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

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A B S T R A C T

The efficient 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 μm^2) 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 x 10^7 CFU/ml in the -20 °C treatment, to approximately 2 x 10^8 and 3 x 10^8 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.

46 *Keywords:* Spodoptera frugiperda multiple nucleopolyhedrovirus; biological insecticide
47 production; insecticidal activity; qPCR; OB size

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,

72 virus production studies aim to achieve the highest yield of OBs in a short period of time,
73 fidelity of amplification of the inoculum, high insecticidal activity of OBs and low levels of
74 microbial contaminants that usually originate from the host insect microbiota (Tang et al.,
75 2012). Where possible, these factors should be optimized at the same time as labor and the
76 costs of materials and overheads are minimized (Shieh 1989; Hunter-Fujita et al., 1998;
77 Grzywacz et al., 2014).

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

90 In the present study we asked whether there was any benefit to extended post-
91 mortem incubation at low temperatures for the yield or the insecticidal characteristics of the
92 resulting OBs. To examine these possibilities, we compared production of SfMNPV OBs in
93 *S. frugiperda* larvae that were frozen immediately following death or were subjected to
94 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 x 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 ± 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 x 4 x 4 mm) that had been treated with 8 x 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 to 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.

136 To produce OB suspensions each larva was thawed in its centrifuge tube and
137 transferred to a glass homogenizer. The plastic centrifuge tube was washed with 300 µl of
138 0.05% (v/v) Tween 80 to remove any insect remains and these washings were transferred to
139 the homogenizer. Larvae were homogenized and the resulting suspension was filtered
140 through a stainless steel mesh with an 80 µm pore size. This procedure was repeated for
141 each of the ten larvae in each replicate. At the end of this process the homogenizer was
142 washed with 4 ml of 0.05% Tween 80 that was also passed through the mesh filter. The
143 resulting suspension was placed in a 15 ml sterile plastic centrifuge tube and adjusted to a
144 total volume of 14 ml. The weight of the insect remains retained by the steel mesh was
145 determined by drying in an oven at 50 °C for 24 h and then weighed using an analytical
146 balance (Explorer EX124, Ohaus, USA).

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

157 *2.4 OB size measurement*

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

162 Each sample was then coated with gold-palladium in a Quorum Q150R sputter-
163 coater and observed at x10,000 magnification in a FEI Quanta 250 FEG scanning electron
164 microscope at an accelerating voltage of 10 kV. Images of OBs (~40 OBs/image) were
165 taken at 18 - 25 different points within each sample at a fixed resolution of 1536 x 1103
166 pixels, 37 ppi. The cross-sectional area of each OB was then estimated with reference to the
167 20 µm scale bar using the ImageJ program (https://imagej.net).

168 *2.5 Quantification of viral genomes*

169 Samples of 2x10^8 OBs in 225 µl sterile water were obtained for all treatments and
170 replicates. Virions were released from the OBs by adding 50 µl of 0.5 M Na_2CO_3 and 25 µl
171 of 10% SDS for 5 min at 55 °C. Debris was pelleted at 8000 rpm for 5 min and was
172 discarded, whereas virion-containing supernatant was incubated with 12.5 µl proteinase K
173 (20 mg/ml) at 55 °C for one hour. Viral genomic DNA was then extracted with phenol and
174 twice with a phenol-chloroform-isopropylalcohol and precipitated by addition of 3 M
175 sodium acetate (pH 5.2) and ice-cold ethanol. This precipitate was centrifuged at 15000
176 rpm for 15 min, washed with 70% ethanol and dissolved in 200 µl of Milli-Q water. The
177 concentration of DNA in each sample was then determined by calculating the average
178 reading from triplicate samples in a spectrophotomer (BioSpec-Nano, Shimadzu, Japan).

179 Quantitative PCR (qPCR) based on SYBR fluorescence was performed in a
180 Mx3005P qPCR System real-time (Stratagene) in 96-well reaction plates. For viral load
181 detection, specific primers were designed based on the polyhedrin gene sequence for
182 SfMNPV-NIC genotype B (Simón et al., 2011), using Primer3 software (Untergasser et al.,
183 2007). The forward primer Sfpolh1 (5'-GAACCTTCACTCTGAGTACACGCAC) and
184 reverse primer Sfpolh2 (5'-AGACGATGGGTTTGTAGAAGTTCTCC) amplified an 82 bp
185 region of the *polh* gene. Amplifications were performed in a final volume of 10 µl,
186 comprising 5 µl of iQ SYBR Green Supermix (Bio-Rad), 3.6 µl of sterile Milli-Q water, 0.2
187 µl of each of the primers (forward and reverse, 10mM) and 1 µl of DNA (0.301-3.786 ng).
188 Three reactions with a negative control were included in each analysis and the standard
189 curve (range 5 x 10^{-6} ng to 5 ng viral DNA) was performed in triplicate to determine the
190 efficiency of each reaction. The qPCR protocol consisted of an initial denaturation step at
191 95 °C for 3 min followed by 45 amplification cycles of 95 °C for 15 s and 60 °C for 30 s
192 and, to determine the melting curve, a cycle of 30 s with increments of 0.5° C between 60
193 °C and 95 °C. Data acquisition and processing was performed using MxPro software
194 (Stratagene, La Jolla, USA).

195 *2.6 Quantification of microbial contaminants*

196 To quantify microbial contaminants in the OB suspension, samples from the five
197 even-numbered replicates, representing half of the total number of replicates, were serially
198 diluted in sterile Milli-Q water (10^{-3} - 10^{-7}) and 100 µl volumes of each dilution were
199 spread over LB agar plates (LB agar, Bioxon) using sterile glass beads. Inoculated plates
200 were incubated in darkness at 37 ± 1 °C in a laboratory incubator for 24 h. The number of
201 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 ± 1 °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 mins were individually transferred to 30 ml plastic cups with diet, maintained in darkness at 25 ± 0.5 °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 ~80% of inoculated insects. Larvae that drank the suspension in 10 mins were individually incubated on diet in darkness at 25 ± 0.5 °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

223 Counts of OBs/group of ten larvae and OBs/mg of larval weight and CFU counts at
224 24 h and 48 h were log-transformed, whereas OB cross-sectional area values were
225 normalized by square root transformation ($\sqrt{x}$) prior to one-way analysis of variance
226 (ANOVA). The quantities of viral DNA in each qPCR reaction were subjected to one-way
227 ANOVA without prior transformation. Treatment means were compared by Tukey test. The
228 weights of tissue debris remaining following OB extraction and total DNA extracted from
229 crude OB suspensions lacked homoscedasticity and were subjected to Kruskal-Wallis
230 analysis followed by Dwass-Steel-Critchlow-Fligner (DSCF) pairwise comparisons
231 (Hollander and Sethuraman 2015). All ANOVAs and non-parametric tests were performed
232 using the R-based program Jamovi v.0.9.1.12 (Jamovi 2018). The frequencies of OBs of
233 different size classes were subjected to contingency table analysis. Concentration-mortality
234 results were subjected to logit regression with the Generalized Linear Interactive Modeling
235 (GLIM 4) program with a binomial error distribution specified (Numerical Algorithms
236 Group 1993). Minor overdispersion in the mortality results was taken into account by
237 scaling the error distribution where necessary (Crawley 1993). Mean time to death values
238 were estimated by Weibull survival analysis in GLIM 4.

239 **3. Results**

240 *3.1 Quantification of OBs following post-mortem incubation*

241 Inoculation of larvae with 8 x 10^6 OBs resulted in >90% larval mortality in all
242 replicates. No mortality was observed in water-inoculated controls. The weight of groups of
243 10 randomly-selected virus-killed larvae that comprised each replicate varied between 2.1

244 and 2.4 g and did not differ significantly among groups assigned to each incubation
245 treatment ($F = 0.987$, $df = 2, 27$; $P = 0.386$).

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

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

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

258 *3.2 OB size measurement*

259 The total number of OBs measured was 1029, 963 and 967 for the -20°, 5° and 15
260 °C treatments, respectively. Estimated cross-sectional area varied between 0.278 and 7.32
261 μm^2. OBs extracted from larvae that experimented post-mortem incubation at 5 °C had a
262 significantly lower cross-sectional area than OBs extracted from the other treatments ($F =$
263 17.4; $df = 2, 2964$; $P < 0.001$), but the difference was small, comprising a reduction of
264 approximately 0.18 μm^2 or ~10% of the cross-sectional area (Fig. 2A). To investigate this

265 effect, we examined the distribution of OB areas in each treatment (Fig. 2B). The
266 prevalence of small OBs was significantly higher in the 5 °C treatment than the other
267 treatments (χ^2 = 7.598; df = 3; P = 0.022). Specifically, the number of OBs with an area of
268 less than 1 μm^2 comprised 16.3% of the total OBs measured in the 5 °C treatment
269 compared to 12.5 and 12.6% of the OBs measured in the -20 °C and 15 °C treatments,
270 respectively. An alternative explanation, in which differences in mean OB size were due to
271 a paucity of particularly large OBs in the 5 °C treatment, was not supported by the data, as
272 OBs of over 4.0 μm^2 comprised less than 1.5% of measured OBs across all treatments and
273 did not differ significantly in prevalence among treatments, with 9 - 15 OBs per treatment
274 in total (χ^2 = 1.205; df = 3; P = 0.547).

275 *3.3 Quantification of viral genomes*

276 The quantity of total DNA extracted from a crude suspension of $2x10^8$ OBs varied
277 between 30.1 and 378.6 ng/µl. The quantity of total DNA, comprising host, virus and
278 microbiota DNA, differed significantly among the three post-mortem incubation regimes,
279 with decreasing total DNA extracted as post-mortem incubation temperature increased
280 (Kruskal-Wallis H = 23.7; df = 2; P <0.001) (Fig. 3A). The quantity of SfMNPV genomic
281 DNA/µl also differed significantly among the incubation regimes with amount of viral
282 DNA in the -20 °C treatment approximately 3.6-fold higher than the 5 °C treatment and
283 1.7-fold higher than the 15 °C treatment (F = 41.0; df = 2, 27; P <0.001) (Fig. 3B).

284 *3.4 Quantification of microbial contaminants*

285 The abundance of microorganisms differed significantly among the post-mortem
286 treatments for microbes counted at 37 °C after 24 h (F = 20.1; df=2, 10; P <0.001), or when

287 re-incubated at 28 °C for an additional 24 h period ($F = 22.1$, $df = 2, 10$, $P < 0.001$)(Fig. 4).
288 Initial aerobic CFU counts on LB agar at 24 h were only slightly increased when re-
289 incubated 48 h in all cases (Fig. 4). Mean CFU counts (±SE) in pooled OB suspensions
290 from larvae frozen at -20 °C varied between 3.3 x 10^7 and 3.7 x 10^7 CFU/ml following
291 incubation at 37 and 28 °C, respectively, which were significantly lower than for OB
292 suspensions from the 5 °C or 15 °C regimes, in which microbial contaminants were
293 approximately six-fold and nine-fold more abundant, respectively (Fig. 4).

294 *3.5 Insecticidal activity of OBs*

295 The concentration-mortality responses of insects that consumed OBs from the
296 different incubation treatments did not differ significantly ($F = 0.70$; $df = 2, 53$; $P = 0.50$).
297 Similarly, the LC_{50} values and the corresponding 95% CI values overlapped broadly among
298 the incubation regimes (Table 1).

299 Mean time to death values were based on larvae that succumbed to an LC_{80}
300 concentration of inoculum that killed 77.8, 79.2 and 82.6% of experimental insects in the -
301 20°, 5° and 15 °C treatments respectively (Table 1). Mean time to death values did not
302 differ significantly among incubation regimes and varied between 96.7 h and 99.7 h,
303 depending on post-mortem incubation temperature.

304 **4. Discussion**

305 Post-mortem incubation at 15 °C was demonstrated to result in a clear increase in
306 OB production in SfMNPV-killed larvae. No such increase was observed in the 5 °C
307 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 from the host and associated microbiota.

The quantity of OBs per larva and per mg larval weight both increased by ~40% during post-mortem incubation at 15 °C, suggesting that the processes involved in OB production, such as ODV occlusion 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 μm^2) 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

353 protecting them from physical assaults in the environment outside of the host (Gross et al.,
354 1994; Sajjan and Hinchigeri 2016). In the absence of the PE, viral OBs appear small and
355 pitted, probably due to the loss of ODVs from the polyhedrin matrix (Li et al., 2015).
356 Importantly, the use of 0.5% sodium dodecyl sulfate during OB extraction can result in the
357 loss of the PE structure, especially in OBs grown in cell culture (Lua et al., 2003).
358 However, in present study we used a very low concentration (0.05%) of Tween 80 for OB
359 extraction and used pure water for OB dilution and the large majority of OBs appeared
360 undamaged during electron microscopic examination.

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

376 require further study on the dynamics of OB development and maturation following the
377 death of the host.

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

388 Interestingly, OBs harvested from living larvae are several times less pathogenic
389 than OBs from virus-killed insects (Ignoffo and Shapiro 1978; Shapiro and Bell 1981),
390 which may be related to OB development within cell nuclei as OB size increases during
391 infection until host death (Takatsuka et al., 2007). The results of our study demonstrate that
392 unpurified OBs did not lose any insecticidal activity over the 9-day incubation period,
393 whereas under natural conditions the half-life of OBs released from larval cadavers on tree
394 foliage has been estimated at 1.7 - 1.9 days, depending on virus and plant species
395 (calculated from decay rates given in Fuller et al., 2012 [2008 data] and Polivka et al.,
396 2017). Environmental factors such as solar radiation and interactions with plant phylloplane
397 compounds and microorganisms are likely responsible for these marked differences in OB
398 persistence (reviewed by Williams 2018).

399 Although not statistically significant, the LC_{50} value estimated for the 15 °C
400 treatment was approximately 30% higher than the corresponding value for the -20 °C
401 treatment. If this difference were real, it would largely cancel out the ~40% increase in OB
402 production observed following post-mortem incubation. High variation in the insect
403 mortality response to OB inoculum often hinders accurate determination of LC_{50} values
404 (Grzywacz and Moore 2017). However, in the case of the present study, logit regression
405 revealed that OBs from the different treatments elicited similar mortality responses across
406 the entire range of inoculum concentrations, strengthening our assertion that post-mortem
407 incubation was not detrimental to the insecticidal activity of OBs. That said, the usefulness
408 of adding a post-mortem incubation step to a virus insecticide production system will
409 depend critically on the relationship between the value of the additional OBs produced and
410 the cost of the incubation step, which will increase as the duration of the incubation period
411 increases. This relationship is likely to differ for each virus production system and requires
412 a cost-benefit analysis applicable to commercial scale production.

413 The abundance of aerobic microbes in experimental suspensions of SfMNPV OBs
414 increased from ~3 x 10^7 CFU/ml in the -20 °C treatment, to 2 x 10^8 and 3 x 10^8 CFU/ml in
415 the 5 °C and 15 °C incubation treatments, respectively. Very similar levels of contaminants
416 have been reported for SfMNPV (Ruiz et al., 2015) and other nucleopolyhedroviruses
417 produced in insects, including the gypsy moth virus LdMNPV (Podgwaite et al., 1983;
418 Shapiro 1986), and viruses of armyworms SeMNPV (Smits and Vlak, 1988; Lasa et al.,
419 2008) and SlitNPV (Grzywacz et al., 1997). The majority of microbial contaminants are
420 usually fecal *Enterococcus* spp., with lower numbers of Enterobacteriaceae, *Bacillus* spp.
421 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 associated with this article can be found in Supplemental Information I.

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622
623

624 **Figure legends**

625 Fig. 1. Occlusion body (OB) production in larvae that were immediately frozen (-20 °C)
626 following death or subjected to post-mortem incubation at 5 or 15 °C. (A) Mean OB
627 production from each group (replicate) of 10 virus-killed larvae, (B) mean OB
628 production per mg larval weight, (C) median weight of larval remains following
629 filtration of larval homogenate in each group of 10 larvae. Vertical bars indicate SE
630 (A and B) or interquartile range (C). Values above columns indicate means or
631 medians. Values followed by identical letters did not differ significantly (Tukey,
632 $P>0.05$ for A and B, or DSCF pairwise comparisons for C).

633

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

640

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

648

649 Fig. 4. Quantification of aerobic microbial contaminants in OB suspensions subjected to
650 immediate freezing of larvae following death (-20 °C) or post-mortem incubation at
651 5 or 15 °C. Mean numbers of colony forming units were determined following 24 h
652 incubation at 37 °C and 24 h additional incubation at 28 °C. Vertical bars indicate
653 SE based on five replicate samples from each treatment. Mean values above

654 columns followed by identical letters did not differ significantly for comparisons of
655 lower case (37 °C) and upper case (28 °C) values (Tukey, P>0.05).
656

Table 1. Logit regression of concentration-mortality responses, 50% lethal concentrations (LC_{50}) 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 LC_{80} inoculum.

Incubation temperature	**Slope ± SE**	**Intercept ± SE**	**LC_{50} (95% CI) (OBs/ml x 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).

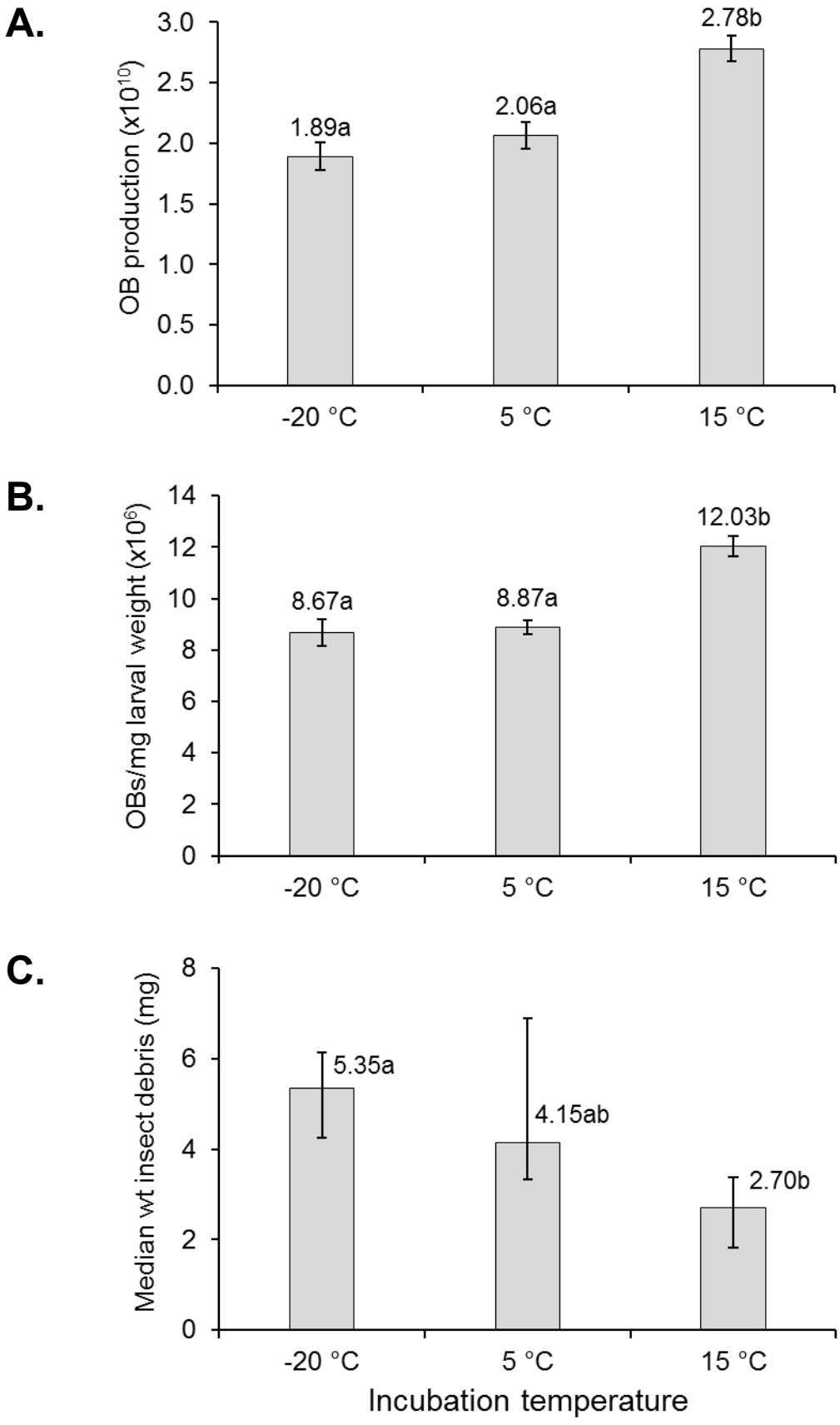

A.
OB production (x10^10)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.89a
2.06a
2.78b
-20 °C
5 °C
15 °C
B.
OBs/mg larval weight (x10^6)
14
12
10
8
6
4
2
0
8.67a
8.87a
12.03b
-20 °C
5 °C
15 °C
C.
Median wt insect debris (mg)
8
6
4
2
0
5.35a
4.15ab
2.70b
-20 °C
5 °C
15 °C
Incubation temperature

Fig. 2

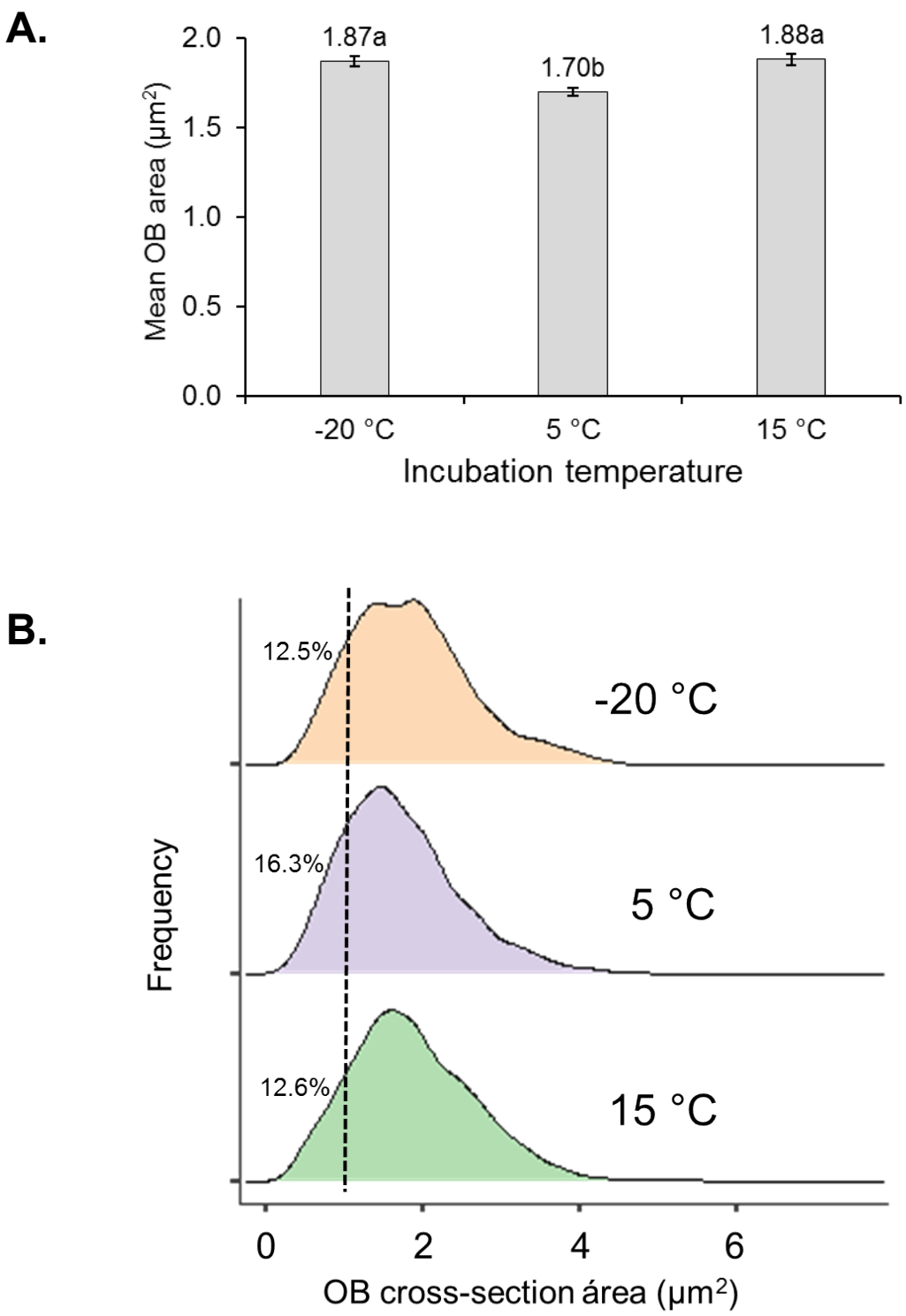

A.
2.0
1.5
1.0
0.5
0.0
Mean OB area (µm²)
1.87a
1.70b
1.88a
-20 °C
5 °C
15 °C
Incubation temperature
B.
12.5%
-20 °C
16.3%
5 °C
12.6%
15 °C
Frequency
0
2
4
6
OB cross-section área (µm²)

Fig. 3

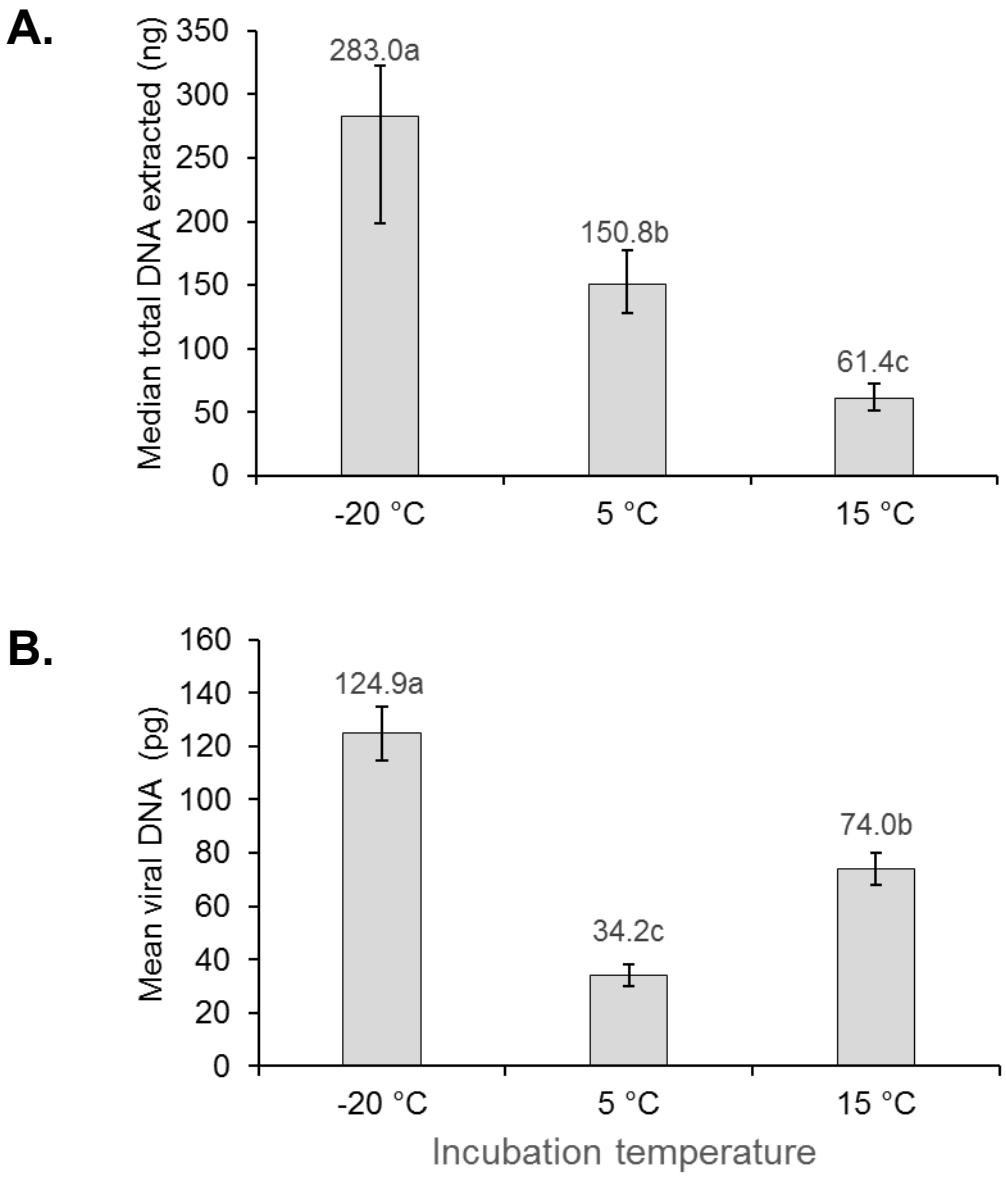
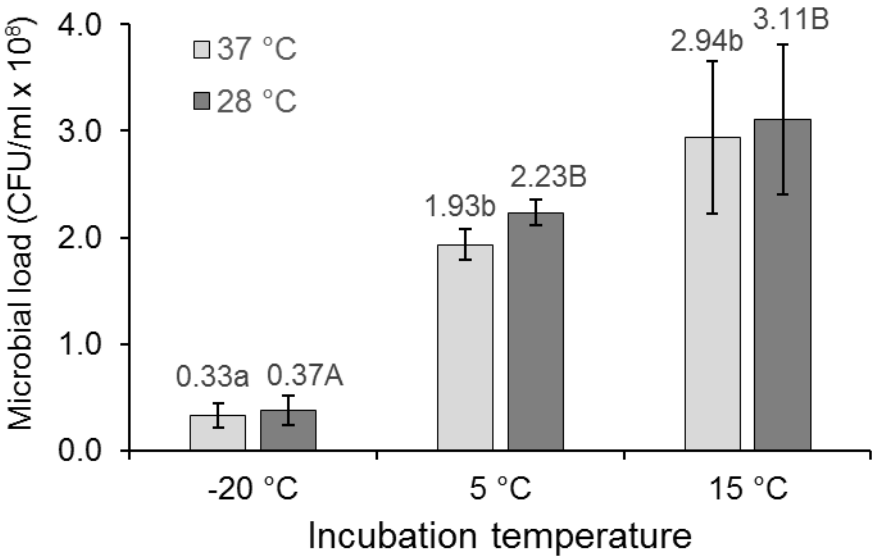
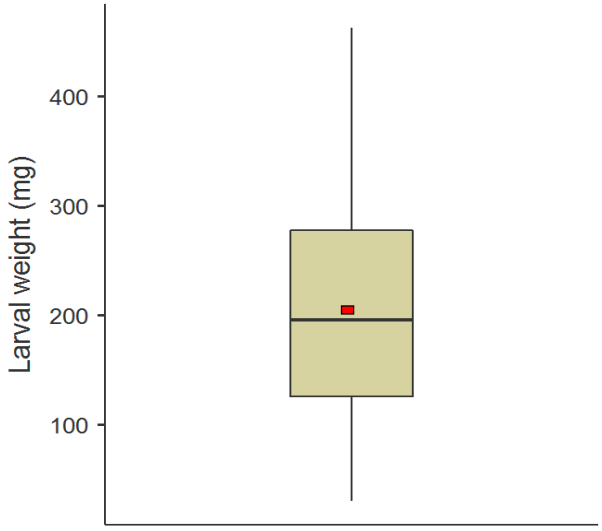

A.
Median total DNA extracted (ng)
350
300
250
200
150
100
50
0
283.0a
150.8b
61.4c
-20 °C
5 °C
15 °C
B.
Mean viral DNA (pg)
160
140
120
100
80
60
40
20
0
124.9a
34.2c
74.0b
-20 °C
5 °C
15 °C
Incubation temperature

Fig. 4

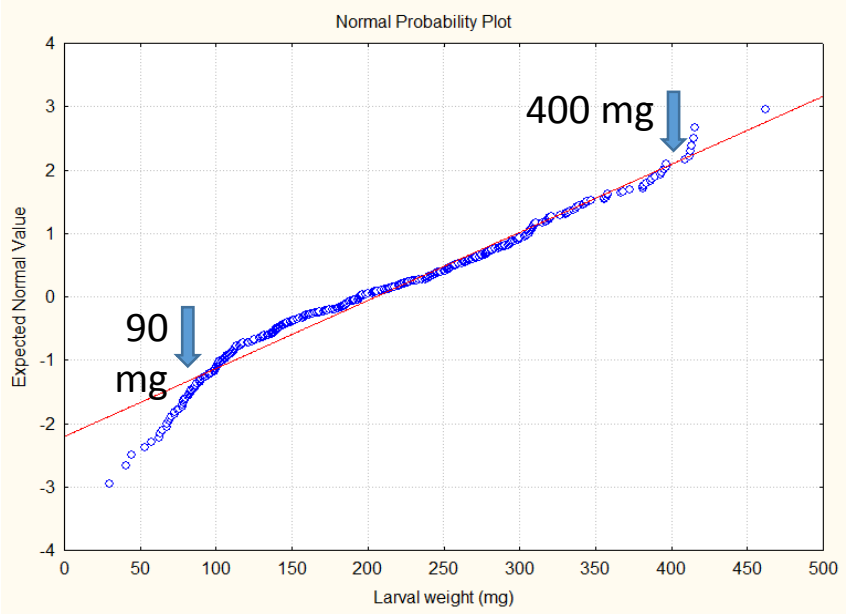

Microbial load (CFU/ml x 10⁸)
4.0
3.0
2.0
1.0
0.0
37 °C
28 °C
0.33a
0.37A
1.93b
2.23B
2.94b
3.11B
-20 °C
5 °C
15 °C
Incubation temperature

Supplemental material. Distribution of larval weights at death of virus-killed insects prior to post-mortem incubation period. **(A)** Box-plot of mean (red rectangle), median (horizontal line), 25-75% interquartile range (shaded box) and range (vertical bar) of larval weights. **(B)** Normal probability plot (P-P plot) of weights indicating deviation from normal distribution for weights above 400 mg (7 individuals) and below 90 mg (40 individuals) out of a total of 429 virus-killed insects (see Table).

A.



B.



Number of larvae of <90 mg or >400 mg in weight that were excluded from each treatment prior to incubation and subsequent analyses.

Treatment	Small larvae (<90 mg)	Large larvae (>400 mg)
-20 °C	10	5
5 °C	17	2
15 °C	13	0
Total:	40	7

Fernando G. Ramírez-Arias: Data curation; Formal analysis; Investigation; Writing – original draft; Writing – review & editing.

Rodrigo Lasa: Conceptualization; Methodology; Supervision; Validation; Writing – original draft; Writing – review & editing.

Rosa Murillo: Conceptualization; Methodology; Supervision.

Laura Navarro-de-la-Fuente: Formal analysis; Investigation; Methodology.

Gabriel Mercado: Methodology; Project administration; Resources.

Trevor Williams: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Supervision; Visualization; Writing – original draft; Writing – review & editing.