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4 **Evidence for covert baculovirus infections in a *Spodoptera exigua* laboratory**
5 **culture**

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Summary

A laboratory culture of *Spodotera exigua* was examined to assess covert (latent or persistent) baculovirus infections and spontaneous disease outbreaks. Two nucleopolyhedrovirus (NPV) species were found to be reactivated from a covert state in a laboratory culture of *S. exigua* to fully lethal forms. These were identified as *S. exigua* (Se) MNPV and *Mamestra brassicae* NPV using restriction enzyme analysis of purified viral DNA. Sequence data derived from both overtly and covertly virus-infected insects revealed highly conserved sequences for *lef-8*, *lef-9* and *polyhedrin* gene sequence (98 - 100 % nucleotide identity to SeMNPV published sequence). By monitoring spontaneous overt infections and quantifiying viral DNA (q-PCR) in asymptomatic individuals over two generations we identified fluctuating trends in viral DNA levels from covert SeMNPV and MbNPV within a *S. exigua* host population. Virus levels per insect life stage ranged from $3.51 \pm 0.101 \times 10^5$ to 0.29 ± 0.036 pg (detection limit at 0.06 pg). Bioassays performed with this culture of larvae showed a differential susceptibility to SeMNPV-like or MbNPV-like viruses, with SeMNPV super-infections being extremely virulent. The data presented has broad implications relating to our understanding of transmission patterns of baculovirus in the environment and the role of covert infections in host-pathogen interaction dynamics.

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58   **Introduction**

59   Baculoviruses are pathogenic insect viruses producing infections commonly causing

60   fatalities in Lepidoptera host larvae. Although the horizontal route is thought to be the

61   major pathway for baculovirus transmission (Cory & Myers, 2003), some studies

62   support the idea that their transmission also involves vertical transfer in field

63   populations (See Kukan 1999 for a review). Individuals exposed to low virus doses may

64   acquire a non fatal sub lethal infection, but transmit the infection vertically to the next

65   generation (Andrealis, 1987; Cory & Myers, 2003). This may affect insect health and

66   fecundity (Rothman & Myers, 1996; Myers *et al.*, 2000; Vilaplana *et al.*, 2008). Insects

67   may also have a covert infection that does not produce visible symptoms. In this state it

68   is suggested that the virus remains either latent in a non-replicating form, or in a

69   persistent state with low level gene expression (Hughes *et al.*, 1997, Burden *et al.*,

70   2003). Covert infections may not be critical for maintaining virus populations in nature

71   but could enable the parasite to persist during periods of low host population density

72   especially in seasonal environments (Burden *et al.*, 2003). Another advantage to the

73   virus of being in a covert state is that it could be carried long distances during host

74   migration (Anderson & May, 1981). Interestingly, vertical transmission appears to be

75   frequent in more mobile insect species such as those within the genus *Spodoptera*

76   (Swaine, 1966; Abul-Nasr *et al.,* 1979; Smits & Vlak 1988, Fuxa & Richter 1991,

77   Vilaplana *et al.*, 2008).

78   Spontaneous emergence of overt infections from a covertly infected population

79   suggests that the viruses remain replication competent during the quiescent state.

80   Common features of persistent viral infections include the selection of cell subsets ideal

81   for long-term maintenance of the viral genome (Kane & Golovkina 2010). Studies

82   targeting baculoviruses detected the presence of viral DNA in adult tissues such as the

83   fat body (Hughes *et al.,* 1993, 1997) and gonads (Burden *et al.*, 2002, Khurad *et al.,*

84   2004), which ultimately are involved in transmitting virus to subsequent generations.

85 Spontaneous NPV outbreaks have been recorded in laboratory cultures reared under

86 virus-free conditions over several generations. Karpov (1979) reported a *Bombyx mori*

87 NPV outbreak to be a problem in practical sericulture. Few studies have been

88 conducted to elucidate the underlying phenomena (Hughes *et al.,* 1997, Fuxa *et al.,*

89 1999), so little is known about the mechanism(s) involved when a covert virus is

90 triggered into an overt infection (Cory & Myers 2003). Advances in molecular

91 techniques have improved the detection of viral DNA or its transcripts in asymptomatic

92 insects. For instance, a laboratory culture of cabbage moths (*Mamestra brassicae)* was

93 reported to harbour a covert baculovirus infection without prior exposure to such

94 viruses (Hughes *et al.,* 1993). This virus was activated to an overt state by challenging

95 the host with a heterologous baculovirus and the covert virus identified as *M. brassicae*

96 NPV (MbNPV). Using PCR the polyhedrin gene was detected in individuals of this

97 population at all ages indicating this population maintained MbNPV-like persistent virus

98 (Hughes *et al.,* 1997). Similar results were obtained using the immediate early gene 1

99 as a target (Burden *et al.*, 2006) although the presence of the entire virus genome in

100 the covert state has yet to be demonstrated. A serendipitous observation reported

101 here leads us to conclude that a laboratory population of *Spodoptera exigua* also

102 contains covert baculoviruses.

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104 **Results**

105 **Virus reactivation in fourth instar *S. exigua*.**

106 Inserting whole baculovirus genomes in low copy number bacmid vectors in *E. coli* was

107 used to clone *S. frugiperda* multinucleopolyhedrovirus (SfMNPV) (Simón *et al.*, 2008)

108 and SeMNPV DNA (Pijlman *et al.,* 2002, Pijlman *et al.*, 2003). The Se.BAC/G26

109 genomes derived from cloning SeMNPV-G26 in this study were *polyhedrin* negative

110 and used to transfect fourth instar (L_4) *S. exigua* by DNA injection into haemocoels.

111 Insect mortality recorded after injections ranged from 2 % to 50 % (Fig. 1a and b) in six

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112   replicates. Similar variation in mortality was found in groups of insects injected with the

113   wild type Se-G26 (0 - 40%). More surprisingly water-injected control and non treated

114   insects also suffered significant mortalities (10-44% and 5-30% respectively).

115   A total of 86 cadavers with symptoms of NPV infection after *in vivo* transfection were

116   inspected for OBs under a microscope and their DNA analysed by restriction enzyme

117   digestion. Despite Se.BAC/G26 genomes being polyhedrin negative (Fig 1c), OBs were

118   consistently observed in all samples. The DNA analysis confirmed the viruses (Fig 2,

119   lane 3-6) did not contain the BAC replicon in the original vector (Fig. 2, lane 2).

120   Additional fragments were present in the progeny DNA profiles after *in vivo* transfection

121   with Se.BAC/G26, suggesting that the SeBAC clones had not simply lost the vector

122   sequences after growth in insects.   Three variants were identified in different

123   proportions (Fig. 2c) after digestion of virus DNA with *Bgl*II. Two variants had similar

124   profiles to SeMNPV-G26 and were named Se-OX1 and Se-OX2. These were observed

125   from 73 and 4 viral deaths, respectively from a total of the 86 profiled. Another variant

126   (Se-OX3) found in 7 insects showed an identical profile to MbNPV, while a mixture of

127   SeMNPV and MbNPV (Fig. 2, lane 6) was found in 2 cases (Fig. 2c).   The Se-OX3

128   variant had the same profile as MbNPV when digested with *Pst*I, *Xho*I, *Eco*RV, *Eco*RI,

129   *Cla*I, and *Hind*III (data not shown).

130   Se-OX1 and Se-OX2 variants were observed in larvae injected with Se.BAC/G26,

131   SeMNPV-G26 and water. Se-OX1, Se-OX2, and Se-OX3 were also found in non

132   challenged insects (non-treated controls), suggesting they had been reactivated

133   spontaneously in those cases.

134   The L_4 *S. exigua* larvae thought to be virus-free were infected with approximately 10^8

135   OB/ml of MbNPV. Nine individuals succumbed to overt infection, virus was isolated and

136   their DNA profiles after restriction enzyme digestion compared with MbNPV (Fig. 2,

137   lanes 8-16). Seven insects produced viruses with DNA profiles typical of MbNPV but

138   one resembled Se-OX1 (Fig. 2, lane 8) and another a mixed population of MbNPV and

139   SeMNPV (Fig. 2, lane 15) respectively.

140   **Quantification of covert viruses in *S. exigua* breeding cultures.**

141   Spontaneous outbreaks of virus disease in *S. exigua* were indicative of high levels of

142   virus DNA. However, PCR methods where products were detected by electrophoresis

143   failed to detect covert virus consistently when *lef8*, *lef9* and *rr1-pol* primers were used

144   to test DNA samples from asymptomatic L_4 larvae. Hence, a more sensitive approach

145   using q-PCR was optimized. Sensitivity and specificity were tested by using serial

146   dilutions of either SeMNPV or MbNPV DNAs (Table 1). This demonstrated that

147   SeVP80 and MbP87 primers could detect 0.06 pg virus DNA (3×10^{-6} ng/μl in 20 μl

148   reaction) which equated to 490 and 366 for SeMNPV and MbNPV genomes,

149   respectively with no cross-reactivity (Table 1). Host DNA did not interfere with the PCR

150   (data not shown).

151   This q-PCR method was used to quantify covert virus DNA throughout insect

152   metamorphosis to correlate it with NPV mortality records in the host population. Twenty

153   breeding couples were used to establish separate F1 populations for rearing 25 adults

154   (Supplementary Figure 1). Four matings produced no offspring and two derived larvae

155   where 96% died from baculovirus infection. None of these could be maintained further.

156   Two other populations (1 and 12) with 8% virus deaths in the larvae (Table 2) were

157   used to produce three F2 populations (1a-c and 12a-c), while others (7a-c, 16a-c and

158   18a-c) derived from F1 populations with no virus deaths. However, not all breeding

159   pairs produced offspring (Supplementary Figure 1). Ten samples from each

160   developmental stage (eggs, L1-L5 and adults) were screened for virus genomes using

161   q-PCR (Table 2). The average F2 viral DNA levels across developmental stages varied

162   from 1.72 ± 0.020 to 0.29 ± 0.036 pg (Table 2) for five lines, significantly higher than in

163   water controls ($1.80 \pm 0.071 \times 10^{-2}$ pg) (t-test pairwise, $p < 0.05$). Offspring from line

164   12b recorded the highest virus DNA levels of $3.51 \pm 0.101 \times 10^{5}$ pg with 45 positive

165 insects from a total of 80 tested insects (t-test pairwise, $p < 0.05$). Similar proportions

166 of insects harbouring viral DNA were found across the rest of the lines tested (Table 2)

167 and no trends for prevalence of virus between developmental stages was observed

168 (Supplementary Table 1).

169 Covert viral DNA levels monitored throughout the host development cycle were further

170 studied in the offspring from line 12b as 10 cases of apparent NPV-induced mortality

171 were found in L_4 (Table 2). To distinguish SeMNPV- from MbNPV-like virus we used

172 the SeVP80 and MbP87 primer sets with the SeEF primer set as an internal host DNA

173 control. SeMNPV DNA was detected in eggs, L_1, L_2, L_3, L_5, pupae and adults. MbNPV

174 DNA was only detected in eggs, L_1 and L_2 at levels considerably less than SeMNPV,

175 although 50% of eggs were positive. Healthy L4 individuals were not tested for virus.

176 REN analysis of larvae that died showed exclusively SeMNPV profiles. When virus

177 DNA levels were normalized to host DNA (SeEF primer set) and log transformed,

178 maximum viral levels were apparent in eggs and first larval instars (Fig. 3). During the

179 larval stage the ratio of virus/host cell DNA decreased in L_2 but increased dramatically

180 in L_3 indicating the virus was replicated actively, and produced overt infections at L_4

181 instars. L_5, pupae, and adults recorded the lowest ratios (Fig. 3), corresponding to

182 similar viral DNA values as those quantified for eggs and L_1, suggesting viral replication

183 was minimal. Restriction enzyme analysis of virus DNA from 10 L_4 larvae resulted in

184 profiles resembling the Se-OX1 genotype.

185 **Sequencing data from covertly and overtly infected insects.**

186 Sequences for *lef-8* and *lef-9* were generated from 10 insects with covert infection (C_1-

187 C_{10}) or overt infection resulting from spontaneous virus reactivation (O_1-O_{10})

188 (Supplementary Fig. 2). All twenty individuals had SeMNPV-specific infections.

189 Comparison of a 533 nucleotide region from *lef-8* showed 100% identity in all samples

190 with Se-G26 (data not shown). For *lef-9* (730 nucleotides), pairwise comparison to Se-

191 G26 showed between 98 to 99 % nucleotide identity, due to some nucleotide

192 mismatches that did not affect the gene transcription and functionality. *lef-8* and *lef-9*

193 sequence showed a 100% and 98% nucleotide identity to the SeMNPV published

194 sequence (GenBank accession no AF169823) respectively.

195 For the 990 bp *rr1-polh* intergenic region, a single repeat of 12 bp was found to be a

196 consensus sequence in samples C_2, C_3, C_5, C_6, C_7, C_9, O_4, O_5, O_9, and O_{10} while it did

197 not appear in samples C_1, C_4, C_8, C_{10}, O_1, O_2, O_3, O_6, O_8, and G26. The repeat was

198 positioned in the non-coding region between the orf 139 and 1 corresponding to *rr1* and

199 *polyhedrin* genes at the nucleotide positions 135,169 -135,181 bp of the GenBank

200 accession no AF169823 genome (supplementary Fig. 1).

201 **Biological response to reactivated viruses**

202 Virus stocks (Se-G26, Se-OX1, Se-OX2, Se-OX3 and MbNPV) were freshly amplified

203 in L_4 *S. exigua* larvae. q-PCR carried out with either SeVP80- or MbP87-specific

204 primers showed no detectable presence of SeMNPV-like viruses in MbNPV and Se-

205 OX3 stocks or MbNPV in SeMNPV preparations (Table 1). Bioassays were used to

206 assess the five virus stocks in early second instar *S. exigua* larvae. Non-viral deaths

207 occurred in less than 5 % across the four replicates for untreated control insects and

208 were not included in subsequent analysis.

209 Overall mortality response for the five viruses was significantly affected by the dose (F

210 = 24.54, f.d. = 1, p < 0.001), and virus variant species (F = 18.46, f.d. = 4, p < 0.001).

211 As the interaction dose × virus was also significant (F = 5.84, f.d. = 4, p < 0.005) the

212 data was split into SeMNPV-like and MbNPV-like virus groups which showed a

213 different trend from one another (Fig. 4). For SeMNPV-like virus all viral doses

214 produced over 75 % mortality in all four replicates. Neither the dose (F = 3.05, fd = 1, p

215 = 0.110) or the virus variant (F = 0.55, f.d. = 2, p = 0.592) significantly affected the

216 mortality response in this group.

217 Mortality was found to increase with dose for MbNPV-like viruses (F = 159.72, f.d. = 1;

218 p < 0.001). No significant differences were found between both viruses in pathogenicity

219 (χ^2 = 10.78, f.d. = 8: p = 0.214) with LC_{50} values of 9,015 OB/ml (5,796 – 13,875) and

220 13,437 OB/ ml (8,628 - 20,685) for Se-OX3 and MbNPV respectively. Neither these

221 viruses had time to death responses significantly different from one another: Se-OX3

222 required 69.83 (62.11 – 78.06) h to kill 50% of a treated population, while MbNPV

223 needed 82.94 (78.99 – 88.20) h (Weibull, r = 0.861).

224 Time to mortality curve patterns consistently revealed two different trends for each

225 species groups (Fig. 5). SeMNPV-like virus induced deaths peaked at 40 h.p.i. and all

226 insects had succumbed to viral death by 48 h.p.i. Deaths caused by MbNPV-like

227 viruses resulted in a distribution with two peaks at 40 h.p.i and at 80 h.p.i., with

228 different proportion of deaths caused by MbNPV or Se-OX3 (Fig. 5a). q-PCR

229 quantification was performed to confirm causes of death for 5 larvae fed with each virus

230 for each mortality record points. Proportions of specific viral DNA were estimated as an

231 average of pooled DNA quantities of 5 larval-samples (SeVP80 or MbP87) normalized

232 to the total viral DNA (SeVP80 + MbP87 readings). All dead larvae collected at 40 h.p.i.

233 harboured SeNPV-like virus predominantly (r = 0.99), irrespective of the virus used to

234 inoculate. Dead larvae sampled at later time records (80 h, 88 h, 96 h, or 104 h)

235 revealed MbNPV-like virus in different proportions (Fig. 5c and 5d), being the only virus

236 detectable in samples collected at 88, 96, and 104 h.p.i.

237 **Discussion**

238 *Spodopera exigua* larvae injected with polyhedrin negative SeMNPV genomes

239 amplified using low copy number bac vectors died with symptoms typical of a

240 baculovirus infection but also produced occluded virus. This might have been a

241 consequence of spontaneous bac vector excision from its insertion site within

242 *polyhedrin*, thus restoring native gene function. However, analysis of virus genomes

243 revealed restriction fragment length polymorphisms elsewhere in the virus genome

244   inconsistent with this hypothesis. Further, control insects suffered spontaneous virus

245   infection despite virus-free rearing conditions. This same population of insects was

246   used as negative controls in studies to detect covert infections in *M. brassicae*

247   populations (Burden *et al.,* 2003, Burden *et al.,* 2006) and considered virus-free.

248   However, the primers used in the PCR-based tests in these earlier studies were

249   optimised for MbNPV and may have failed to amplify SeMNPV DNA. Covert infections

250   were postulated to explain the different REN profiles of progeny after cross-infections

251   with heterologous NPVs (Longworth & Cunningham, 1968; Fuxa *et al.*, 1992; Hughes

252   *et al.,* 1993; Cooper *et al.,* 2003; Kouassi *et al.,* 2009) or a sudden occurrence of overt

253   infection in healthy populations (Fuxa *et al.*, 1999). In this study, production of a

254   different viral progeny was observed after *in vivo* transfection with Se.BAC genomes or

255   *per os* OB infections with MbNPV. It was also observed in untreated larvae (water-

256   injected and non-injected controls) so there may have been a stress in rearing

257   conditions during the experiment acting as a trigger. Previously, stress factors such as

258   crowded rearing conditions and high humidity were reported to induce higher

259   prevalence of NPVs in the progeny of a *Trichoplusia ni* laboratory culture (Steinhaus,

260   1958: Fuxa & Richter, 1999). However, the mortality induced after *in vivo* transfections

261   in our studies fluctuated between groups of insects reared under similar conditions.

262   This suggests that despite genetic in-breeding of the *S. exigua* population, certain

263   individuals are more likely to suffer triggering of covert to overt infection or simply that

264   levels of virus vary between them.

265   We compared different viruses from *S. exigua* using REN analysis and DNA

266   sequencing. Interestingly, we observed a degree of genotypic variability among

267   SeNPV-like reactivated virus and a second species resembling MbNPV. Sequencing

268   generated data for *lef*-8, *lef*-9 of SeMNPV-like viruses that not only showed high

269   similarity to the published SeMNPV genome, but no major differences between DNA

270   from overtly and covertly infected insects. Only the *rr1-polh* intergenic region revealed

271   that 10 out of 20 covert and overt DNA infections contained a 12 bp repeat not present

272   in the wild type initially used for cloning. Although we analysed a very small part of the

273   genome (approximately 2,173 bp), these results suggest that the viral genome might

274   not suffer major changes when transformed from a fully lethal form into a covert form or

275   *vice versa*.

276   Assuming a high degree of similarity at the nucleotide level between the virus genomes

277   in covert and overt infections we developed a PCR-based technique capable of

278   detecting small quantities of viral DNA specific for any of the identified reactivated

279   NPVs. Regarding sensitivity, we detected as little as 0.06 pg of viral DNA which

280   equates to 490 and 366 genomes of SeMNPV or MbNPV respectively. Previous

281   studies carried out on different baculovirus species reported a wide range of detection

282   limits for standard PCR (i.e. 10 pg for *Plodia internpunctella* GV DNA Burden *et al.,*

283   2002; 0.0038 pg for MbNPV DNA, Burden *et al.*, 2003) or nested PCR (0.1 pg for *S.*

284   *exempta* NPV, Vilaplana *et al.*, 2010). In this study, q-PCR enabled us to assess viral

285   load in insects and trends over time, as opposed to expression studies that focus on

286   the activity of the virus using RT-PCR detection.

287   Vertical transmission of SeMNPV was induced in *S. exigua* when L_5 larvae were fed

288   virus (Smits & Vlak, 1988). A persistent, low-level infection of *Plodia interpunctella* GV

289   was established in progeny derived from infected adults (Burden *et al.*, 2002). We

290   assessed the prevalence of covert virus over two generations in *S. exigua* both of

291   which had seen some insects succumb to a spontaneously reactivated NPV. Across

292   the six lines tested, 5 to 56% of 80 insects were shown to harbour the virus as a covert

293   infection, with only one line registering NPV-induced deaths. It was possible to

294   demonstrate that levels of viral DNA fluctuated considerably throughout development.

295   In an earlier study, spontaneous NPV-induced deaths were reported to occur in mature

296   larvae but rarely in early instars (Karpov, 1979). Consistently, in this study the levels of

297   covert virus DNA dramatically increased with larval development up to 10^4 fold,

298   suggesting an extremely active viral replication period that eventually caused the

299   deaths observed at L_4. Very low viral levels relative to host DNA were found at later

300   instars, pupae and adults when the virus may not be actively replicating and the host

301   may have suppressed a productive infection.

302   Larvae from *S. exigua* laboratory culture with a covert infection were subjected to a

303   super-infection with each reactivated virus and two SeMNPV and MbNPV wild types.

304   Unexpectedly, no correlation between dose and mortality was observed for the SeNPV-

305   like virus tested on L_2. A dose as low as 9.9×10^3 OBs/ml (3 OBs/L_2) produced over

306   50% mortality although there was no NPV-induced mortality in controls and virtually all

307   second instar larvae fed with SeNPV-like viruses died by 48 hpi. Murillo *et al.*, 2006

308   previously estimated the pathogenicity and virulence of the Se-G26 in similar bioassay

309   conditions, with the LD_{50} value of 9.3×10^4 OBs/ml (31 OBs/L_2) and the MTD value of

310   104 h.p.i.. It seems plausible that the covert virus became active when the larvae were

311   challenged with SeMNPV-like virus derived from an overt infection. Interestingly, the

312   MbNPV-like viruses, Se-OX3 and the reference MbNPV which were indistinguishable

313   by RFLP presented close response in terms of their LD_{50} and MTD. Remarkably,

314   MbNPV-like viruses as a group produced a very different response comparing to

315   SeMNPV-like viruses. Only ~ 10% of the OB progeny from MbNPV-challenged larvae

316   resulted in SeMNPV-like reactivation detected by qPCR.

317   Our study indicates that a *S. exigua* laboratory culture may harbour a covert infection

318   caused by two distinct NPV species. Covert infections have been proposed as the viral

319   strategy in response to variation in transmission opportunities, through fluctuating host

320   population (Burden *et al.*, 2003, Cooper *et al.*, 2003). Theoretically, low levels of covert

321   infections may be explained as an evolved parasite strategy when transmission

322   opportunities vary (Sorrel *et al.,* 2009). However, high prevalence of NPV covert

323   infections has been consistently found in field lepidopteran populations (Burden *et al.*,

324   2003, Vilaplana *et al.,* 2010). The apparent loss of persistent virus infection observed

325   in some of the experiments in this study is intriguing, but may simply reflect the

326   difficulties in detecting the pathogen, particularly when there is no associated

327   phenotype. In other systems used to study vertical virus transmission, such as

328   *Drosophila melanogaster* and sigma virus, the pathogen renders the host susceptible

329   to CO_2 (L'Heritier, 1970, Brun and Plus, 1980), which provides a convenient marker of

330   virus infection. Yampolsky *et al.* (1999) showed that CO_2 sensitivity was lost from two

331   female populations, suggesting the virus was no longer present. In *S. exigua*,

332   persistent baculovirus infections may be lost spontaneously in some individuals but in

333   our breeding population it must be maintained in the majority. This putative virus loss

334   may be a consequence of antiviral immunity in the host. Information on host immunity

335   is well developed for *Drosophila* (Wang *et al.*, 2010) but less so for lepidopteran

336   species. This should be a productive area for future studies on persistent baculovirus-

337   host interactions.

338

339   **Methods**

340   **Virus and insects.** *S. exigua* were reared on diet (Hunter-Fujita *et al.,* 1998) at 25°C,

341   16:8 light: dark photoperiod, and 60 – 70 % humidity. The SeMNPV (SeMNPV-G26)

342   was originally collected from greenhouses in Almeria (Spain) (Murillo *et al.,* 2007;

343   Murillo *et al.,* 2006). MbNPV was used as a comparative control.

344   **Cloning of the SeMNPV into BAC vector.** A bacmid containing the SeMNPV-G26

345   genome was constructed by vector insertion at the polyhedrin locus. Two µg of CsCl-

346   purified viral DNA (King & Possee, 1992) and 100 ng pBAC.SanDI vector based on

347   pBAC.3.6 (Hitchman, 2002) were digested with *San*DI (Stratagene, UK). The reactions

348   were treated for 15 min at 65°C and the vector dephosphorylated with CIP (New

349   England Biolabs, UK). Digested SeMNPV-G26 and gel-purified vector were ligated

350   using 5 units of T4 DNA ligase (Promega, UK) at a ratio of 1:20 and dialysed against

351   TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 4°C. One tenth of this reaction was used

352 to electroporate Gene-hog cells (Invitrogen, UK), which were amplified on agar plates

353 containing chloramphenicol (12.5 µg/ml). SeMNPV bacmids were selected from 100

354 clones and designated SeBAC-G26.

355 **Transfection of *S. exigua* larvae with BAC genome DNA.** SeBAC-G26 DNA was

356 mixed with Lipofectin (300 µg/ml; Invitrogen) and injected into early L_4 *S.exigua* using a

357 micro-applicator fitted to a syringe and Microfine needle (G30). 100 ng of circular viral

358 DNA in 6µl was injected into the haemocoel. Injected and control larvae were incubated

359 at 26 ± 2°C and checked daily for infection. Dead larvae were smeared on slides and

360 inspected under a contrast phase microscope (x 400) for OBs.

361 **Viral DNA isolation and RFLP analysis.** OBs and virus particles were purified as

362 described by King and Possee (1992). Virus particles were lysed by addition of 0.5 M

363 Na_2CO_3, 0.1 % SDS and incubated for 2 hours at 65º C with proteinase K (200 µg/ml).

364 DNA was purified by phenol/chloroform extractions and ethanol precipitation,

365 resuspended in 30 µl water, digested with *Bgl*II, fractionated on 0.6% or 1.2% agarose

366 gel with TBE buffer, (40 mM Tris-HCl; 40 mM Boric Acid, 1 mM EDTA) and stained with

367 SYBR Gold (Invitrogen, UK).

368 **Detection of covert infections by q-PCR.** A SYBR Green based PCR method was

369 used to quantify baculovirus DNA. Oligonucleotide primers were designed targeting the

370 VP80 and P87 genes in SeMNPV and MbNPV respectively (Table 1). The *S. exigua*

371 Elongation Factor (EF) Alfa gene sequence (Gene bank accession no. AF151624) was

372 used as a host reference (Table 1). All reactions were performed using Platinium SYBR

373 Green q-PCR SuperMix-UDG in 20 µl containing 1 × reaction buffer, 10 pmoles/µl of

374 each primer and 5 µl of template DNA. Standard curves were generated using $7 \times \log_{10}$

375 dilutions of CsCl - purified virus DNA (6×10^5 – 0.06 pg). Six water (minus template)

376 controls were included in each batch of 72 samples. The q-PCR was performed in a

377 Rotor Gene 6000 thermal cycler (Corbett Research, Cambridge, UK) and fluorescence

378 recorded on the Green / FAM channel with a gain setting of 5. Cycling conditions were

379 determined for each primer pair against its target DNA. A common hold step of 2 min at

380 95 °C was followed by 40 cycles of amplification involving a denaturation step of 30 sec

381 at 95 °C, an annealing step of 30 sec at 62 °C, and elongation step of 17 sec at 72 °C.

382 Melting curves were generated by fluorescence readings over ramped temperatures at

383 the end of cycling between 50 to 99°C. The specificity of primers was assessed by

384 testing for cross reactivity against MbNPV, SeMNPV, or host DNA. Quantification was

385 by comparison of cycle time at which the fluorescence exceeded the threshold level

386 generating the best fit regression with respect to the standards. Only standard curves

387 in which the regression ratios (R^2) exceeded 0.99 were considered sufficiently accurate

388 for determination of persistent virus levels. The readings obtained for the primer set

389 SeEFalfa were used for the standardization of the viral DNA with host DNA.

390 **Levels of covert virus during *S. exigua* development.** Fifty fourth instar *S. exigua*

391 were individually reared on virus-free diet in a UV-sterilized incubator and sexed at

392 pupation. Twenty couples were mated in paper bags with water feeding pots. Females

393 laid eggs for 2 - 3 days and then parents were frozen for subsequent analysis. Eggs

394 were placed in 300 ml polypots containing artificial diet. When neonates emerged, 25

395 insects were individually reared to adults (offspring F1) in multi-well plates with diet.

396 Insects were observed daily for signs of NPV infection (Supplementary Figure 1). The

397 survivors in lines from which some F1 offspring died of spontaneous overt NPV

398 infections were allowed to produce F2 offspring. Three more F1 lines with no deaths

399 were also allowed to generate an F2. Ten asymptomatic individuals at each

400 development stage of F2 were frozen for subsequent DNA analysis by q-PCR.

401 Remaining individuals were monitored for NPV deaths. The NPV-killed progeny were

402 stored at -20°C for subsequent REN analysis as described above.

403 Total DNA from insect development stages was extracted using a Tepnel robot (Life

404 Sciences, UK) with the Nucleoplex plant DNA kit. Half of each L5, pupae and adult

405 samples were used to avoid column overload, while eggs, L_1, L_2 and L_3 samples were

406   processed in their entirety. Extractions were performed in a 96 well format in tubes

407   which contained a metal ball bearing. Tissue disruption was facilitated by using a bead

408   beater (Biorad, USA) at 1.5 Hz for 2 min once the lysis buffer samples were dispensed.

409   The homogenized samples were incubated in a water bath at 65°C for 2 h prior to DNA

410   extractions

411   **PCR and sequencing of reactivated and persistent virus.** To characterize virus in

412   *S. exigua*, sequence data from three well conserved genes was assessed (Jehle *et al.,*

413   2006). Forward and reverse oligonucleotide primers sets were designed targeting three

414   SeMNPV genome regions (GenBank accession no AF169823): the *rr1-polyhedrin*

415   intergenic region (5' cgacgactttgtgcaccagc 3' and 5' ttcatcgtgtccggtttgacg 3'), *lef-9* (5'

416   gccacgttgtcgtttttgta 3' and 5' aactcagcacccaagtctcg 3') and *lef-8* (5'

417   aacgagctgtcgtatctgcg 3' and 5' agccaatcttgacgttggac 3') for the amplification of 910,

418   730, and 533 bp fragments respectively. Each reaction contained 5 µl of template viral

419   DNA, 0.3 µl of 2 mM dNTP, 5 µl of 10x buffer, 1 unit Taq polymerase enzyme, and 1 µl

420   of primer (10 pM) in a volume of 50 µl. The thermocycling program comprised 30

421   cycles of 94 ºC (4 minutes), 58 ºC and 72 ºC (1 minute each)in a Primus 96

422   thermocycler (MWG-Biotech). The DNA products were purified and sequenced with

423   primers used for the generation of the DNA plus a second internal primer for the *rr1-pol*

424   intergenic region (Re 5' ccatcgtcgttgttcgt 3') and *lef-9* (Re 5' cgctaacacggactgcac 3').

425   Sequencing reactions were performed according to the supplier's recommendation and

426   analysed on an Applied Biosystems 3730. Trace files (forward and reverse) were

427   aligned to generate a consensus sequence for each PCR product (Staden *et al.*, 2003).

428   Sequences were aligned and compared using Clustal X (Thompson *et al.,* 1997).

429   **Bioassay.** Prior to bioassays fresh stocks of Se-OX1, Se-OX2, Se-OX3 and wild types

430   SeMNPV-G26 and MbNPV were amplified by droplet feeding L_4 *S. exigua* with 10^7

431   OBs/ml. The identity of viral inocula was confirmed by REN and PCR.

432   Dose-mortality responses of Se-OX1, Se-OX2, Se-OX3 were measured using

433   bioassays with second instar *S. exigua* fed on virus droplets (Hughes and Wood,

434   1981). OBs were counted in a haematocytometer and used immediately to make

435   dilutions in sterile water containing 5% blue food dye (Langdale). The doses were

436   estimated to produce mortalities between 5 and 95%: 9.9×10^3, 2.7×10^4, 8.1×10^4,

437   2.5×10^5, and 7.4×10^5 OBs/ml and 3×10, 3×10^2, 3×10^3, 3×10^4 and 3×10^5

438   OBs/ml for SeMNPV-like and MbNPV-like viruses, respectively.   Second instar *S.*

439   *exigua* were selected by head capsule size, moulted and starved for 16 hours prior to

440   virus dosing. Seven groups of larvae containing 30 individuals for each virus treatment

441   were used. Larvae that ingested droplets within 10 min were individually transferred to

442   25 - well plates containing diet and reared at 25 ± 2°C. Mortality was recorded every 12

443   h for 7 days. Bioassays were performed on four occasions.

444   A single dose bioassay was carried out to determine the dose-time response following

445   the same method. A group of 75 L_2 *S. exigua* were dosed with each virus to produce in

446   excess of 95% mortality. Mortality was recorded every 8h for 5 days.

447   **Statistical analyses.** Dose-mortality responses were subjected to General Linear

448   Models using Minitab (2006) by fitting the data in the minimal possible model for

449   proportionate mortality as response, the virus as fixed factor with 5 levels and log dose

450   as a covariant. Mortality data not fitting normal distribution were subjected to squared

451   transformation previously. Model behaviour was checked by examination of the

452   distribution of residual and fitted values.

453   To compare relative virulence Median lethal concentration (LC_{50}) and Mean time to

454   death (MTD) were estimated for viruses showing significantly positive dose-virus

455   response. Probit analyses assuming logic data distribution were used to estimate the

456   LC_{50}. MTD was estimated fitting mortality-time data as a Weibull distribution as right

457   censored data excluding individuals that did not die from virus infection from the

458   analysis (Farrar & Ridgway, 1998).

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463

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581   **Table 1.** Oligonucleotides used for quantification of SeMNPV and MbNPV by Q-PCR.

Primer	Primer Sequence (Fw / Re)	*Range of detection		Amplicon melting temperature for specific template (°C)		
		pg	viral genomes	MbNPV	SeMNPV	*S. exigua*
SeVP80	5'-CGAGCGCTGTTGATGAAATAG-3' 5'-GAATTTAACGGCCATCAACG-3'	6×10^5 - 6×10^{-2}	490	n.p.	86	n.p.
MbP87	5'-GCATTAGGGTGTCGTTATCG-3' 5'-CCATCGTTATTAGCCTCGACA-3'	6×10^5 - 6×10^{-2}	366	83	n.p.	n.p.
SeEF	5'-GGCTGGTATCTCGAAGAACGG-3' 5'-GCTTGACACCGAGTGTGAAAGC-3'	2×10^5 - 2×10^{-2}	n.a.	n.p.	n.p.	82

582   * The detection range was determined from amplifications of ten–fold dilution of purified

583   virus DNA. The specificity of the primers was indicated by the generation of a single

584   amplicon with a specific melting temperature and no cross reactivity to the

585   heterologous virus.

586   n.a. = not applicable

587   n.p.= no detectable product for the template range tested

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592 **Table 2.** Frequency of spontaneous NPV-mortality in F1 and F2 offspring, and q-PCR

593 quantification of Se-like persistent DNA for F2 offspring across six lines of *S. exigua*

594 insects generated by breeding two generation (supplementary figure).

595

Breeding line (F2)	Number of spontaneous overtly Infected insects		SeNPV-like persistent viral DNA	
	[1]F1 (parent line)	[2]F2	[3]Mean ± se (pg)	N (q-PCR positives)
Lines in which spontaneous NPV infections were recorded in F1				
Line 1-b	2	0	1.26 ± 0.039 b	5/80
Line 1-c	2	0	1.72 ± 0.020 b	4/80
Line 12-b	5	10	$3.51 \pm 0.101 \times 10^2$ a	45/80
Lines in which spontaneous infections were not recorded in F1				
Line 7-b	0	0	1.73 ± 0.053 b	6/80
Line 16-a	0	0	0.31 ± 0.035 b	6/80
Line 18-a	0	0	0.29 ± 0.036 b	8/80
Background levels in water controls			$1.80 \pm 0.071 \times 10^{-2}$ c	n.a

596 [1]N=25

597 [2]N=75

598 [3]Average of q-PCR positive insects (N) data pooled from different development

599 stages. Different letters denoted significant differences by the t test (p < 0.005).

600

601

24

Figure legends

603 **Fig. 1.** Percentage NPV Mortality following intrahaemocelic injections with SeMNPV

604 DNA in fourth instar *S. exigua* larvae. Six repetitions were carried out on different days

605 and included three different treatments: Se-G26, Se-BAC/G26 and water as control (a)

606 or Se.BAC/G26, water, and non treated controls (b). The percentage mortality was

607 calculated from 75 L_4 treated larvae at each repetition. The absence or presence of a

608 functional polyhedral gene in the virus genomes used as inocula or generated in the

609 bioassays is shown. (c).

610

611 **Fig. 2:** Genome profiles of *Bgl*II-digested virus DNA. Lane 1, wild type Se-G26; lane 2,

612 Se.Bac-G26; lanes 3-6, viral progeny from larvae transfected with Se.Bac-G26; lane 7,

613 MbNPV; lanes 8-16, viruses from larvae infected per os with MbNPV. DNA digests

614 were fractionated in 0.6% (panel a) or 1.2% agarose (panel b) . Three different variants

615 Se-OX1 (lanes 3 and 8), Se-OX2 (lane 4), Se-OX3 (MbNPV-like) (lane 5), and the

616 mixed population Se/Mb (lanes 6, and 15) were detected. BAC vector fragments are

617 denoted by grey arrows in lane 2. Positions of additional bands that do not appear in

618 Se.BAC/G26 (lane 2) are denoted by white arrows. Fragment sizes in kilobase pairs on

619 the left. Number of individuals containing each *Bgl*II variant (N=86) is showed in panel

620 c.

621

622 **Fig. 3**: Log covert SeMNPV-like DNA (SeVP80, open diamonds) or MbNPV-like

623 (MbP87, solid diamonds) DNA standardized by host DNA as measures in

624 asymptomatic insects from sampled as eggs, L_1, L_2, L_3, L_5, pupae, and adult of line 12-

625 b (F2 offspring). Viral DNA levels for fully lethal infected L_4 larvae were included as a

626 reference assuming a value of 1 for host DNA.

627

628

629   **Fig. 4.** Logit mortality of second-instar *S. exigua* larva challenged with Se-G26 (solid

630   square), Se-OX1 (solid diamond), Se-OX2 (solid triangle), Se-OX3 (open square), and

631   MbNPV (open diamond). Data are given as logit mortality where logit (mortality) = ln

632   (p/1-p) and p is the proportionate mortality. The solid line shows the fitted value for Se-

633   OX3 challenged insects: logit (mortality) = - 7.646 + 0.647 $\log_{10}$ (OB/ml) and the dotted

634   line for MbNPV challenged insects: logit (mortality) = - 5.128 + 0.242 $\log_{10}$ (OB/ml).
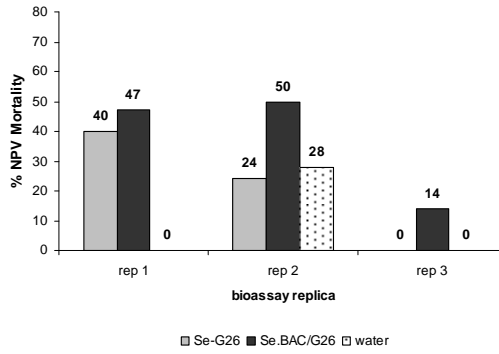
635

636   **Fig. 5.** Mortality of second-instar *S. exigua* larvae over time following inoculation with

637   the SeMNPV-like viruses Se-OX1, Se-OX2, Se-G26 (dotted lines) the MbNPV-like

638   virus Se-OX3, MbNPV (solid lines) (a). Proportion of Se-like and Mb-like viruses in

639   larvae collected at 40, 80, 88, 96, and 104 h.p.i (pooled data of 5 insects per record

640   time) measured by q-PCR (SeVP80 and MbP87 primers used for SeNPV-like and

641   MbNPV-like virus detection respectively) for insect inoculated with Se-OX1, Se-OX2,

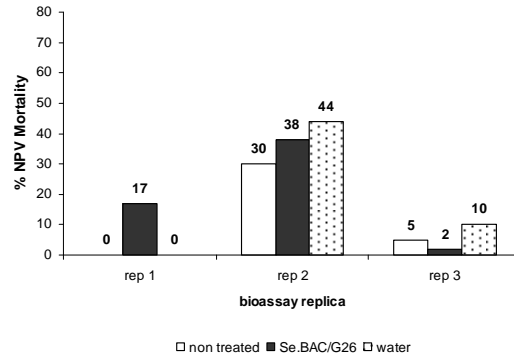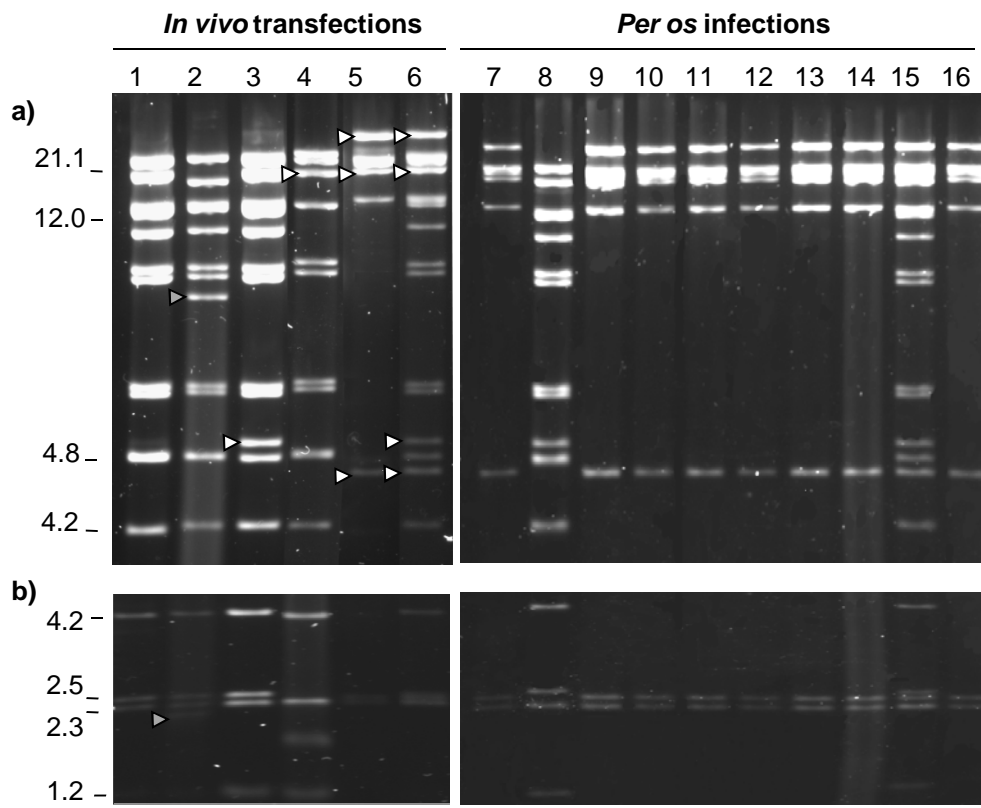642   Se-G26 (b) Se-OX3 (c), and MbNPV (MbNPV) (d).

643

644

a)

Chart a) — y-axis: % NPV Mortality (0–80); x-axis: bioassay replica (rep 1, rep 2, rep 3)

rep 1: Se-G26 = 40, Se.BAC/G26 = 47, water = 0
rep 2: Se-G26 = 24, Se.BAC/G26 = 50, water = 28
rep 3: Se-G26 = 0, Se.BAC/G26 = 14, water = 0

Legend: ☐ Se-G26 ■ Se.BAC/G26 ☐ water

b)

Chart b) — y-axis: % NPV Mortality (0–80); x-axis: bioassay replica (rep 1, rep 2, rep 3)

rep 1: non treated = 0, Se.BAC/G26 = 17, water = 0
rep 2: non treated = 30, Se.BAC/G26 = 38, water = 44
rep 3: non treated = 5, Se.BAC/G26 = 2, water = 10

Legend: ☐ non treated ■ Se.BAC/G26 ☐ water

c)

Treatment	Polyhedrin +/- genome	OBs progeny under microscope
SeBAC/G26	-	+
Se-G26	+	+
Water control	n.a.	+
Non treated control		+

a)

In vivo transfections   *Per os* infections

1 2 3 4 5 6   7 8 9 10 11 12 13 14 15 16

21.1
12.0
4.8
4.2

b)

4.2
2.5
2.3
1.2

c)

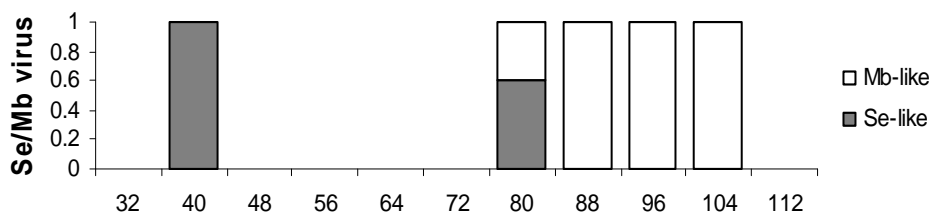| *Bgl*II REN variants	NPV-like	Number
Se-OX1	SeMNPV-like	73
Se-OX2	SeMNPV-like	4
Se-OX3	MbNPV-like	7
Mixed populations	SeMNPV /MbNPV-like	2

a)
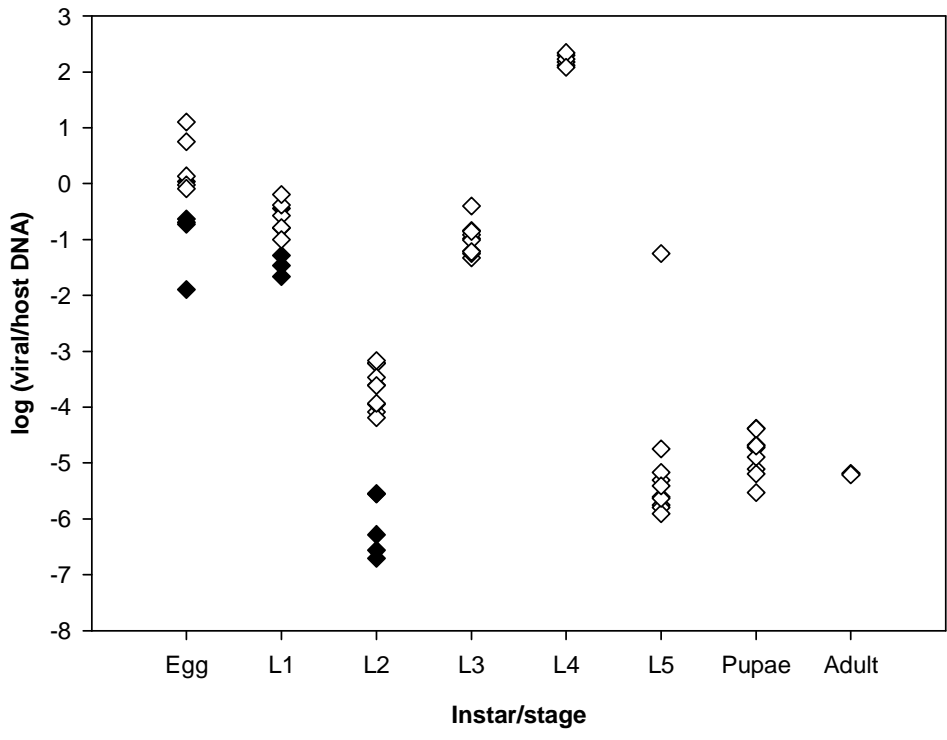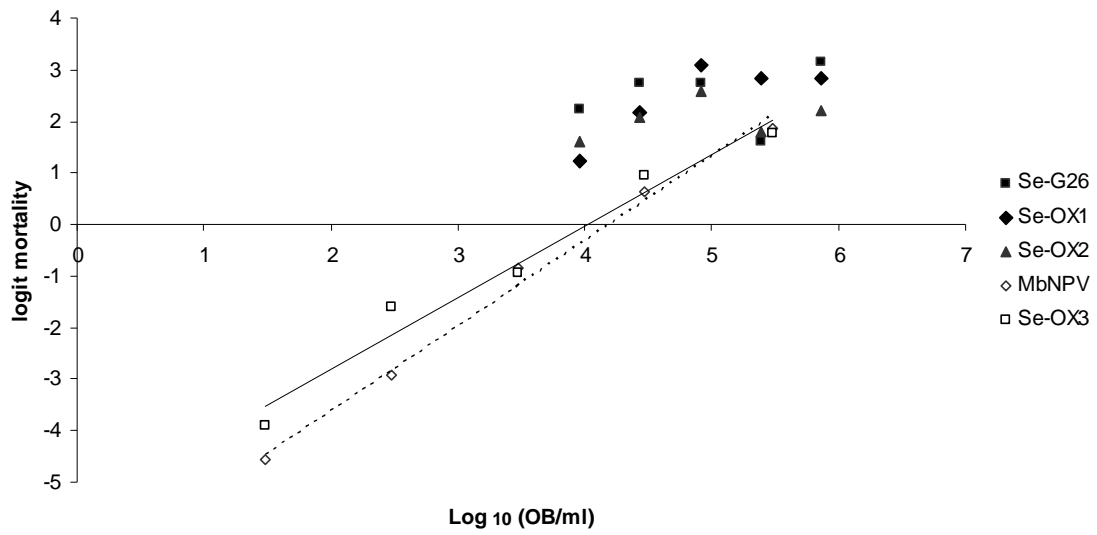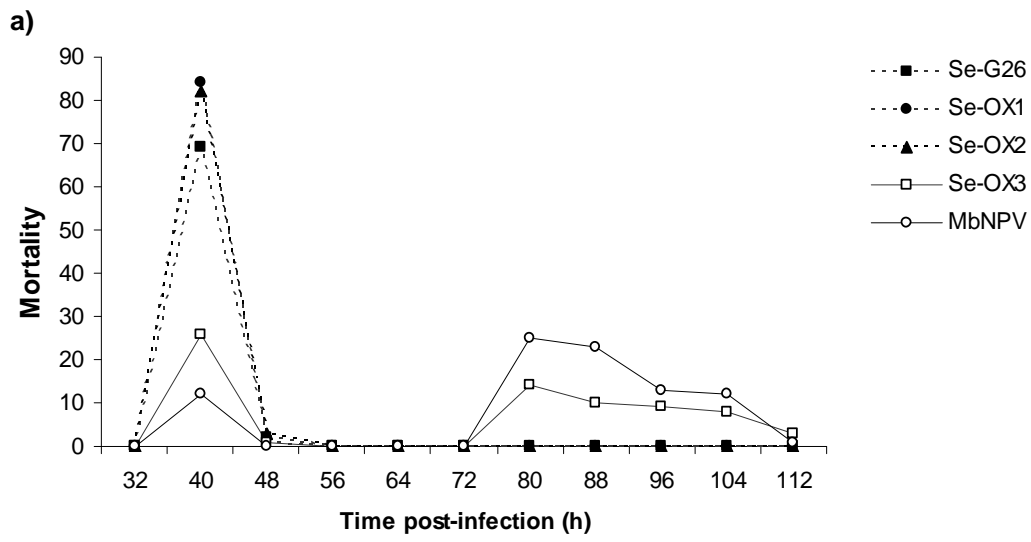
b) Se-G26/Se-OX1/Se-OX2

c) Se-OX3

d) MbNPV