



Natural populations of *Spodoptera exigua* are infected by multiple viruses that are transmitted to their offspring



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ABSTRACT

Sublethal infections by baculoviruses (Baculoviridae) are believed to be common in Lepidoptera, including *Spodoptera exigua*. In addition, novel RNA viruses of the family *Iflaviridae* have been recently identified in a laboratory population of *S. exigua* (*S. exigua* iflavirus-1: SeIV-1; *S. exigua* iflavirus-2: SeIV-2) that showed no overt signs of disease. We determined the prevalence of these viruses in wild populations and the prevalence of co-infection by the different viruses in shared hosts. Infection by *S. exigua* multiple nucleopolyhedrovirus (SeMNPV) and iflaviruses in *S. exigua* adults ($N = 130$) from horticultural greenhouses in southern Spain was determined using qPCR and RT-PCR based techniques respectively. The offspring of these insects ($N = 200$) was reared under laboratory conditions and analyzed to determine virus transmission. Overall, 54% of field-caught adults were infected by SeMNPV, 13.1% were infected by SeIV-1 and 7.7% were infected by SeIV-2. Multiple infections were also detected, with 8.4% of individuals harboring SeMNPV and one of the iflaviruses, whereas 2.3% of adults were infected by all three viruses. All the viruses were transmitted to offspring independently of whether the parental female harbored covert infections or not. Analysis of laboratory-reared insects in the adult stage revealed that SeIV-1 was significantly more prevalent than SeMNPV or SeIV-2, suggesting high transmissibility of SeIV-1. Mixed infection involving three viruses was identified in 6.5% of laboratory-reared offspring. We conclude that interspecific interactions between these viruses in co-infected individuals are to be likely frequent, both in the field, following applications of SeMNPV-based insecticides, or in laboratory colonies used for SeMNPV mass production.

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1. Introduction

The beet armyworm *Spodoptera exigua* is a pest that causes important losses in horticultural crops worldwide. Populations of *S. exigua* are infected, both in natural and in controlled laboratory conditions, by entomopathogenic DNA and RNA viruses (Caballero et al., 1992; Choi et al., 2012; Millán-Leiva et al., 2012). In a recent transcriptome analysis of a laboratory population of *S. exigua*, the simultaneous presence of transcripts from putative DNA (*S. exigua* multiple nucleopolyhedrovirus, SeMNPV) and RNA viruses (mainly iflavirus but also cytovirus and noravirus)

were identified (Pascual et al., 2012). SeMNPV (genus *Alphabaculovirus*, family *Baculoviridae*) is a highly specific pathogen of *S. exigua* with notable insecticidal properties against this pest (Kolodny-Hirsch et al., 1997; Lasa et al., 2007; Smits and Vlask, 1994). This virus constitutes the active ingredient of a number of bioinsecticides, including: SPOD-X[®] (Certis, USA), SPEXIT[®] (Andermatt Bio-control, Switzerland) and VIR-EX[®] (Biocolor, Spain). In Europe, SeMNPV-based insecticides are being incorporated into pest management programs including those in Europe's largest area of horticultural production, in Almería, southern Spain (Lasa et al., 2007).

The intra- and inter-generational transmission of nucleopolyhedroviruses (NPVs) among individuals in a population of insects is a key factor in understanding the ecology of the virus, and for the efficient use of these pathogens as pest control agents. The highly persistent virus occlusion bodies (OBs) are responsible for

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horizontal transmission to healthy susceptible larvae that consume OB-contaminated plant material. However, when host population densities are low and conditions for horizontal transmission are unfavourable, vertical transmission, from parents to offspring, plays an important role in the survival of the virus (Cory and Myers, 2003). Vertically-transmitted infections also permit virus dispersal and the colonization of new areas of habitat through the migration of infected adult hosts (Vilaplana et al., 2010; Burand et al., 2011). For vertical transmission to occur the virus must persist in the adult host as a covert or sublethal infection which does not prevent adult reproduction. Sublethal baculovirus infections have been reported in a number of lepidopteran species (Burden et al., 2002, 2003; Cabodevilla et al., 2011a; Vilaplana et al., 2010). Vertical transmission has been reported in the *S. exigua*-SeMNPV pathosystem (Bianchi et al., 2001; Smits and Vlak, 1988), but only recently PCR-based quantification has been employed to estimate the importance of this transmission route in host populations (Cabodevilla et al., 2011b; Murillo et al., 2011). Moreover, a recent study on *S. exigua* demonstrated transovarial transmission of SeMNPV, and the role of the parental female in the persistence of the virus population from one generation to the next (Virto et al., 2013).

The iflaviruses are positive-stranded RNA viruses responsible for both lethal and asymptomatic infections in insects. Some iflaviruses are well known economically-important pathogens of silkworms (Infectious flacherie virus, IFV) and honeybees (Sacbrood virus, SBV, and Deformed wing virus, DWV) (van Oers, 2010). Inapparent infections are frequent and these viruses are capable of vertical transmission (Yue et al., 2007). Recently novel iflaviruses have been identified from the transcriptome analysis of *S. exigua* laboratory cultures (Choi et al., 2012; Millán-Leiva et al., 2012; Pascual et al., 2012). Although relatively little is known about iflavivirus pathology, these viruses have been isolated from insect corpses that succumbed to baculovirus infection (Wang et al., 1999, 2004), and have also been reported in association with nucleopolyhedroviruses in studies that predate the development of molecular techniques (Vail et al., 1983a). Apparently, these viruses did not cause lethal infection, but resulted in reduced larval weight gain (Vail et al., 1983a,b).

Evidence of an association between SeMNPV and SeIV has been detected in OBs produced in our laboratory insect colonies, where in both virus apparently were co-occluded (*S. Herrero, A. Jakubowska, P. Caballero, R. Murillo, A. Carballo* unpublished data). Further analysis revealed that this association increased SeVI infectivity and resistance to UV radiation and elevated temperature; two of the main factors affecting virus persistence outside the host. However, such an association was not detected between SeMNPV and other RNA viruses (cypovirus, noravirus, etc.). For this reason, we examined whether iflavivirus infections occur in natural *S. exigua* populations that are subjected to control measures that include the use of SeMNPV-based insecticides. The aim of this study was to evaluate the prevalence of baculovirus and iflavivirus inapparent infections in a field population of *S. exigua* present in the horticultural greenhouse agroecosystem of Almería, and to determine their ability for vertical transmission.

2. Materials and methods

2.1. Field collection of *S. exigua* insects

S. exigua adults were sampled in the horticultural area of Almería (southern Spain) during the 2011 sweet pepper growing season (September – October). Moths were collected from three experimental greenhouses, 100 m² in area, planted with sweet pepper that was naturally infested by *S. exigua*. Samples were

taken at intervals of 2–7 days over three consecutive weeks in October, during the peak of the pest infestation. Two different methods were used to capture moths inside greenhouses around sunset. The first method involved collecting adults that landed on a white sheet placed vertically behind a UV light source. These adults were confined individually in 25 ml plastic cups containing a piece of filter paper for oviposition in the case of females. The second method involved a funnel placed under a UV lamp and connected to a collecting box at the bottom. In this case the adults fell into the funnel and the collecting box after being attracted to the UV light. Adults remained together inside the collecting box overnight and were separated the next morning as described above. All gravid females were allowed to lay eggs for 2 days and then all adults of both sexes were individually frozen at –80 °C until required for PCR analyses. Eggs were not surface decontaminated because a previous study determined that surface decontamination did not influence the prevalence of transmission of infection to progeny insects (Virto et al., 2013). From the eggs of each female, a group of 24 neonate larvae (1–24 h post-hatching) was collected and reared individually on semi-artificial diet (Elvira et al., 2010) through to the adult stage (F₁), under standard laboratory conditions (25 ± 2 °C, 50 ± 10% RH, in a continuously dark room). F₁ adults were frozen at –80 °C for subsequent analysis.

2.2. Total DNA and RNA extraction

For detection of viral covert infections, total DNA and RNA were purified from both field-caught and F₁ adults after being sexed by observation of the external genitalia. Master Pure Complete DNA and RNA Purification kit (Epicentre Biotechnologies) protocols were used for total DNA and RNA extraction. The abdomens of frozen adults were dissected and placed individually in a 2 ml microfuge tube with ceramic beads, 300 µl tissue lysis solution and 1 µl proteinase K (50 ng/µl). Samples were homogenized using MP FastPrep-24 tissue cell homogenizer at 4 m/s for 20 s and incubated at 65 °C for 15 min at constant 1100 rpm orbital agitation. Samples were divided in two 150 µl aliquots. One aliquot was used for DNA extraction and treated with 1 µl RNase at 37 °C for 30 min. Debris was pelleted by adding protein precipitation reagent. The supernatant was washed with isopropanol, twice with 70% ethanol, and the pellet was resuspended in 30 µl milli-Q water and stored at –20 °C. For RNA extraction, a protein precipitation reagent was added to the 150 µl aliquot, centrifuged at maximum speed for 13 min and the supernatant washed with isopropanol to precipitate the nucleic acids. Pellets were treated with RNase-free DNase buffer and 5 µl of DNase for 30 min at 37 °C. A volume of 200 µl of 2 × T and C lysis solution was added and vortexed for 5 s followed by 200 µl of protein precipitation reagent and vortex for 10 s. The debris was pelleted by centrifugation and the supernatant was washed once with isopropanol and twice with 70% ethanol. Finally, RNA was resuspended in 30 µl DEPC (diethylpyrocarbonate) water and stored at –20 °C. Blank extraction samples containing only water were processed in parallel to detect cross-contamination during the extraction process. All equipment and reagents were previously sterilized and treated with DEPC to remove RNases.

2.3. Detection of SeIV and SeMNPV by RT-PCR and qPCR

The presence of two single-stranded RNA viruses belonging to the *Iflaviridae* family, named SeIV-1 and SeIV-2, was determined by multiplex RT-PCR. Specific primers were designed to amplify a 457-bp and 297-bp in the RNA-dependent RNA polymerase (RdRp) region (SeIV1-Fw: 5'-CATTCAAGACGGTTACACCATTC-3'; SeIV1-Rv: 5'-GACTTTGAATACACGGGACGG-3'; SeIV2-Fw: 5'-GAGTCCATCGTTCATCTTGGC-3'; SeIV2-Rv: 5'-TAGGAGAGCCACAGAGGACTTG-3')

designed using the genomic sequences of SeIV-1 (Millán-Leiva et al., 2012) and SeIV-2 (Choi et al., 2012), respectively. Before reverse transcription, an 8 μ l volume of RNA solution was treated with 1 μ l DNase and 1 μ l DNase buffer (Promega) at 37 °C for 30 min to remove DNA contamination. Following this, 1 μ l DNase stop (Promega) was added and incubated at 65 °C for 10 min. Finally, a 4 μ l volume of the resulting RNA solution was incubated at 70 °C for 5 min with 1 μ l dT primer. The reverse transcription mix consisted of 2 μ l 5 \times buffer (Promega), 1.2 μ l MgCl₂ (25 mM), 0.5 μ l dNTP mix (10 mM), 0.8 μ l DEPC water and 1 μ l ImProm-II reverse transcriptase (Promega). The mix was added to RNA samples and incubated at 25 °C for 5 min, followed by 42 °C for 60 min and 70 °C for 15 min. For PCR amplification, 1 μ l cDNA was used as template and mixed with 2.5 μ l NH₄ (10 \times), 1.25 μ l MgCl₂ (50 mM), 0.25 μ l dNTPs mix (10 mM), 0.5 μ l of both SeIV1-Fw and SeIV1-Rv primers (10 μ M), 0.3 μ l of both SeIV2-Fw and SeIV2-Rv primers (10 μ M), 18.15 μ l sterile milliQ water and 0.25 μ l Taq DNA polymerase (Bioline). The PCR protocol consisted of an initial denaturation cycle at 95 °C for 1 min, 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and an extension cycle of 72 °C for 5 min. PCR products were visualized by electrophoresis in 1% agarose gels containing ethidium bromide. A Bioline HyperLadder IV size marker was used for size determination. cDNA fragments were visualized in a UV transilluminator, Chemi Doc (Syngene). Primer specificity and the identity of amplified fragments were validated previously in samples of virus-free insects and virus-infected insects. The sensitivity of the reaction was estimated using 10-fold serial dilutions of a mixture of SeIV-1 and SeIV-2 cDNA containing from 1.66 pg/ μ l to 1.66 \times 10⁻⁵ pg/ μ l and from 1.50 \times 10⁻² pg/ μ l to 1.50 \times 10⁻⁷ pg/ μ l of SeIV-1 and SeIV-2, respectively. This mixture originated from viral transcription of iflavirus infected larvae, kindly provided by A. Jakubowska (unpublished results). The limit of detection was defined as the lowest concentration producing a clear electrophoresis band, corresponding to 1.66 \times 10⁻⁴ pg/ μ l and 1.50 \times 10⁻⁴ pg/ μ l, which equated to 18 and 29 genome copies per reaction for SeIV-1 and SeIV-2 respectively, according to the reported genome sizes (Choi et al., 2012; Millán-Leiva et al., 2012). cDNA of each iflavirus was used as positive control in all multiplex reactions to ensure correct identification of the amplified fragments.

To detect SeMNPV infections we used a qPCR-based method described by Cabodevilla et al. (2011b) and slightly modified by Virto et al. (2013). Briefly, specific primers (DNApol149-Fw: 5'-CCGCTCGCCAACACTACATTAC-3'; DNApol149-Rv: 5'-GAATCCGTGT CGCCGTATATC-3') were designed to amplify a 149-bp region within the DNA polymerase gene based on the full genome sequence of SeMNPV-AI1 (Thézé et al., 2014). qPCR based on SYBR Green fluorescence was carried out in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in 96-well reaction plates. A Mastermix containing 10 μ l SYBR Premix Ex Taq (2 \times), 0.4 μ l ROX Reference Dye (50 \times) and 0.4 μ l of both DNApol149Fw and DNApol149Rv primers (10 μ M) was added to a 1 μ l template DNA. A blank extraction and four non-template reactions were included in each run. For the standard curve, CsCl-purified SeMNPV-AI1 DNA was quantified using a spectrophotometer (Eppendorf BioPhotometer plus). Ten-fold serial dilutions in sterile MilliQ water (from 100 to 1 \times 10⁻³ pg/ μ l) were used to construct the standard curve in duplicate. The amplification reaction consisted of denaturation at 95 °C for 30 s, followed by 45 amplification cycles at 95 °C for 5 s and 60 °C for 30 s. Finally, a melting curve analysis, involved a dissociation stage of 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s was added to confirm a single peak of the target product. The regression parameters of the standard curve were R² = 0.997 and slope = -3.570 (approximately 91% efficiency) (Bustin et al., 2009). The limit of detection was defined as the last standard concentration showing correct amplification

curves and the expected melting temperature (83.5 °C) point for the specific amplification product. This limit was determined at 10⁻³ pg/ μ l, representing 6.8 SeMNPV genomes per reaction. By extrapolation against the standard curve, this corresponded to a critical Cq (quantification cycle) value of 33.3 cycles. Data acquisition and analyses were performed using Sequence Detector Version 2.2.2 software (Applied Biosystems).

The frequencies of the different viruses in field-caught adults were compared for sampling method and adult gender using Pearson's χ^2 test in the SPSS Statistics package (v.19 IBM). The prevalence of infection in the progeny of covertly infected and non-infected parental females was examined by fitting generalized linear models (GLM) using the GLIM 4 program (Numerical Algorithms Group, 1993) with a binomial error specified. For this, the progeny of each female was considered as a distinct group. Changes in model deviance following sequential steps of model simplification approximate to a χ^2 distribution. Means separation was achieved by *t*-test (Crawley, 1993). The prevalence of infections among the sexes in progeny insects was compared by χ^2 test.

3. Results

3.1. Prevalence of SeMNPV and SeIV covert infections in field-caught adults

To evaluate the presence of SeMNPV and SeIV infections in *S. exigua* field-caught adults, abdomens of 130 moths were analyzed by qPCR and RT-PCR. A total of 70 (53.8%) insects were positive for the DNA polymerase SeMNPV gene, whereas the prevalence of iflaviruses was significantly lower ($\chi^2 = 38.75$, *df* = 1, *P* < 0.05), with 17 (13.1%) and 10 (7.7%) adults infected by SeIV-1 and SeIV-2, respectively (Fig. 1). Co-infections involving both virus families were also detected, with 11 individuals (8.4%) harboring both SeMNPV and one of the iflaviruses, and three adults carrying the three viruses (Table 1). Similar proportions of males and females were infected by SeMNPV ($\chi^2 = 0.331$, *df* = 1, *P* = 0.56) or the iflaviruses ($\chi^2 = 0.625$, *df* = 1, *P* = 0.20) (Fig. 1).

Sample methods were compared to assess whether the virus could be transmitted during overnight contact of adults in funnel traps. Similar frequencies of SeMNPV infections were detected in moths caught in funnel traps or individually attracted to the white sheet ($\chi^2 = 0.663$, *df* = 1, *P* = 0.20). SeIV-2 was the only RNA virus detected in trap-collected insects (Fig. 1). The proportion of SeIV1-infected insects was significantly greater when moths were captured individually compared to those caught and held overnight in funnel traps ($\chi^2 = 22.82$, *df* = 1, *P* < 0.05).

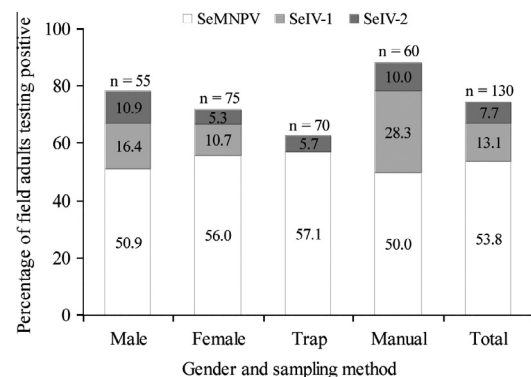


Fig. 1. Percentage of SeMNPV, SeIV-1 or SeIV-2 covert infection in field caught adults according to gender and sampling method. Numbers in the columns indicate the percentage of individuals that tested positive for each virus. Numbers above the columns indicate the number of individuals tested.

Table 1

Number of adults infected by multiple viruses in both field adults and F₁ generation. A total of 330 individuals were tested by qPCR and RT-PCR to detect a SeMNPV, SeIV-1 or SeIV-2 infection, respectively. Numbers in brackets indicate the number of individuals analyzed.

Mixed infection	Field adults (130)	F ₁ generation (200)
SeMNPV + SeIV-1 + SeIV-2	3	13
SeMNPV + SeIV-1	8	8
SeMNPV + SeIV-2	3	1
SeIV-1-SeIV-2	2	17

3.2. Transgenerational transmission

Virus transmission to offspring was investigated by detection of SeMNPV and SeIVs in offspring (F₁) adults. Ten field-caught females, either infected or non-infected by SeMNPV and proved to be RT-PCR negative for SeIV-1 or SeIV-2, were selected at random from those that had produced offspring. Groups of ten offspring that reached the adult stage were analyzed for each of the 10 maternal females. All descendents included in the analysis were reared individually from neonate larvae through to adulthood. Although we could not rule out the possibility of horizontal transmission in neonate insects, we did not observe overt infection by either virus (SeMNPV or SeIV) in any of the offspring included in this study.

The three viruses were detected in the laboratory-reared offspring (F₁). Overall, SeMNPV was detected in 21.5% of F₁ adults, whereas the overall prevalence of the iflaviruses (58%) was markedly higher in offspring (F₁) compared to that of field-caught adults (GLM: $\chi^2 = 3.94$, $df = 1$, $P < 0.05$).

Among the progeny of field-caught insects, the overall prevalence of infection did not differ according to the infection of the parental female (GLM: $\chi^2 = 0.009$, $df = 1$, $P > 0.05$), although the prevalence of SeIV-2 infection was significantly lower than that of SeMNPV in the progeny of females that were infected by SeMNPV (Fig. 2). The prevalence of SeIV-1 infection in progeny insects (39%) was consistently higher than that of SeMNPV or SeIV-2 (GLM: $\chi^2 = 23.68$, $df = 2$, $P < 0.05$) (Fig. 2). The prevalence of SeMNPV ($\chi^2 = 0.57$, $df = 1$, $P = 0.45$) or SeIV ($\chi^2 = 0.14$, $df = 1$, $P = 0.71$) infections did not differ significantly in male and female F₁ adults.

Mixed infections involving both virus families were detected in the F₁ individuals. All three viruses were detected in 13 out of 200

individuals tested (6.5%), whereas nine (4.5%) harbored two viruses (SeMNPV plus one of the SeIV variants). Both SeIV variants were detected in the same host in 17 out of 200 individuals (8.5%) (Table 1).

4. Discussion

Infections by single or multiple baculoviruses and iflaviruses, were detected both in *S. exigua* field-collected adults and in laboratory-reared offspring. The findings of the present study reveal that both types of virus naturally co-infect field populations of *S. exigua*. Moreover, laboratory rearing favoured this association, particularly with respect to the elevated transmission of the SeIV-1 variant. The natural association of the two viruses could have major implications for the mass production of SeMNPV-based insecticides and possibly for the efficacy of these products in pest control.

Covert infections by baculoviruses have been detected in field-caught adults of Lepidoptera, such as *S. exigua* (Cabodevilla et al., 2011a), *Spodoptera exempta* (Vilaplana et al., 2010), *Mamestra brassicae* (Burden et al., 2003), *Choristoneura fumiferana* (Kemp et al., 2011) *Operopthera brumata* (Burand et al., 2011) and in phytophagous larvae of the hymenopteran sawfly *Neodiprion sertifer* (Krokene et al., 2013). Similarly, iflaviruses produce sublethal infections in the European honeybee *Apis mellifera* (Yue et al., 2007), the Varroa mite, *Varroa destructor* (Ongus et al., 2004), the brown planthopper *Nilaparvata lugens* (Murakami et al., 2013) and *S. exigua* (Choi et al., 2012; Millán-Leiva et al., 2012). The viruses studied here were selected from those RNA viruses with potential to influence *S. exigua* population dynamics (Pascual et al., 2012). PCR-based techniques had been successfully used to detect inapparent infection by these viruses in laboratory *S. exigua* colonies (Jakubowska et al., 2014; Millán-Leiva et al., 2012).

Field-collected adults were found to harbor SeIV and SeMNPV, alone and in mixed infections, in reproductively active moths. A high prevalence of sublethal infection was detected; overall 62% of moths had one or more of the viruses, the majority of which were individuals infected by SeMNPV (54%). A previous study using RT-PCR to detect SeMNPV transcripts in adults, performed using insects from the same region, reported a prevalence of sublethal infection of 16% (Cabodevilla et al., 2011a). This difference in reported prevalence may reflect the greater sensitivity of the qPCR (6.8 genome copies per reaction, Virto et al., 2013) technique that we employed compared to the RT-PCR technique (35 genome copies per reaction; Cabodevilla et al., 2011a), or could also be a result of within-season or year-to-year variations in the prevalence of SeMNPV infections in *S. exigua* populations, that tend to increase during sequential cropping cycles (Cabodevilla et al., 2011a). Here we used qPCR due to its high sensitivity, as demonstrated in previous studies by us on SeMNPV covert infections (Cabodevilla et al., 2011b; Virto et al., 2013). For the first time, iflaviruses were detected in field-collected *S. exigua* adults, although the prevalence of infection (~20%) was lower than that of SeMNPV. Assuming that the qPCR-based method is more sensitive than RT-PCR, the greater prevalence of SeMNPV covert infection compared to that reported in previous studies is possibly explained by the technique used or by spatial or temporal variation in the prevalence of SeMNPV covertly infected insects. However by using multiplex RT-PCR the presence of mixed infections involving both iflaviruses was demonstrated. Although the difference in sensitivity of these techniques may have influenced the results, it is clear that the iflaviruses, particularly SeIV-1, were prevalent in natural populations of *S. exigua*.

We examined whether the presence of SeMNPV might influence the transmission of the iflaviruses (or *vice versa*) from field-collected insects to their laboratory-reared offspring. Unexpectedly,

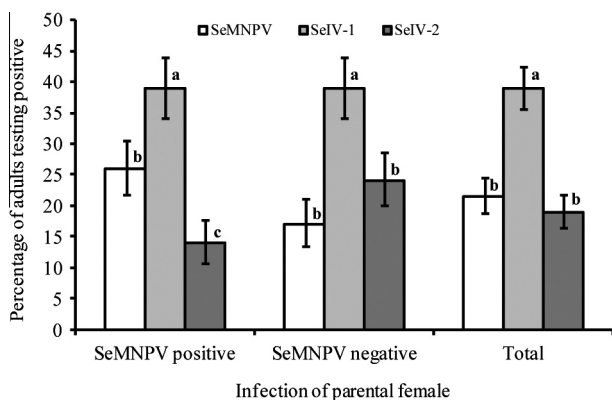


Fig. 2. Mean percentage of F₁ adults positive for SeMNPV, SeIV-1 or SeIV-2 among the offspring of field collected females that were classified as infected (SeMNPV positive) or non-infected (SeMNPV negative) and that were negative for iflavirus infection. Columns labeled with identical letters did not differ significantly for comparisons of the prevalence of each type of virus within each maternal infection status (GLM, *t*-test, $P > 0.05$).

the prevalence of iflavirus infection increased dramatically in F_1 insects, as high percentages of the offspring of iflavirus-negative females were found to be positive for SeIV-1 (39%) or SeIV-2 (19%) infection. A combination of highly efficient vertical and horizontal transmission could explain these results, since under laboratory conditions the transition from apparently healthy *S. exigua* colonies to 100% infection by the SeIV-1 was achieved in a single host generation (Millán-Leiva et al., 2012). Horizontal transmission of iflavirus most likely occurs via regurgitation or the production of feces that contaminate the larval diet, especially in laboratory reared insects (Murakami et al., 2013; van Oers, 2010). This is because the midgut is the most abundantly infected larval tissue and gregarious rearing conditions could lead to rapid contamination of diet by iflavirus particles in frass (Millán-Leiva et al., 2012). For this reason, F_1 neonates were individualized, reared individually and confined in the adult stage until qPCR analysis, in our study. The contribution of the male lineage to vertical transmission has been demonstrated in *Apis mellifera* eggs that were artificially inseminated with DWV-contaminated semen (Yue et al., 2007). In our study, the male contribution to virus transmission was not examined, but as males and females were infected at similar frequencies with the iflaviruses, it is possible that males could contribute to transgenerational transmission. In line with previous studies, the sex-specific distribution of SeMNPV infections in field-caught adults and their offspring was similar between male and female moths (Virto et al., 2013).

Co-infection by SeMNPV and SeIV in both field-collected populations and in the F_1 generation was detected at low prevalence. Also, SeIV-1 and SeIV-2 mixed infections rarely occurred in field-caught adults (2/130) but were more frequent in laboratory-reared individuals (17/200). Intriguingly, both *Ectropis obliqua* iflavirus and *Perina nuda* iflavirus were isolated from lepidopteran pests that had died from an associated baculovirus infection (Wang et al., 1999, 2004). These observations provided support for previous findings by Vail et al. (1983a,b) in which AcMNPV OB preparations were found to be contaminated with an iflavirus-like pathogen that persisted in the baculovirus population. Similarly, mixed infections caused by alphabaculoviruses and betabaculovirus were detected at low prevalence in a laboratory population of *C. fumiferana*, but not in field-sampled insects (Kemp et al., 2011). It seems that laboratory insect colonies often harbor persistent infections (Hughes et al., 1993; Kemp et al., 2011; Kouassi et al., 2009; Murillo et al., 2011). Interactions between co-infecting microorganisms are invariably complex and their consequences unpredictable. For instance, *Wolbachia* infection of dipteran species seemed to protect the host against RNA viruses (Glaser and Meola, 2010), whereas mortality due alphabaculovirus infection of *S. exempta* was 6–14-fold higher in *Wolbachia* infected hosts compared to healthy conspecific populations (Graham et al., 2012). Previous studies on *S. exigua* indicated that covert infections by SeMNPV affect host fitness by increasing their susceptibility to superinfection (Cabodevilla et al., 2011b). In line with this result, SeMNPV pathogenicity differed when bioassayed in covertly infected insects in comparison with virus-free insect lines (Cabodevilla et al., 2011a). Increased susceptibility to alphabaculovirus infections may be desirable in pest populations targeted for virus-based biological control, but ongoing laboratory bioassays will reveal whether covert infections by SeIV modify insect responses following consumption of lethal or sublethal doses of SeMNPV OBs.

5. Conclusions

Inapparent iflavirus infections of field captured insects used to start laboratory colonies for *in vivo* production of baculovirus have

the potential to influence the fidelity of the mass production process and the efficacy of baculovirus-based insecticides. Previous findings on iflaviruses in association with lethal baculovirus infections underline the value of determining whether susceptibility to baculoviruses is modulated by sublethal iflavirus infections or whether iflaviruses can affect the insecticidal properties of baculovirus insecticides. These studies are currently being performed by us.

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References

- Bianchi, F.J.J.A., van Essen, P.H.A., van der Werf, W., Smits, P.H., 2001. Transmission dynamics of the multicapsid nucleopolyhedrovirus SeMNPV in *Spodoptera exigua* populations in greenhouse chrysanthemum. *Proc. Exper. Appl. Entomol.* 12, 91–96.
- Burand, J., Kim, W., Welch, A., Elkinton, J.S., 2011. Identification of a nucleopolyhedrovirus in winter moth populations from Massachusetts. *J. Invertebr. Pathol.* 108, 217–219.
- Burden, J.P., Griffiths, C.M., Cory, J.S., Smith, P., Sait, S.M., 2002. Vertical transmission of sublethal granulovirus infection in the Indian meal moth, *Plodia interpunctella*. *Mol. Ecol.* 11, 547–555.
- Burden, J.P., Nixon, C.P., Hodgkinson, A.E., Possee, R.D., Sait, S.M., King, L.A., Hails, R.S., 2003. Covert infections as a mechanism for long-term persistence of baculoviruses. *Ecol. Lett.* 6, 524–531.
- Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55, 611–622.
- Caballero, P., Aldebis, H.K., Vargas-Osuna, E., Santiago-Alvarez, C., 1992. Epizootics caused by a nuclear polyhedrosis virus in populations of *Spodoptera exigua* in southern Spain. *Biocontrol Sci. Technol.* 2, 35–38.
- Cabodevilla, O., Ibañez, I., Simón, O., Murillo, R., Caballero, P., Williams, T., 2011a. Occlusion body pathogenicity, virulence and productivity traits vary with transmission strategy in a nucleopolyhedrovirus. *Biol. Control* 56, 184–192.
- Cabodevilla, O., Villar, E., Virto, C., Murillo, R., Williams, T., Caballero, P., 2011b. Intra- and intergenerational persistence of an insect nucleopolyhedrovirus: Adverse effects of sublethal disease on host development, reproduction, and susceptibility to superinfection. *Appl. Environ. Microbiol.* 77, 2954–2960.
- Choi, J.Y., Kim, Y., Wang, Y., Shin, S.W., Kim, I., Tao, X.Y., Liu, Q., Roh, J.Y., Kim, J.S., Je, Y.H., 2012. Complete genome sequence of a novel picorna-like virus isolated from *Spodoptera exigua*. *J. Asia-Pac. Entomol.* 15, 259–263.
- Cory, J.S., Myers, J.H., 2003. The ecology evolution of insect baculoviruses. *Ann. Rev. Ecol. Syst.* 34, 239–272.
- Crawley, M.J., 1993. *GLIM for Ecologists*. Blackwell Sci., Oxford, UK.
- Elvira, S., Williams, T., Caballero, P., 2010. Juvenile hormone analog technology: Effects on larval cannibalism and the production of *Spodoptera exigua* (Lepidoptera: Noctuidae) nucleopolyhedrovirus. *J. Econ. Entomol.* 103, 577–582.
- Glaser, R.L., Meola, M.A., 2010. The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile Virus infection. *PLoS ONE* 5, e11977.
- Graham, R.L., Grzywacz, D., Mushobozi, W.L., Wilson, K., 2012. *Wolbachia* in a major African crop pest increases susceptibility to viral disease rather than protects. *Ecol. Lett.* 15, 993–1000.
- Hughes, D.S., Possee, R.D., King, L.A., 1993. Activation and detection of a latent baculovirus resembling *Mamestra brassicae* nuclear polyhedrosis virus in *M. brassicae* insects. *Virology* 194, 608–615.
- Jakubowska, A.K., D'Angiolo, M., González-Martínez, R.M., Millán-Leiva, A., Carballo, A., Murillo, R., Caballero, P., Herrero, S., 2014. Simultaneous occurrence of covert infections with small RNA viruses in the lepidopteran *Spodoptera exigua*. *J. Invertebr. Pathol.*, <http://dx.doi.org/10.1016/j.jip.2014.06.009>.
- Kemp, E.M., Woodward, D.T., Cory, J.S., 2011. Detection of single and mixed covert baculovirus infections in eastern spruce budworm, *Choristoneura fumiferana* populations. *J. Invertebr. Pathol.* 107, 202–205.
- Kolodny-Hirsch, D.M., Sitchawat, T., Jansari, J., Chenrchaivachirakul, A., Ketunuti, U., 1997. Field evaluation of a commercial formulation of the *Spodoptera exigua* (Lepidoptera: Noctuidae) nuclear polyhedrosis virus for control of beet armyworm on vegetable crops in Thailand. *Biocontrol Sci. Technol.* 7, 475–488.
- Kouassi, L.N., Tsuda, K., Goto, C., Mukawa, S., Sakamaki, Y., Kusigemati, K., Nakamura, M., 2009. Prevalence of latent virus in *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) and its activation by a heterologous virus. *Appl. Entomol. Zool.* 44, 95–102.

- Krokene, P., Heldal, I., Fossdal, C.G., 2013. Quantifying *Neodipron* nucleopolyhedrovirus DNA from insects, foliage and forest litter using the quantitative real-time polymerase chain reaction. *Agric. Forest Entomol.* 15, 120–125.
- Lasa, R., Ruiz-Portero, C., Alcazar, M.D., Belda, J.E., Caballero, P., Williams, T., 2007. Efficacy of *Spodoptera exigua* multiple nucleopolyhedrovirus as a biological insecticide for beet armyworm control in greenhouses of southern Spain. *Biocontrol Sci. Technol.* 17, 221–232.
- Millán-Leiva, A., Jakubowska, A.K., Ferre, J., Herrero, S., 2012. Genome sequence of SelV-1, a novel virus from the *Iflaviridae* family infective to *Spodoptera exigua*. *J. Invertebr. Pathol.* 109, 127–133.
- Murakami, R., Suetsugu, Y., Kobayashi, T., Nakashima, N., 2013. The genome sequence and transmission of an iflavirus from the brown planthopper, *Nilaparvata lugens*. *Virus Res.* 176, 179–187.
- Murillo, R., Hussey, M.S., Possee, R.D., 2011. Evidence for covert baculovirus infections in a *Spodoptera exigua* laboratory culture. *J. Gen. Virol.* 92, 1061–1070.
- Numerical Algorithms Group, 1993. In: Francis, B., Green, C., Payne, C. (Eds.), *The GLIM System: Release 4 Manual*. Clarendon Press, Oxford, UK.
- Ongus, J.R., Peters, D., Bonmatin, J.M., Bengsch, E., Vlák, J.M., van Oers, M.M., 2004. Complete sequence of a picorna-like virus of the genus *Iflavirus* replicating in the mite *Varroa destructor*. *J. Gen. Virol.* 85, 3747–3755.
- Pascual, L., Jakubowska, A.K., Blanca, J.M., Cañizares, J., Ferré, J., Gloeckner, G., Vogel, H., Herrero, S., 2012. The transcriptome of *Spodoptera exigua* larvae exposed to different types of microbes. *Insect Biochem. Mol. Biol.* 42, 557–570.
- Smits, P.H., Vlák, J.M., 1988. Biological activity of *Spodoptera exigua* nuclear polyhedrosis virus against *S. exigua* larvae. *J. Invertebr. Pathol.* 52, 107–114.
- Smits, P.H., Vlák, J.M., 1994. Registration of the first viral insecticide in the Netherlands: the development of Spod-X based on *Spodoptera exigua* nuclear polyhedrosis virus. *Meded. Fac. Landbouwwet., Rijksuniv. Gent* 59, 385–392.
- Thézé, J., Cabodevilla, O., Palma, L., Williams, T., Caballero, P., Herniou, E.A., 2014. Genomic Diversity in European *Spodoptera exigua* multiple nucleopolyhedrovirus isolates. *J. Gen. Virol.* <http://dx.doi.org/10.1099/vir.0.064766-0>.
- Vail, P.V., Morris, T.J., Collier, S.S., Mackey, B., 1983a. An RNA virus in *Autographa californica* nuclear polyhedrosis virus preparations: Incidence and influence on baculovirus activity. *J. Invertebr. Pathol.* 41, 171–178.
- Vail, P.V., Morris, T.J., Collier, S.S., 1983b. An RNA virus in *Autographa californica* nuclear polyhedrosis virus preparations: Gross pathology and infectivity. *J. Invertebr. Pathol.* 41, 179–183.
- van Oers, M.M., 2010. Genomics and biology of Iflaviruses. In: Asgari, S., Johnson, K. (Eds.), *Insect Virology*. Caister Academic Press, Norfolk, UK, pp. 231–250.
- Vilaplana, L., Wilson, K., Redman, E., Cory, J., 2010. Pathogen persistence in migratory insects: High levels of vertically-transmitted virus infection in field populations of the African armyworm. *Evol. Ecol.* 24, 147–160.
- Virto, C., Zárate, C.A., López-Ferber, M., Murillo, R., Caballero, P., Williams, T., 2013. Gender-mediated differences in vertical transmission of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV). *PLoS ONE* 8, e70932.
- Wang, C.H., Wu, C.Y., Lo, C.F., 1999. A new picorna-like virus, PnPV, isolated from *Ficus* Transparent wing moth, *Perina nuda* (Fabricius). *J. Invertebr. Pathol.* 74, 62–68.
- Wang, X., Zhang, J., Lu, J., Yi, F., Liu, C., Hu, Y., 2004. Sequence analysis and genomic organization of a new insect picorna-like virus, *Ectropis obliqua* picorna-like virus, isolated from *Ectropis obliqua*. *J. Gen. Virol.* 85, 1145–1151.
- Yue, C., Schroder, M., Gisder, S., Genersch, E., 2007. Vertical-transmission routes for deformed wing virus of honeybees (*Apis mellifera*). *J. Gen. Virol.* 88, 2329–2336.