significantly in pathogenicity, measured as concentration-mortality metrics, or speed of kill. A 10% reduction in OB cross-sectional area in the 5 °C treatment (Fig. 2A) would equate to a 15% reduction in OB volume assuming that OBs were roughly spherical. As the number of ODVs occluded within an OB is proportional to OB size (Allaway, 1983), we may have expected a reduced concentration-mortality response in insects that consumed OBs from this treatment, but this was not observed, possibly because a 15% variation in the number of ODVs ingested by a larva was insufficient to affect the likelihood of primary infection of midgut cells and the subsequent development of lethal polyhedrosis disease.

Interestingly, OBs harvested from living larvae are several times less pathogenic than OBs from virus-killed insects (Ignoffo and Shapiro, 1978; Shapiro and Bell, 1981), which may be related to OB development within cell nuclei as OB size increases during infection until host death (Takatsuka et al., 2007). The results of our study demonstrate that unpurified OBs did not lose any insecticidal activity over the 9-day incubation period, whereas under natural conditions the half-life of OBs released from larval cadavers on tree foliage has been estimated at 1.7–1.9 days, depending on virus and plant species (calculated from decay rates given in Fuller et al., 2012 [2008 data] and Polivka et al., 2017). Environmental factors such as solar radiation and interactions with plant phytochemical compounds and microorganisms are likely responsible for these marked differences in OB persistence (reviewed by Williams, 2018).

Although not statistically significant, the LC₅₀ value estimated for the 15 °C treatment was approximately 30% higher than the corresponding value for the –20 °C treatment. If this difference were real, it

would largely cancel out the ~40% increase in OB production observed following post-mortem incubation. High variation in the insect mortality response to OB inoculum often hinders accurate determination of LC₅₀ values (Grzywacz and Moore, 2017). However, in the case of the present study, logit regression revealed that OBs from the different treatments elicited similar mortality responses across the entire range of inoculum concentrations, strengthening our assertion that post-mortem incubation was not detrimental to the insecticidal activity of OBs. That said, the usefulness of adding a post-mortem incubation step to a virus insecticide production system will depend critically on the relationship between the value of the additional OBs produced and the cost of the incubation step, which will increase as the duration of the incubation period increases. This relationship is likely to differ for each virus production system and requires a cost-benefit analysis applicable to commercial scale production.

The abundance of aerobic microbes in experimental suspensions of SfMNPV OBs increased from $\sim 3 \times 10^7$ CFU/ml in the -20°C treatment, to 2×10^8 and 3×10^8 CFU/ml in the 5°C and 15°C incubation treatments, respectively. Very similar levels of contaminants have been reported for SfMNPV (Ruiz et al., 2015) and other nucleopolyhedroviruses produced in insects, including the gypsy moth virus LdMNPV (Podgwaite et al., 1983; Shapiro, 1986), and viruses of armyworms SeMNPV (Smits and Vlak, 1988; Lasa et al., 2008) and SlitNPV (Grzywacz et al., 1997). The majority of microbial contaminants are usually fecal *Enterococcus* spp., with lower numbers of *Enterobacteriaceae*, *Bacillus* spp. and yeasts (Krieg et al., 1979; Grzywacz et al., 1997; Lasa et al., 2008). The insect-associated microbiota does not usually include significant levels of human pathogens and most phytosanitary regulatory authorities have established acceptable levels of microbial contaminants in baculovirus-based insecticides (Jenkins and Grzywacz, 2000).

In conclusion, post-mortem incubation at 15°C resulted in increased OB production, probably due to processes of ODV occlusion and OB maturation. This did not occur at 5°C , in which a significant proportion of the OB population likely did not mature and may have undergone enzymatic degradation of viral genomes. Post-mortem incubation did not adversely affect the insecticidal properties of OBs and levels of microbial contaminants remained within normal limits. A deeper understanding of post-mortem processes in nucleopolyhedrovirus-killed insects will require examining specific aspects of ODV occlusion, OB maturation and the fate of non-occluded virions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biocontrol.2019.05.003>.

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