Evidence for covert baculovirus infections in a *Spodoptera exigua* laboratory 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 quantifying 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 $0.29 \pm 0.036$ 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.

Key words: NPVs reactivation, persistent/latent infections, SeMNPV, MbNPV, baculovirus
Introduction

Baculoviruses are pathogenic insect viruses producing infections commonly causing fatalities in Lepidoptera host larvae. Although the horizontal route is thought to be the major pathway for baculovirus transmission (Cory & Myers, 2003), some studies support the idea that their transmission also involves vertical transfer in field populations (See Kukan 1999 for a review). Individuals exposed to low virus doses may acquire a non-fatal sub-lethal infection, but transmit the infection vertically to the next generation (Andrealis, 1987; Cory & Myers, 2003). This may affect insect health and fecundity (Rothman & Myers, 1996; Myers et al., 2000; Vilaplana et al., 2008). Insects may also have a covert infection that does not produce visible symptoms. In this state it is suggested that the virus remains either latent in a non-replicating form, or in a persistent state with low level gene expression (Hughes et al., 1997, Burden et al., 2003). Covert infections may not be critical for maintaining virus populations in nature but could enable the parasite to persist during periods of low host population density especially in seasonal environments (Burden et al., 2003). Another advantage to the virus of being in a covert state is that it could be carried long distances during host migration (Anderson & May, 1981). Interestingly, vertical transmission appears to be frequent in more mobile insect species such as those within the genus Spodoptera (Swaine, 1966; Abul-Nasr et al., 1979; Smits & Vlak 1988, Fuxa & Richter 1991, Vilaplana et al., 2008).

Spontaneous emergence of overt infections from a covertly infected population suggests that the viruses remain replication competent during the quiescent state. Common features of persistent viral infections include the selection of cell subsets ideal for long-term maintenance of the viral genome (Kane & Golovkina 2010). Studies targeting baculoviruses detected the presence of viral DNA in adult tissues such as the fat body (Hughes et al., 1993, 1997) and gonads (Burden et al., 2002, Khurad et al., 2004), which ultimately are involved in transmitting virus to subsequent generations.
Spontaneous NPV outbreaks have been recorded in laboratory cultures reared under virus-free conditions over several generations. Karpov (1979) reported a *Bombyx mori* NPV outbreak to be a problem in practical sericulture. Few studies have been conducted to elucidate the underlying phenomena (Hughes *et al.*, 1997, Fuxa *et al.*, 1999), so little is known about the mechanism(s) involved when a covert virus is triggered into an overt infection (Cory & Myers 2003). Advances in molecular techniques have improved the detection of viral DNA or its transcripts in asymptomatic insects. For instance, a laboratory culture of cabbage moths (*Mamestra brassicae*) was reported to harbour a covert baculovirus infection without prior exposure to such viruses (Hughes *et al.*, 1993). This virus was activated to an overt state by challenging the host with a heterologous baculovirus and the covert virus identified as *M. brassicae* NPV (MbNPV). Using PCR the polyhedrin gene was detected in individuals of this population at all ages indicating this population maintained MbNPV-like persistent virus (Hughes *et al.*, 1997). Similar results were obtained using the immediate early gene 1 as a target (Burden *et al.*, 2006) although the presence of the entire virus genome in the covert state has yet to be demonstrated. A serendipitous observation reported here leads us to conclude that a laboratory population of *Spodoptera exigua* also contains covert baculoviruses.

### Results

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

Inserting whole baculovirus genomes in low copy number bacmid vectors in *E. coli* was used to clone *S. frugiperda* multinucleopolyhedrovirus (SfMNPV) (Simón *et al.*, 2008) and SeMNPV DNA (Pijlman *et al.*, 2002, Pijlman *et al.*, 2003). The Se.BAC/G26 genomes derived from cloning SeMNPV-G26 in this study were *polyhedrin* negative and used to transfect fourth instar (L4) *S. exigua* by DNA injection into haemocoels. Insect mortality recorded after injections ranged from 2 % to 50 % (Fig. 1a and b) in six...
replicates. Similar variation in mortality was found in groups of insects injected with the wild type Se-G26 (0 - 40%). More surprisingly water-injected control and non treated insects also suffered significant mortalities (10-44% and 5-30% respectively).

A total of 86 cadavers with symptoms of NPV infection after in vivo transfection were inspected for OBs under a microscope and their DNA analysed by restriction enzyme digestion. Despite Se.BAC/G26 genomes being polyhedrin negative (Fig 1c), OBs were consistently observed in all samples. The DNA analysis confirmed the viruses (Fig 2, lane 3-6) did not contain the BAC replicon in the original vector (Fig. 2, lane 2). Additional fragments were present in the progeny DNA profiles after in vivo transfection with Se.BAC/G26, suggesting that the SeBAC clones had not simply lost the vector sequences after growth in insects. Three variants were identified in different proportions (Fig. 2c) after digestion of virus DNA with BglII. Two variants had similar profiles to SeMNPV-G26 and were named Se-OX1 and Se-OX2. These were observed from 73 and 4 viral deaths, respectively from a total of the 86 profiled. Another variant (Se-OX3) found in 7 insects showed an identical profile to MbNPV, while a mixture of SeMNPV and MbNPV (Fig. 2, lane 6) was found in 2 cases (Fig. 2c). The Se-OX3 variant had the same profile as MbNPV when digested with PstI, XhoI, EcoRV, EcoRI, Clal, and HindIII (data not shown).

Se-OX1 and Se-OX2 variants were observed in larvae injected with Se.BAC/G26, SeMNPV-G26 and water. Se-OX1, Se-OX2, and Se-OX3 were also found in non challenged insects (non-treated controls), suggesting they had been reactivated spontaneously in those cases.

The L4 S. exigua larvae thought to be virus-free were infected with approximately $10^8$ OB/ml of MbNPV. Nine individuals succumbed to overt infection, virus was isolated and their DNA profiles after restriction enzyme digestion compared with MbNPV (Fig. 2, lanes 8-16). Seven insects produced viruses with DNA profiles typical of MbNPV but
one resembled Se-OX1 (Fig. 2, lane 8) and another a mixed population of MbNPV and SeMNPV (Fig. 2, lane 15) respectively.

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

Spontaneous outbreaks of virus disease in *S. exigua* were indicative of high levels of virus DNA. However, PCR methods where products were detected by electrophoresis failed to detect covert virus consistently when *lef8*, *lef9* and *rr1-pol* primers were used to test DNA samples from asymptomatic L4 larvae. Hence, a more sensitive approach using q-PCR was optimized. Sensitivity and specificity were tested by using serial dilutions of either SeMNPV or MbNPV DNAs (Table 1). This demonstrated that SeVP80 and MbP87 primers could detect 0.06 pg virus DNA (3×10^{-6} ng/µl in 20 µl reaction) which equated to 490 and 366 for SeMNPV and MbNPV genomes, respectively with no cross-reactivity (Table 1). Host DNA did not interfere with the PCR (data not shown).

This q-PCR method was used to quantify covert virus DNA throughout insect metamorphosis to correlate it with NPV mortality records in the host population. Twenty breeding couples were used to establish separate F1 populations for rearing 25 adults (Supplementary Figure 1). Four matings produced no offspring and two derived larvae where 96% died from baculovirus infection. None of these could be maintained further. Two other populations (1 and 12) with 8% virus deaths in the larvae (Table 2) were used to produce three F2 populations (1a-c and 12a-c), while others (7a-c, 16a-c and 18a-c) derived from F1 populations with no virus deaths. However, not all breeding pairs produced offspring (Supplementary Figure 1). Ten samples from each developmental stage (eggs, L1-L5 and adults) were screened for virus genomes using q-PCR (Table 2). The average F2 viral DNA levels across developmental stages varied from 1.72 ± 0.020 to 0.29 ± 0.036 pg (Table 2) for five lines, significantly higher than in water controls (1.80 ± 0.071 ± 10^{-2} pg) (t-test pairwise, p < 0.05). Offspring from line 12b recorded the highest virus DNA levels of 3.51 ± 0.101 ± 10^{5} pg with 45 positive
insects from a total of 80 tested insects (t-test pairwise, p < 0.05). Similar proportions of insects harbouring viral DNA were found across the rest of the lines tested (Table 2) and no trends for prevalence of virus between developmental stages was observed (Supplementary Table 1).

Covert viral DNA levels monitored throughout the host development cycle were further studied in the offspring from line 12b as 10 cases of apparent NPV-induced mortality were found in L4 (Table 2). To distinguish SeMNPV- from MbNPV-like virus we used the SeVP80 and MbP87 primer sets with the SeEF primer set as an internal host DNA control. SeMNPV DNA was detected in eggs, L1, L2, L3, L5, pupae and adults. MbNPV DNA was only detected in eggs, L1 and L2 at levels considerably less than SeMNPV, although 50% of eggs were positive. Healthy L4 individuals were not tested for virus.

REN analysis of larvae that died showed exclusively SeMNPV profiles. When virus DNA levels were normalized to host DNA (SeEF primer set) and log transformed, maximum viral levels were apparent in eggs and first larval instars (Fig. 3). During the larval stage the ratio of virus/host cell DNA decreased in L2 but increased dramatically in L3 indicating the virus was replicated actively, and produced overt infections at L4 instars. L5, pupae, and adults recorded the lowest ratios (Fig. 3), corresponding to similar viral DNA values as those quantified for eggs and L1, suggesting viral replication was minimal. Restriction enzyme analysis of virus DNA from 10 L4 larvae resulted in profiles resembling the Se-OX1 genotype.

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

Sequences for *lef*-8 and *lef*-9 were generated from 10 insects with covert infection (C1-C10) or overt infection resulting from spontaneous virus reactivation (O1-O10) (Supplementary Fig. 2). All twenty individuals had SeMNPV-specific infections. Comparison of a 533 nucleotide region from *lef*-8 showed 100% identity in all samples with Se-G26 (data not shown). For *lef*-9 (730 nucleotides), pairwise comparison to Se-G26 showed between 98 to 99 % nucleotide identity, due to some nucleotide
mismatches that did not affect the gene transcription and functionality. *lef-8* and *lef-9* sequence showed a 100% and 98% nucleotide identity to the SeMNPV published sequence (GenBank accession no AF169823) respectively.

For the 990 bp *rr1-polh* intergenic region, a single repeat of 12 bp was found to be a consensus sequence in samples C2, C3, C5, C6, C7, C9, O4, O5, O9, and O10 while it did not appear in samples C1, C4, C8, C10, O1, O2, O3, O6, O8, and G26. The repeat was positioned in the non-coding region between the orf139 and 1 corresponding to *rr1* and *polyhedrin* genes at the nucleotide positions 135,169 -135,181 bp of the GenBank accession no AF169823 genome (supplementary Fig. 1).

**Biological response to reactivated viruses**

Virus stocks (Se-G26, Se-OX1, Se-OX2, Se-OX3 and MbNPV) were freshly amplified in L4 *S. exigua* larvae. q-PCR carried out with either SeVP80- or MbP87-specific primers showed no detectable presence of SeMNPV-like viruses in MbNPV and Se-OX3 stocks or MbNPV in SeMNPV preparations (Table 1). Bioassays were used to assess the five virus stocks in early second instar *S. exigua* larvae. Non-viral deaths occurred in less than 5% across the four replicates for untreated control insects and were not included in subsequent analysis.

Overall mortality response for the five viruses was significantly affected by the dose (F = 24.54, f.d. = 1, p < 0.001), and virus variant species (F = 18.46, f.d. = 4, p < 0.001). As the interaction dose × virus was also significant (F = 5.84, f.d. = 4, p < 0.005) the data was split into SeMNPV-like and MbNPV-like virus groups which showed a different trend from one another (Fig. 4). For SeMNPV-like virus all viral doses produced over 75% mortality in all four replicates. Neither the dose (F = 3.05, fd = 1, p = 0.110) or the virus variant (F = 0.55, f.d. = 2, p = 0.592) significantly affected the mortality response in this group.
Mortality was found to increase with dose for MbNPV-like viruses ($F = 159.72$, f.d. = 1; $p < 0.001$). No significant differences were found between both viruses in pathogenicity ($\chi^2 = 10.78$, f.d. = 8; $p = 0.214$) with LC$_{50}$ values of 9,015 OB/ml (5,796 – 13,875) and 13,437 OB/ml (8,628 - 20,685) for Se-OX3 and MbNPV respectively. Neither these viruses had time to death responses significantly different from one another: Se-OX3 required 69.83 (62.11 – 78.06) h to kill 50% of a treated population, while MbNPV needed 82.94 (78.99 – 88.20) h (Weibull, $r = 0.861$).

Time to mortality curve patterns consistently revealed two different trends for each species groups (Fig. 5). SeMNPV-like virus induced deaths peaked at 40 h.p.i. and all insects had succumbed to viral death by 48 h.p.i. Deaths caused by MbNPV-like viruses resulted in a distribution with two peaks at 40 h.p.i and at 80 h.p.i., with different proportion of deaths caused by MbNPV or Se-OX3 (Fig. 5a). q-PCR quantification was performed to confirm causes of death for 5 larvae fed with each virus for each mortality record points. Proportions of specific viral DNA were estimated as an average of pooled DNA quantities of 5 larval-samples (SeVP80 or MbP87) normalized to the total viral DNA (SeVP80 + MbP87 readings). All dead larvae collected at 40 h.p.i. harboured SeNPV-like virus predominantly ($r = 0.99$), irrespective of the virus used to inoculate. Dead larvae sampled at later time records (80 h, 88 h, 96 h, or 104 h) revealed MbNPV-like virus in different proportions (Fig. 5c and 5d), being the only virus detectable in samples collected at 88, 96, and 104 h.p.i.

**Discussion**

*Spodopera exigua* larvae injected with polyhedrin negative SeMNPV genomes amplified using low copy number bac vectors died with symptoms typical of a baculovirus infection but also produced occluded virus. This might have been a consequence of spontaneous bac vector excision from its insertion site within *polyhedrin*, thus restoring native gene function. However, analysis of virus genomes revealed restriction fragment length polymorphisms elsewhere in the virus genome.
inconsistent with this hypothesis. Further, control insects suffered spontaneous virus
infection despite virus-free rearing conditions. This same population of insects was
used as negative controls in studies to detect covert infections in *M. brassicae*
populations (Burden et al., 2003, Burden et al., 2006) and considered virus-free.
However, the primers used in the PCR-based tests in these earlier studies were
optimised for MbNPV and may have failed to amplify SeMNPV DNA. Covert infections
were postulated to explain the different REN profiles of progeny after cross-infections
with heterologous NPVs (Longworth & Cunningham, 1968; Fuxa et al., 1992; Hughes
et al., 1993; Cooper et al., 2003; Kouassi et al., 2009) or a sudden occurrence of overt
infection in healthy populations (Fuxa et al., 1999). In this study, production of a
different viral progeny was observed after *in vivo* transfection with Se.BAC genomes or
*per os* OB infections with MbNPV. It was also observed in untreated larvae (water-
injected and non-injected controls) so there may have been a stress in rearing
conditions during the experiment acting as a trigger. Previously, stress factors such as
crowded rearing conditions and high humidity were reported to induce higher
prevalence of NPVs in the progeny of a *Trichoplusia ni* laboratory culture (Steinhaus,
1958: Fuxa & Richter, 1999). However, the mortality induced after *in vivo* transfections
in our studies fluctuated between groups of insects reared under similar conditions.
This suggests that despite genetic in-breeding of the *S. exigua* population, certain
individuals are more likely to suffer triggering of covert to overt infection or simply that
levels of virus vary between them.

We compared different viruses from *S. exigua* using REN analysis and DNA
sequencing. Interestingly, we observed a degree of genotypic variability among
SeNPV-like reactivated virus and a second species resembling MbNPV. Sequencing
generated data for *lef*-8, *lef*-9 of SeMNPV-like viruses that not only showed high
similarity to the published SeMNPV genome, but no major differences between DNA
from overtly and covertly infected insects. Only the *rr1-polh* intergenic region revealed
that 10 out of 20 covert and overt DNA infections contained a 12 bp repeat not present in the wild type initially used for cloning. Although we analysed a very small part of the genome (approximately 2,173 bp), these results suggest that the viral genome might not suffer major changes when transformed from a fully lethal form into a covert form or vice versa.

Assuming a high degree of similarity at the nucleotide level between the virus genomes in covert and overt infections we developed a PCR-based technique capable of detecting small quantities of viral DNA specific for any of the identified reactivated NPVs. Regarding sensitivity, we detected as little as 0.06 pg of viral DNA which equates to 490 and 366 genomes of SeMNPV or MbNPV respectively. Previous studies carried out on different baculovirus species reported a wide range of detection limits for standard PCR (i.e. 10 pg for *Plodia interpunctella* GV DNA Burden *et al.*, 2002; 0.0038 pg for MbNPV DNA, Burden *et al.*, 2003) or nested PCR (0.1 pg for *S. exempta* NPV, Vilaplana *et al.*, 2010). In this study, q-PCR enabled us to assess viral load in insects and trends over time, as opposed to expression studies that focus on the activity of the virus using RT-PCR detection.

Vertical transmission of SeMNPV was induced in *S. exigua* when L₅ larvae were fed virus (Smits & Vlak, 1988). A persistent, low-level infection of *Plodia interpunctella* GV was established in progeny derived from infected adults (Burden *et al.*, 2002). We assessed the prevalence of covert virus over two generations in *S. exigua* both of which had seen some insects succumb to a spontaneously reactivated NPV. Across the six lines tested, 5 to 56% of 80 insects were shown to harbour the virus as a covert infection, with only one line registering NPV-induced deaths. It was possible to demonstrate that levels of viral DNA fluctuated considerably throughout development. In an earlier study, spontaneous NPV-induced deaths were reported to occur in mature larvae but rarely in early instars (Karpov, 1979). Consistently, in this study the levels of covert virus DNA dramatically increased with larval development up to 10⁴ fold,
suggesting an extremely active viral replication period that eventually caused the
deaths observed at L4. Very low viral levels relative to host DNA were found at later
instars, pupae and adults when the virus may not be actively replicating and the host
may have suppressed a productive infection.

Larvae from *S. exigua* laboratory culture with a covert infection were subjected to a
super-infection with each reactivated virus and two SeMNPV and MbNPV wild types.
Unexpectedly, no correlation between dose and mortality was observed for the SeNPV-
like virus tested on L2. A dose as low as $9.9 \times 10^3$ OBs/ml (3 OBs/L2) produced over
50% mortality although there was no NPV-induced mortality in controls and virtually all
second instar larvae fed with SeNPV-like viruses died by 48 hpi. Murillo *et al.*, 2006
previously estimated the pathogenicity and virulence of the Se-G26 in similar bioassay
conditions, with the LD$_{50}$ value of $9.3 \times 10^4$ OBs/ml (31 OBs/L2) and the MTD value of
104 h.p.i.. It seems plausible that the covert virus became active when the larvae were
challenged with SeMNPV-like virus derived from an overt infection. Interestingly, the
MbNPV-like viruses, Se-OX3 and the reference MbNPV which were indistinguishable
by RFLP presented close response in terms of their LD$_{50}$ and MTD. Remarkably,
MbNPV-like viruses as a group produced a very different response comparing to
SeMNPV-like viruses. Only $\sim$10% of the OB progeny from MbNPV-challenged larvae
resulted in SeMNPV-like reactivation detected by qPCR.

Our study indicates that a *S. exigua* laboratory culture may harbour a covert infection
caused by two distinct NPV species. Covert infections have been proposed as the viral
strategy in response to variation in transmission opportunities, through fluctuating host
population (Burden *et al.*, 2003, Cooper *et al.*, 2003). Theoretically, low levels of covert
infections may be explained as an evolved parasite strategy when transmission
opportunities vary (Sorrel *et al.*, 2009). However, high prevalence of NPV covert
infections has been consistently found in field lepidopteran populations (Burden *et al.,
2003, Vilaplana *et al.*, 2010). The apparent loss of persistent virus infection observed
in some of the experiments in this study is intriguing, but may simply reflect the
difficulties in detecting the pathogen, particularly when there is no associated
phenotype. In other systems used to study vertical virus transmission, such as
*Drosophila melanogaster* and sigma virus, the pathogen renders the host susceptible
to CO₂ (L’Heritier, 1970, Brun and Plus, 1980), which provides a convenient marker of
virus infection. Yampolsky *et al.* (1999) showed that CO₂ sensitivity was lost from two
female populations, suggesting the virus was no longer present. In *S. exigua*,
persistent baculovirus infections may be lost spontaneously in some individuals but in
our breeding population it must be maintained in the majority. This putative virus loss
may be a consequence of antiviral immunity in the host. Information on host immunity
is well developed for *Drosophila* (Wang *et al.*, 2010) but less so for lepidopteran
species. This should be a productive area for future studies on persistent baculovirus-host interactions.

**Methods**

**Virus and insects.** *S. exigua* were reared on diet (Hunter-Fujita *et al.*, 1998) at 25°C,
16:8 light: dark photoperiod, and 60 – 70 % humidity. The SeMNPV (SeMNPV-G26)
was originally collected from greenhouses in Almeria (Spain) (Murillo *et al.*, 2007;
Murillo *et al.*, 2006). MbNPV was used as a comparative control.

**Cloning of the SeMNPV into BAC vector.** A bacmid containing the SeMNPV-G26
genome was constructed by vector insertion at the polyhedrin locus. Two µg of CsCl-
purified viral DNA (King & Possee, 1992) and 100 ng pBAC.SanDI vector based on
pBAC.3.6 (Hitchman, 2002) were digested with SanDI (Stratagene, UK). The reactions
were treated for 15 min at 65°C and the vector dephosphorylated with CIP (New
England Biolabs, UK). Digested SeMNPV-G26 and gel-purified vector were ligated
using 5 units of T4 DNA ligase (Promega, UK) at a ratio of 1:20 and dialysed against
TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 4°C. One tenth of this reaction was used
to electroporate Gene-hog cells (Invitrogen, UK), which were amplified on agar plates containing chloramphenicol (12.5 µg/ml). SeMNPV bacmids were selected from 100 clones and designated SeBAC-G26.

**Transfection of *S. exigua* larvae with BAC genome DNA.** SeBAC-G26 DNA was mixed with Lipofectin (300 µg/ml; Invitrogen) and injected into early L₄ *S.exigua* using a micro-applicator fitted to a syringe and Microfine needle (G30). 100 ng of circular viral DNA in 6µl was injected into the haemocoel. Injected and control larvae were incubated at 26 ± 2°C and checked daily for infection. Dead larvae were smeared on slides and inspected under a contrast phase microscope (x 400) for OBs.

**Viral DNA isolation and RFLP analysis.** OBs and virus particles were purified as described by King and Possee (1992). Virus particles were lysed by addition of 0.5 M Na₂CO₃, 0.1 % SDS and incubated for 2 hours at 65°C with proteinase K (200 µg/ml). DNA was purified by phenol/chloroform extractions and ethanol precipitation, resuspended in 30 µl water, digested with *Bgl*II, fractionated on 0.6% or 1.2% agarose gel with TBE buffer, (40 mM Tris-HCl; 40 mM Boric Acid, 1 mM EDTA) and stained with SYBR Gold (Invitrogen, UK).

**Detection of covert infections by q-PCR.** A SYBR Green based PCR method was used to quantify baculovirus DNA. Oligonucleotide primers were designed targeting the VP80 and P87 genes in SeMNPV and MbNPV respectively (Table 1). The *S. exigua* Elongation Factor (EF) Alfa gene sequence (Gene bank accession no. AF151624) was used as a host reference (Table 1). All reactions were performed using Platinium SYBR Green q-PCR SuperMix-UDG in 20 µl containing 1 × reaction buffer, 10 pmoles/µl of each primer and 5 µl of template DNA. Standard curves were generated using 7 × log₁₀ dilutions of CsCl - purified virus DNA (6 × 10⁵ – 0.06 pg). Six water (minus template) controls were included in each batch of 72 samples. The q-PCR was performed in a Rotor Gene 6000 thermal cycler (Corbett Research, Cambridge, UK) and fluorescence recorded on the Green / FAM channel with a gain setting of 5. Cycling conditions were
determined for each primer pair against its target DNA. A common hold step of 2 min at 95 °C was followed by 40 cycles of amplification involving a denaturation step of 30 sec at 95 °C, an annealing step of 30 sec at 62 °C, and elongation step of 17 sec at 72 °C. Melting curves were generated by fluorescence readings over ramped temperatures at the end of cycling between 50 to 99°C. The specificity of primers was assessed by testing for cross reactivity against MbNPV, SeMNPV, or host DNA. Quantification was by comparison of cycle time at which the fluorescence exceeded the threshold level generating the best fit regression with respect to the standards. Only standard curves in which the regression ratios (R²) exceeded 0.99 were considered sufficiently accurate for determination of persistent virus levels. The readings obtained for the primer set SeEFalfa were used for the standardization of the viral DNA with host DNA.

**Levels of covert virus during *S. exigua* development.** Fifty fourth instar *S. exigua* were individually reared on virus-free diet in a UV-sterilized incubator and sexed at pupation. Twenty couples were mated in paper bags with water feeding pots. Females laid eggs for 2 - 3 days and then parents were frozen for subsequent analysis. Eggs were placed in 300 ml polypots containing artificial diet. When neonates emerged, 25 insects were individually reared to adults (offspring F1) in multi-well plates with diet. Insects were observed daily for signs of NPV infection (Supplementary Figure 1). The survivors in lines from which some F1 offspring died of spontaneous overt NPV infections were allowed to produce F2 offspring. Three more F1 lines with no deaths were also allowed to generate an F2. Ten asymptomatic individuals at each development stage of F2 were frozen for subsequent DNA analysis by q-PCR. Remaining individuals were monitored for NPV deaths. The NPV-killed progeny were stored at -20°C for subsequent REN analysis as described above.

Total DNA from insect development stages was extracted using a Tepnel robot (Life Sciences, UK) with the Nucleoplex plant DNA kit. Half of each L5, pupae and adult samples were used to avoid column overload, while eggs, L₁, L₂ and L₃ samples were
processed in their entirety. Extractions were performed in a 96 well format in tubes which contained a metal ball bearing. Tissue disruption was facilitated by using a bead beater (Biorad, USA) at 1.5 Hz for 2 min once the lysis buffer samples were dispensed. The homogenized samples were incubated in a water bath at 65°C for 2 h prior to DNA extractions.

**PCR and sequencing of reactivated and persistent virus.** To characterize virus in *S. exigua*, sequence data from three well conserved genes was assessed (Jehle *et al.*, 2006). Forward and reverse oligonucleotide primers sets were designed targeting three SeMNPV genome regions (GenBank accession no AF169823): the *rm1-polyhedrin* intergenic region (5’ cgacgactttgtgcaccagc 3’ and 5’ ttcatcgtgtccggtttgacg 3’), *lef-9* (5’ gccaagtgtcttttgtta 3’ and 5’ aactcagcacccaagtctcg 3’) and *lef-8* (5’ aacgagtctgtatctctg 3’ and 5’ agccaatcttgacctggac 3’) for the amplification of 910, 730, and 533 bp fragments respectively. Each reaction contained 5 µl of template viral DNA, 0.3 µl of 2 mM dNTP, 5 µl of 10x buffer, 1 unit Taq polymerase enzyme, and 1 µl of primer (10 pM) in a volume of 50 µl. The thermocycling program comprised 30 cycles of 94 °C (4 minutes), 58 °C and 72 °C (1 minute each)in a Primus 96 thermocycler (MWG-Biotech). The DNA products were purified and sequenced with primers used for the generation of the DNA plus a second internal primer for the *rr1-pol* intergenic region (Re 5’ ccatcgtctttctgct 3’) and *lef-9* (Re 5’ cgaatacagactgc 3’). Sequencing reactions were performed according to the supplier’s recommendation and analysed on an Applied Biosystems 3730. Trace files (forward and reverse) were aligned to generate a consensus sequence for each PCR product (Staden *et al.*, 2003). Sequences were aligned and compared using Clustal X (Thompson *et al.*, 1997).

**Bioassay.** Prior to bioassays fresh stocks of Se-OX1, Se-OX2, Se-OX3 and wild types SeMNPV-G26 and MbNPV were amplified by droplet feeding L4 *S. exigua* with 10⁷ OBs/ml. The identity of viral inocula was confirmed by REN and PCR.
Dose-mortality responses of Se-OX1, Se-OX2, Se-OX3 were measured using bioassays with second instar *S. exigua* fed on virus droplets (Hughes and Wood, 1981). OBs were counted in a haematocytometer and used immediately to make dilutions in sterile water containing 5% blue food dye (Langdale). The doses were estimated to produce mortalities between 5 and 95%: $9.9 \times 10^3$, $2.7 \times 10^4$, $8.1 \times 10^4$, $2.5 \times 10^5$, and $7.4 \times 10^5$ OBs/ml and $3 \times 10$, $3 \times 10^2$, $3 \times 10^3$, $3 \times 10^4$ and $3 \times 10^5$ OBs/ml for SeMNPV-like and MbNPV-like viruses, respectively. Second instar *S. exigua* were selected by head capsule size, moulted and starved for 16 hours prior to virus dosing. Seven groups of larvae containing 30 individuals for each virus treatment were used. Larvae that ingested droplets within 10 min were individually transferred to 25-well plates containing diet and reared at $25 \pm 2^\circ$C. Mortality was recorded every 12 h for 7 days. Bioassays were performed on four occasions.

A single dose bioassay was carried out to determine the dose-time response following the same method. A group of 75 L$_2$ *S. exigua* were dosed with each virus to produce in excess of 95% mortality. Mortality was recorded every 8h for 5 days.

**Statistical analyses.** Dose-mortality responses were subjected to General Linear Models using Minitab (2006) by fitting the data in the minimal possible model for proportionate mortality as response, the virus as fixed factor with 5 levels and log dose as a covariant. Mortality data not fitting normal distribution were subjected to squared transformation previously. Model behaviour was checked by examination of the distribution of residual and fitted values.

To compare relative virulence Median lethal concentration (LC$_{50}$) and Mean time to death (MTD) were estimated for viruses showing significantly positive dose-virus response. Probit analyses assuming logic data distribution were used to estimate the LC$_{50}$. MTD was estimated fitting mortality-time data as a Weibull distribution as right censored data excluding individuals that did not die from virus infection from the analysis (Farrar & Ridgway, 1998).
Acknowledgements

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References


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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>*Range of detection</th>
<th>Amplicon melting temperature for specific template (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Fw / Re)</td>
<td>pg</td>
<td>viral genomes</td>
</tr>
<tr>
<td>SeVP80</td>
<td>5'-CGAGCGCTGTTGATGAAATAG-3' 5'-GAATTTAACGGCCATCAACG-3'</td>
<td>$6 \times 10^5 - 6 \times 10^{-2}$</td>
<td>490</td>
</tr>
<tr>
<td>MbP87</td>
<td>5'-GCAATAGGCGTCTCTTTTATTG-3' 5'-CCATCGTTATTAGCCTCGAC-3'</td>
<td>$6 \times 10^5 - 6 \times 10^{-2}$</td>
<td>366</td>
</tr>
<tr>
<td>SeEF</td>
<td>5'-GGCTGGTATCTCGAAGAACGG-3' 5'-GCTTGACACCGAGTGTGAAAGC-3'</td>
<td>$2 \times 10^5 - 2 \times 10^{-2}$</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

* The detection range was determined from amplifications of ten–fold dilution of purified virus DNA. The specificity of the primers was indicated by the generation of a single amplicon with a specific melting temperature and no cross reactivity to the heterologous virus.

n.a. = not applicable
n.p. = no detectable product for the template range tested
Table 2. Frequency of spontaneous NPV-mortality in F1 and F2 offspring, and q-PCR quantification of Se-like persistent DNA for F2 offspring across six lines of *S. exigua* insects generated by breeding two generation (supplementary figure).

<table>
<thead>
<tr>
<th>Breeding line (F2)</th>
<th>Number of spontaneous overtly infected insects</th>
<th>SeNPV-like persistent viral DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1 (parent line)</td>
<td>F2</td>
</tr>
<tr>
<td>Lines in which spontaneous NPV infections were recorded in F1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Line 1-b</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Line 1-c</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Line 12-b</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Lines in which spontaneous infections were not recorded in F1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Line 7-b</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Line 16-a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Line 18-a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Background levels in water controls</td>
<td></td>
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</table>

1^N=25
2^N=75
3Average of q-PCR positive insects (N) data pooled from different development stages. Different letters denoted significant differences by the t test (p < 0.005).
Figure legends

Fig. 1. Percentage NPV Mortality following intrahaemocelic injections with SeMNPV DNA in fourth instar *S. exigua* larvae. Six repetitions were carried out on different days and included three different treatments: Se-G26, Se-BAC/G26 and water as control (a) or Se.BAC/G26, water, and non treated controls (b). The percentage mortality was calculated from 75 L4 treated larvae at each repetition. The absence or presence of a functional polyhedral gene in the virus genomes used as inocula or generated in the bioassays is shown. (c).

Fig. 2: Genome profiles of *Bgl*II-digested virus DNA. Lane 1, wild type Se-G26; lane 2, Se.Bac-G26; lanes 3-6, viral progeny from larvae transfected with Se.Bac-G26; lane 7, MbNPV; lanes 8-16, viruses from larvae infected per os with MbNPV. DNA digests were fractionated in 0.6% (panel a) or 1.2% agarose (panel b) . Three different variants Se-OX1 (lanes 3 and 8), Se-OX2 (lane 4), Se-OX3 (MbNPV-like) (lane 5), and the mixed population Se/Mb (lanes 6, and 15) were detected. BAC vector fragments are denoted by grey arrows in lane 2. Positions of additional bands that do not appear in Se.BAC/G26 (lane 2) are denoted by white arrows. Fragment sizes in kilobase pairs on the left. Number of individuals containing each *Bgl*II variant (N=86) is showed in panel c.

Fig. 3: Log covert SeMNPV-like DNA (SeVP80, open diamonds) or MbNPV-like (MbP87, solid diamonds) DNA standardized by host DNA as measures in asymptomatic insects from sampled as eggs, L1, L2, L3, L5, pupae, and adult of line 12-b (F2 offspring). Viral DNA levels for fully lethal infected L4 larvae were included as a reference assuming a value of 1 for host DNA.
Fig. 4. Logit mortality of second-instar *S. exigua* larva challenged with Se-G26 (solid square), Se-OX1 (solid diamond), Se-OX2 (solid triangle), Se-OX3 (open square), and MbNPV (open diamond). Data are given as logit mortality where logit (mortality) = ln(p/1-p) and p is the proportionate mortality. The solid line shows the fitted value for Se-OX3 challenged insects: logit (mortality) = -7.646 + 0.647 log10 (OB/ml) and the dotted line for MbNPV challenged insects: logit (mortality) = -5.128 + 0.242 log10 (OB/ml).

Fig. 5. Mortality of second-instar *S. exigua* larvae over time following inoculation with the SeMNPV-like viruses Se-OX1, Se-OX2, Se-G26 (dotted lines) the MbNPV-like virus Se-OX3, MbNPV (solid lines) (a). Proportion of Se-like and Mb-like viruses in larvae collected at 40, 80, 88, 96, and 104 h.p.i (pooled data of 5 insects per record time) measured by q-PCR (SeVP80 and MbP87 primers used for SeNPV-like and MbNPV-like virus detection respectively) for insect inoculated with Se-OX1, Se-OX2, Se-G26 (b) Se-OX3 (c), and MbNPV (MbNPV) (d).
**a)**

% NPV Mortality vs bioassay replica

- Se-G26
- Se.BAC/G26
- Water

**b)**

% NPV Mortality vs bioassay replica

- Se-G26
- Se.BAC/G26
- Water

**c)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Polyhedrin +/- genome</th>
<th>OBs progeny under microscope</th>
</tr>
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<tbody>
<tr>
<td>SeBAC/G26</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Se-G26</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Water control</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Non treated control</td>
<td>n.a.</td>
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</table>
In vivo transfections

Per os infections

a)  

b)  

c)

<table>
<thead>
<tr>
<th>BgM REN variants</th>
<th>NPV-like</th>
<th>Number</th>
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<tbody>
<tr>
<td>Se-OX1</td>
<td>SeMNPV-like</td>
<td>73</td>
</tr>
<tr>
<td>Se-OX2</td>
<td>SeMNPV-like</td>
<td>4</td>
</tr>
<tr>
<td>Se-OX3</td>
<td>MbNPV-like</td>
<td>7</td>
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<tr>
<td>Mixed populations</td>
<td>SeMNPV /MbNPV-like</td>
<td>2</td>
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