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# Occlusion body pathogenicity, virulence and productivity traits vary with transmission strategy in a nucleopolyhedrovirus

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ABSTRACT

The prevalence of sublethal infections of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) was quantified in natural populations of *S. exigua* in Almería, Spain, during 2006 and 2007. Of 1045 adults collected, 167 (16.1%) proved positive for viral *polyhedrin* gene transcripts by RT-PCR. The prevalence of covert infection varied significantly according to sex and sample date. Of 1660 progeny of field-collected insects, lethal disease was observed in 10–33% of offspring of transcript-positive females and 9–49% of offspring of transcript-negative females. Isolates associated with vertically transmitted infections were characterized by restriction endonuclease analysis using *Bgl*II or *Eco*RV and compared with isolates originating from greenhouse soil-substrate believed to be horizontally transmitted. Insects from a sublethally infected Almerian colony were between 2.3-fold and 4.6-fold more susceptible to infection than healthy insects from a Swiss colony, depending on isolate. Horizontally transmitted isolates were significantly more pathogenic than vertically transmitted isolates in insects from both colonies. Mean speed of kill in second instars (Swiss colony) varied between isolates by >20 h, whereas mean occlusion body (OB) production in fourth instars (Swiss colony) varied by 3.8-fold among isolates. Intriguingly, all three horizontally transmitted isolates were very similar in speed of kill and OB production, whereas all three vertically transmitted isolates differed significantly from one another in both variables, and also differed significantly from the group of horizontally transmitted isolates in speed of kill (one isolate) or both variables (two isolates). We conclude that key pathogenicity and virulence traits of SeMNPV isolates vary according to their principal transmission strategy.



## 1. Introduction

The survival of virus pathogens between host generations is of critical importance in the dynamics of infection (Dieckmann et al., 2002). The transmission of virus pathogens in natural populations of insects may occur by horizontal transmission, from infected to healthy individuals of each generation, and by vertical transmission, from parents to offspring (Rothman and Myers, 2000). The efficiency of the virus transmission process within and between generations will influence both the dynamics of viruses in natural insect populations (Cooper et al., 2003; Fuxa, 2004) and their effectiveness as biological insecticides (Zhou et al., 2005; de Souza et al., 2007).

Baculovirus infections may become persistent or latent after a sublethal dose of occlusion bodies (OBs) has been ingested by the host. Latent viral infections have been defined as those in which the virus exhibits minimal gene expression, whereas persistent viral infections are those in which the virus is replicating and a range of viral genes are expressed without signs of disease (Burden et al., 2002, 2003). Both latent and persistent viral infections may be classed as covert in that neither results in obvious signs of disease. The covert infection strategy is likely to be advantageous for pathogen survival when opportunities for horizontal transmission are limited, such as during periods of low host density. Experimental and anecdotal evidence suggest that sublethal infections can switch to patent lethal disease under the appropriate conditions of crowding, physiological stress, or the presence of additional pathogens (Fuxa et al., 1999). Covert infection may also offer opportunities for vertical transmission of the pathogen to the offspring of infected parents (Kukan, 1999).

Baculovirus DNA and transcripts have been detected and found to persist in the immature and adult stages of a number of moth species reared over multiple generations (Hughes et al., 1993, 1997; Burden et al., 2002; Khurad et al., 2004). Natural insect

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populations also appear susceptible to sublethal virus infections (Burden et al., 2003; Vilaplana et al., 2010), and a theoretical framework has been developed to study the impact of sublethal infections on host-pathogen population dynamics (Boots and Norman, 2000; Sorrell et al., 2009).

The *Spodoptera exigua* multiple nucleopolyhedrovirus (Family *Baculoviridae*, Genus *Alphabaculovirus*, Species *Spodoptera exigua multiple nucleopolyhedrovirus*, SeMNPV) has been intensively studied as a biopesticide for use in greenhouse and field crops in the United States, Europe, southern and south-east Asia (Smits and Vlak, 1994; Kolodny-Hirsch et al., 1997; Cunningham, 1998), and recently in southern Spain (Lasa et al., 2007a,b). SeMNPV populations are often characterized by high genetic heterogeneity (Gelernter and Federici, 1986; Caballero et al., 1992; Hara et al., 1995; Muñoz et al., 1998). Some of the genotypes present within a field isolate, named SeMNPV-SP2, that originated from infected larvae collected during a natural epizootic in Almería, southern Spain, were characterized by Muñoz et al. (1999). A number of additional genotypes were subsequently identified from reservoirs of OBs present in Almerian greenhouse soil-substrate samples (Murillo et al., 2007). Because OBs in the soil can achieve transmission only if they are transported back onto plant surfaces and then eaten by susceptible insects (Fuxa and Richter, 2001), the genotypes found in OB reservoirs were suspected to be horizontally transmitted. In contrast, field-collected insects and laboratory colonies of *S. exigua* (Hübner) are frequently reported to succumb to spontaneous NPV disease which is believed to originate from vertically transmitted infections. However, the identity of genotypes involved in vertically transmitted infections is uncertain and the diversity of genotypes present in SeMNPV populations led us to hypothesize that certain genotypes may be specialized for different routes of transmission.

To assess SeMNPV abundance and diversity present in natural *S. exigua* populations, we analyzed field caught insects and performed a preliminary characterization of genotypes associated with covert infections that are likely to be vertically transmitted. To provide insights into the relationship between genetic diversity and transmission strategy.

The main objectives of the present study were to quantify the incidence of covert infections and the prevalence of vertical transmission of SeMNPV in natural insect populations in Almerian greenhouses. Genotypes involved in vertical transmission were subsequently characterized for insecticidal properties and the ability to cause lethal or sublethal disease was compared in isolates with likely different transmission strategies; specifically we examined the hypothesis that vertically and horizontally transmitted genotypes would differ in their ability to cause disease and in their patterns of host exploitation. We also postulated that the host response to each genotype would depend on its infection status.

## 2. Materials and methods

### 2.1. Insect cultures and virus stock

*S. exigua* larvae were obtained from three laboratory populations and each was maintained on artificial diet and virus-free conditions in a rearing room exclusively used for that purpose at the insectary facilities of the Universidad Pública de Navarra, Pamplona, Spain. Each colony was started using the larvae from one of three origins: (i) a short-term culture that had been maintained in the laboratory for four generations and that was started using eggs laid by field-collected adults collected in Almería (Spain), which we named the Almerian colony (RT-PCR studies described in Section 2.3, indicated that ~80% of the adults from this culture were sublethally infected by SeMNPV), (ii) a long-term laboratory culture for which evidence of a persistent infection had been observed in at least 21% of insects (R.D. Possee and R. Murillo, unpublished data) from the Center for Ecology and Hydrology, Oxford (UK), which we named the Oxford colony, (iii) a virus-free culture provided by Andermatt Biocontrol AG (Grossdietwil, Switzerland), which we named the Swiss colony. This colony consistently proved negative for SeMNPV infection in RT-PCR analyses performed during the period of the experiment and in qPCR studies performed subsequently. The Oxford colony was established in December 2003 and originated from a laboratory colony maintained in the insectary of Syngenta UK (Jealott's Hill International Research Center, Bracknell, UK), whereas the Swiss colony is of uncertain origin. Prior to the start of this study the Swiss, Almerian and Oxford colonies were reared on the same diet and conditions for 2, 4, and >10 generations, respectively.

The different strains of SeMNPV were obtained either from our virus collection or were isolated in this study. The SeMNPV strains SeG24, SeG25, and SeG26 were obtained from OBs present in the greenhouse soil-substrate environment (Murillo et al., 2007); they were considered to be horizontally transmitted and are hereafter prefixed with the letters HT. These strains each comprise a single dominant genotype (as indicated by an absence of submolar fragments in restriction endonuclease profiles), although none of them have been subjected to cloning procedures. Other isolates used in this study were each obtained from the patently diseased progeny of field collected or laboratory insects and are considered to be vertically transmitted isolates, and are prefixed with the letters VT.

### 2.2. Field collection of S. exigua and identification of vertically transmitted viruses

Adult *S. exigua* of both sexes were attracted by UV light tramps and caught on a white sheet shortly after sunset in July and September of 2006 and 2007 in the greenhouse zone around Almería, within a 20 km radius of the sites where the horizontally transmitted isolates were collected. Females were placed in 25 ml perforated plastic cups containing a damp cotton pad and were allowed to lay eggs for 1–2 days. Egg masses were not subjected to surface decontamination. Twenty neonate larvae from each female were selected at random, individualized in cups containing diet and reared through to the adult stage. Larvae were monitored daily for signs of viral disease and those that died were stored at −20 °C for restriction endonuclease analysis (REN) of viral DNA. Each NPV-killed larva was considered to have succumbed to a vertically transmitted infection. Additional vertically transmitted isolates used in this study were obtained from the Oxford insect colony. For this, 15 pairs of adults were selected at random, allowed to mate and the incidence of NPV disease in their progeny was monitored as described above.

### 2.3. Detection of SeMNPV transcripts in S. exigua using RT-PCR

Total RNA was extracted from the adults using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer instructions. Briefly, adults that had been stored individually at −80 °C were allowed to thaw and their abdomens were dissected with sterilized toothpicks on a sterile plate. A 50–100 mg tissue sample was taken from each abdomen and homogenized in 1 ml of Trizol reagent. Then 100 µl chloroform was added to separate two phases by centrifugation at 12,000 × *g* for 15 min at 4 °C. The aqueous phase was precipitated using 2-propanol and centrifuged at 12,000 × *g* for 10 min at 4 °C. RNA pelleted was washed with 75% ethanol and resuspended in 50 µl deionized water. All equipment and reagents were previously sterilized and treated with diethylpyrocarbonate (DEPC) to remove RNases.

Total RNA samples extracted from adults and a virus infected moribund larva as positive control were used for RT-PCR reactions to detect transcripts of the *polyhedrin* and *DNA-polymerase* genes using specific primers. RNA samples were previously treated with DNase to avoid DNA contamination. Similar amounts of RNA (160–400 ng) were used in each reaction. RT-PCR was performed in two steps. First, cDNA synthesis was performed using the ImProm-II reverse transcriptase (Promega, Madison, WI, USA) and the *polyhedrin* reverse primer Sepolh.2 (5′ TGTCTTCCATGAAACGCGTC 3′) that amplified in the SeMNPV *polyhedrin* stop codon, or *DNA-polymerase* reverse primer Sednapol.2 (5′ TAGCACGTCGTCTTAGCGTG 3′) that amplified in the *DNA-polymerase* stop codon, according to the manufacturer's instructions. One quarter volume of the reaction was then subjected to PCR amplification with *Taq* DNA polymerase (Bioline) and the two internal primers for the *polyhedrin* gene: Sepolh.1 (5′ ATGTATTACTCGCTACAGCTA 3′) that amplified 319 bp upstream of the stop codon and Sepolh.2. Alternatively, the *DNA-polymerase* gene primer pair was used: Sednapol.1 (5′ ATGACTTCTTCGTCGTCGTC 3′) that amplified 320 bp upstream of the *DNA-polymerase* stop codon and Sednapol.2. Samples from each insect were subjected to PCR analysis in triplicate. PCR calibration studies performed in triplicate indicated that the consistent detection limit of this technique was $5 \times 10^{-6}$ ng of genomic DNA, equivalent to ~35 virus genome copies. PCR products were electrophoresed in 1% agarose gels, alongside a 100 bp marker ladder (Invitrogen). DNA fragments were stained with ethidium bromide, visualized in a UV transilluminator, photographed and examined using the molecular analyst program Chemi doc (Bio-Rad, Hercules, CA, USA). To determine the effect of collection data and sex on the prevalence of infection in field-collected adults and their progeny, RT-PCR results were subjected to Pearson's $\chi^2$ test (SPSS version 10.0).

### 2.4. Genotypic variability of vertically transmitted isolates

The progeny of Oxford colony insects that died from polyhedrosis disease were individually subjected to REN analysis of viral DNA. For this, cadavers were individually homogenized in 0.1% SDS, filtered through muslin and washed twice with 0.1% SDS by centrifugation. OBs were suspended in 300 μl distilled water, 150 μl 0.5 M $Na_2CO_3$ and 50 μl 1% SDS to dissolve the OB matrix. The released virions were incubated with proteinase K for 1 h at 50 °C. DNA was extracted by two passes of phenol and a final step with chloroform. DNA was precipitated by the addition of 2.5 volumes of ice-cold ethanol and 0.1 volumes of sodium acetate. DNA was washed with 70% ethanol and resuspended in 50 μl of distilled sterile milli-Q water. Samples of 2 μg of viral DNA were treated with *Bgl*II, loaded in 0.7% agarose gel with TAE buffer (40 mM Tris–acetate; 1 mM EDTA) and electrophoresed at 16 V overnight. *Bgl*II was used because it allowed clear discrimination among SeMNPV genotypes (Murillo et al., 2007). Ethidium bromide stained gels were then visualized on a UV transilluminator, photographed and examined.

The genomes of the Oxford colony isolate VT-SeOx4 and the vertically and horizontally transmitted isolates originating from Almerian greenhouses were sequenced (O. Cabodevilla and E.A. Herniou, unpublished data) and subjected to digestion *in silicio* using *Bgl*II, and *Eco*RV in the case of VT-SeAl1 and HT-SeG26, using the NEBcutter V2.0 program (Vincze et al., 2003).

### 2.5. Bioassay for the induction of covert infection

Pre-molt *S. exigua* fourth instars from the Swiss and Almerian colonies were starved overnight and, having molted, were inoculated by the droplet feeding method (Hughes and Wood, 1981) with an OB suspension containing $9 \times 10^3$ OB/ml in sterile distilled water, 10% sucrose (w/v) and 0.001% (w/v) of the food dye Fluorella Blue. Virus treatments involved inoculation with one of three horizontally transmitted (HT-SeG24, HT-SeG25 and HT-SeG26) and three vertically transmitted (VT-SeAl1, VT-SeAl2, VT-SeOx4) isolates. Fourth instars ingested 3.3 μl during droplet feeding (Smits et al., 1987), that equates to 30 OBs/larva. Larvae were allowed to drink for 10 min or until they moved away from the droplet and were then transferred individually to a 24-compartment plate containing diet. Twenty-four larvae were dosed for each virus-treatment and a negative control was treated with sterile distilled water instead of virus. Larvae were reared at 25 ± 2 °C and mortality was recorded daily. Adult survivors were subjected to RT-PCR to detect transcripts of the *polyhedrin* and *DNA-polymerase* gene. Bioassays were performed independently on three occasions. The prevalence of RT-PCR positive insects was analyzed by fitting generalized linear models with a binomial error structure specified in GLIM 4 (Numerical Algorithms Group, 1993). When necessary, models were scaled to account for overdispersion in the error distribution (Crawley, 1993).

### 2.6. Determination of OB concentration–mortality response

The pathogenicity of the horizontally and vertically transmitted isolates was determined by inoculating *S. exigua* second instars as described above with one of the following concentrations of OBs: $2.54 \times 10^5$, $8.18 \times 10^4$, $2.72 \times 10^4$, $9.09 \times 10^3$ and $3.03 \times 10^3$ OBs/ml. This range of concentrations killed between 95% and 5% of the test larvae in preliminary bioassays. A cohort of 24 larvae was allowed to drink from an OB-free suspension and served as a control. Larvae were reared at 25 ± 2 °C and mortality was recorded for 7 days post-inoculation. Bioassays were performed three times for each isolate. Data were subjected to logit regression using GLIM 4. To compare the responses of healthy and covertly infected insects, the entire bioassay was performed using the Swiss and Almería colony insects, respectively.

### 2.7. Determination of mean time to death

Second instars from the healthy Swiss colony were allowed to drink for 10 min from droplets of a single concentration of OBs of each isolate that was previously estimated to result in ~90% mortality. Twenty-four inoculated larvae were transferred individually to diet and monitored at 8 h intervals for 5 days. Bioassays were performed on three occasions. Time mortality results of individuals that died due to NPV infection by different isolates were subjected to Weibull survival analysis in GLIM. The validity of the Weibull model was determined by comparing fitted values with Kaplan–Meier survival function estimated values (Crawley, 1993).

### 2.8. Determination of OB production

Groups of 48 fourth instars from the healthy Swiss colony were starved overnight and then allowed to drink for 10 min from a suspension of $5 \times 10^7$ OBs/ml of each of the isolates. Inoculated larvae were individualized in 24 well culture plates containing diet and checked daily for virus-induced mortality. Virus-killed larvae were frozen to avoid liquefaction and OBs were collected and individually homogenized in 1 ml sterile distilled water. Each homogenate was filtered through a fine wire mesh to remove debris and the resulting suspension was counted in triplicate in a Neubauer chamber at 400× magnification. Counts were performed on 30 randomly selected larvae for each isolate. OB production data were $\log_e$-transformed and analyzed by fitting generalized linear models in GLIM. Model estimates and corresponding SEs were used to perform pairwise *t*-tests on differences between isolates in $\log_e$ OB production/larva.

## 3. Results

### 3.1. Incidence of SeMNPV infection in S. exigua field populations

From a total of 1045 adults (males and females) collected during four sampling periods and analyzed by RT-PCR, 167 (16.1%) proved positive for transcripts of the viral *polyhedrin* gene (Fig. 1A). The prevalence of covert infection varied according to sex and sample date. The prevalence of infection did not differ significantly between males and females in 2006 ($\chi^2 = 0.847$; d.f. = 1; $P > 0.05$), whereas in 2007, infections in males were significantly more frequent than those in females ($\chi^2 = 39.1$; d.f. = 1; $P < 0.001$) (Fig. 1B). The prevalence of sublethal infections was significantly reduced in insects captured in 2007 compared to 2006 ($\chi^2 = 123.2$; d.f. = 1; $P < 0.001$).

The females tested by RT-PCR were classified as transcript-positive or negative and batches of 10–20 offspring from each female were individually reared under clean conditions in the laboratory. Overall, lethal NPV disease was observed in 333 (20%) out of 1660 progeny obtained from field collected females (Fig. 1B). Lethal polyhedrosis disease was observed in the offspring of both transcript-positive and transcript-negative females. The proportion of transcript-positive females that produced diseased progeny varied from zero to 91% (11 out of 12 insects) but sample sizes were too small to draw conclusions (Fig. 1B). Among the transcript-negative females, 37% (7 out of 19 females) and 90% (18 out of 20 females) produced diseased cohorts of offspring in the samples taken in July and September 2006, respectively, compared to 27% (42 out of 155 females) and 11% (18 out of 164 females) in samples taken in July and September 2007, respectively.

The prevalence of polyhedrosis disease among offspring of transcript-positive females was 10% (1 out of 10 offspring) in the July 2006 sample, 33% (43 out of 130 offspring) in the September 2006 sample, whereas no offspring of transcript-positive females died of NPV disease in the 2007 samples. In contrast, the prevalence of disease among offspring of transcript-negative females collected in 2006 was 11% (8 out of 70 offspring) in the July sample and 49% (122 out of 250 offspring) in the September sample, compared to 15% (126 out of 840 offspring) and 9% (33 out of 360 offspring) in the July and September 2007 samples, respectively.

### 3.2. Identification of virus genotypes involved in vertical transmission

OBs isolated from the virus diseased offspring of field-collected and laboratory colony insects were subjected to extraction of viral DNA and REN analysis, either *in vitro* or *in silicio* following genome sequencing. NPV-killed larvae from the offspring of field-collected adults were analyzed and two different isolates were identified, VT-SeAl1 (8/14, 57%) and VT-SeAl2 (6/14, 43%) (Fig. 2A). The *Bgl*II profile of VT-SeAl1 comprised 20 fragments (named *Bgl*II-A to *Bgl*II-T) and was identical to HT-SeG26, but could be differentiated from this isolate by treatment with *Eco*RV (Fig. 2B). The profile of VT-SeAl2 was also identical to that of VT-SeAl1 except the *Bgl*II-K fragment was shorter (~4.8 kb) (Fig 2A).

Of a total of many thousands of larvae reared from the Oxford colony, 45 died from polyhedrosis disease. Of these, 42 insects (93%) were found to have succumbed to a single genotype, named VT-SeOx1 that was identical in terms of *Bgl*II profile to the Spanish isolate VT-SeAl2 (Fig. 2C). Additional genotypes were each isolated

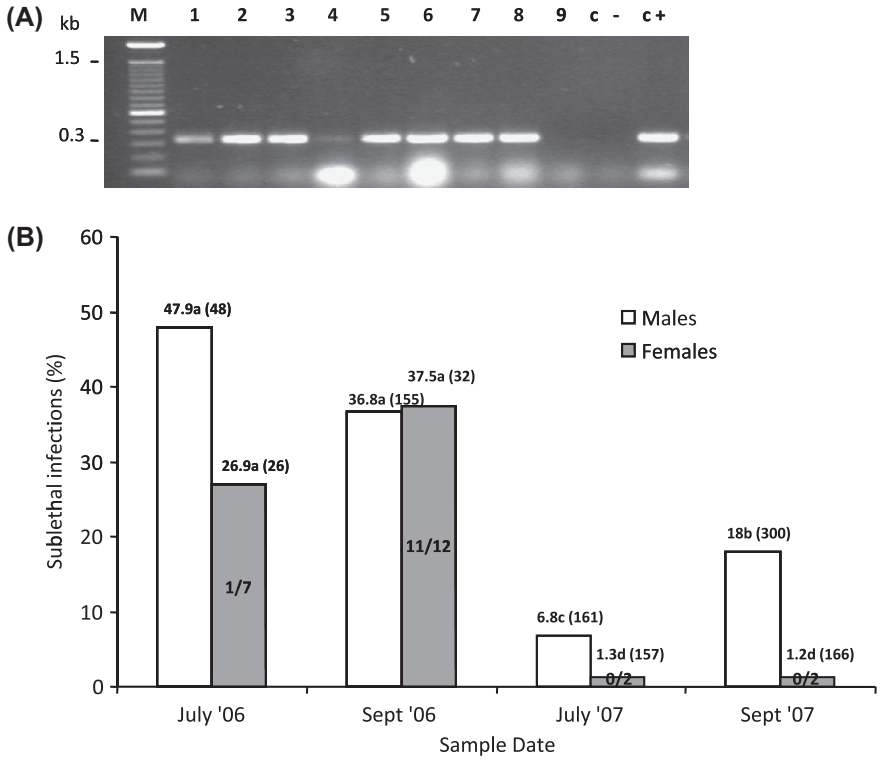


**Fig. 1.** (A) Detection of SeMNPV *polyhedrin* gene expression using RT-PCR. Viral transcripts were identified using the RNA extracted from adults collected in the field. RT products were used as a template for PCR amplification of a 319-bp fragment of the *polyhedrin* gene (lanes 1–9). M, Marker; 1–9, individual *Spodoptera exigua* adults from field; water control (c−) and positive control using total RNA extracted from a laboratory-infected moribund larva (c+). (B) Percentage of field adults (males and females) with positive amplification for SeMNPV *polyhedrin* by RT-PCR and the number of tested individuals in parentheses. Values followed by identical letters did not differ significantly (Pearson's $\chi^2$, $P > 0.05$). Values within shaded columns indicate number of transcript-positive females that produced diseased offspring out of the total number of transcript-positive females in each sample.

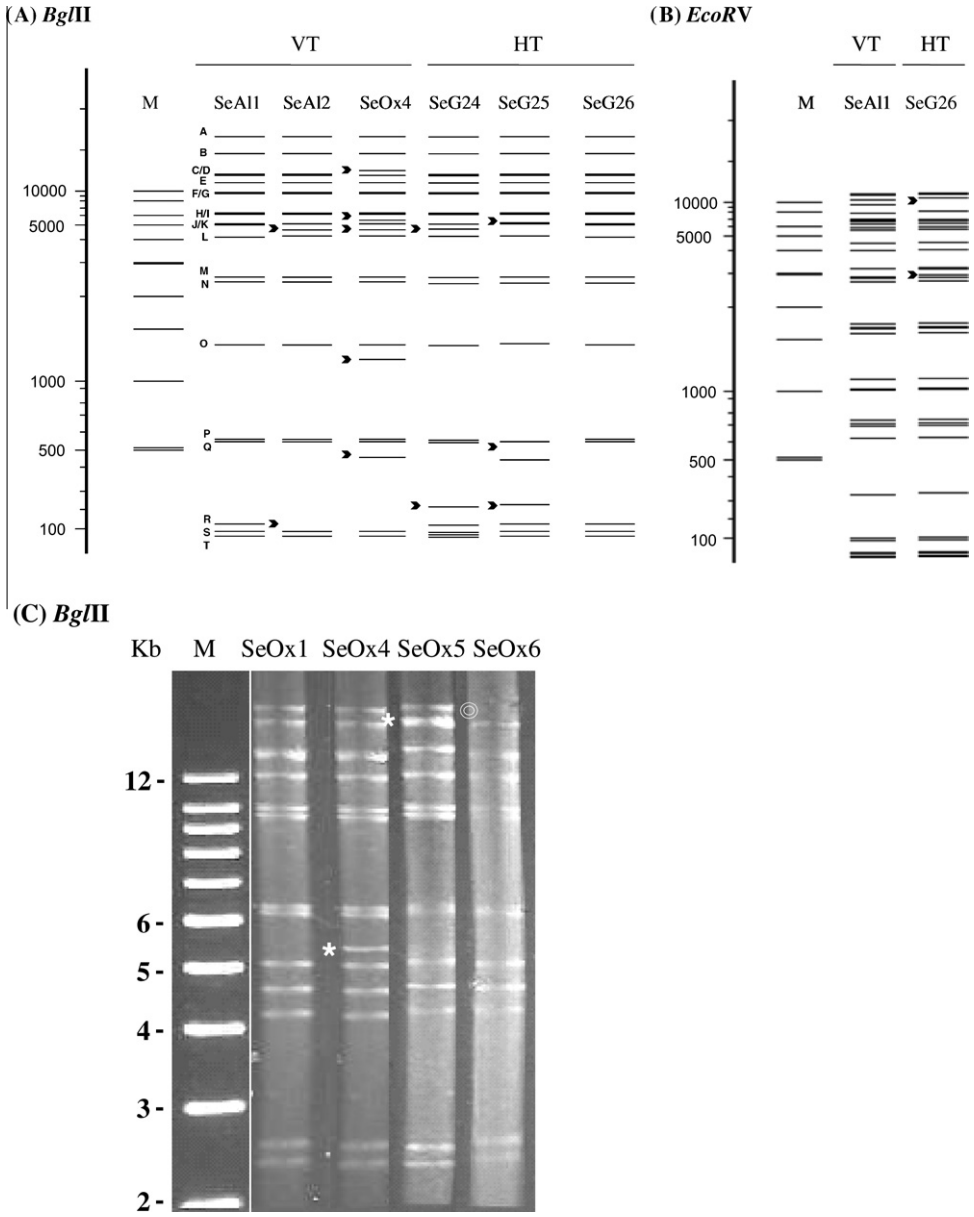


**Fig. 2.** Restriction endonuclease (REN) profiles of SeMNPV DNA originating from vertically (VT) and horizontally (HT) transmitted genotypic variants from *Spodoptera exigua* collected from greenhouses in Almeria, Spain and isolated in the laboratory, or from a laboratory colony originating from Oxford, UK. Viral DNA was subjected to digestion *in silicio* using (A) *Bgl*II or (B) *Eco*RV. For the SeAl1 variant, the different fragments are labeled A to T in (A) and arrowheads indicated REN profile differences with respect to the SeAl1 variant. (C) DNA extracted from OBs was subjected to digestion *in vitro* using *Bgl*II for the variants originating from the Oxford insect colony (Se-Ox1, Se-Ox4, Se-Ox5, Se-Ox6). Asterisks indicate polymorphic fragments and the position of a single fragment absent with respect to isolate Se-Al1 is indicated by a circle. The position of molecular markers (M) is shown in all cases. The vertical transmission (VT) or horizontal transmission (HT) prefixes for isolate names have been omitted for clarity.

from single diseased larvae: VT-SeOx4 and VT-SeOx5 were characterized by a unique fragment of ~5.3 kb and 21 kb, respectively, whereas VT-SeOx6 lacked the *Bgl*II-A (~23 kb) fragment. The VT-SeOx4 isolate was selected for further study as it was found to be stable on passage in insects, whereas a degree of genetic instability was observed in VT-SeOx5 and VT-SeOx6.

### 3.3. Induction of covert infections

Fourth instars from both the Swiss and Almerian colonies were infected with ~30 OBs of one of the three vertically (VT-SeAl1, VT-SeAl2, VT-SeOx4) or horizontally transmitted isolates (HT-SeG24, HT-SeG25, HT-SeG26), in groups of 24 per treatment. The prevalence of NPV mortality varied between 25% and 59% of inoculated larvae and did not differ significantly between isolates ($\chi^2 = 7.68$, d.f. = 5, $P = 0.18$). Overall, insects from the Swiss population experienced an average 53% mortality that was significantly higher than the 40% mortality experienced by insects from the Almerian colony ($\chi^2 = 9.90$, d.f. = 1, $P < 0.05$). No mortality was observed in controls in either host population.

Adult survivors of the virus challenge were analyzed by RT-PCR using both *polyhedrin* and *DNA-polymerase* primer sets alternatively. *DNA-polymerase* transcripts were detected in different proportions, but *polyhedrin* transcripts were not detected. No positive amplifications were detected in adults from control larvae of the Swiss population, whereas over 80% of control larvae from the Almerian colony were RT-PCR positive for *DNA-polymerase* transcripts (data not shown). The prevalence of RT-PCR positive insects differed significantly between isolates ($F_{5,34} = 5.23$, $P < 0.05$) and between insect populations ($F_{1,34} = 5.01$, $P < 0.05$). No clear

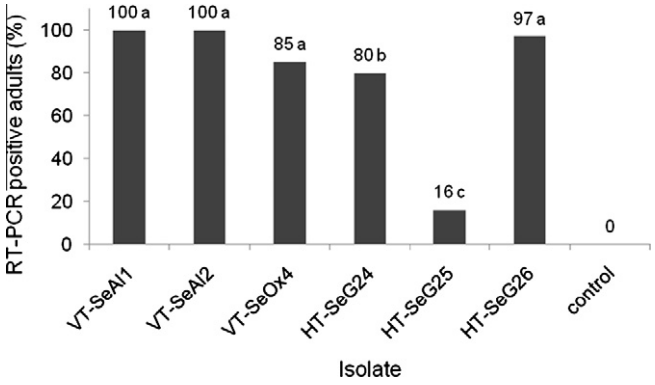


**Fig. 3.** Mean percentage of *DNA-polymerase* transcript positive adults by RT-PCR from the healthy Swiss population inoculated as larvae with vertically and horizontally transmitted isolates. Values above columns followed by different letters are significantly different (*t*-test, $P < 0.05$). Bars indicated SE.

pattern among either horizontally or vertically transmitted isolates was apparent in their ability to induce persistent infections in the Swiss insect colony (Fig. 3); 100% of the adults infected as larvae with the vertically transmitted VT-SeAl1 or VT-SeAl2 isolates were positive for *DNA-polymerase* transcripts, whereas only 16% of adults from larvae inoculated with the horizontally transmitted HT-SeG25 were transcript positive, all other isolates were intermediate between these values.

### 3.4. Pathogenicity of horizontally and vertically transmitted isolates

To compare the OB pathogenicity characteristics of vertically and horizontally transmitted isolates, concentration–response assays were performed using insects from the Almerian and Swiss populations (Table 1). In all cases, mortality increased significantly with OB concentration ($F_{1,53} = 521$, $P < 0.001$, scale parameter 1.36). Insects from the Almerian colony were significantly more susceptible to infection than the Swiss colony insects (data pooled across all genotypes) ($F_{1,53} = 80.1$, $P < 0.001$, scale parameter 1.36). The magnitude of colony differences in susceptibility, based on the ratio of $LC_{50}$ values (Table 1), ranged from 2.3-fold for the VT-SeAl2 isolate to 4.6-fold for the HT-SeG26 isolate. Genotypes differed significantly in their pathogenicity ($F_{5,52} = 47.7$, $P < 0.001$, scale parameter 1.36), with HT-SeG25 being the most pathogenic isolate of those tested for both colonies, and VT-SeAl2 being the least pathogenic for both colonies, although these differences were only significant in insects from the Almerian colony (Table 1). When grouped according to transmission strategy, horizontally transmitted isolates were significantly more pathogenic than vertically transmitted isolates in insects from both colonies ($F_{1,57} = 18.7$, $P < 0.001$, scale parameter 1.82). No virus mortality was registered in mock-infected control insects from either colony.

### 3.5. Speed of kill and OB production

The mean (±SE) speed of kill in second instars from the healthy Swiss colony varied significantly between isolates ($\chi^2_1 = 1516$; $P < 0.001$; Weibull hazard function $\alpha = 10.787$) from a minimum of 89.5 ± 1.9 h for insects infected by VT-SeOx4 to a maximum of 110.0 ± 2.5 h for insects infected by VT-SeAl1. Similarly, mean OB production per larva varied significantly among isolates ($F_{5,193} = 15.4$; $P < 0.001$) with a minimum of $2.99 \times 10^8 \pm 4.59 \times 10^7$ OBs/larvae for VT-SeOx4 to a maximum of $1.14 \times 10^9 \pm 1.26 \times 10^8$ OBs/larvae for VT-SeAl2. Intriguingly, a plot of speed of kill in second instars against OB production in fourth instars (Fig. 4) revealed that all three horizontally transmitted isolates were very similar in these characteristics (indicated by the shaded region in Fig. 4), whereas all three vertically transmitted isolates differed significantly from one another in both variables; vertically transmitted isolates also differed significantly from the cluster of horizontally transmitted isolates in speed of kill (VT-SeAl1) or both variables (VT-SeOx4, VT-SeAl2).

## 4. Discussion

The main objectives of the present study were to quantify the incidence of covert infections and the prevalence of vertical transmission of SeMNPV in natural insect populations in Almerian greenhouses, and to compare the pathogenicity and virulence characteristics of NPV isolates with likely different transmission strategies.

RT-PCR studies revealed that overall, 16% of field-collected *S. exigua* adults harbored a persistent NPV infection. These insects were found to contain viral transcripts of the *polyhedrin* gene, suggesting that the virus was actively replicating. Studies on other lepidopteran species suggest that PCR studies targeting viral DNA tend to result in higher estimates of the prevalence of covert infection than RT-PCR studies that target viral transcripts (Burden et al.,

**Table 1**
Logit regression of concentration–mortality results of vertically and horizontally transmitted isolates in insects from the covertly infected Almerian and the healthy Swiss colonies.

| Insect colony and isolate | $LC_{50}$ ($\times 10^4$) (OBs/ml) | Range of 95% C.I. ($\times 10^4$) | Intercept ± SE | Potency | *P* |
|---|---|---|---|---|---|
| I. Almerian colony[a] | | | | | |
| VT-SeAl1 | 1.40 | 1.01–1.94 | −6.490 ± 0.3516 | 1 | – |
| VT-SeAl2 | 3.25 | 2.34–4.48 | −7.067 ± 0.3620 | 0.43 | 0.015 |
| VT-SeOx4 | 1.06 | 0.76–1.47 | −6.308 ± 0.3735 | 1.32 | 0.285 |
| HT-SeG24 | 0.85 | 0.60–1.19 | −6.158 ± 0.3465 | 1.65 | 0.088 |
| HT-SeG25 | 0.59 | 0.41–0.83 | −5.909 ± 0.3435 | 2.38 | 0.015 |
| HT-SeG26 | 0.66 | 0.46–0.93 | −5.988 ± 0.3444 | 2.12 | 0.025 |
| II. Swiss colony[b] | | | | | |
| VT-SeAl1 | 4.95 | 3.13–7.92 | −7.950 ± 0.523 | 1 | – |
| VT-SeAl2 | 7.46 | 4.30–16.1 | −8.146 ± 0.5285 | 0.66 | 0.429 |
| VT-SeOx4 | 4.75 | 3.03–8.08 | −7.920 ± 0.5218 | 1.04 | 0.901 |
| HT-SeG24 | 3.59 | 2.27–5.87 | −7.714 ± 0.5158 | 1.38 | 0.341 |
| HT-SeG25 | 2.27 | 1.46–3.45 | −7.377 ± 0.5064 | 2.18 | 0.046 |
| HT-SeG26 | 3.03 | 1.94–4.81 | −7.597 ± 0.5125 | 1.63 | 0.173 |

Potencies were calculated as the ratio of effective concentrations relative to the VT-SeAl1 isolate.
[a] Regressions were fitted with a common slope (±SE) of 0.681 ± 0.0335.
[b] Regressions were fitted with a common slope (±SE) of 0.736 ± 0.0474.

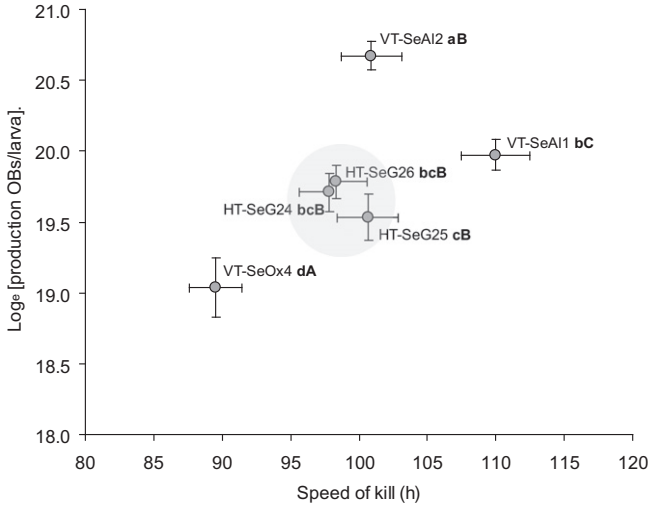


**Fig. 4.** Mean time to kill in second instars and $\log_e$ OB production/larva in fourth instars from the healthy Swiss colony that were infected with vertically or horizontally transmitted isolates of SeMNPV. Each point is labeled with the name of the isolate. Isolate names followed by identical lower case bold letters did not differ significantly in $\log_e$ production of OBs/larva (Generalized linear model, $P > 0.05$), whereas those followed by identical upper case bold letters did not differ significantly in speed of kill (Weibull survival analysis, $P > 0.05$). Horizontal and vertical bars indicate SE. Grey shaded area indicates cluster of horizontally transmitted isolates and is defined arbitrarily.

2002; Vilaplana et al., 2010). A likely explanation is that in a sublethal infection, only a fraction of the total virus population is actively replicating and producing transcripts, so that the concentration of RT-PCR targets may be close to the limits of detection in some or most of the covertly infected insects. As our study was based on detection of viral transcripts it is likely that the estimates of covert infection were conservative in nature. The use of primers that target *polyhedrin* transcripts has proved useful in other studies aimed at quantifying the prevalence of covert infection in lepidopteran populations (Martínez et al., 2005; Vilaplana et al., 2010). However, detection of *DNA-polymerase* transcripts appeared to be a more sensitive indicator of infection than polyhedrin transcripts, so the values given in Fig. 1B are likely to be conservative estimates of the prevalence of covert infection in the *S. exigua* population. We designed these primer sets based on the published SeMNPV genome sequence (Ijkel et al., 1999), but did not test the specificity of the primers in other NPVs and cannot exclude the possibility that some infections we detected were caused by different viruses.

Both sample date and sex significantly influenced the prevalence of persistent virus infections in *S. exigua* field-collected adults. The percentage of infected males (21.8%) was significantly higher than that of females (6.0%) and the proportion of diseased offspring was higher for negative transcripts females. This intriguing observation that males might be more influential than females in the transmission of viral infections merits additional study, and finds a number of similarities with the baculovirus-like nudivirus infections that are sexually transmitted in certain lepidopteran and orthopteran populations (Wang et al., 2007). Moreover, a particularly high prevalence of infection was observed in the progeny of insects captured in September 2006 compared to other sample dates, for reasons that are unclear, but that may have been related to population density or environmental stressors. For example, high-density field populations were found to harbor inapparent baculovirus infections, whereas low-density populations did not (Cooper et al., 2003). The prevalence of patent NPV disease can change rapidly from one year to the next, particularly during or following epizootics (Fuxa, 2004), whereas the dynamics of covert infections in host populations remain largely unknown.

Vertical transmission of baculovirus infections appears to be a common phenomenon in Lepidoptera (Kukan, 1999), although the mechanisms and factors that regulate this process are not well understood. Vertical transmission has been suggested as a survival strategy for the virus which persists in sublethally infected insects during periods of low host density when opportunities for horizontal transmission are highly restricted (Cory and Myers, 2003). The combination of covert infection and vertical transmission may also be an effective strategy for viral dispersal via host migration, especially for moths such as *Spodoptera* spp. in which adults may fly long distances in search of suitable host plants for oviposition. In the case of *S. exigua*, moth populations annually arrive in Almería from the North Africa region during the months of April through to August. Previous studies on the incidence of vertical transmission in *Spodoptera* spp. that survived virus inoculation in the larval stage also reported a wide variation (~5–50%) in the prevalence of lethal infection in the progeny (Abul-Nasr et al., 1979; Smits and Vlak, 1988; Fuxa and Richter, 1991). More recent molecular studies have confirmed the presence of virus in the progeny of field-collected insects (Cooper et al., 2003; Vilaplana et al., 2008). Burden et al. (2002) detected viral mRNA in 60–80% of the samples derived from mating between infected and non-infected individuals in *Plodia interpunctella* (Hübner), although none of the progeny larvae died of virus infection. Similarly, Vilaplana et al. (2010) detected viral DNA in 78% of *S. exempta* (Walker) progeny of field-collected adults. In the present study, overall 25% of females (that were positive or negative for infection by RT-PCR) produced cohorts of offspring, some of which died of NPV disease. The average prevalence of lethal disease in these progeny larvae was 20.1%, although considerable variation was present across sampling dates (from 9% to 49%), including the progeny of females in which viral transcripts were not detected by RT-PCR analysis.

It is unclear how virus-transcript-negative females were able to transmit the infection, but possible explanations include: (i) that those females had previously copulated with males carrying a persistent infection, (ii) that the virus could be in a non-replicating state undetectable by RT-PCR, (iii) the abundance of viral transcripts may be below the detection threshold levels for detection by RT-PCR in some covertly infected insects. Nonetheless, it is clear that vertical transmission of SeMNPV infections are a common feature of the natural populations of *S. exigua* in Almería. The results of our study did not provide clues as to the mechanism by which virus is transmitted from parents to offspring; this may be transovum for virus that contaminates the outside of the egg, or transovarial for virus transmitted within the egg (Fuxa et al., 2002). Transovum transmission may explain the observation that virus transcript-negative females produced offspring that died of polyhedrosis disease, as replication and tissue trophism may differ for viruses adopting each transmission strategy. However, surface decontamination using formalin or sodium hypochlorite did not marked affect that incidence of vertical transmitted NPV infections in a laboratory colony of *S. exempta* compared to field collected material, suggesting that transmission is mostly transovarial rather than transovum in this species (Vilaplana et al., 2010).

REN characterization of vertically transmitted NPVs has been performed for *Mamestra brassicae* (L.) (Burden et al., 2006), *Malacasoma* spp. (Cooper et al., 2003) and *S. exempta* (Vilaplana et al., 2008), but the present study significantly advances previous findings in that distinct genotypic variants have been found to be associated with each route of infection. Previous studies of genotypic variability with SeMNPV in both field-collected insect cadavers (Muñoz et al., 1999) and soil samples (Murillo et al., 2007) suggest that genotypes may be adapted to particular habitats or routes of transmission. We attempted to determine the isolates associated with vertically transmitted infections either from the progeny of field-collected adults and from laboratory-reared adults. Two

different virus genotypes were detected in the progeny of field-collected adults (VT-SeAl1 and VT-SeAl2) and four in the larval progeny of laboratory-reared adults (VT-SeOx1, VT-SeOx4, VT-SeOx5 and VT-SeOx6).

We hypothesized that horizontally transmitted isolates would be likely to be more pathogenic, in terms of concentration–mortality metrics than vertically transmitted genotypes, and this was indeed the case. This is because horizontal transmission depends entirely on achieving peroral infection. In contrast, vertically transmitted isolates are not limited to a single transmission mechanism; they may kill their host and adopt a horizontal route when conditions are favorable for peroral infection and vertical route when they are not. Higher insecticidal capacity of OBs of horizontally transmitted isolates was observed in insects from both covertly infected and healthy *S. exigua* colonies. Interestingly, infections in the healthy Swiss colony insects, in which no interference was possible due to covert infections, vertically transmitted genotypes were markedly more heterogeneous in their speed of kill and OB production characteristics compared to horizontally transmitted genotypes that clustered as a single group in Fig. 4.

This suggests an intriguing possibility that vertically transmitted isolates present phenotypically disparate characteristics in response to adverse conditions that may favor unusual virulence and productivity traits that differ from those of horizontally transmitted genotypes, because at certain moments environmental or ecological conditions are not favorable for horizontal transmission. The genetic differences in the insect colonies used and the limited geographical area from which our samples were collected limit our ability to generalize the results of our study. Verification of this hypothesis will require studies on vertically and horizontally transmitted baculovirus isolates collected over a broad geographical range (Erlandson, 2009), at different host densities (Reeson et al., 1998) and from a range of host plants (Hodgson et al., 2002), that can affect the transmission of these viruses.

The infected Almerian population was significantly more susceptible to the genotypes that we tested than the healthy Swiss colony. It remains to been determined whether this is because covertly infected insects are intrinsically more susceptible to lethal infection following the consumption of OBs, or whether this result reflects the fact that in the present study, five out of six genotypes tested originated from Almería. NPV isolates tend to be more pathogenic to local host populations, in terms of dose–mortality metrics, than insects from geographically distinct origins (Shapiro and Robertson, 1991; Del Rincón-Castro and Ibarra, 1997). Alternatively, the differences in virus susceptibility between the Swiss and Almerian insect colonies may be due to the genetic background of the insects rather than their infection status.

This study confirms that covert infections in adults occur as a result of surviving SeMNPV challenge, as occurs in other baculovirus systems (Burden et al., 2002). RT-PCR results suggested a differential ability to establish covert infection among the different isolates tested. It seems that VT-SeAl1, VT-SeAl2 and HT-SeG26 had the highest capacity to produce covert infection, whereas few of the survivors of HT-SeG25 proved positive for covert infection by RT-PCR. These isolates are now being studied to identify possible genetic characteristics associated in each transmission strategy.

In conclusion, the prevalence of covert SeMNPV infection in field-collected adults was found to vary with sex and sampling date. Vertical transmission of SeMNPV resulted in significant levels of mortality in the offspring of infected parents. As hypothesized, genotypes associated with horizontally transmitted infections were more pathogenic to insects from covertly infected or healthy colonies than vertically transmitted genotypes. Improved understanding of the genetic and ecological factors that modulate vertical transmission of NPV disease could have clear applications for trans-generational biological control of *S. exigua* infestations in greenhouse and field crops.

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