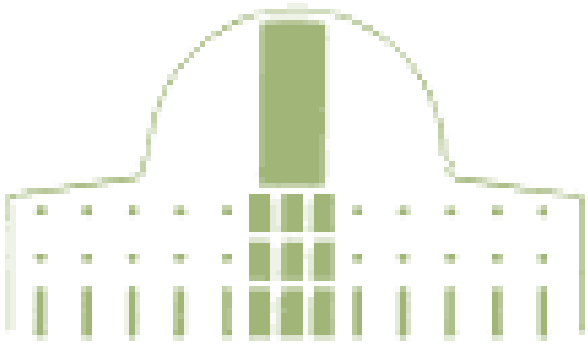
Vertical transmission of the *Spodoptera exigua multiple nucleopolyhedrovirus* and its application in biological control

CRISTINA VIRTO GARAYOA

Pamplona, 2016

Programa de Doctorado en Biotecnología
Universidad Pública de Navarra



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Memoria presentada por

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para optar al grado de Doctora por la Universidad Pública de Navarra

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INFORMAN:

que la presente memoria de Tesis Doctoral titulada **"Vertical transmission of the *Spodoptera exigua multiple nucleopolyhedrovirus* and its application in biological control"** elaborada por Dña. **CRISTINA VIRTO GARAYOA** ha sido realizada bajo nuestra dirección, y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor.

Y para que así conste, firman la presente en Pamplona a 7 de septiembre de 2016,

Fdo. Dr. Primitivo Caballero Murillo Fdo. Dra. Rosa Murillo Pérez Fdo. Dr. Trevor Williams

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RESUMEN

Las infestaciones de larvas de *Spodoptera exigua* (Lepidoptera: Noctuidae) son muy frecuentes en los cultivos de pimiento de los invernaderos de Almería. En estudios previos, la caracterización molecular e identificación de los aislados del *nucleopoliedrovirus múltiple* de *S. exigua* (SeMNPV; *Baculoviridae*) con mayor potencial insecticida así como el desarrollo de otras tecnologías (producción masiva y formulación) permitieron la obtención de un bioinsecticida que es más efectivo que los plaguicidas químicos convencionales para combatir las plagas de *S. exigua* en las condiciones de dichos invernaderos. Las aplicaciones de formulados de baculovirus se han realizado principalmente utilizando la modalidad de suelta inundativa, en la cual sólo cabe esperar que ejerza un efecto de control el inóculo liberado. Sin embargo, tras la aplicación de un tratamiento con baculovirus, además de la mortalidad producida por el inóculo liberado, se producen otros efectos sobre las sucesivas generaciones del insecto cuya repercusión en la regulación de las plagas que causa han sido poco estudiados. En esta tesis, básicamente se han analizado y cuantificado algunas interacciones huésped-baculovirus y se aportan datos cualitativos y cuantitativos que pueden servir de base para definir una metodología intermedia entre las sueltas de tipo inundativo e inoculativo.

En primer lugar se evaluó la incidencia de infecciones encubiertas causadas por el SeMNPV y dos virus de RNA pertenecientes a la familia *Iflaviridae* (*S. exigua* iflavirus-1: SeIV-1; *S. exigua* iflavirus-2: SeIV-2) en adultos de *S. exigua* capturados en los invernaderos de Almería. El SeMNPV fue detectado en un 54% de los insectos analizados, mientras que un 13% y 8% estaban infectados por el SeIV-1 y SeIV-2, respectivamente. Se encontraron infecciones múltiples en las que el 8% de los individuos eran portadores del SeMNPV y uno de los iflavirus, mientras que solamente el 2% de los adultos albergaba una infección triple. En la descendencia de las hembras de campo evolucionadas en laboratorio (F_1) se encontró un incremento en la prevalencia del SeIV-1 (39%) y SeIV-2 (19%) respecto a los parentales, mientras que la infección por SeMNPV fue transmitida al 21% de la descendencia. La co-infección producida por virus pertenecientes a distintas familias en la descendencia fue baja (4%) y solamente el 6% de los individuos fueron portadores de una infección triple.

Tras comprobar que las infecciones encubiertas por SeMNPV pueden llegar a afectar a más del 50% de individuos de poblaciones naturales de su huésped homólogo, se evaluó la contribución del género en la transmisión transgeneracional de dichas infecciones, mediante apareamientos entre adultos portadores de infecciones encubiertas del SeMNPV y otros libres de virus (machos sanos x hembras sanas, machos infectados x hembras

sanas, machos sanos x hembras infectadas, machos infectados x hembras infectadas). El análisis por qPCR del ADN extraído de adultos permitió detectar específicamente la presencia del virus en los parentales y en su descendencia (F_1). Tras comprobar que la prevalencia de la infección en los individuos parentales supervivientes a la inoculación del virus fue alta (65-85%), se determinó la incidencia de la infección en la progenie de cada uno de los apareamientos estudiados. La presencia del virus fue detectada en adultos descendientes (F_1) de cualquiera de los apareamientos con individuos parentales infectados, independientemente de su sexo. No obstante, la línea materna fue el doble de eficiente en la transmisión del virus (49%) que la paterna (26%) en términos de incidencia del virus en la descendencia. Además se determinó, mediante descontaminación superficial de los huevos, que la transmisión del virus se produce internamente (transovarial) y no superficialmente (transovum). En los adultos descendientes de individuos subletalmente infectados se encontró una correlación positiva entre la carga viral por insecto infectado y el porcentaje de individuos positivos del grupo parental del que provenían, es decir, que cuantos más insectos infectados había en un grupo, más carga viral albergaban dichos individuos.

Recientemente se ha demostrado que algunos genotipos del SeMNPV aparecen asociados a distintas vías de transmisión. El genotipo Se-Al1 (asociado a la transmisión vertical: TV) es capaz de producir una infección encubierta en un elevado porcentaje de los insectos que ingieren una dosis subletal y, además, dicha infección se transmite verticalmente durante al menos 5 generaciones sucesivas. El genotipo Se-G25 (asociado a la transmisión horizontal: TH), produce porcentajes de infecciones encubiertas significativamente menores pero, en cambio, presenta valores de patogenicidad y virulencia mejores que el genotipo Se-Al1. A la vista de estos resultados, se planteó estudiar mezclas de OBs de ambos genotipos en las siguientes proporciones (TV:TH): 25:75, 50:50 y 75:25, con la finalidad de determinar su efecto sobre las propiedades insecticidas del inóculo viral y la capacidad de producir infecciones encubiertas. Las mezclas de genotipos que contienen un 25 o un 75% del genotipo de TH mejoraron la patogenicidad respecto al genotipo de TV, sin embargo, no hubo diferencias significativas en cuanto a virulencia y producción de OBs/larva para ninguno de los tratamientos. Tras la aplicación de una dosis subletal, el genotipo de TV produjo un 90% de infecciones encubiertas en los insectos supervivientes, dato significativamente superior al 51% producido por el genotipo de TH. Todas las mezclas evaluadas (75:25, 50:50 y 25:75) fueron igual de eficientes que el genotipo de TV en cuanto a su capacidad de producir infecciones subletales. En condiciones de invernadero la capacidad de producir infecciones encubiertas fue menor, para todos los tratamientos, que en condiciones de laboratorio. El genotipo de TV produjo un 76% de adultos infectados, mientras que la infección producida por el de TH fue significativamente inferior (45%), obteniéndose valores intermedios para las mezclas 75:25

y 25:75. En cuanto a la eficiencia de dichos tratamientos en la protección del cultivo se determinó el daño directo e indirecto producido por las larvas en plantas de pimiento tras la aplicación de 5 x 10^8 OBs/l de los genotipos de TV, TH y la mezcla 75:25. Los tratamientos virales, que fueron igual de efectivos entre ellos, resultaron ser casi tan eficaces como el insecticida químico metoxifenocida cuando se evaluó el porcentaje de hojas dañadas. Al evaluar el daño directo en fruto los genotipos puros y la mezcla 75:25 fueron tan eficaces como la metoxifenocida. Por tanto, la incorporación de genotipos del SeMNPV con buenas cualidades para la TV en los formulados de bioinsecticidas, no sólo serían un método de control eficaz frente a plagas causadas por *S. exigua* sino que a su vez podría contribuir a disminuir la cantidad de inóculo viral necesario para el control de las siguientes generaciones del insecto.

El conocimiento de los factores que regulan la reactivación de infecciones encubiertas producidas por el SeMNPV, para producir infecciones francas en larvas de *S. exigua,* sería muy útil para el control de la plaga y podría contribuir a disminuir el número de aplicaciones necesarias de bioinsecticidas basados en el SeMNPV. En este sentido, se estudió la aplicación de diferentes materias químicas y biológicas (otros entomopatógenos) como factores de reactivación de una infección encubierta para convertirla en una infección letal en larvas de segundo estadio que albergaban una infección encubierta del SeMNPV. Los tratamientos con sulfato de cobre (0,1%), sulfato de hierro (1%) y selenito de sodio (1 ppm) produjeron una mortalidad por baculovirus de 12, 15 y 41%, respectivamente, en condiciones de laboratorio. La capacidad de activación de sulfato de cobre (0,1%) y selenito de sodio (1 ppm) también se evaluó en condiciones de invernadero sobre larvas con infección encubierta que se encontraban infestando plantas de pimiento. Sin embargo, menos del 3% de las larvas recolectadas murieron por baculovirus lo cual sugiere que estas sustancias tienen una baja capacidad para activar infecciones encubiertas en las condiciones descritas en este ensayo.

Finalmente, en esta tesis se han estudiado las infecciones encubiertas y su transmisión vertical causadas por el SeMNPV y se discute la aplicación que pueden tener estos resultados en el diseño de nuevas estrategias de control de *S. exigua* como alternativa a las aplicaciones inundativas de los bioinsecticidas a base de SeMNPV que hasta el momento se realizan en los invernaderos de Almería.

SUMMARY

The beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae) is an important pest of pepper crops in Almerian greenhouses. In previous studies, the identification and molecular characterization of native strains of the *S. exigua multiple nucleopolyhedrovirus* (SeMNPV; *Baculoviridae*) with great insecticide potential, as well as the development of additional technologies (mass-production and formulation procedures) has led to the creation of a baculovirus-based bioinsecticide which provides growers with alternative options that can be more effective than conventional chemical insecticides to suppress the damage of *S. exigua* larvae in Almerian greenhouses. So far, applications of baculovirus-based insecticides are almost invariably based on inundative methods, similar to the strategy of applications based on the use of chemical products. In the present thesis, the SeMNPV vertical transmission and long-term persistence infections in natural populations of *S. exigua* and their impact on successive generations of infected insects were examined. These findings could constitute the basis for a new strategy of inoculative applications.

First, the incidence of covert infections caused by the SeMNPV and two RNA viruses belonging to the *Iflaviridae* family (*S. exigua* iflavirus-1: SeIV-1; *S. exigua* iflavirus-2: SeIV-2) was evaluated in *S. exigua* adults collected in Almerian greenhouses. Overall, 54% of field-caught adults were infected by SeMNPV, whereas 13% and 8% were infected by SeIV-1 and SeIV-2 respectively. Multiple infections were also detected, with 8% of individuals harbouring SeMNPV and one of the iflaviruses, while just 2% of adults were infected by all three viruses. In the offspring of field collected females reared under laboratory conditions (F_1), the prevalence of SeIV-1 and SeIV-2 increased to 39% and 19%, respectively, in relation to the parental generation, whereas the prevalence of SeMNPV infection in the progeny was 21%. Co-infection produced by viruses belonging to different family groups was as low as 4%, and mixed infections involving three viruses was only detected in 6% of the insects.

After verifying that SeMNPV covert infections may affect more than 50% of the individuals belonging to natural populations of their homologous host, the contribution of gender in transgenerational transmission of these infections was assessed. For this, four mating groups involving SeMNPV covertly infected and virus-free insects were performed (healthy males x healthy females, infected males x healthy females, healthy males x infected females, and infected males x infected females). qPCR analysis of DNA extracted from adults revealed that the virus was present in both parents and their offspring (F_1). After verifying that the prevalence of covert infection in parental individual survivors to a virus challenge as larvae was high (65-85%), the prevalence in offspring was examined. Viral

DNA was detected in adult descendants (F_1) of any of the infected parental mating group, independently of gender. However, female-mediated vertical transmission (49%) was approximately twice as efficient as male-mediated transmission (26%) in terms of virus incidence in the progeny. Furthermore, egg surface decontamination did not have significant effect on the level of viral transmission suggesting that the main route of transmission was transovarial rather than transovum. A positive relationship was found between the percentage of infected adults in the F_1 generation and their viral load, suggesting that adults that transmit the virus to a high proportion of their offspring tend to transmit greater amounts of viral DNA.

Recently, it has been demonstrated that certain SeMNPV genotypes are associated with different routes of transmission. The Se-Al1 genotype (associated with vertical transmission: VT) produced a high percentage of covert infections in survivors of a virus challenge and was transmitted through five successive generations. In contrast, the Se-G25 genotype (associated with horizontal transmission: HT), showed greater insecticidal properties in terms of pathogenicity and virulence. Interactions between these genotypes was determined using mixtures of OBs in the following proportions (VT:HT): 25:75, 50:50 and 75:25, in order to select a mixture that had a useful combination of insecticidal properties and was capable of producing covert infections. OBs mixed populations involving 25 and 75% of the HT genotype improved pathogenicity compared to VT genotype, whereas no significant difference was found in virulence and productivity (OBs/larva) for any of the treatments. The ability to produce covert infections in insect survivors of a sublethal dose was significantly higher using the VT genotype (90%) than the HT genotype (45%), whereas mixtures comprising 75:25, 50:50 and 25:75 were as efficient as the VT genotype in producing sublethal infections. Overall, under greenhouse conditions the ability to produce covert infections was lower compared to that observed in laboratory assays. VT genotype produced 76% of infected adults, while the infection produced by HT genotype was significantly lower (45%), intermediate values were obtained by the 75:25 and 25:75 mixtures. Regarding crop protection efficiency of the mixtures, direct and indirect injury caused by survivors to an application of 5×10^8 OB/l of the VT, HT and 75:25 mixture were compared with a chemical insecticide (methoxyfenozide) in field trials in pepper crops. Viral treatments, which were all equally effective, were nearly as effective as the methoxyfenozide treatment in terms of foliar feeding damage. Regarding direct damage to fruits, pure genotypes and the 75:25 mixture were as effective as methoxyfenozide. Therefore, the addition of SeMNPV genotypes with the capacity for vertical transmission into baculovirus-based bioinsecticides could provide effective crop protection against *S. exigua* larvae and may also contribute to extend the timing between field applications due to transgenerational control capacity of the virus.

The activation of covert infections into lethal diseases in *S. exigua* larvae could contribute to initiate epizootics, and therefore reduce the number of applications of SeMNPV-based bioinsecticides. Different chemicals and entomopathogens were studied as a trigger factors in covertly infected *S. exigua* second instars. Virus activation was observed in insects treated with 0.1% copper sulfate, 1% iron (II) sulfate, and 1 ppm sodium selenite that resulted in 12, 15, and 41% of lethal polyhedrosis disease, respectively. The rate of activation of 0.1% copper sulfate and 1 ppm sodium selenite was also evaluated in greenhouse trials using covertly infected larvae for artificially infesting sweet pepper plants. However, less than 3% of the collected larvae died by baculovirus suggesting that these substances did not activate covert infections under the conditions described in this assay.

Finally, in this thesis the use of SeMNPV covert infection and vertical transmission have been discussed as the basis of novel control strategies for *S. exigua* and for pest damage reduction. These findings could contribute to the design of alternative virus control strategies to the inundative applications that are presently used in Almerian greenhouses.

CHAPTER I

Introduction

1. General introduction and scope of research

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is a polyphagous pest responsible for great economical losses in both open-field and greenhouse crops worldwide (CABI, 2016). In southern Spain *S. exigua* populations frequently attain pest status in greenhouses of pepper crops between the months of June and September (Belda, 1994; Moreno et al., 1992). The excessive use of chemical pesticides against this pest over a period of decades has generated a number of phytosanitary problems associated with the presence of insecticidal residues in pepper fruits (Glass and Egea, 2012), the selection of *S. exigua* populations resistant to a variety of chemical insecticides (Ahmad and Arif, 2010; Moulton et al., 2002; Smagghe et al., 2003; Torres Vila et al., 1998; Wang et al., 2006) and the incompatibility of broad spectrum insecticides with biological control methods (Lara and Urbaneja, 2002; Stansly et al., 2005). All this, coupled with the demand of Almerian growers for effective control products, compatible with the use of natural enemies, has lead to the development of biopesticides based on the *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV; family *Baculoviridae*; genus *Alphabaculovirus*).

The Microbial Bioinsecticides Group (UPNA-CSIC) has conducted an extensive study on SeMNPV as a biological control agent, including the genotypic and phenotypic characterization of native virus populations (Muñoz et al, 1999; Murillo et al., 2007), the selection of genotypes and mixtures of genotypes with different insecticidal properties (Murillo et al., 2006), field trials of efficacy, mass-production methods and formulation procedures (Lasa, 2007). These studies resulted in the registration and commercialization of a biopesticide based on SeMNPV under the trade name of Virex® (BioColor S.L.). This product provides better protection than chemical insecticides or *Bacillus thuringiensis* based products and has been used widely by growers in integrated pest management (IPM) programs in Almería (Caballero et al., 2009).

However, we should not forget the risk of the emergence of resistance among treated insects due to the repeated use of high concentrations of this virus. An example of this situation is the case of the *Cydia pommonella granulovirus* (GV; family *Baculoviridae*; genus *Betabaculovirus*) in Europe. In 2005, *C. pomonella* populations were reported to have developed 10,000-fold reduction in

their susceptibility to their homologous granulovirus (CpGV) (Asser-Kaiser et al., 2007). Fortunately, the selection of new genotypes from distinct natural isolates (Eberle et al., 2008) or successive passages in resistant insects (Berling et al., 2009) proved effective in overcoming resistance to the virus.

Recent studies on the molecular ecology of SeMNPV have revealed that covert infections in *S. exigua* populations are frequent and seem to have an important role in the vertical transmission of the virus (Cabodevilla et al., 2011a; Virto et al., 2014). Covert or non-lethal infections have been described as a strategy for virus persistence in response to a variable environment or the lack of opportunities for horizontal transmission (Cory and Myers, 2003). New genotypes of SeMNPV that differed in their biological characteristics were isolated from soils of Almerian crops (Murillo et al., 2007) and from an insect colony that originated from individuals collected in greenhouses of this area (Cabodevilla et al., 2011a). Interestingly, genotypes from soil samples had favorable insecticidal properties, whereas genotypes obtained from larvae that died spontaneously in the colony tended to produce a higher prevalence of covert infections and pass on the virus to progeny (Cabodevilla et al., 2011a; Cabodevilla et al., 2011b). Therefore, the former genotypes were considered to be horizontally transmitted genotypes and the latter, vertically transmitted genotypes.

Given this scenario, the aim of this thesis was to examine some aspects of SeMNPV vertical transmission and covert infections, in order to provide field application effectiveness of SeMNPV-based insecticides for extended control programs. These studies have been specifically focused on: i) the incidence of SeMNPV covert infections and its transmission, as well as other viruses that covertly infect field populations of *S. exigua,* ii) the influence of host gender on vertical transmission, iii) the use of mixing vertically and horizontally transmitted genotypes in OB mixtures to improve viral transmissibility and the chance of transgenerational host mortality, and iv) the evaluation of biological and chemical stressors as trigger factors of lethal disease that could activate persistent infections under laboratory and field conditions.

2. *Spodoptera exigua*

2.1 Morphology

Spodoptera exigua adults are medium-sized moths with a wingspan of 25 to 30 mm, have brownish to grey forewings and semi-transparent hindwings crossed by dark veins (Figure 1A) (Capinera, 2008). Males and females can be distinguished by their abdomen shape, being wider the latter than the former. The eggs, 0.35-0.37 mm in diameter, are laid in clusters of 30-200 eggs, and often cover by female scales. The egg color varies from yellowish-white to greenish and gradually changes to dark brown as embryos develop, becoming black shortly before hatching (Figure 1B) (Amaldoss and Hsue, 1989).

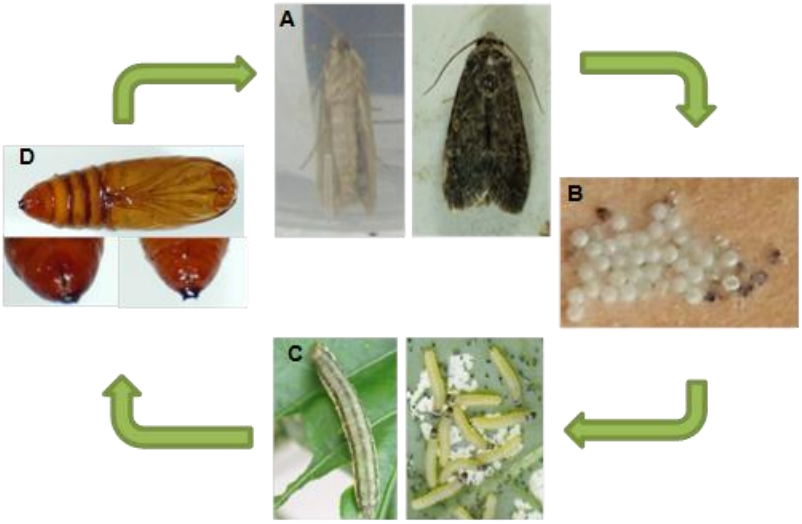

Figure 1. Development stages of the *S. exigua* life cycle: A) ventral and dorsal view of adult, B) eggs, C) fifth instar larva (left) and pre-molt second instar larvae (right), D) pupa (top), last segments on the ventral surface of female (bottom on the left) and male pupae (bottom on the right).

During the first and second instars, larvae are pale green or yellow, gregarious and may feed on foliage in large groups (Figure 1C, right panel). At the third instar they acquire pale stripes, and at the fourth instar they turn dark grey, or yellowish green showing dark stripes runs dorsally and laterally. During the fifth instar color varies depending on the food source, between green and dark grey with white spiracles that contain a narrow black border (Figure 1C, left panel)

(Capinera, 2008; Cayrol, 1972). Eruciform larvae develop throughout five instars reaching up to 3.6 cm long (Cayrol, 1972). Additional instars have been reported under certain conditions. Mature larvae develop to 15 to 20 mm long, reddish-brown, fusiform pupae (Figure 1D, top panel) (Capinera, 2008). Sexual dimorphism is easily identified by observing the last segments of the pupa abdomen. Females may be distinguished from males by the presence of a longitudinal vent on the ventral surface of the penultimate segment, whereas males possess two circular notches on the last segment (Figure 1D, bottom panel).

2.2 Geographical distribution and host plants

The beet armyworm *S. exigua* is a migratory species originates from South-East Asia and currently worldwide distributed in all tropical and subtropical areas (Figure 2) (CABI, 2016). In Europe, due to the cold winter conditions and the lack of diapause, *S. exigua* can only successfully overwinter under greenhouses conditions (Sunderland et al., 2010) or in the warmest regions such as southern Spain (Belda et al., 1994). Nevertheless, because of its dispersal abilities, in summer regular migrations are common from Africa to Europe reaching the British Isles and Scandinavia (French, 1969).

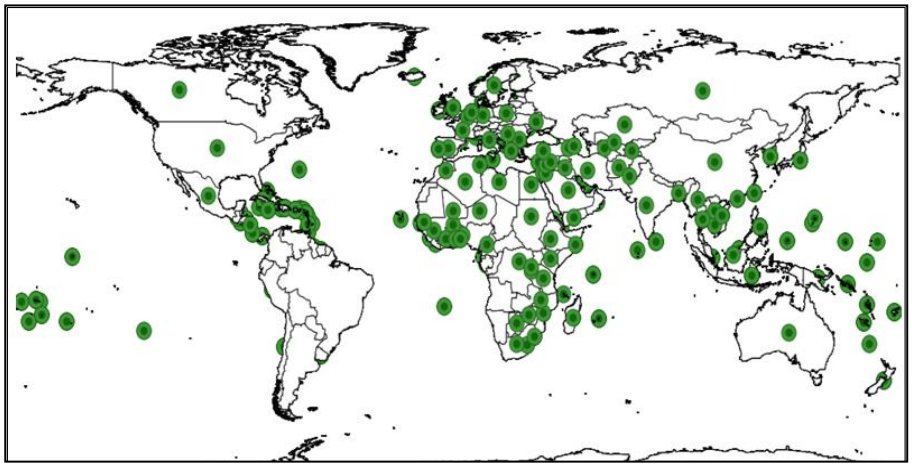

Figure 2. Geographic distribution of *S. exigua* in the world. Green circles represent countries where *S. exigua* is present (CABI, 2016).

The beet armyworm is a polyphagous species that produces serious economic losses worldwide (Brady and Ganyard, 1972). It has an extremely wide host range of more than 200 plant species (Brown and Dewhurst, 1975). Larval feeding behavior produces both indirect and direct damage by consuming leaves or fruits and flowers, respectively. In southern Spain, *S. exigua* represents a key pest in both horticultural crops like peppers, courgette, melon or watermelon (Moreno et al., 1992) and in open-field crops like cotton, sunflower or alfalfa (Belda, 1994). It has also been observed attacking ornamental crops such as chrysanthemum.

2.3 Biology and ecology

As adults *S. exigua* are extremely mobile and can fly, mainly at night, up to 3500 km in just 9 or 11 days (Mikkola, 1970). Mating occurs principally at night, soon after emergence of the moths, and oviposition begins within two to three days. Oviposition extends over a three to seven day period, and the moths usually die within nine to ten days after emergence (Amaldoss and Hsue, 1989). Female egg production ranges between 600 and 1700 eggs (Chu and Wu, 1992). Under field conditions females deposit the eggs over the underside of leaves to prevent desiccation and predation of the eggs. During warm weather eggs hatch in two to three days. Larval development is temperature-dependent and varies between 14.5 days at 33 °C and 120.5 days at 15 °C (Karimi-Malati et al., 2014). Late instar larvae enter the soil where pupation occurs. Duration of the pupal stage is six to seven days during warm weather. The duration of the life cycle varies according to climate; under favorable conditions the life cycle can be completed in just twenty days (Belda, 1994).

The number of *S. exigua* generations per year is variable depending of the geographical region and host plant (Ali and Gaylor, 1992). A maximum of eleven generations per year has been observed in China (Amaldoss and Hsue, 1989). In southern Spain, three to four generations per year are estimated to occur in outdoor crops. Greenhouses conditions involving low competition for food, high nutritional quality, a limited number of natural enemies and high temperatures and relative humidity, results in up to seven or eight generations per year (Belda, 1994).

2.4 Feeding damage

The larvae of *S. exigua* cause damage in at least 60 cultivated plant species from 23 families (Brown and Dewhurst, 1975), including crops of major economic importance such as pepper, cucumbers, pumpkins, melon or watermelon, and field crops including cotton, sunflower or alfalfa as well as ornamental plants such as chrysanthemums and other non-commercial plants (Belda, 1994).

Early instar larvae feed gregariously on the undersides of leaves removing parenchyma and reducing the photosynthetic area as a result (Figure 3A). As they grow they perforate the leaves, causing defoliation (Figure 3B), and move up to the top of the plant as solitary larvae, leaving upper leaves skeletonized (Figure 3C). High infestations often result in larval feeding on fruits, resulting in scaring, boreholes, and rotting, that leads to a significant reduction in the commercial value of the crop (Figure 3D) (Lasa, 2007). Occasionally larvae are observed inside the flower buds of some species where they are protected from predators and contact insecticides (Figure 3E).

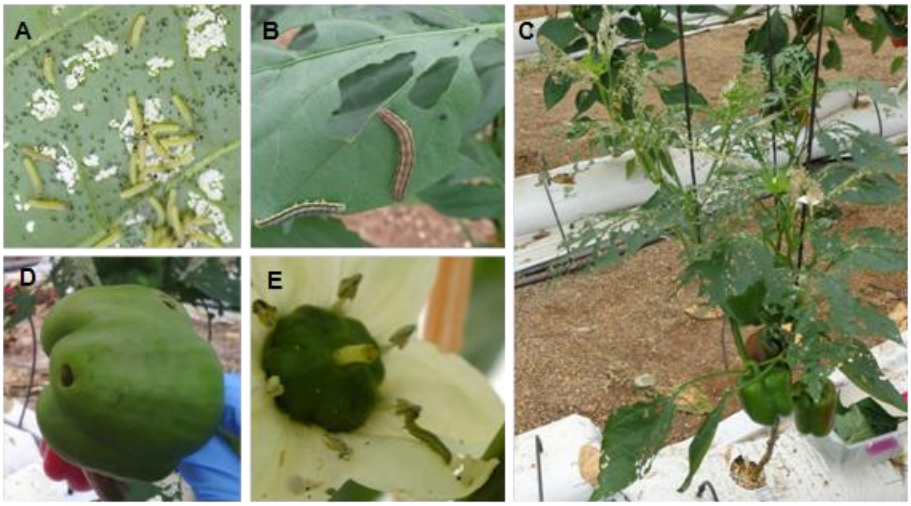

Figure 3. Feeding damage caused by *S. exigua* in pepper crops: A) early instar larvae producing superficial feeding damage, B) defoliation caused by final instar larvae, C) hard defoliation caused by *S. exigua* in a pepper plant, D) pepper fruit damaged by *S. exigua*, and E) *S. exigua* larva feeding on a pepper flower.

2.5 *S. exigua* impact in greenhouses of southern Spain

The province of Almería comprises an estimated area of 29,500 ha of intensive greenhouse production that due to the double cultivation reaches the 44,000 ha per year (Figure 4) (Junta de Andalucía, 2015). Almerian growers supply the European Union with the principal salad vegetables, being this province the leading vegetable exporter during 2015 (http://www.freshplaza.com). The 2014-15 production cycle exceeded 2.6 million tons, of which 1.9 were exported with value of 1,771 million € (Junta de Andalucía, 2015). Intensive horticulture takes place nearly all year round. Sweet pepper is one of the main crops in which *S. exigua* pest pressure occurs from June to October (Belda, 1994). Annually, over 6 million euros have been spent on chemical insecticides to control this pest in the region (Lasa, 2007).

Figure 4. Aerial view of Almeria showing area covered by greenhouse horticultural production. Picture from Glass and Egea (2012).

2.6 Control methods

2.6.1. Chemical control

Control of *S. exigua* has been achieved by spraying synthetic insecticides (Chandler and Ruberson, 1996; Ishtiaq and Saleem, 2011). Because of its polyphagous nature this pest has been exposed to an intensive use of a variety of chemical products, which have generated a series of problems such as the

appearance of resistance associated to a lack of an efficacy control (Ahmad and Arif, 2010; Moulton et al., 2002; Smagghe et al., 2003), the generation and accumulation of chemical residues on fruits (Glass and Egea, 2012), and the incompatibility between chemical control measures and natural enemies utilized against other pests that coexist with *S. exigua* (Caballero et al., 2009). Over the past 50 years, crop protection has relied on synthetic chemical pesticides, consequently, so far resistance to at least 39 active ingredients of insecticides (http://www.pesticideresistance.org) including abamectin, cypermethrin, endosulfan or spinosad has been reported for *S. exigua* (Ahmad and Arif, 2010; Che et al., 2015; Ishtiaq and Saleem, 2011; Osorio et al., 2015; Wang et al., 2006). Insect growth regulators (IGR) such as methoxyfenozide, flufenuxoron, lufenuron or tebufenozide have become very popular, but insect resistance selection has also been demonstrated as a consequence of abusive used that lead to a dramatic effectiveness reduction of field applications (Smagghe et al.,2003; Osorio et al., 2008; Zhou et al., 2011).

The same scenario was described in Almería some years ago mainly because of pest resistance to insecticides (Torres-Vila et al., 1998), and due to the European Union 2009 directive based on Integrated Pest Management (IPM) measures and focused on achieving a sustainable use of pesticides (European Commission, 2009). Moreover, residues of isofenphos-methyl were found in peppers from Almería during 2006, a pesticide that had not been registered for horticultural use, prevented their commercialization in Europe (Glass and Egea, 2012). On the other hand, the fact that other phytophagous pests had being controlled by biological control agents (Lara and Urbaneja, 2002; Stansly et al., 2005) made the search for a safe and effective control agent for *S. exigua* as a priority in the Almería region, and particularly in sweet pepper crop (Lasa, 2007; van der Bloom, 2010).

In the last ten years there has been a real “green revolution” in the province of Almería, thousands of crops hectares are becoming from conventional agriculture to integrated production with biological control. Initially, at a small scale and with unpredictable results, however, due to an early pest detection, release of predators and parasitic, the use of entomopathogen agents and the grown experience, the implementation of IPM programs became technically viable and economically feasible (Glass and Egea, 2012; van der Blom, 2010). For instance,

the use of chemical insecticides was drastically reduced and the greenhouse area passed from 1,400 biologically-controlled hectares in 2007 to 26,372 ha in 2014 (Chandler et al., 2011; Sanchez et al., 2014). Although biological control is currently implemented in the greenhouse sector of Almería, chemical pesticides were recorded in growing crops of tomato, pepper, cucumber and aubergine during 2011/2012 season (Glass and Egea, 2012). This highlights the need for maintaining an IPM programs using a combination of the best control methods available.

2.6.2. Pheromone traps

Several investigations of *S. exigua* sex pheromones have been carried out to assess whether they can be useful to capture males and consequently disrupt mating and inhibit reproduction (Chandler et al., 2011; Trumble and Baker, 1984). A series of acetates and alcohols have been isolated from virgin females, identified as sex pheromones and tested in field conditions to catch males (Takai and Wakamura, 1990; Tumlinson et al., 1990). Synthesized compounds based on the most successful pheromones have been developed, being the most effective compound a blend of (Z, E)-9,12-tetradecadienyl acetate (Z9E12-14:OAc) and (Z)-9-tetradecenyl alcohol (Z9-14:OAc) in a 10:1 proportion respectively, which was as effective as virgin females in trapping males (López, 1998; Mitchell et al., 1983). However, blends without compounds described above were not effective as traps, remarking the importance of these two pheromones (Tumilnson et al., 1990).

In spite of being useful for monitoring and mass trapping, sex pheromones are applied at low levels in IPM programs (Chandler et al., 2011). Egg masses and young larvae were reduced to 6 and 1% after Z9E12-14:OAc and Z9-14:OAc blend application in field conditions (Takai and Wakamura, 1990). However, when beet armyworm density increases pheromones are ineffective to pest control and additional control methods are required (Kerns, 2000).

2.6.3. Biological control

Large complexes of natural enemies parasitize or prey on *S. exigua* populations at different moments of its life cycle. Eggs and small larvae are especially susceptible to predation by adults and nymphs of *Lygus hesperus* (Hemiptera: Miridae), *Nabis americoferus* (Hemiptera: Nabidae) and *Orius*

tristicolor (Hemiptera: Anthocoridae), and adults of *Collops vittatus* (Coleoptera: Melyridae) and *Hippodamia convergens* (Coleoptera: Coccinellidae) (Ehler, 2007). The endoparasitoid *Chelonus insularis* (Hymenoptera: Braconidae) is the most abundant parasitoid of eggs and young larvae (Caballero et al., 1990; Sertkaya et al., 2004) whereas some parasitoids of larvae such as *Hyposoter exiguae* (Hymenoptera: Ichnemonidae), *Pristomerus spinator* (Hymenoptera: Ichnemonidae) and *Microplitis pallidipes* (Hymenoptera; Braconidae) (Jiang, 2010) have also been reported frequently.

Among the most abundant parasitoid species found in southern Spain are the braconid *Meteorus pulchricornis* (Hymenoptera: Braconidae), the ichneumonid *Hyposoter didymator* (Hymenoptera: Ichneymonidae) and the tachinid *Gonia bimaculata* (Diptera: Tachinidae) producing considerable levels of mortality in non-treated crops outside greenhouses (Caballero et al., 1990; Cabello, 1989). These natural enemies are important mortality agents in open field crops (Ehler 2004), but not to a commercially acceptable level (Sunderland et al., 2010).

2.6.4. Microbial control

Entomopathogens known as Microbial Control Agents (MCA), are by far the most promising groups of biological control agents developed against *S. exigua* (Cory and Franklin, 2012). Fungal species such as *Beauveria bassiana* (Hypocreales: Clavipitaceae), *Nomurea rileyi* (Moniliales: Moniliaceae) and *Metarrhizium anisopliae* (Hypocreales: Clavicipitaceae) (Hung and Boucias, 1992; Kao and Tsai, 1989), and the entomopathogenic nematodes *Steinernema carpocapsae* (Nematoda: Steinermatidae) have been evaluated (Gothama et al., 1996; Gothama et al., 1995) but their application is unusual in greenhouses of southern Spain due to the high temperatures and a lack of moisture on the crop (Glass and Egea, 2012).

Several strains of *Bacillus thuringiensis* (Bt) are known to infect and kill *S. exigua* larvae (Estruch et al., 1996; Xue et al., 2005). In Spain until 2007, the only MCA registered and marketed against *S. exigua* was Bt *ser. aizawai* and *ser. kustaki* but its efficacy has diminished because of intensive use and the development of pest resistance (Herrero et al., 2005).

In contrast, the *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV; family *Baculoviridae;* genus *Alphabaculovirus*), has proven to be highly

efficient against the beet armyworm (Kolodny-Hirsch et al., 1993). This pathogen naturally regulates host population by causing epizootics that reduce *S. exigua* populations by up to 80% in both open field and greenhouses crops (Caballero et al., 1992a). Native SeMNPV genotypes were studied as the active ingredient of the first bioinsecticide based on a native baculovirus registered in Spain (Figure 5) (Caballero et al., 2009; Lasa, 2007).

Figure 5. Commercial product based on a Spanish strain of the SeMNPV and used to control *S. exigua* in southern Spain.

3. Baculovirus morphology and taxonomy

Baculoviruses are arthropod-specific viruses isolated from insect species belonging to the orders Lepidoptera, Hymenoptera and Diptera (Caballero and Williams, 2008; Herniou et al., 2003), and are practically ubiquitous. Nowadays, they are well known for their ability as biological insecticides (Moscardi, 1999), gene expression vectors for transduction of mammalian cells and gene therapy (Clem and Passarelli, 2013). The baculovirus genome consists of a single, double-stranded, circular, supercoiled DNA molecule. So far over 60 baculovirus genomes are fully sequenced, with sizes varying from about 80 to over 180 Kb, that encode between 90 and 180 genes (Rohrmann, 2013). The members of this family produce occlusion bodies (OBs), which contain, protein-lipid enveloped virions comprising in turn, rod-shaped nucleocapsids of 40-60 nm in diameter and 230-385 nm in length (Ackermann and Smirnoff, 1983; Boucias and Pendland, 1998; Federici, 1986; Herniou et al., 2012).

During the infection cycle two types of virions are formed that exhibit identical genomic material, similar nucleocapsid structure, but differ in their function and envelopes composition: occlusion derived virus (ODV) responsible for the primary infection, and budded virus (BV) responsible for the secondary infection (Figure 6).

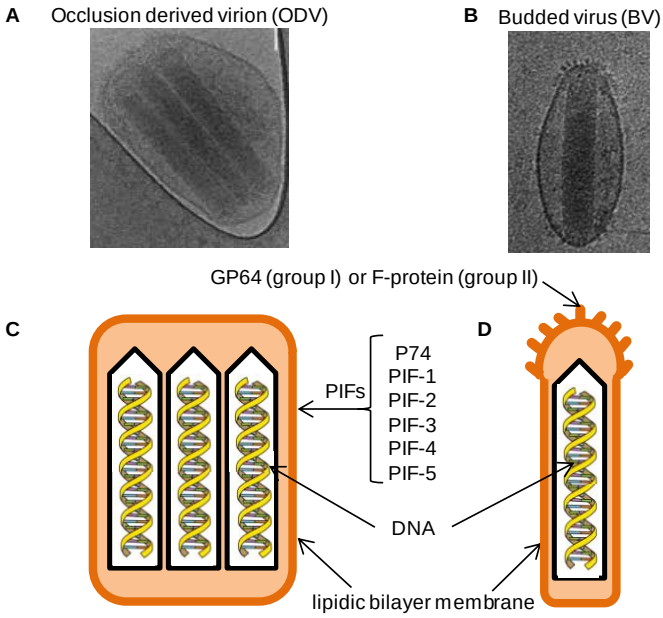

Figure 6: Cryo-electron microscope images (A and B) and structural composition (C and D) of the two virion phenotypes produced during the baculovirus infection cycle. Occlusion derived virions (ODVs) (A and C) and budded virus (BVs) (B and D) contain identical nucleocapsids but differ in the lipid and protein composition of their envelopes. Images from Wang et al. (2016).

ODVs are contained in the occlusion bodies (OBs) where they preserve their infectivity capacity outside the host. ODVs are formed in the nucleus of the virus-infected cell and may contain a variable number of nucleocapsids. The membrane of the ODVs is synthesized *de novo* (Stoltz et al., 1973) and has proteins recognized as envelope components and other proteins designated as important *per os* infectivity factors (PIFs) (Figure 6C) (Braunagel et al., 1996; Fang et al., 2009; Faulkner et al., 1997; Haas-Stapleton et al., 2004; Kikhno et al., 2002; Lapointe et al., 2004; Li and Blissard, 2009; Ohkawa et al., 2005; Pijlman et al., 2003; Zhang et al., 2005). These proteins play an essential role in the ODV

infectivity to the midgut cells. Late in the replication cycle ODVs are surrounded by large amounts of a protein matrix (polyhedrin or granulin) to form the OBs. OBs are highly stable and can persist in the environment outside the host for long periods of time and are responsible for horizontal transmission of the virus (Rohrmann, 2013). BVs are involved in cell to cell infection and systemic spread of disease. They always contain a single nucleocapsid that buds out through the plasma membrane of the insect cell thereby acquiring a lipoprotein envelope containing a virus-encoded fusion protein known as GP64 (in group I NPVs) or F protein (in group II NPVs), that allows budding and entry into new target cells (Figure 6D) (Blissard and Wenz, 1992; Garry and Garry, 2008; Monsma et al., 1996; Oomens and Blissard, 1999; Pearson et al., 2000).

Formerly baculoviruses were classified into two genera according to OB morphology: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Figure 7) (Murphy et al., 1995). NPVs produce large polyhedron-shaped structures (0.15 to 15 μm in diameter) called occlusion bodies (OBs) that are composed of a polyhedral protein matrix surrounding several ODVs that can comprise a single nucleocapsid (SNPV) or multiple nucleocapsids (MNPV) (Figure 7) (Slack and Arif, 2007). GVs are smaller than NPVs, with a granular shaped structure, 150 nm in diameter and 400-600 nm in length. They comprise a protein matrix of granulin that always contains one ODV with a single nucleocapsid (Figure 7) (Slack and Arif, 2007).

Since 2006, baculovirus classification has been based on genome sequence-based phylogeny and the family is now divided into four genera: *Alphabaculovirus* (lepidopteran-specific NPVs), *Betabaculovirus* (lepidopteran-specific GVs), *Gammabaculovirus* (hymenopteran-specific NPVs) and *Deltabaculovirus* (dipteran-specific NPVs) (Jehle et al., 2006; King et al., 2012).

Phylogenetic analysis indicated that the genus *Alphabaculovirus* can also be divided into two groups (I and II), so that the envelope fusion protein GP64 or the F protein is present in the BVs of members of group I and group II, respectively (Pearson and Rohrmann, 2002; Rohrmann, 2013), although this subdivision is not officially recognized by the International Committee on Taxonomy of Viruses (ICTV) in the current division. SeMNPV belongs to group II, together with *Spodoptera frugiperda* MNPV and *Lymantria dispar* MNPV. The last revision of the

ICTV includes 49 species of which 32 are *Alphabaculovirus*, 14 are *Betabaculovirus,* 1 is a *Deltabaculovirus* and 2 are *Gammabaculovirus* (ICTV, 2014), but this classification is continuously being updating.

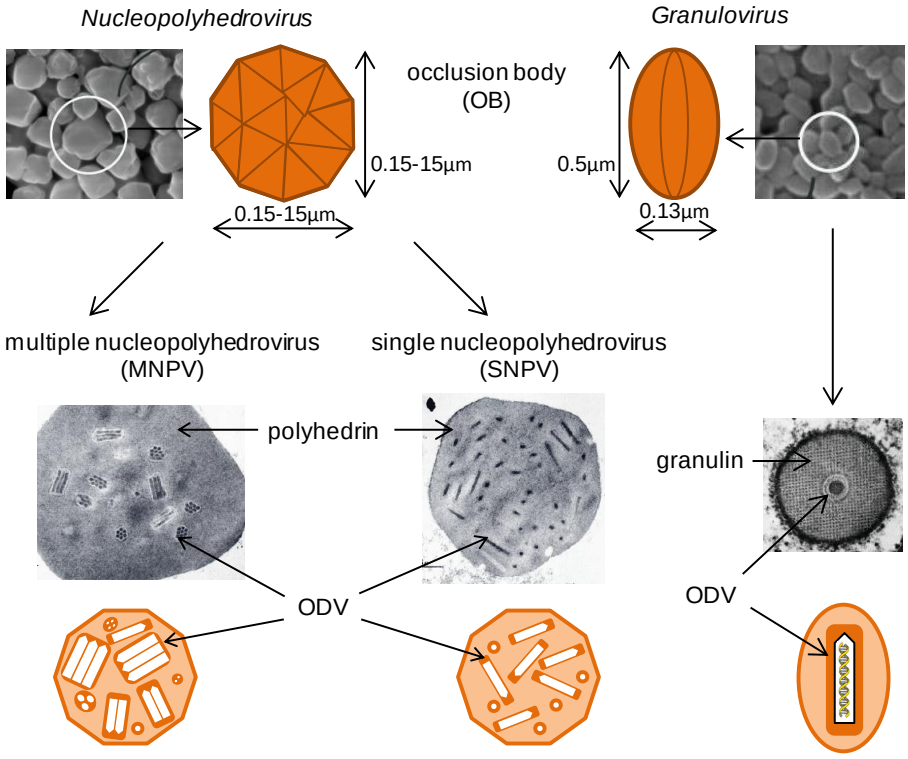

Figure 7: Electron microscope images and schematic representation of the two former genera of baculovirus: *Nucleopolyhedrovirus* and *Granulovirus*, and cross section of two nucleopolyhedroviruses and one granulovirus showing occlusion derived virions (ODVs). Images from Rohrmann, (2013).

4. The baculovirus infection cycle and pathogenesis

The baculovirus infection cycle varies according to the genus of the virus (Jehle et al., 2006). Alphabaculovirus and betabaculovirus infections are generalized with all host tissues affected, whilst the replication of deltabaculoviruses and gammabaculoviruses are restricted to the insect midgut cells (Jehle et al., 2006). Since SeMNPV is a member of the *Alphabaculovirus* genus, this replication cycle is described in detail.

The alphabaculovirus infection cycle starts when a susceptible larva ingests the OBs present on the foliar surface (Figure 8A). When the OBs reach the insect midgut, its alkaline pH dissolves the OB matrix and numerous ODVs are released (Figure 8B). The first barrier that the ODVs encounter in the insect midgut is the peritrophic membrane (PM), a chitin and glycoprotein matrix that protects the epithelial cells. Although it is not completely clear how the ODVs pass through the PM, it is known that they are helped by host cell proteinases and viral enhancins that digest the mucin component (Lepore et al., 1996; Slavicek and Popham, 2005; Toprak et al., 2012; Wang and Granados, 1997). ODVs then infect the midgut epithelial cells, where primary infection takes place. ODV envelope proteins, namely PIFs, fuse with the membrane of the epithelial cells and nucleocapsids are released into the cell cytoplasm (Figure 8C). Nucleocapsids are transported to the nuclear membrane in a process that involves actin polymerization (Goley et al., 2006; Ohkawa et al., 2010) and pass directly through nuclear pores into the nucleus of the cell (van Loo et al., 2001).

Once in the nucleus, viral DNA replication (Figure 8D) is initiated that eventually results in the production of new nucleocapsids which finally bud out of the nucleus and then exit the cell to become BV for the systemic phase of infection (Figure 8E). In addition some nucleocapsids may bypass the nuclear replication phase and may bud out of the basal side of the cell to continue viral spread (Slack and Arif, 2007). Newly formed BVs use tracheal cell projections that penetrate through the basal lamina to access the tracheal system, an excellent pathway to propagate the secondary infection to other tissues (fat body, muscle, trachea, hemocytes, epithelial cells) (Figure 8F) (Engelhard et al., 1994; Flipsen et al., 1995; Maina, 1989). Part of the new assembled nucleocapsids that remain in the nucleus acquire an envelope synthetized *de novo* to form ODVs (Figure 8G). At the end of the cycle high levels of polyhedrin are produced, which accumulates in nuclei and at some point condenses and crystallizes around the ODVs to form the occlusion bodies (Figure 8H) (Hamblin et al., 1990; Wood et al., 1994). At the end of the infection cycle the nuclear and the plasma membrane breakdown releasing the OBs into the hemocoel.

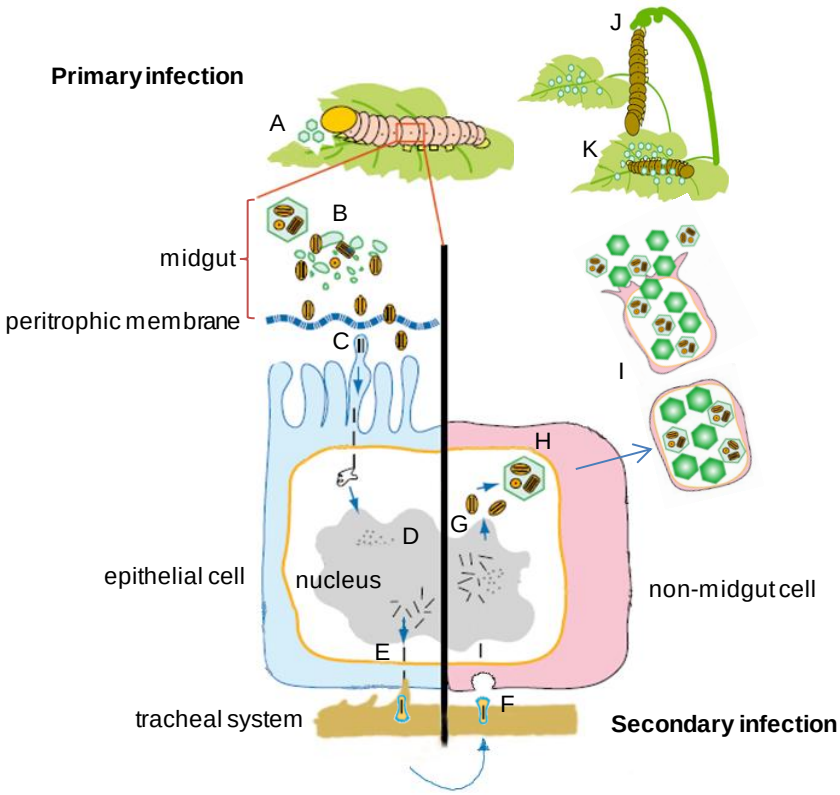

Figure 8: Alphabaculovirus infection cycle. Sequential steps of alphabaculovirus multiplication during primary and secondary infection are numered by letters. Adapted from Ikeda et al. (2015).

Some days after OBs have been ingested the typical signs and symptoms of infection start to be visible: larvae lose their appetite and become less active, molting is blocked and the color and brightness of the tegument changes (Granados and Williams, 1986). As the infection progresses, an enormous production of OBs occurs within cells of the insect host (Figure 8I). Shortly before death host behavioral changes modulated through *egt* and *ptp* viral genes, induce larval to climb to the upper parts of the plants (Figure 8J) (Hoover et al., 2011; Katsuma et al., 2012, van Houte et al, 2012). Upon death they typically hang by their last abdominal pseudopods. At this point tissue liquefaction and cuticle rupture occurs mediated by viral encoded chitinase and cathepsin proteins, favoring OBs dispersion into the environment (Federici, 1997).

5. Baculovirus diversity

Large DNA viruses evolved several million years ago along with their host and consequently as different insects proliferated so did their viruses (Thezé et al., 2015). Baculoviruses have been isolated from more than 700 insect species from a variety of ecosystems including aquatic and terrestrial habitats (Caballero and Williams, 2008). Molecular and biological characterization has revealed a large genotypic and phenotypic diversity, both between different baculovirus species (interspecific diversity) (Figure 9A), but also between isolates belonging to certain baculovirus species (intraspecific diversity) (Figure 9B). The advent of molecular tools in genomic sequencing together with restriction endonuclease analysis (REN) and PCR-based techniques have increasingly revealed the high degree of alphabaculovirus genotypic diversity (Erlandson, 2009; Muñoz and Caballero, 2001), which might explain differences in phenotypic characteristics linked to insecticidal properties (Serrano et al., 2015).

5.1 Interspecific diversity

The comparative analyses of different baculovirus genome sequences have provide information on viral genome size, gene content (Herniou et al., 2003; Miele et al., 2011) and genomic organization (Serrano et al., 2013). The comparison of 57 genome sequences of baculoviruses has revealed that 31 core genes are responsible for the main biological functions, such as transcription of viral late genes, virion structure and primary and systemic infections (Hayakawa et al., 2000; Rohrmann, 2013; van Oers and Vlak, 2007). Non-core genes are specific to certain genera or species and provide diversity within baculovirus populations (Miele et al., 2011; van Oers and Vlak, 2007). A phylogenetic approach based on specific sequences has been developed for classification and nomenclature of viral species (Jehle et al., 2006). In this sense, the phylogenetic distance of conserved genomic regions has been proposed as the principal criterion to assign species status, based on Kimura 2-parameter values derived from concatenated *polh*, *lef-8* and *lef-9* nucleotide sequences. Using this system isolates are considered different species when the K-2 distance exceeds 0.05, whilst they belong to the same species when the parameter is less than 0.015. Intermediate values between 0.015 and 0.05 require additional information on the isolates phenotypic and ecological characteristics (Kimura, 1980).

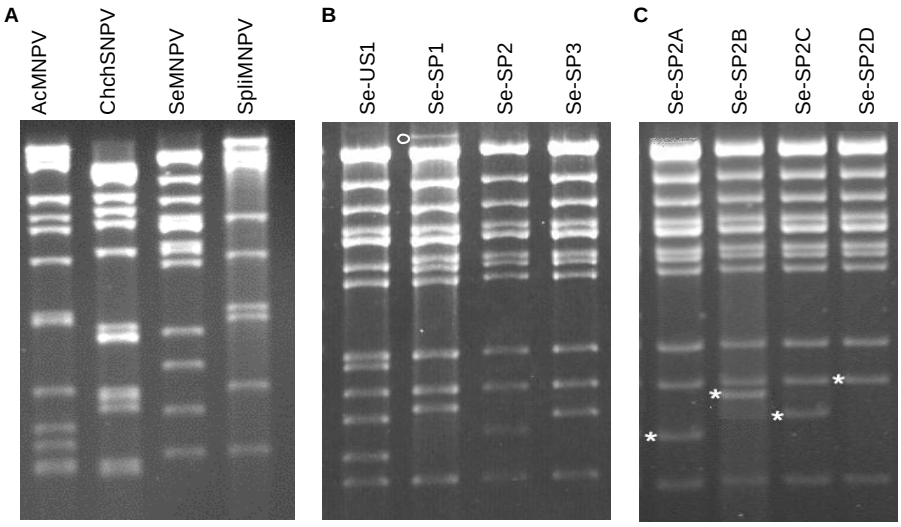

Figure 9. *Pst*I restriction endonuclease profiles of the genomic DNA of A) four different baculovirus species: *Autographa californica* MNPV (AcMNPV), *Chrysodeixis chalcites* SNPV (ChchSNPV), *Spodoptera exigua* MNPV (SeMNPV) and *Spodoptera littoralis* MNPV (SpliMNPV); B) four different geographical isolates of the SeMNPV: SeMNPV-US1 (Se-US1), SeMNPV-SP1 (Se-SP1), SeMNPV-SP2 (Se-SP2), SeMNPV-SP3 (Se-SP3); and C) four genotypic variants of the SeMNPV-SP2 purified by an *in vivo* method Se-SP2A, Se-SP2B, Se-SP2C, and Se-SP2D (adapted from Muñoz et al., 1999). White circle indicate submolar fragments and asterisks indicate characteristic fragments of genotypes.

Host range and insecticidal properties are also important for classification (Cory and Myers, 2003). Baculoviruses receive the name of the species from which they were first isolated. The host range defined as the spectrum of insect species susceptible to the virus, is a crucial aspect for the use of baculovirus-based bioinsecticides and the associated risks. Alphabaculovirus specificity ranges from a single host species, such as the SeMNPV, to a broader host spectrum as seen in AcMNPV (and related *Anagrapha falcifera* MNPV and *Rachiplusia ou* MNPV; Harrison and Bonning, 1999), which infects up to 50 species from 15 different families. But, not all host species are equally susceptible to a particular virus. Host permissivity to the infection can be grouped into three categories: permissive, semi-permissive and non-permissive (Bishop et al., 1995). When more than one baculovirus species simultaneously infect the same host, genetic material might be exchanged and recombination could occur. For this reason, heterologous infections represent a source of variability in virus

populations that may result advantageous for evolution, adaptation and virus survival in the field (Cory and Myers, 2003).

Baculoviruses have demonstrated highly levels of recombination in both culture cells (Croizier and Ribeiro, 1992; Erlandson 2009) and *in vivo* (Muñoz et al, 1997). Also, the morphological structure of NPVs can facilitate transmission of high levels of heterogeneity since genetically different variants can be package within the same OB (Bull et al., 2001) or even in the same ODV (Clavijo et al., 2010). Interestingly, recent studies demonstrated the possibility of generating ODVs containing mixtures of different virus species (Beperet, 2014). The development of the termed "co-occlusion technology" opened a door to explore the possibility for virus improvement without the use of recombinant technology, and to customize products according host range requirements in particular crops (Arrizubieta, 2015).

5.2 Intraspecific diversity

High degrees of genotypic and phenotypic variability have also been reported within baculovirus species (Erlandson, 2009; Muñoz and Caballero, 2001), likely as a consequence of host-pathogen adaptation and evolution (Cory and Myers, 2003). Genotypic variants have usually been distinguished by restriction (REN) profiles of DNA, in which the presence of submolar bands are frequently found, indicating the presence of mixtures of genotypic variants (Figure 9B). Genetic heterogeneity has been widely demonstrated between different geographical isolates (Barrera et al., 2011; Figueiredo et al., 1999; Gettig and McCarthy, 1992; Shapiro et al., 1991), and within a viral population associated with a region (Murillo et al., 2006; Cooper et al., 2003a). The genotypic structure of NPV isolates has been studied in depth, by separating the variants using *in vitro* (Lynn et al., 1993; Ribeiro et al., 1997; Simón et al., 2004a; Arrizubieta et al., 2013) or *in vivo* cloning techniques (Figure 9C) (Muñoz et al., 1999; Smith and Crook, 1988) or more recently, Bacterial Artificial Chromosome (BAC) technology (Wang et al., 2003). By *in vitro* cloning techniques up to twenty five genotypes of the *Panolis flammea* NPV were identified from a single infected caterpillar (Cory et al., 2005). Interestingly, non-autonomous replicative genotypes were identified as part of mixed genotype isolates of SeMNPV (Muñoz et al., 1998) and SfMNPV (Simón et al., 2004a, 2005). These defective genotypic variants were unable to

infect orally because they lacked essential genes (*pif*). They were first thought to act as parasitic variants because their presence reduced the virulence and pathogenicity of the wild type population (Muñoz and Caballero, 2000). However subsequent studies reported advantages in the transmissibility in mixtures comprising defective and other variants (Simón et al., 2005; Serrano et al., 2013), confirming the functional role of these deleted variants and explaining their persistence in natural virus populations.

The genotypic heterogeneity is attributable to a number of mechanisms such as DNA deletions (Muñoz et al., 1998; Pijlman et al., 2001) or insertions (Muñoz et al., 1998), duplication events, point mutations or transposon insertions (Jehle et al., 1995; Jehle et al., 1998). In principle, genetic variation might occur anywhere in the genome, but it is frequently confined in certain regions termed hypervariable regions (Cory et al., 2005; Muñoz et al., 1999), related to homologous regions (*hrs*) and baculovirus repeat ORFs (*bro* genes). Recombination seems to be the main force involved in the evolution of baculoviruses (Cory and Myers, 2003; Cory, 2010; Hajos et al., 2000), but this phenomenon is important when its produces differences in the phenotype. Cloned genotypes from a single isolate exhibit differences associated with their biological characteristics (Muñoz et al., 1998; Muñoz et al., 1999; Simón et al., 2004; Simón et al., 2008, Erlandson, 2009), even when the genotypes hardly differ at the genomic level.

More interestingly, recent studies have focused on the genomics behind phenotypic traits in relation to insecticidal properties. Five SeMNPV genotypic variants associated with different routes of transmission and different insecticidal properties (Cabodevilla et al., 2011) were fully sequenced and were shown to have an identity of 97.3% (Thezé et al., 2014). A number of ORFs such as *se04*, *se05*, *se76* and *se129* identified as likely to be involved in pathogenic and virulence traits, since genomes deleted in those genes resulted in decreased pathogenicity. Specifically, *se05*-deleted genomes were 10-fold less pathogenic and less virulent than the corresponding wild type isolate (Serrano et al., 2015).

5.3 Maintenance of diversity

The genotypic variation in natural baculovirus populations is maintained over the time, suggesting that this heterogeneity is important for survival and

evolution (Cory, 2010; Hodgson et al., 2003). Some mechanisms or strategies involved in the maintenance of this heterogeneity may include trade-offs between different phenotypes, differential selection, interactions between genotypes, or interspecific competition (Cory, 2010; Cory and Myers, 2003; Hitchman et al., 2007; Hodgson et al., 2001, 2003). The trade-off theory proposes that pathogen traits with opposing effects on fitness are likely to be correlated. The most obvious case is the correlation between speed of kill and OB production that allow the coexistence of fast-killing and low yield genotypes with slow-killing and high yield genotypes (Hernandez-Crespo et al., 2001; Muñoz et al., 2000). Recently, pathogenicity and virulence (speed of kill) have been suggested to be associated with virus transmission pathways. The most pathogenic genotypes are likely to favor horizontal transmission to facilitate rapid and efficient exploitation of each infected insect, whereas the less pathogenic genotypes are may be more capable of persisting in the host and be transmitted to the progeny (Cabodevilla et al., 2011a).

Another source of variation is related to variant selection inherent in better performance of a particular genotype under certain environmental conditions (Cory, 2010). Hence, the passage of a wild type isolate comprising a mixture of variants through alternative hosts might alter the relative abundance of individual variants, indicated by changes in the REN profile (Kolodny-Hirsch and VanBeek, 1997; Weitzman et al., 1992) and eventually its phenotypic characteristics (Hitchman et al., 2007; Espinel et al., 2010). Similarly the host plant species may also influence the overall biological characteristics of the viral population. When *P. flammea* larvae were fed on two host plants and inoculated with two different genotypes, each genotype had higher pathogenicity and virulence for each plant species, promoting the maintenance of distinct genotypes in different environments (Hodgson et al., 2002). Also abiotic factors such as the sensitivity to ultraviolet irradiation (UV) or temperature could result in a differential performance of certain genotypes (Brassel and Benz, 1979).

Mixed infection, involving more than one genotype, might lead to three different scenarios: i) an antagonistic interaction in which mixed genotypes compete resulting in a reduction in viral fitness compared with the performance of single genotypes (Arends et al., 2005; Barrera et al., 2013; Muñoz and Caballero, 2000; Muñoz et al., 1998), ii) neutral interaction when the properties of the

genotypes and the wild type are equal (Milks et al., 2001), and iii) synergistic interaction in which the traits of mixtures performed better than its individual components (Bernal et al., 2013, Hodgson et al., 2004; Simón et al., 2005). The outcome of such interactions is unpredictable and depends on the combination of the genotypes tested. More interesting is the case reported by Lopez-Ferber et al. (2003) in which a mixture of a complete genotype and a genotype defective in *per os* infectivity factors resulted in a phenotype with increased OB pathogenicity. This result derives from the fact that different genotypes can replicate in the same cell, and may even be enveloped in the same ODV so that defective genotypes need to replicate in the presence of complete genotypes (that provide PIF factors) to achieve transmission. The co-occlusion of multiple genotypes within the same ODV or OB has been described as one of the most important mechanisms for maintaining phenotype heterogeneity in NPVs (Clavijo et al., 2010).

The possibility of selecting the most promising genotypes based on insecticidal properties such as OB pathogenicity, speed of kill, and OB production is fundamental for biopesticide development, since the production of specific mixtures of genotypes allows researchers to improve the biological properties of the active ingredient compared to those of wild type isolates. The co-occlusion of different genotypic variants has also resulted in improvements in insecticidal characteristics compared to mixtures of OBs of the individual component genotypes (Bernal et al., 2013). For this reason, co-occlusion technology has been adopted to enhance the insecticidal properties of recently patented baculovirus-based insecticides (Arrizubieta, 2015; Beperet, 2014).

6. Baculovirus persistence and dispersion

Baculoviruses persist outside the host via OB formation thanks to the proteinaceous coat that allows viral particles to remain stable in the environment. Conventional wisdom is that OB persistence is the key for viral transmission between susceptible hosts (Fuxa, 2004). The capacity of baculoviruses to persist and disperse in the environment is directly related with the probability of successfully infecting a new susceptible host, so the better the persistence and dispersion of the virus the more likely to encounter a suitable host. OBs can persist in environmental reservoirs protected from ultraviolet light (UV) during

years (Fuxa, 1974) or even decades (Olofsson, 1998). Abiotic agents like precipitation and gravity transport the OBs from the corpses on the canopy of plants to the bottom of plants, crevices in bark or to the soil (Fuxa and Richter, 2001). Therefore the soil is the main reservoir that provides protection from UV and allows OB populations to persist for years in crops without losing their insecticidal properties (Jaques, 1967). Conversely, OBs exposed to solar radiation on plant foliage are rapidly inactivated and may lose their insecticidal activity completely within hours (Young, 2001; Fuxa, 2004). Other abiotic factors such as high temperatures or alkaline pH on the phylloplane also inactivate NPVs (Young, 2001). From the soil the OBs can be transported by rain splash (Fuxa and Richter, 2001), wind (Olofsson, 1988) or tillage (Fuxa and Richter, 2001) to the plant surface where they can infect susceptible insects (Young and Yearian, 1986). This step is a key issue in understanding the initiation of new epizootics (Fuxa, 2004), but is not fully understood in all types of ecosystems. Thus, this cycling model might become unpredictable in ephemeral ecosystems, and could be strongly influenced by variation in host population dynamics or migratory movement. Alternative theories on viral persistence are also gaining acceptance to explain virus persistence and dispersal, involving the presence of viral particles or naked DNA in the host cells that somehow escape host immune system clearance (Kane and Golovkina, 2010).

Baculovirus non-lethal infections can persist for a long time within their insect host retaining the capacity to cause lethal infections in the future (Burden et al., 2003; Cooper et al., 2003b; Hughes et al., 1993). An increasing number of studies reported that lepidopteran natural populations carried a non-pathogenic form of baculovirus (Burand et al., 2011; Burden et al., 2003; Cabodevilla et al., 2011a; Kemp et al., 2011; Krokene et al., 2013; Vilaplana et al., 2010; Virto et al., 2014). Interestingly this asymptomatic infection is transmitted to the next generations in many cases (Burden et al., 2003; Cabodevilla et al., 2011a; Vilaplana et al., 2010; Virto et al., 2014) suggesting a mode of long-persistence strategy in response to a changeable environment like high UV conditions, fluctuation of host population densities (Cooper et al., 2003b), highly mobile migratory species (Burand et al., 2011) or low transmission potential (Cory, 2010; Cory and Myers, 2003; Sorrell et al., 2009).

Dispersion abilities of the virus determine the chances to remain in contact with a changing host population in time and space. Environmental dispersion of baculoviruses has been categorized locally as within agricultural fields, and long-distance between fields or even between distinct geographic areas comprising hundreds of kilometers (Fuxa, 2004).

6.1 Baculovirus transmission

The process by which a pathogen passes from an infected to healthy individual is called transmission (Anderson and May, 1981). Two main routes of transmission are described for baculovirus, vertical transmission occurring from the parents to their offspring, and horizontal transmission, occurring among individuals who are not parents and offspring (Fuxa and Tanada, 1987). In field populations of insects, baculovirus transmission probably consists of a combination of horizontal and vertical transmission. For decades it has been assumed that the former is the most common route (Cory and Myers, 2003), but recently a mixed mode of transmission where the prevalence of one or another route depends on each virus-host system and environmental conditions has been proposed (Cory, 2015).

Horizontal transmission often occurs when an infected insect dies and millions of viral particles (OBs) are released into the environment increasing the likelihood that a new susceptible host ingests enough OBs to begin a new infection (Figure 10). Viral dispersion in small areas (within agricultural fields) occurs mainly via horizontal transmission, when susceptible hosts ingest OBs from infected cadavers, foliage or OB-contaminated soil (Fuxa, 2004; Krokene et al., 2013), feces or regurgitate of diseased larvae (Vasconcelos, 1996a), by cannibalism of infected insects (Vasconcelos, 2001), or after application of baculovirus-based insecticides (Fuxa and Ritcher, 1999).

On the other hand, long-distance dispersion is favored by migratory species that move from sources of inoculum to new areas. This implies long flights of adults and a life stage that is invulnerable to infection. Therefore, this supposes that the insect has been previously infected during the egg or larval stage and the virus is transmitted to the offspring (Figure 10) (Burden et al., 2002; Khurand et al., 2004). Vertical transmission involves the passage of the virus from adults to their

progeny by either superficial contamination of eggs or the transfer of non-lethal infections (Kukan, 1999).

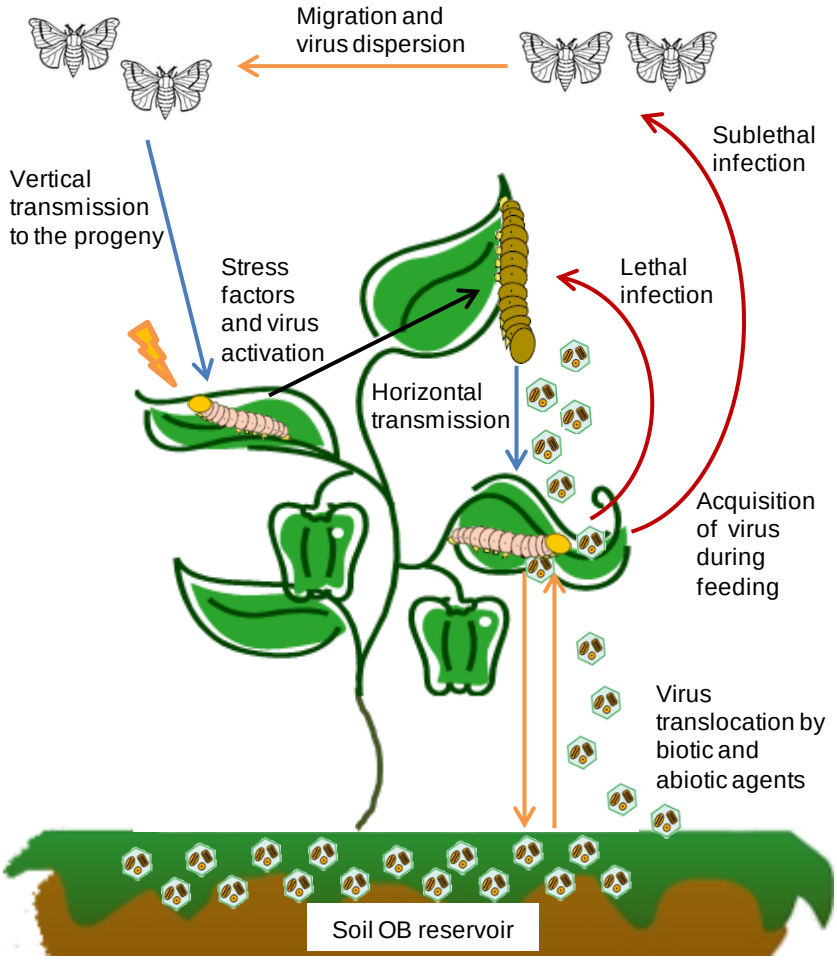

Figure 10. Baculovirus transmission routes, mode of infection and dispersal pathways in the environment. After larvae ingest OBs while feeding, either lethal or sublethal infections are produced (red arrows). Then, the overtly infected larvae eventually release OBs onto the host plant where can reach a susceptible host (blue arrow), thereby producing one or more infection cycles. The virus is dispersed by biotic or abiotic agents to or from the main reservoir, the soil (orange arrow). Alternatively a sublethally-infected adult may pass the virus to the offspring which eventually can disperse over long distances via migration (orange arrow). Vertical transmission might occur throughout generations (blue arrow) until a trigger factor switches the covert form to an overt infection (black arrow).

The efficiency of horizontal transmission depends principally on host behavior, the virus pathogenicity (infectivity), virulence and productivity (Cory, 2010; Cory, 2015). It is noticeable that baculoviruses modulate host behavior to maximize their transmission and dispersion. Baculovirus infection induces hyperactivity and climbing (tree-top) behavioral changes regulated by certain viral genes. For instance, the *ptp* gene is responsible for enhanced mobility that helps to spreading the virus locally (Kamita et al., 2005; Katsuma et al., 2012; van Houte et al., 2012). This behavior was quantified for the first time in *Mamestra brassicae* in which infected larvae moved three to five times further than healthy ones (Vasconcelos et al., 1996a). Climbing behavior is promoted by the *egt* gene leading infected larvae to move to the top of the plant prior to death followed by the dissemination of millions of OBs over the foliage below (Hoover et al., 2011). The comparison of wild-type and a non-functional *egt* gene recombinant virus showed that this gene also interferes in the normal insect development by inactivating the molting process (O'Reilly and Miller, 1989), thereby prolonging the larval time to death and allowing pre-death climbing behavior (Van Houte et al., 2015). Finally the role of the *chitinase* and *cathepsin*, genes that appear to act together, is linked with liquefaction or the “melting” of the cadaver thereby enhancing the release and dispersal of OBs from insect cadavers (Hodgson et al., 2011).

Another viral-mediated host behavior relates to cannibalism or intraspecific predation. Cannibalism is observed in many lepidopteran species during the late instars and can lead to efficient virus transmission among conspecifics (Elvira et al., 2010; Bernal, 2013). An NPV-infected cadaver has been associated to chemical emission that results in fatal attraction for conspecific larvae (Matsumoto, 2015). Necrophagy, contact or consumption of conspecific cadavers was studied in *S. exigua* where encounters with NPV-infected larvae resulted in high rates of lethal infection (82-93%). Additionally, necrophagy of infected cadavers was observed to be favored by the climbing behavior associated with late-stage baculovirus infection (Rebolledo et al., 2015).

Factors such as host plant, host nutrition or host density modulate the vigor of the host and consequently the ease with which the virus can infect a new host (Cory and Hoover, 2006). Plant-host-pathogen tritrophic interactions have been addressed to explain viral transmission traits. Food quality affects the host-

parasite interaction in different ways: poor quality food can result in detrimental effects both on immune response and disease resistance (Shikano et al., 2010). Also, to compensate for low quality diets, larvae need to increase their food intake thereby increasing the probability of ingesting OBs in the environment (Cory, 2010). The possibility of food deprivation, with a low nutritional content diet, is also linked to immune function (Siva-Jothy and Thompson, 2002; Yang et al., 2007). On the other hand, plant secondary chemicals can also reduce the effectiveness of baculoviruses. For example, some plants produce alkaline exudates on the leaf surface that can inactivate baculovirus, probably by the premature dissolution of occlusion bodies (Duffey et al., 1995). Other chemicals like phenolics or peroxidases have a direct impact by damaging the occlusion derived virions, virus receptors or by producing oxidative stress (Cory and Hoover, 2006).

Natural populations of Lepidoptera exhibit large fluctuations of density that can affect the transmission of baculovirus. High densities are expected to favor horizontal transmission, whereas low density periods tend to reduce the rate of host encounters with inoculum, but field studies have revealed less clear relationships. For *M. brassicae* and *Plodia interpunctella* larval densities were found to be positively related with NPV transmission efficiency (Vasconcelos, 1996b; Knell et al., 1998). Conversely, transmission of the gypsy moth and its NPV is a non-linear process across host densities (D'Amico et al., 1996), influenced by heterogeneity in the virus distribution (D'Amico et al., 2005). Factors that induce host resistance indirectly affect baculovirus transmissibility. For example, SpexNPV was affected by the degree of resistance to NPV infection that is higher in *S. exempta* larvae reared gregariously compared with those reared solitary (Reeson et al., 1998; Vilaplana et al., 2008).

Another source of NPV dispersion in the field, and consequently of horizontal transmission, is the activity of a wide range of predators and parasites (Lee and Fuxa, 2000; Vasconcelos et al., 1996b). The success of this dispersal mechanism depends mainly on whether these natural enemies are attracted by NPV-infected larvae and the persistence of OBs inside the vector organism. The interaction between coleopteran predators and baculovirus-infected larvae showed no discrimination between healthy and diseased larvae of the cabbage moth *M. brassicae*. OB infectivity was maintained after passage through the predator gut which transported enough virus to cause mortality in different instars of healthy *M.*

brassicae larvae (Vasconcelos et al., 1996b). The predator *Podisus maculiventris*, scavengers *Sarcophaga bullata* and *Acheta domesticus* were able to transport AcMNPV OBs at significant rates in greenhouse conditions (Lee and Fuxa, 2000), while different arthropods, most of them belonging to the order Hemiptera, spread *A. gemmatalis* MNPV from soya crops to the adjacent fields (Fuxa and Richter, 1994).

Parasitoid wasps can act as vectors transmitting the virus from infected to healthy hosts. No consensus results were found between discrimination studies of NPV-infected and healthy larvae by parasitoid females among species (Brooks, 1993; Cossentine, 2009; Escribano et al., 2000a; Kyei-Poku and Kunimi, 1997. In contrast, Jiang et al. (2014) and Stark et al. (1999) observed selective oviposition since parasitoids preferred healthy larvae over infected ones. More noticeable is the role of parasitoids on virus dispersion and transmission. Namely, an experiment carried out with the *M. pallidipes* parasite and the SeMNPV showed that parasitoid females that had developed or oviposited in virus-infected hosts, or that emerged from pupae contaminated with virus, were able to transmit infective doses of virus to healthy host larvae. Furthermore, when parasitoids completed their development in virus-infected hosts before the hosts died from baculovirus, increased mortality of the current *S. exigua* generation was observed as well as an increase in the prevalence of virus disease in the next generation (Jiang et al., 2011).

6.2 Baculovirus covert infections in lepidopteran populations

One aspect of the disease dynamics produced by baculoviruses in natural insect populations, which is increasingly attracting attention, is the maintenance of the virus in the host population when opportunities for horizontal transmission are unfavorable (Cory and Myers, 2003). The advent of molecular techniques has lead to an improved understanding on the prevalence of cryptic sublethal infections caused by viruses (Kane and Golovkina, 2010; Sorrell et al., 2009), including baculoviruses. Although baculoviruses are known for their high pathogenicity and virulence, there is growing evidence on covert baculovirus infections, their persistence and transmission and their influence on the host population dynamics (Burand et al., 2011; Burden et al., 2003; Cabodevilla et al., 2011a; Cory, 2010; Kemp et al., 2011; Vilaplana et al., 2010; Virto et al., 2014). The molecular

mechanisms involved for the switch between latency and active virus replication, or the virus strategies for its maintenance in the host tissues, however, remain poorly understood.

6.2.1. Terminology

Terminology on this issue is controversial and needs to be clearly defined to provide improved precision in the following arguments and explanations.

Covert infection

OB ingestion does not always result in symptomatic fatal infections; sublethal doses can establish covert infections. Fatal disease leads to horizontal transmission events, whereas covert infection opens the opportunity to vertical transmission strategies. Covert infections (also known as inapparent, sublethal, or occult infections) are non-lethal infections involving sophisticated interactions between pathogen and host in which the virus avoids clearance and remains within the host for extended periods of time or almost indefinitely with low biological costs to the host. Baculovirus covert infections are not transmitted between individuals of the same generation although they may or may not be transmitted vertically from parents to offspring (Kukan, 1999). Another characteristic of covert infections is that the pathogen remains fully competent within the host and at a certain moment the infection can activate to produce overt and lethal disease (Burden et al., 2006; Murillo et al., 2011).

Covert infection is a common term in the virology literature whereas the medical community tends to use terms such as ‘silent’ or ‘dormant’ infection (Sorrell et al., 2009). Covert infections are characterized by the absence of visible signs of disease. However, it is convenient differentiate them from inapparent infections that are also asymptomatic, causing no or little disease but note that are transmitted exclusively horizontally (Dimmorck et al., 2007).

Sublethal infection

The mechanism behind the establishment of covert infections is still unclear, but evidence indicates that insect survivors of a virus challenge in the larval stage are capable of transmitting the infection to their offspring (Burden et al., 2002). The larvae, pupae and adults of the inoculated larvae are sublethally infected having controlled the replication and lethal effects of the pathogen.

Cabodevilla et al. (2011b) studied whether host larval stage and OB concentration influenced the prevalence of sublethal infections in the survivors of a virus challenge. They determined the stage and OB concentration that have subsequently been used by other authors to establish sublethal infections in *S. exigua* populations (Cabodevilla et al., 2011a; Virto et al., 2013; Virto et al., 2016).

Persistent vs latent infections

Spontaneous baculovirus deaths in apparently healthy insect cultures suggest the existence of covert infections in laboratory insect colonies. Early studies on covert infection and vertical transmission involved viral treatment of larvae from one generation and the subsequent quantification of baculovirus mortality in the progeny (see review by Kukan, 1999). Nowadays, evidence of covert infection relies on molecular analyses. The used of the reverse transcription polymerase chain reaction (RT-PCR) or the polymerase chain reaction (PCR) is very sensitive and allows differentiation between latent and persistent infections. In the former, the viral genome is present within the host although the virus is in a non-replicating form and therefore viral particles are not formed, whereas the latter is characterized by active virus replication where a range of viral genes are expressed and viral particles can be produced (Chao et al., 1998; Dimmorck et al., 2007). Hughes et al. (1993) amplified by PCR *polyhedrin* gene sequences in asymptomatic insects of all stages demonstrating that latent virus infection was present throughout the life cycle of *M. brassicae* in laboratory culture. Subsequently, in the same pathogen-host system, evidence of a persistent infection was found by RT-PCR as evidence for the expression of viral genes (Hughes et al., 1997). In order to detect and quantify minimal amounts of viral replication in covertly infected insects, novel and more sensitive techniques have been developed such as real-time quantitative PCR (qPCR), that is able to detect as few as 5 or 7 viral genome copies in an insect sample using a hydrolysis (TaqMan) probe or SYBR green based technique, respectively (Graham et al., 2015; Virto et al., 2013).

Persistent infections are characterized by a constant low-level production of virus particles within an infected cell. These infections represent a balance between the host and the virus, which may be maintained thorough the interaction of the cells and the virus alone (Dimmock et al., 2007).

In latent infections, the viral genome and possibly some virus encoded products are present, but infectious virus particles are not formed. This scenario involves a shut-down in viral gene transcription with only those genes involved in maintaining the latent state being expressed (Chao et al., 1998; Dimmock et al., 2007). Latent infections do not represent a dead-end for the virus as, given an appropriate triggering stimulus, the infection can revert to a fully overt infection.

6.2.2. Transgenerational transmission

The passage of virus to a subsequent generation comprises both the transovarial and transovum pathways. Transovarial transmission implies the process of virus passing to progeny within the eggs, whereas the transovum route involves contamination of the egg surface with viral particles that infect neonate larvae when they ingest the chorion (Cory and Myers, 2003; Kukan, 1999). The procedure to distinguish between these transmission pathways consists of superficial decontamination by soaking egg batches in a formalin or sodium hypochlorite solution during 5-10 minutes. A number of studies have reported that egg surface decontamination reduces the number of progeny developing a lethal infection from 2-80% to 0.1-9% (Kukan, 1999). Subsequent studies on the NPVs of *L. dispar* (Myers et al., 2000), *Bombyx mori* (Khurad et al., 2004) and *Spodoptera exempta* (Vilaplana et al., 2008) support the idea that transmission occurs via internal contamination of eggs since similar levels of spontaneous NPV-induced mortality were observed in the offspring from infected adults independently of whether the eggs had been surface decontaminated or not. In line with these results Virto et al. (2013) detected by qPCR a similar prevalence of SeMNPV covert infection in the offspring of superficial decontaminated eggs comparing to those from non-decontaminated eggs. Surprisingly, lethal infections were found in the progeny from *S. exigua* females that were negative for SeMNPV covert infection (Cabodevilla et al., 2011a), raising possible contribution of the male linage to vertical transmission. Both sexes have found to be involved in vertical transmission since the virus persists from one generation to the next when infected females are mated with healthy males or *vice versa* (Burden et al., 2002; Khurad et al., 2004; Vilaplana et al., 2008; Virto et al., 2013). Furthermore, Burden et al. (2002) detected a high prevalence of viral transcripts in testis and ovaries of sublethally infected *P. interpunctella* adults. Similarly, histological observation of *B.*

mori gonads revealed the presence of viral particles in the reproductive organs of both males and females (Khurad et al., 2004). Even so, females seem to play a relevant role in transgenerational transmission. Maternal transmission of SeMNPV was approximately twice as efficient as the paternal route, not only because females transmitted higher percentages of infection, but also because viral loads were higher in the offspring of infected mothers compared to infected fathers (Virto et al., 2013). Similarly, studies with *Drosophila* sigma virus revealed that transmission was more efficient in females than males since a low proportion of infected offspring and low virus titers were present in the progeny of infected males (Fleuriet, 1988; Longdon et al., 2011). Venereal transmission has been demonstrated for different insect viruses during mating including in the nudivirus of *Oryctes rhinoceros* (Zelazny, 1976), the gonad specific nudivirus HzNV-2 of *Helicoverpa zea* (Hamm et al., 1996), a parvovirus in *Aedes albopictus* (Barreau et al., 1997), a sigma virus in *Drosophila* spp. (Longdon and Jiggins, 2012) and iflavirus in honeybees (de Miranda and Fries, 2008).

In view of these results, the organs most affected by covert infections could be those of the reproductive system. Studies carried out in bees detected deformed wing virus (DWV) sequences in testis, seminal vesicles and vesicular glands of drones, and in gut, spermatheca and ovaries of queens previously inseminated with DWV-negative sperm (de Miranda and Fries, 2008; Yue et al., 2007). The manner by which viral DNA remains in the host tissues is unclear, although a few studies have focused on the location of viral genomes during latency. An early study revealed viral DNA in the fat body tissues of covertly infected *M. brassicae* larvae by PCR (Hughes et al., 1993). Later, high levels of viral transcripts were detected in ovaries (90%) and testes (70%), but also in the rest of the body (60-70%) from *P. interpunctella* adult survivors of a GV challenge (Burden et al., 2002). Conversely, Graham et al. (2015) determined that the principal body parts harboring SpexNPV covert infections were wings, head and legs, although low viral titers were also found in the thorax and abdomen. More studies using advanced techniques could lead us to a deeper understanding of covert infection and the organs involved in within-host persistence and vertical transmission.

6.2.3. Maintenance and activation of covert infections

Despite the evidence of baculovirus covert infections, the molecular mechanisms behind viral activation are unclear yet. Viral strategies for achieving covert infections include the selection of cell subset for maintenance of viral genomes, the modulation of viral gene expression, and the avoidance of clearance by the immune system (Kane and Golovkina, 2010). Two main scenarios have been foreseen regarding the presence of viral genomes within host cells; the integration of the viral DNA into the genome of the host cell, as a provirus structure, as occurs in polydnaviruses (Strand, 2010), or the maintenance of viral genomes as independent episomes as in herpesviruses (Mellerick and Fraser, 1987). Certainly covert infections depend on host survival and consequently the virus has to minimize its negative impact on the host to ensure persistence and transmission (Moore, 2002). Studies involving microRNAs have focused on a new aspect of this issue. The *Heliothis zea nudivirus 1* (HzNV-1) produces micro-RNAs to suppress certain viral genes and induce a covert infection (Wu et al., 2011). Also Singh et al. (2010) identified four *B. mori* NPV-encoded microRNAs that appear to be implicated in the immune defense of the host. On the other hand, anti-apoptotic genes (IAPs) have been described as factors involved in the maintenance of the virus by blocking host cells that try to enter apoptosis (Feng et al., 2007; Hughes, 2002; Luo and Pang, 2006). The deletion of the apoptotic suppressor gene *p35* in AcMNPV allowed the persistence of baculovirus covert infection in *S. frugiperda* cells (Lee et al., 1998). Interestingly, certain genotypes of SeMNPV may have adopted a survival strategy based on covert infection and vertical transmission. Genotypes that are frequently transmitted to the offspring are less pathogenic and virulent than those that are horizontally transmitted (Cabodevilla et al., 2011a).

Insects have also developed different mechanisms to protect themselves from, or minimize the impact of, foreign pathogens. Genetic resistance to virus has been documented in both laboratory (Abot et al., 1996; Haas-Stapleton et al., 2005; Hoover et al., 2000) and field populations (Eberle and Jehle, 2006). European *Cydia pomonella* field populations have developed up to 10,000-fold resistance by blocking CpGV replication at an early stage of infection (Asser-Kaiser et al., 2007; Asser-Kaiser et al., 2011; Schmitt et al., 2013; Undorf-Spahn et

al., 2012). Other known mechanisms that the host uses in defense include the development of antimicrobial peptides (Cheng et al., 2006), phagocytosis (Narayan, 2004; Wittig, 1968), cell apoptosis (Blissard, 1996) or cell sloughing (Washburn et al., 2003). A quantitative model of damage has been proposed to explain the occurrence of host resistance or tolerance based on a damage threshold (Moreno-García et al., 2014) that determines whether the host resists and overcomes the infection (Marques and Carthew, 2007), adopts a covert infection strategy (Sorrell et al., 2009) or eventually succumbs to infection and dies.

Covert infections were firstly proposed to explain the spontaneous outbreaks of baculovirus occurring in apparently healthy insects (Cory et al., 1997). Early studies advanced that these events in lepidopteran populations were not random, and lead to examine the factors that can trigger fatal infections, an issue that has received little attention so far. Physical factors, rearing conditions, chemicals or the presence of other pathogens have been associated with the activation of latent virus in the past (reviewed by Podgawaite and Mazzone, 1986). Similarly, overcrowded rearing conditions (Fuxa et al., 1999; Opoku-Debrah et al., 2013), changes in temperature or relative humidity (Fuxa et al., 1999; Guimares et al., 1998), the addition of certain chemical compounds to the diet (Ilyinykh et al., 2004), or changes in nutrient availability (David and Gardiner, 1965; David and Gardiner, 1966), have been examined for their role of activators to overt disease, although few consistent effects have been identified to date. The most consistent reported effect has been a challenge by a second pathogen (superinfection), reported as another source of stress in the past (Jurkovicova, 1979; Longworth and Cunningham, 1968; Smith, 1976), and more recent confirmed by cross-inoculation using heterologous viruses that have consistently triggered fatal infections in several host-virus systems (Cooper et al., 2003b; Fuxa et al., 2002; Hughes et al., 1993; Kouassi et al., 2009).

6.2.4. Sublethal effects of covert infections

The fitness costs required to fight a viral infection may compromise normal insect growth and development (Myers et al., 2000; Rothman and Myers, 1996). In this sense, a number of developmental parameters of the insect have been

reported to adversely be affected by baculovirus sublethal infections, but not many studies have addressed the consequences of long-term covert infection.

The effect of baculovirus infections in virus-challenged insects has been studied by comparison of developmental parameters in healthy and infected insects. For instance, the developmental time for larvae increased in sublethally infected larvae of *S. exigua* (Cabodevilla et al., 2011b), *Trichoplusia ni* (Milks et al., 1998) and *M. brassicae* (Goulson and Cory, 1995), whereas pupal development time was prolonged for *Spodoptera litura* (Monobrullah and Shankar, 2008), *T. ni* (Milks et al., 1998), *M. brassicae* (Goulson and Cory, 1995) and *L. dispar* (Murray et al., 1991). Reductions in fecundity and fertility have been described as two of the most important negative effects of sublethal infections, since individual fitness depends on the viability of the progeny (Rothman and Myers, 1994). In some cases, egg production has been reduced up to 50% (Cabodevilla et al., 2011b), and a correlation between pupal weight and fecundity per infected female has been observed in several species of insects including *S. exigua* (Cabodevilla et al., 2011b; Smits et al., 1987), *S. exempta* (Vilaplana et al., 2008), *Lasiommata megera* (Karlsson and Wiklund, 1984) and *Antheraea polyphemu* (Miller et al., 1982). The preoviposition period, was longer in *S. exigua* (Cabodevilla et al., 2011b), *S. exempta* (Vilaplana et al., 2008) and *Pieris brassicae* (Sood et al., 2010) infected females, so that they might travel longer distances than healthy conspecifics before starting to lay eggs, resulting in greater dispersal of covertly infected progeny. Interestingly, alterations in sex ratio in emerging adults of pupae treated with virus in favor of females have been reported for *S. exigua* (Cabodevilla et al., 2011b), *S. littoralis* (Scheepens and Wysoki, 1989; Vargas-Osuna and Santiago-Alvarez, 1988), *M. brassicae* (Goulson and Cory, 1995) and *Mythimna separata* (Patil et al., 1989).

Long term host-pathogen interactions might convey some benefits to the host defense against further infection (Jones et al., 2011). For example, some vertically transmitted bacterial symbionts (i.e. *Wolbachia*), can provide protection to their hosts against superinfection (Hedges et al., 2008; Himler et al., 2011). For the insect-baculovirus pathosystems the benefits of covert infection on infection by additional pathogens is not clear. The *Helicoverpa armigera* densovirus-1 appears to protect its host against a second infection of baculovirus or Bt biopesticide (Xu et al., 2014). Conversely, *S. exempta* larvae infected by *Wolbachia* resulted 6-14

times more susceptible to SpexNPV than their counterparts (Graham et al., 2012). For *S. exigua*-SeMNPV pathosystem, host susceptibility increased in SeMNPV-infected larvae two-fold when the same virus was used to produce a superinfection (Cabodevilla et al., 2011b). Furthermore, a *S. exigua* colony derived from field collected adults that were covertly infected was 3.4-fold more susceptible than a laboratory virus free colony when both populations were subjected to bioassays using various SeMNPV genotypes (Cabodevilla et al., 2011a). A logical explanation is that the endogenous virus is activated when the second infection occurs, as described for heterologous NPV infections (Burden et al., 2003; Cooper et al., 2003b; Kouassi et al., 2009; Murillo et al., 2011).

6.2.5. Environmental relevance of covert infections

The advent of molecular techniques led to a growing number of reports of widespread latent or persistent baculovirus infections that involved both laboratory and field populations of Lepidoptera (Table 1). Despite the techniques used for detection or quantification, the detection threshold, the prevalence of covert infections and their transmission vary considerably between virus-host species. Notably, the prevalence of covert infection can attain values close to 100% of tested individuals, such as *S. exigua* laboratory populations (Cabodevilla et al., 2011b), and in *S. exempta* (Vilaplana et al., 2010) and *M. brassicae* (Burden et al., 2003) field and laboratory cultures (Table 1). Several studies have examined vertical transmission in highly mobile or migratory species such as those belonging to the genus *Spodoptera* (Abul-Nasr et al., 1979; Fuxa and Richter, 1991; Smits and Vlak, 1988; Swaine, 1966). This is consistent with the hypothesis that the viruses employ covert infection and vertical transmission to efficiently disperse the virus long-distances in migratory host species (Burand et al., 2011; Vilaplana et al., 2010). However mathematical models that consider covert infections as a parasite virulence strategy predict lower rates of covert infections than those found in field populations (Sorrell et al., 2009). This suggests that additional factors not taken into account so far, might influence the role of covert infections in host population dynamics.

From a pest control perspective, sublethal effects in insect survivors after field application may be desirable and benefit the pest control in subsequent generations as covertly infected insects might be more susceptible to a second

virus application and lower rates of OBs applications would be necessary for effective pest control.

Table 1. Summary of prevalence of insect populations that harbor a covert infection for different host species, origin of the population, techniques used to detect covert infection, and number of generations analyzed to detect vertical transmission.

Population	Technique	Infected insects (%)	Generations analyzed	Reference
Field				
S. exigua	RT-PCR	16	1st	Cabodevilla et al., 2011a
	qPCR	54	1st	Virto et al., 2014
	" "	21.5	2nd	" "
S. exempta	Nested RT-PCR	60	1st	Vilaplana et al., 2010
	" "	50	2nd	" "
	" "	25	7th	" "
	Nested PCR	97	1st	" "
		90	2nd	" "
		100	7th	" "
M. brassicae	Nested PCR	50-100	1st	Burden et al., 2003
	" "	75-80	2nd	" "
	" "	100	6th	" "
Operophtera brumata	PCR	19-28	1st	Burand et al., 2011
Choristoneura fumiferana	PCR	70	1st	Kemp et al., 2011
Laboratory				
S. exigua	RT-PCR	15	1st to 5th	Cabodevilla et al., 2011b
	qPCR	70-100	" "	" "
S. exempta	Nested RT-PCR	38	Stock culture	Vilaplana et al., 2010
	Nested PCR	93	" "	" "
P. interpunctella	RT-PCR	30-70	1st	Burden et al., 2002
	PCR	30	1st	" "
Choristoneura fumiferana	PCR	28	Stock culture	Kemp et al., 2011

All insects were analyzed in the adult stage, except the study carried out by Burand et al. (2003) that analyzed larvae and pupae.

7. *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV)

Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) was first isolated by Steinhaus, (1949) and some years later was described by Hunter and Hall (1968). Since then, numerous SeMNPV isolates have been reported from *S. exigua* larvae collected worldwide, including California (Gelernter and Federici, 1986), Florida (Muñoz et al., 1998), Egypt and Netherlands (Vlak et al., 1981), Japan (Kondo et al., 1994), Thailand (Hara et al., 1995) and Spain (Caballero et al., 1992a; Cabodevilla et al., 2011a; Murillo et al., 2001). The characterization of

SeMNPV isolates by REN analysis has revealed high genotypic diversity among geographically different origins (Caballero et al., 1992b; Gelernter and Federici, 1990; Muñoz et al., 1998; Muñoz et al., 1997; Murillo et al., 2001), but also among closely related variants cloned from a single isolate (Muñoz et al., 1998). Phenotypically both isolates (Muñoz et al., 1997; Murillo et al., 2006) and genotypic variants (Muñoz et al., 1999) differ in their biological characteristics, especially those related to insecticidal properties. The value of this virus as a biological control agent has been tested in *S. exigua* populations from distinct geographical areas under laboratory (Caballero et al., 2009; Cabodevilla et al., 2011a) and field conditions (Kolodny-Hirsch et al., 1997; Lasa, 2007). In some studies local virus isolates have been found to be more effective against local insect populations than against geographically distant insect populations (Caballero et al., 1992b). Hence, prior to the development of an SeMNPV-based insecticide, the biological activity of native isolates should be checked in local insect populations.

SeMNPV is a totally host-specific virus (Caballero et al., 1992a; Simón et al., 2004b) that presents an a high degree of pathogenicity and virulence against its natural host (Bianchi et al., 2000; Smits et al., 1987; Smits and Vlak, 1988) and which can be mass-produced *in vivo* at a reasonable cost (Lasa, 2007). For these reasons it has been developed as the basis of a biological control agent in different countries of the world including United States, Netherlands, several southern Asian countries and Spain (Bianchi et al., 2002; Cunningham, 1998; Kolodny-Hirsch et al., 1997; Lasa et al., 2007b; Smits and Vlak, 1994). Currently three bioinsecticides based on different strains are commercialized under the trade-names of Spod-X® (Certis Corp., Columbia, USA), Spexit® (Andermatt Biocontrol, Grossdietwil, Switzerland) and Virex® (Biocolor, Almeria, Spain). A SeMNPV isolate from Florida (SeMNPV-US2) constitutes the active ingredient of Spod-X® which is registered in United States, Netherlands and Thailand. This isolate comprises at least seven different genotypes, of which two have defective genomes which act as parasitic genomes decreasing the pathogenicity of the isolate (Muñoz et al., 1998). The active ingredient of Spexit® is a SeMNPV isolate collected in California (Gelernter and Federici, 1986), the genotypic composition of which was studied in depth by Heldens et al. (1996). Finally, Virex® is based on a mixture of three SeMNPV genotypes that were selected from a large number of

SeMNPV isolates collected from greenhouses of Almeria, Spain (Murillo et al., 2006). These isolates differed in their genotypic composition and phenotypic characteristics, but interestingly a specific mixture of certain genotypes resulted in improved pathogenicity and virulence in a local population from Almería (Murillo et al., 2006). This mixture has been used as the active ingredient in the Virex® biopesticide (Caballero et al., 2009). The development of a mass-production system (Lasa et al., 2007a) and formulation procedures (Lasa et al., 2007c) lead to the registration of this bioinsecticide in Spain. Field studies in Almería demonstrated that Virex® applications to greenhouse sweet pepper crops provided efficient control of the pest at levels that exceeded those of chemical insecticide treatments (Lasa et al., 2007b; Lasa et al., 2007c). Consequently, it has been successfully implemented in biological control pest programs in horticultural crops in Almería (Caballero et al., 2009).

Subsequent studies have examined the incidence of SeMNPV in natural *S. exigua* populations from that area. A total of 16% of the insects collected in 2006 and 2007 from Almería greenhouses harbored a persistent infection and 20% of the offspring succumbed to lethal NPV disease. REN analysis of these insects revealed two new SeMNPV genotypes that later were classified as vertically transmitted genotypes (Cabodevilla et al., 2011a). Further insect captures in 2011 lead to monitoring SeMNPV covert infections in this region, revealing that NPV covert infections were detectable in 54% of the population (Virto et al., 2014). This time, analysis was performed by qPCR, a more sensitive technique that detects viral DNA at low copy numbers. The widespread use of SeMNPV-based insecticides in greenhouses of Almería probably had an influence on the high percentages of SeMNPV infected insects obtained in 2011.

A comparative study thoroughly characterized SeMNPV genotypes obtained either from the offspring of field-collected adults or from soil samples of Almerian greenhouses, including insecticidal properties and their ability to produce covert infections. The most pathogenic and virulent genotypes (characteristics related to horizontal transmission) showed a low capacity to produce covert infections and were considered to be horizontally transmitted genotypes. In contrast, the less virulent and pathogenic genotypes were transmitted to the offspring at a high frequency, and therefore were considered vertically transmitted genotypes (Cabodevilla et al., 2011a). The analysis and comparison of the

complete nucleotide sequence of vertically and horizontally transmitted genotypes identified differences in the *Se4* and *Se5* genes that could explain their biological traits (Serrano et al., 2015; Theze et al., 2014).

8. Other viruses that infect *S. exigua* populations

Recent transcriptomic analysis of an *S. exigua* population revealed the presence of sequences belonging to RNA viruses, especially picorna-like viruses (Pascual et al., 2012). The order *Picornavirales* is divided in five families, *Dicistroviridae*, *Iflaviridae*, *Marnaviridae*, *Picornaviridae* and *Sequiviridae,* and two genera unassigned to any family: *Bacillarnavirus* and *Labyrnavirus* (Le Gall et al., 2008). Iflaviruses have been described infecting exclusively insects from the orders Lepidoptera, Hymenoptera and Hemiptera, and in bee parasitic mites (class Arachnida, order Acarina) (van Oers, 2010). Iflaviruses form non-enveloped icosahedral particles of approximately 30 nm in diameter, which contain a single-stranded RNA genome of positive polarity with length of 8.6 – 10.3 kb (van Oers, 2010). Initially, they were discovered causing fatal infections and important economic losses in insects belonging to the apiculture sector (*Apis mellifera*) (Ribière et al., 2010) and silk production (*B. mori*) (Aizawa et al., 1964). The pathology of these viruses varies widely, for instance sacbrood virus (SBV) produces a failure to pupate in *A. mellifera* larvae (Bailey and Ball, 1991; Gochnauer, 1990), whereas deformed wing virus (DWV) causes wing deformities, shortened abdomens and miscoloring of adult bees (Bailey and Ball, 1991). Infectious flacherie virus (IFV) causes diarrhea in the silkworm *B. mori* (Aizawa et al., 1964), but many iflaviruses can also remain in the host without showing clear symptoms of disease (Yue et al., 2007). Improvements in sequencing technologies and the use of very sensitive detection techniques have allowed the identification of a large number of RNA viruses that persist within the insect host in the absence of visible symptoms of infection.

The study of the *S. exigua* transcriptome resulted in the identification of two novel iflaviruses (*S. exigua* iflavirus-1: SeIV-1 and *S. exigua* iflavirus-2: SeIV-2) that were covertly infecting a laboratory population of *S. exigua* (Choi et al., 2012; Millan-Leiva et al., 2012; Pascual et al., 2012). Jakubowska et al. (2014) confirmed that four laboratory *S. exigua* populations from geographically distinct origins

harbored abundant iflavirus infections. Also, in field insects captured in Almería, covert infections of two iflaviruses were detected causing both single and mixed infections comprising either two SeIV species, or alternatively SeMNPV and an iflavirus (Virto et al., 2014). Apparently, these infections do not have marked biological costs to the host and are not fatal to lepidopteran species (Vail et al., 1983a). Interestingly, IFV has been isolated from dead insects that succumbed to granulovirus (Wang et al., 2004) or nucleopolyhedrovirus infections (Wang et al., 1999). Recent transmission electron microscopy (TEM) images and RT-qPCR analysis of SeMNPV OBs preparations showed evidence of a physical association between SeMNPV and SeIV (Jakubowska et al., 2016). Icosahedral particles with a similar size to the iflaviruses, embedded in the polyhedrin matrix of OBs, suggest that the SeIV could be occluded *within* the SeMNPV OBs (Figure 11) (Jakubowska et al., 2016). Previous studies had found signs of iflavirus-like particles persistence in AcMNPV OBs population (Vail et al., 1983a; Vail et al., 1983b).

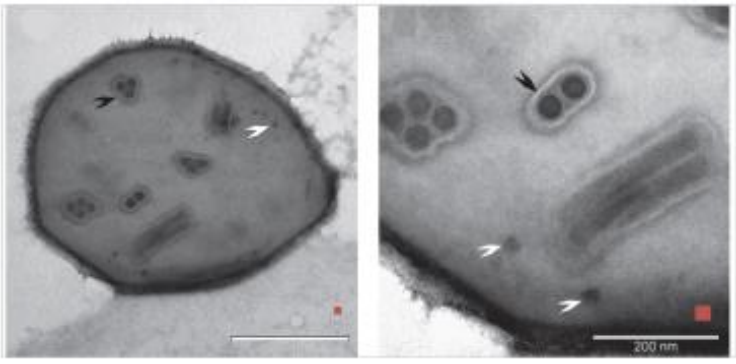

Figure 11. TEM images of purified OBs from SeMNPV. Black arrows indicate ODVs of SeMNPV and white arrows SeIV-like particles. A red square of 25 nm per side is shown in each figure for size comparison (Jakubowska et al., 2016).

Iflavirus infections can be transmitted both vertically and horizontally. DWV and SBV are examples in which horizontal transmission vectored by the mite *Varroa destructor* is the most virulent mode of transmission causing high bee mortalities (Nordström et al., 1999). However horizontal transmission through, for example, contaminated food, feces or regurgitate (Shen et al., 2005) and vertical transmission from queens to both worker and drone offspring have also been detected (Yue et al., 2006). High horizontal transmissibility in SeIV was suspected

following the observation that a virus-free colony was contaminated in just one generation by another population carrying SeIV that had been reared independently in the same growth chamber (Jakubowska et al., 2014; Millan-Leiva et al., 2012), even when insects were individually reared from very early instars (Virto et al., 2014). Vertical transmission was demonstrated from *S. exigua* field caught adults to their offspring, when individuals were carefully reared in a virus-free environment, and the SeIV load was found to be higher in the progeny than in the parents (Virto et al., 2014). SeIV was also detected in individuals from eggs that had been previously surface-decontaminated, suggesting transovarial transmission (Jakubowska et al., 2014). Finally, we have to take into account that insects can also be infected by SeMNPV OBs that contain embedded iflavirus particles (Jakubowska et al., 2016).

Mixed infections between iflaviruses and other groups of virus appear to be frequent in insect populations. Flacherie disease in the silkworm is often produced by mixed infections of IFV and densovirus (Tanada and Kaya, 1993). Bee infections by DWV with *Varroa destructor virus-1* (Ongus et al., 2004) or dicistroviruses (Chen et al., 2004) have also been reported. In fact 93% of the queens studied by Chen et al. (2005) carried multiple infections. Due to the fact that multiple infections are frequent, sometimes it is difficult to attribute the pathology of diseased insects to a specific virus. In early studies, a treatment of AcMNPV containing iflavirus-like particles in *T. ni* larvae resulted in reduced larval weight (Vail et al., 1983a; Vail et al., 1983b). The pathogenicity of SeMNPV was reduced when OBs were contaminated with SeIV (Jakubowska et al., 2016). Currently some studies are being carrying out in *S. exigua* to examine in depth the fitness cost of harboring SeIV covert infections, and the implications of the SeIV on the insecticidal properties of SeMNPV OBs.

9. Aims of the thesis

The aim of this thesis was to investigate the role of covert infection and its vertical transmission in the *S. exigua* host-NPV pathosystem, as the basis for new application strategies for pest control using SeMNPV, which might include inoculative field application.

In **chapter II**, the incidence of covert infections of SeMNPV and two iflavirus (*Iflaviridae*) species (SeIV-1, SeIV-2) was quantified in a natural *S. exigua* population from a horticultural greenhouse agroecosystem in Almería. Vertical transmission from collected adults to their offspring was demonstrated for the three viruses although the prevalence of infection in the offspring differed between them. Co-infections involving both virus families were also analyzed and whether iflaviruses can affect the efficiency of baculovirus-based insecticides was discussed.

To assess the importance of parental gender in baculovirus transgenerational transmission, four *S. exigua* mating groups (healthy males x healthy females, infected males x healthy females, healthy males x infected females, and infected males x infected females) were performed in **chapter III**. Viral load was quantified in parents and offspring of each mating group, confirming that females were twice as efficient as males in transmitting SeMNPV covert infection to the progeny. Also, a positive relationship was found between the proportion of infected insects and the viral DNA load. The study demonstrates that the main route of transmission is probably transovarial rather than transovum. Evidence for venereal transmission was not detected.

Chapter IV is focused on the evaluation of OB mixtures of vertically (VT) and horizontally transmitted (HT) genotypes, to select those which retain both the best insecticidal properties and the ability to produce a high prevalence of covert infections for transgenerational host suppression. Three mixtures comprising different proportions of VT and HT genotypes (75:25, 50:50 and 25:75 respectively) were evaluated for their insecticidal properties and their ability to produce covert infections in survivors of a virus challenge. Finally, the mixture with the best performance for insecticidal properties and capable of producing a high prevalence of covert infections, was tested as a crop protection agent in greenhouse assays. The mixture comprising 75% of the VT genotype was as effective as a chemical insecticide for pest control in greenhouse trials.

In **chapter V** the use of different biological and chemical stressors was investigated to determine whether SeMNPV covert infection could be triggered into fatal overt disease. The value of this strategy to initiate an epizootic was assessed in greenhouse conditions using compounds that previously had activated covert

infections in laboratory tests. Metal salts such as copper sulfate, iron sulfate and sodium selenite induced NPV mortality in persistently infected larvae in the laboratory, but not under greenhouse conditions.

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CHAPTER II

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

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 harboured 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 cypovirus 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 Vlak, 1994). This virus constitutes the active ingredient of a number of bioinsecticides, including: SPOD-X® (Certis, USA), SPEXIT® (Andermatt Biocontrol, 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 Almeria, 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 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; Burden et al., 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 iflavirus pathology, these viruses have been isolated from insect corpses that sucumbed to baculovirus infection (Wang et al., 1999; Wang et al., 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; Vail et al., 1983b).

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-ocluded (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 iflavirus 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 iflavirus inapparent infections in a field population of *S. exigua* present in the horticultural greenhouse agroecosystem of Almeria, and to determine their ability for vertical transmission.

2. Material 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 m^2 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 two days and then all adults of both sexes were individually frozen at -80ºC 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 hours post-hatching) was collected and reared individually on semi-artificial diet (Elvira et al., 2010) through to the adult stage (F_1), under standard laboratory conditions (25 ± 2ºC, 50 ± 10% RH, in a continuously dark room). F_1 adults were frozen at -80ºC 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 F_1 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 65ºC 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 37ºC 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 -20ºC. 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 37ºC. 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 (di-ethylpyrocarbonate) water and stored at -20ºC. 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 µl volume of RNA solution was treated with 1 µl DNAse and 1 µl DNase buffer (Promega) at 37ºC for 30 min to remove DNA contamination. Following this, 1 µl DNase stop (Promega) was added and incubated at 65ºC for 10 min. Finally, a 4 µl volume of the resulting RNA solution was incubated at 70ºC for 5 min with 1 µl dT primer. The reverse transcription mix

consisted of 2 µl 5× buffer (Promega), 1.2 µl $MgCl_2$ (25 mM), 0.5 µl dNTP mix (10 mM), 0.8 µl DEPC water and 1 µl ImProm-II reverse transcriptase (Promega). The mix was added to RNA samples and incubated at 25ºC for 5 min, followed by 42ºC for 60 min and 70ºC for 15 min. For PCR amplification, 1 µl cDNA was used as template and mixed with 2.5 µl NH_4 (10×), 1.25 µl $MgCl_2$ (50 mM), 0.25 µl dNTPs mix (10 mM), 0.5 µl of both SeIV1-Fw and SeIV1-Rv primers (10 µM), 0.3 µl of both SeIV2-Fw and SeIV2-Rv primers (10µM), 18.15 µl sterile milliQ water and 0.25 µl Taq DNA polymerase (Bioline). The PCR protocol consisted of an initial denaturation cycle at 95ºC for 1 min, 35 cycles of 95ºC for 30 sec, 60ºC for 30 sec and 72ºC for 2 min, and an extension cycle of 72ºC for 5 min. PCR products were visualized by electrophoresis in 1% agarose gels containing ethidium bromide. A Bioline Hyper-Ladder 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/µl to 1.66×10^{-5} pg/µl and from 1.50×10^{-2} pg/µl to 1.50×10^{-7} pg/µl 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 1.66×10^{-4} pg/µl and 1.50×10^{-4} pg/µl, 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'-CCGCTCGCCAACTACATTAC-3'; DNApol149-Rv: 5'-GAATCCGTGTCGCCGTATATC-3') were designed to amplify a 149-bp region within the *DNA polymerase* gene based on the full genome sequence of SeMNPV-Al1 (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 µl SYBR Premix Ex Taq (2×), 0.4 µl ROX Reference Dye (50×) and 0.4 µl of both DNApol149Fw and DNApol149Rv primers (10 µM) was added to a 1 µl template DNA. A blank extraction and four non-template reactions were included in each run. For the standard curve, CsCl-purified SeMNPV-Al1 DNA was quantified using a spectrophotometer (Eppendorf BioPhotometer plus). Ten-fold serial dilutions in sterile MilliQ water (from 100 to 1×10^{-3} pg/µl) were used to construct the standard curve in duplicate. The amplification reaction consisted of denaturation at 95ºC for 30 s, followed by 45 amplification cycles at 95ºC for 5 s and 60ºC for 30 s. Finally, a melting curve analysis, involved a dissociation stage of 95 ºC for 15 s, 60ºC for 15 s and 95ºC for 15 s was added to confirm a single peak of the target product. The regression parameters of the standard curve were $R^2 = 0.997$ 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 (83.5ºC) point for the specific amplification product. This limit was determined at 10^{-3} pg/µl, 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 (χ^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 (Figure 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 (χ^2 = 0.331, *df* = 1, *P* = 0.56) or the iflaviruses (χ^2 = 0.625, *df* = 1, *P* = 0.20) (Figure 1).

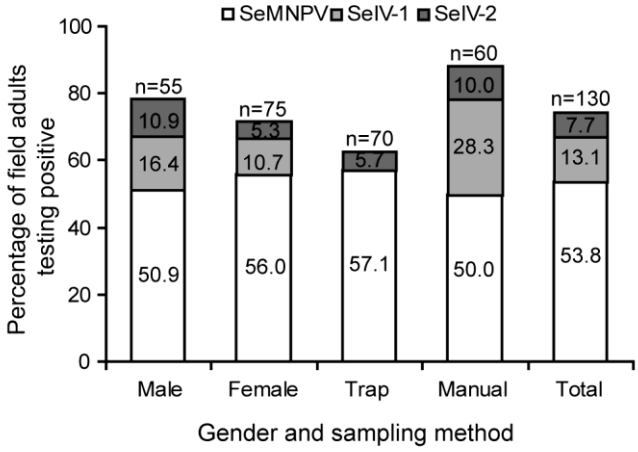

Figure 1. Percentage of SeMNPV, SeIV-1 or SeIV-2 covert infection in field caught-adults according to gender and sampling method. Numbers in the columns indicate the percentage of individuals that tested positive for each virus. Numbers above the columns indicate the number of individuals tested.

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 (χ^2 = 0.663, *df* = 1, *P* = 0.20). SeIV-2 was the only

RNA virus detected in trap-collected insects (Figure 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 (χ^2 = 22.82, *df* = 1, *P* < 0.05).

Table 1. Number of adults infected by multiple viruses in both field adults and F_1 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 (F_1) 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 (F_1). Overall, SeMNPV was detected in 21.5% of F_1 adults, whereas the overall prevalence of the iflaviruses (58%) was markedly higher in offspring (F_1) compared to that of field-caught adults (GLM: χ^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: χ^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 not infected by SeMNPV (Figure 2). The prevalence of SeIV-1 infection in progeny insects (39%) was consistently higher than that of SeMNPV or SeIV-2 (GLM: χ^2 =

23.68, *df* = 2, *P* < 0.05) (Figure 2). The prevalence of SeMNPV (χ^2 = 0.57, *df* = 1, *P* = 0.45) or SeIV (χ^2 = 0.14, *df* = 1, *P* = 0.71) infections did not differ significantly in male and female F_1 adults.

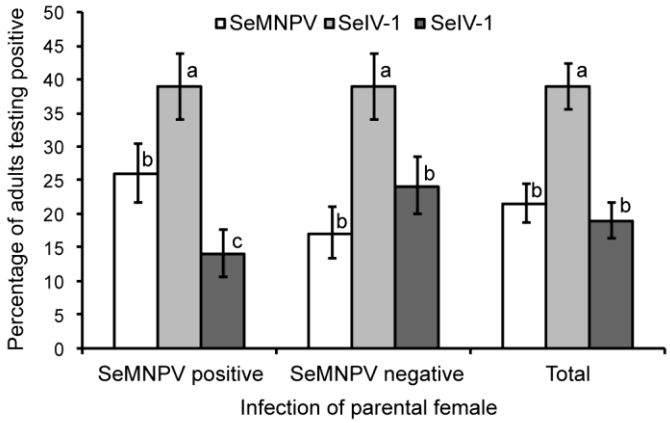

Figure 2. Mean percentage of F_1 adults positive for SeMNPV, SeIV-1 or SeIV-2 among the offspring of field collected females that were classified as infected (SeMNPV positive) or non-infected (SeMNPV negative) and that were negative for iflavirus infection. Columns labeled with identical letters did not differ significantly for comparisons of the prevalence of each type of virus within each maternal infection status (GLM, *t*-test, *P* > 0.05).

Mixed infections involving both virus families were detected in the F_1 individuals. All three viruses were detected in 13 out of 200 individuals tested (6.5%), whereas nine (4.5%) harboured 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 harbour 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, 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; Wang et al., 2004). These observations provided support for previous findings by Vail et al. (1983a; 1983b) 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.

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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CHAPTER III

Gender-mediated differences in vertical transmission of a nucleopolyhedrovirus

Abstract

With the development of sensitive molecular techniques for detection of low levels of asymptomatic pathogens, it becoming clear that vertical transmission is a common feature of some insect pathogenic viruses, and likely to be essential to virus survival when opportunities for horizontal transmission are unfavorable. Vertical transmission of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) is common in natural populations of *S. exigua*. To assess whether gender affected transgenerational virus transmission, four mating group treatments were performed using healthy and sublethally infected insects: i) healthy males (H♂) × healthy females (H♀); ii) infected males (I♂) × healthy females (H♀); iii) healthy males (H♂) × infected females (I♀) and iv) infected males (I♂) × infected females (I♀). Experimental adults and their offspring were analyzed by qPCR to determine the prevalence of infection. Both males and females were able to transmit the infection to the next generation, although female-mediated transmission resulted in a higher prevalence of infected offspring. Male-mediated venereal transmission was half as efficient as maternally-mediated transmission. Egg surface decontamination studies indicated that the main route of transmission is likely transovarial rather than transovum. Both male and female offspring were infected by their parents in similar proportions. Incorporating vertically-transmitted genotypes into virus-based insecticides could provide moderate levels of transgenerational pest control, thereby extending the periods between bioinsecticide applications.

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1. Introduction

Nucleopolyhedroviruses (genus *Alphabaculovirus*, family *Baculoviridae*) are arthropod-specific viruses that have been used in many parts of the world as biological insecticides due to their insecticidal properties towards certain insect pests and their outstanding biosafety characteristics (Eberle et al., 2012). They are also commonly employed in biotechnological applications for the production of recombinant proteins (Hitchman et al., 2011).

Nucleopolyhedrovirus populations adopt one of two transmission pathways to infect susceptible host insects. Horizontal transmission occurs when virus occlusion bodies (OB) from an infected cadaver are consumed in sufficient quantity by a healthy conspecific larva. This is, by far, the best understood mechanism of transmission (Cory and Myers, 2003). Little is known about vertical transmission of entomopathogenic viruses from infected parents to their offspring, but this has been proposed as a survival strategy to overcome periods of host scarcity when opportunities for horizontal transmission are limited (Cory and Myers, 2003). Clearly the ability to adopt horizontal or vertical transmission routes depends on the virulence of the infection; only persistent sublethal infections with reduced virulence will be capable of vertical transmission (Burden et al., 2002). Persistent infections have been reported in a number of lepidopteran species from field-collected (Burand et al., 2011; Vilaplana et al., 2010), and laboratory populations (Fuxa et al., 2002; Fuxa et al., 1999; Kukan, 1999; Murillo et al., 2011).

The beet armyworm, *Spodoptera exigua* is a major pest of greenhouse crops in many parts of the world (Belda, 1994). The multiple nucleopolyhedrovirus of *S. exigua* (SeMNPV) has been developed as the basis for several bioinsecticide products (Lasa et al., 2007). Vertical transmission of SeMNPV was a common feature in field-collected adults of *S. exigua* in southern Spain (Cabodevilla et al., 2011a). In that study, a selection of vertically transmitted (VT) genotypes, were isolated and their insecticidal properties were characterized. Among these, the VT-SeAl1 genotype had the greatest capacity to induce persistent infections compared to genotypes associated with the horizontal transmission pathway. Sublethal infections by VT genotypes persisted for at least five generations after their inoculation in a healthy experimental laboratory colony of *S. exigua*

(Cabodevilla et al., 2011b). Transgenerational transmission can involve the transovarial or transovum pathways. Transovarial transmission describes the process of virus passing to progeny within the eggs, whereas the transovum route involves contamination of the egg surface with viral particles that infect neonate larvae when they ingest the chorion (Kukan, 1999; Vilaplana et al., 2008).

Unexpectedly, Cabodevilla et al. (2011a) observed that a fraction of field-caught gravid females produced virus-infected offspring even though no evidence of infection was seen in these females using sensitive PCR methods targeted at the detection of viral transcripts. This led us to suspect that these females may have mated with infected wild males, suggesting that both sexes could contribute to vertical transmission of the pathogen. In the present study we determined the effect of parental gender and the importance of the transovum *vs*. transovarial routes on the transmission efficiency of this virus.

2. Material and methods

2.1 Insects and viruses

A healthy *S. exigua* culture was obtained from Andermatt Biocontrol AG (Grossdietwil, Switzerland) and reared on artificial diet (Elvira et al., 2010) at a constant temperature (25 ± 1ºC), relative humidity (50 ± 5%), and photoperiod (16 h:8 h light:dark cycle) in the insectary facilities of the Universidad Pública de Navarra, Pamplona, Spain. A single genotype of SeMNPV, named VT-SeAl1, was used in the experiment. This genotype was previously isolated from a sublethally infected colony of insects collected in the greenhouses of Almeria (Spain) and was known to be capable of parent to offspring transmission (Cabodevilla et al., 2011a; Cabodevilla et al., 2011b).

2.2 DNA extraction

Total insect DNA was extracted using MasterPure Complete DNA Purification kit (Epicentre Biotechnologies) standard protocol for tissue samples. Abdomens of recently thawed adults were dissected individually, and sexed by observation of external genitalia. The dissected abdomen was placed in a 2 ml tube with ceramic beads and 300 µl of lysis solution with 1 µl of 50 µg/µl Proteinase K added. The tissue was homogenized using MP FastPrep-24 tissue in

a cell homogenizer at 4.0 m/s for 20 s. The mixture was incubated at 65ºC for 15 min with a constant 1100 rpm orbital agitation. A 150 µl volume of the sample was then treated with RNase for 30 min at 37ºC. Debris was pelleted by adding protein precipitation reagent and centrifuged at 10000 × g for 15 min. DNA was precipitated using cold isopropanol, washed twice with 70% ethanol, resuspended in 20 µl Milli-Q water and stored at -20ºC. Blank extraction samples containing only water were processed in parallel to detect cross-contamination during the extraction process.

2.3 Detection of sublethal infections

Quantitative PCR based on SYBR fluorescence was performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in 96-well reaction plates. To detect virus genomic DNA specific primers were designed to amplify a 149-bp region of the SeMNPV *DNA polymerase* gene (DNApol149-Fw: 5'- CCGCTCGCCAACTACATTAC-3'; DNApol149-Rv: 5'-GAATCCGTGTCGCCGTATATC-3') based on the complete genome sequence of the SeMNPV strain VT-SeAl1 (unpublished data). Amplifications were performed in a total reaction of 10 µl containing 5 µl of SYBR Premix Ex Taq (2×), 0.2 µl of ROX Reference Dye (50×), 0.2 µl of both forward and reverse primers (10 pmol/µl), and 1 µl of DNA template containing up to 50 ng of DNA. Three non-template reactions were included in each run and a standard curve was prepared in duplicate to determine the efficiency of each reaction. The qPCR protocol consisted of an initial denaturation step at 95ºC for 30 s, followed by 45 amplification cycles of 95ºC for 5 s, 60ºC for 30 s, and finally added dissociation steps of 95ºC for 15 s, 60ºC for 15 s, 95ºC for 15 s. Data acquisition and analysis were handled by Sequence Detector System version 2.2.2 software (Applied Biosystems). For the standard curve VT-SeAl1 DNA was extracted from OBs, purified thorough CsCl gradients, quantified using a spectrophotometer (Eppendorf BioPhotometer plus) and then serially diluted in sterile MilliQ water to the following concentrations: 10, 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 pg/µl. A total of seven replicates of the DNA dilutions were performed and the average Cq value for each point was calculated and used to fit a linear regression. DNA quantities were consistently estimated per sample by extrapolation of Cq values from the standard curve. Every DNA sample was performed in triplicate and the specificities of PCR

products were monitored by analyzing amplification profiles and the corresponding dissociation curves. Quantified viral DNA was normalized based on the total DNA concentration for each sample and measured using a NanoDrop 2000 (Thermo Scientific).

2.4 Gender effects on vertical transmission

To determine gender effects on vertical transmission of SeMNPV, groups of adults that were either sublethally infected (infected males: I♂ and infected females: I♀) or were not subjected to prior virus treatment (virus-free adults, healthy males: H♂ and healthy females: H♀). For this, two genetically identical subpopulations were generated by inducing sublethal infections in experimental insects (qPCR detection limits 10^{-3} pg of viral DNA), whereas insects from the healthy treatment groups were not subjected to virus inoculation. To produce sublethally infected insects batches of 200 newly molted *S. exigua* fourth instars were fed a virus suspension containing 9×10^3 OBs/ml. In parallel, groups of 100 larvae were treated identically using a suspension without OBs. Larvae that drank the suspension within 10 minutes were individually placed in perforated 25-ml plastic cups containing artificial diet and reared at 25 ± 2ºC and 50 ± 5% RH until pupation or death from virus disease. Pupae that survived inoculation were assigned to separate groups according to their sex and viral treatment. Once the adults emerged, the following mating schedules were performed: i) healthy males (H♂) × healthy females (H♀); ii) infected males (I♂) × healthy females (H♀); iii) healthy males (H♂) × infected females (I♀) and iv) infected males (I♂) × infected females (I♀). Five adult pairs were confined in groups in paper bags provided with a moist cotton water source and maintained at 25 ± 2ºC and 50 ± 5% RH for oviposition during a 2–4 day period. Egg batches from each treatment group were collected using sterilized instruments and adults were frozen at -80ºC for subsequent analysis (F_0 generation). Egg masses laid from each paper bag were divided into two parts and either soaked in a 0.25 ppm sodium hypochlorite solution (surface decontamination) or in sterile distilled water (no decontamination) for 5 minutes. Groups of 24 larvae that emerged from each half of each egg mass were individually placed in 25 ml cups with diet, and reared individually through to the adult stage (F_1) to avoid cross-contamination among insects in this cohort.

Adults were individually stored at -80ºC for subsequent analysis. The whole experiment was performed four times.

2.5 Statistical analysis

In order to determine the influence of parental infection status on the offspring, the prevalence of qPCR positive insects and results of virus loads in the offspring were analyzed by fitting generalized models in GLIM 4 (Numerical Algorithms Group, Oxford, UK) with a binomial or normal error distribution specified, respectively, and with gender or mating group treatment specified as factors. Proportions of infected insects were compared by Fisher's exact test and subjected to *t*-test for pairwise comparison. Values of viral load per infected insect were normalized by log-transformation prior to analysis. The effect of egg surface decontamination on vertical transmission was examined using Pearson's χ^2 test in the SPSS Statistics program (v.19 IBM). The correlation between proportions of F_1 infected insects and their viral load was examined by Spearman's rank correlation.

3. Results

3.1 Establishing qPCR amplification parameters

Following mating and oviposition, parental insects from each of the four mating groups were subjected to qPCR to determine the prevalence of sublethal infection. A linear relationship was established between the critical quantification cycle (Cq) and the log-transformed amount of viral DNA (Figure 1). The regression coefficient (R^2 = 0.995) and slope value (-3.215), indicated very high reaction efficiency (Bustin et al., 2009).

The cut-off value was defined as the lowest concentration detected that fell within the linearity of the regression, in this case 1×10^{-3} pg. This value was used to set a limit of 33.4 cycles; all samples with higher Cq values were treated as negative, whereas all samples with a lesser number of cycles and that showed a single peak at the expected melting temperature (83ºC) in the dissociation curve, were considered as positive. As the genome of VT-SeAl1 was estimated to be 135696 bp (unpublished data) the theoretical detection limit equates to 6.8 genome copies per reaction.

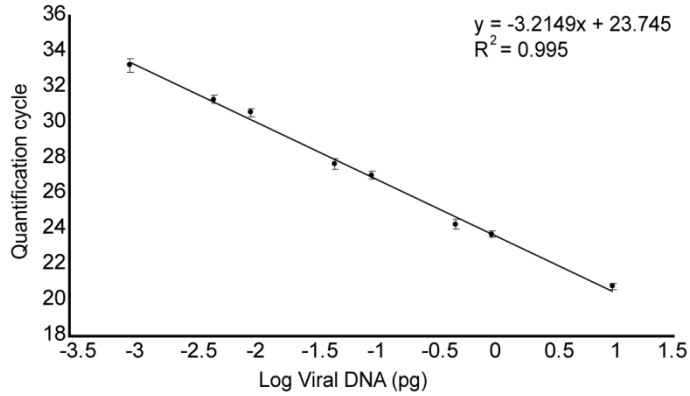

Figure 1. Standard curve for qPCR quantification. Linear regression with different critical quantification PCR (Cq) following serial dilution of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) genomic DNA.

3.2 Detection of sublethal infections in parental (F_0) insects

Overall, 57.6 ± 4.4% of the larvae that consumed VT-SeAl1 OBs succumbed to virus infection, whereas no mortality was registered in mock-infected control larvae. The prevalence of qPCR positive reactions in the insects that survived, following consumption of viral OBs in the larval stage, was clearly higher than that of control insects (Figure 2). Viral load in parental (F_0) adults averaged 1.514 ± 0.287 × 10^{-3} pg viral DNA/µg total DNA per insect (N = 72 positive samples) that represents 10.34 ± 1.96 genome copies per reaction.

Overall, sublethally infected individuals were more abundant in the virus challenged groups of insects than in the mock-infected groups (χ^2 = 60.49, *df* = 1, *P* < 0.001). Between 70 and 85% of adult males that survived OB treatment were classified as sublethally infected, compared with 65 to 80% of adult females (Figure 2). Unexpectedly, 12 out of the 80 insects that had not consumed OBs were positive for sublethal infection, suggestion a low level of inapparent or latent infection in the insect colony from Switzerland (Figure 2). The frequency of infected adults in the control group H♂ × H♀ (5 positive adults (both sexes) out of 40 tested) was similar to that found in apparently healthy groups mated with infected insects (I♂ × H♀ = 4 positive females out of 20 and H♂ × I♀= 3/20 positive males; χ^2 = 0.392, *df* = 1, *P* = 0.531).

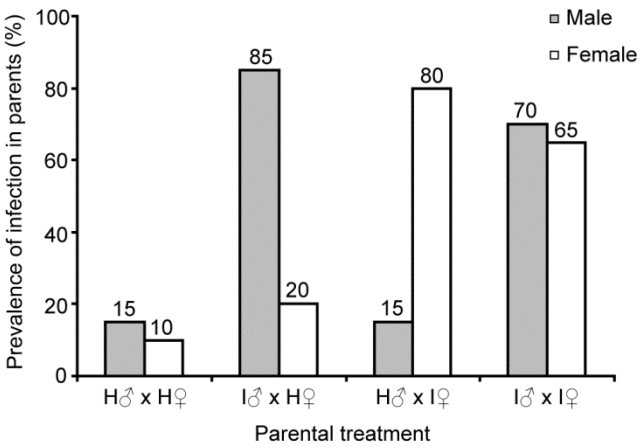

Figure 2. Prevalence of infection in parental adults in each mating group. H♂: Healthy male, H♀: Healthy female, I♂: Infected male, I♀: Infected female. Each mating group comprised 20 male and female *S. exigua* moths.

3.3 Transgenerational transmission

In order to elucidate whether virus was passed to offspring via transovarial or transovum transmission, the prevalence of sublethal infection in F_1 adults was compared between adults from either decontaminated or non-decontaminated eggs. Egg surface decontamination did not significantly affect the prevalence of sublethally infected F_1 adults (decontaminated eggs = 16.5%; non-decontaminated eggs = 14.9%; χ^2 = 0.649, *df* = 1, P = 0.420). Therefore, all results were pooled across decontamination treatments for subsequent analyses. Parental mating group treatment significantly influenced viral transmission to F_1 adults (F = 18.95, *df* = 3, 31, $P < 0.001$; Figure 3). Subletally infected males that mated with healthy females produced offspring with an average prevalence of 26% inapparent infection, compared to 8% in the offspring of the control insect group. In contrast, when infected females mated with healthy males, the prevalence of infection in offspring was 49%, compared to 44% when both parents were infected (Figure 3). These results indicate that female-mediated vertical transmission was approximately twice as efficient as male-mediated transmission. Both sexes of offspring were equally likely to have acquired a sublethal infection from their parents (male mean = 34.9 ± 6.7%; female mean = 27.9 ± 7.9%; F = 0.997, *df* = 1, 28, P = 0.327). Similarly, no significant interaction was observed between parental

mating group and offspring gender in the prevalence of sublethal infection (F = 0.863, df = 3, 27, P = 0.472). Importantly, none of the F_1 generation insects died of patent virus disease during rearing from larva to adult, so that all infections that we detected were present in insects that showed no signs of disease.

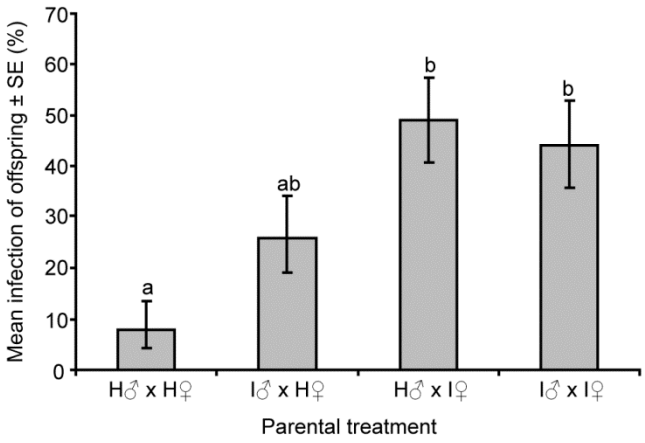

Figure 3. Percentage of offspring positive by qPCR according to parental infection status. H♂: Healthy male, H♀: Healthy female, I♂: Infected male, I♀: Infected female (N = 120). Columns labeled with different letters indicate signficant differences (t-test, $P < 0.05$).

3.4 Virus DNA loads present in offspring

Viral load in F_1 adults was quantified and normalized by total DNA content for each insect sample. Mean viral load values in infected offspring were similar between mating groups (F = 1.31, df = 3, 12, P = 0.316) and ranged from 1.07 ± 0.12 × 10^{-3} to 1.7 ± 0.29 × 10^{-3} pg viral DNA/mg total DNA, i.e., the quantity of viral DNA in each insect was independent of the parental source of the infection (male, female or both).

In order to investigate whether viral loads differed according to offspring the viral load results of F_1 infected adults were pooled and found not to differ significantly according to sex (male mean = 1.74 ± 0.26 pg viral DNA/µg total DNA; female mean = 1.46 ± 0.16 pg viral DNA/µg total DNA; t = 0.639, df = 25, P = 0.474).

Finally, a significant positive relationship was detected between average viral load per F_1 infected insect and the proportion of F_1 infected insects produced

by each mating group (Spearman rank correlation: 0.687, $P < 0.05$), i.e., the prevalence of vertical transmission was positively associated with the number of genome copies in each infected insect (Figure 4).

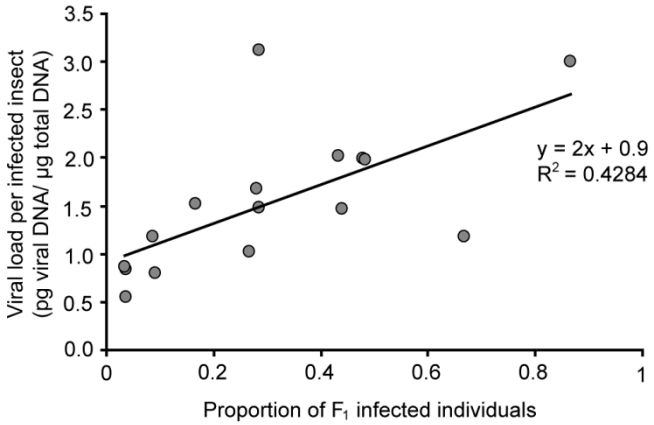

Figure 4. Relationship between viral DNA load per F_1 infected insect and proportion of F_1 infected insects in each cohort. Grey circles indicate different experimental groups taking into account parental treatment and replicate. Spearman rank correlation: 0.687, $P < 0.05$.

4. Discussion

The development of highly sensitive molecular tools has recently allowed insect pathologists to focus attention on the vertical transmission of insect viruses and to assess the role of this strategy in the survival of these pathogens in natural and laboratory insect populations (Vilaplana et al., 2010). It seems that both alphabaculoviruses (Burden et al., 2003; Khurad et al., 2004; Vilaplana et al., 2010), and betabaculoviruses (Burden et al., 2002) can establish sublethal infections in larvae that survive after having consumed OBs. Moreover the prevalence of such infections can be dose-dependent (Cabodevilla et al., 2011b). In the present study between 65 and 85% of sublethal infection was detected in adult survivors of an inoculum that killed 57.6% of experimental insects. A small number of control insects proved positive for sublethal infection by qPCR which suggests a low level infection in what was believed to be a completely healthy insect colony. However, it was clear that deliberately infected insects harbored markedly higher levels of virus than the untreated insects from the laboratory

colony, which led us to believe that the overall findings and conclusions of this study are likely to be valid, despite the low level presence of virus in the host colony. Indeed, apparently healthy laboratory colonies of lepidopteran species are often found to harbor sublethal virus infections as soon as they are subjected to sensitive molecular techniques for pathogen detection (Burden et al., 2002; Hughes et al., 1993; Hughes et al., 1997; Vilaplana et al., 2010). Moreover, latent infections have been reported for all developmental stages of asymptomatic individuals of *S. exigua*, confirming that inapparent infections can be detected in all stages of the host life cycle (Murillo et al., 2011).

Our study demonstrated biparental transmission of SeMNPV to offspring, although the efficiency of maternally-mediated transmission was approximately double that of paternally-mediate transmission. Transmission during mating has been described in a range of insect pathogenic viruses, including a rhabdovirus in a palm beetle (Zelazny, 1976), a parvovirus in a mosquito (Barreau et al., 1997), sigmaviruses of *Drosophila* spp. (Longdon and Jiggins, 2012), an iflavirus in honeybees (de Miranda and Fries, 2008), and nucleopolyhedroviruses of lepidopteran pests (Knell and Webberley, 2004), among others. Sexual transmission has also been demonstrated for the gonad specific nudivirus Hz-2V of the noctuid moth *Helicoverpa zea*, that was transmitted during copulation, through waxy virus-rich secretions at the tip of the abdomen of the infected insect (Hamm et al., 1996).

Virus titers required for transmission were estimated to be very low and were calculated at approximately 10.4 viral genomes per reaction or 208 genomes per infected insect (assuming 100% efficiency in DNA extraction, which is highly unlikely). For the vertically-transmitted sigmavirus of *Drosophila* spp., the transmission of viral particles occurs inside the oocyte, likely due to the size and activity differences between male and female gametes. Infected male sperm may also be not as competitive as non-infected counterparts (López-Ferber et al., 1997). Accordingly, sigmaviruses show marked parental sex differences in the contribution to virus transmission and quantity of virus genomes transmitted to offspring (Fleuriet, 1988; Longdon et al., 2011).

Persistent infections of nucleopolyhedroviruses often have biological costs that include lower developmental rates, lower pupal and adult body weights and reduced reproductive capacity (Cabodevilla et al., 2011b; Goulson and Cory,

1995; Hatem et al., 2011; Kukan, 1999; Myers et al., 2000), although occasionally beneficial effects have been detected (Thomas-Orillard, 1990).

The quantity of viral DNA present in sublethally infected insects of F_1 did not differ significantly according to sex or mating group (infected fathers *vs*. infected mothers, or both), whereas lower titers of sigma virus were detected in *Drosophila* embryos when sigmavirus was paternally transmitted (Longdon et al., 2011).

A positive correlation was detected between the percentage of infected adults in F_1 generation and their viral load, suggesting that the adults that transmitted the virus to a high proportion of their progeny tended to transmit greater amounts of viral DNA. It may be that due to their genotype, nutritional or physiological characteristics, certain hosts provide better conditions for virus multiplication, so that they provide a greater contribution to the number of virus genome copies in the offspring. Further studies are required to investigate this hypothesis.

Previous studies on baculovirus transmission demonstrated that both sexes were involved in vertical transmission for *Bombyx mori* nucleopolyhedrovirus (BmNPV) (Khurad et al., 2004), *Plodia interpunctella* granulovirus (PiGV) (Burden et al., 2002) and *Spodoptera exempta* nucleopolyhedrovirus (SpexNPV) (Vilaplana et al., 2008). Interestingly, viral particles were observed in either testis or ovaries cells, confirming the presence of the virus in gonads of sublethally infected individuals by histological observation (Khurad et al., 2004) or by viral transcript detection (Burden et al., 2002; Khurad et al., 2004). Vilaplana et al. (2008) observed lethal NPV infection in offspring in both cases, when the infected parental was male or female. For *B. mori*, mating pairs with a female infected with BmNPV resulted in higher mortalities of first instar offspring (78%) than observed in offspring from treatments in which the male was responsible for transmission (57%). These authors concluded that transmission occurred principally via the transovarial route rather than transovum transmission (Khurad et al., 2004), as did studies on SpexNPV on *S. exempta* in which the prevalence of infection in the offspring was independent of eggs surface decontamination treatment (Vilaplana et al., 2008). In contrast, in the present study patent disease was not observed in the offspring of covertly infected insects, perhaps as a result of low levels of stress during rearing or another unidentified factor that favored the maintenance of

sublethal infection over the expression of patent lethal disease (Fuxa et al., 2002; Fuxa et al., 1999; Kukan, 1999).

In conclusion, vertical transmission of SeMNPV was observed when male or female parents harbored a sublethal infection, but female-mediated transmission was more efficient than that of males. Improving our knowledge on the factors affecting vertical transmission mechanisms may contribute to the development of optimal strategies for the use of virus-based insecticides. Additional studies on the mechanisms that trigger sublethal infections into lethal patent infections may also provide useful information aimed at reducing the frequency of virus insecticide applications in the field.

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CHAPTER IV

Can mixtures of horizontally and vertically transmitted nucleopolyhedrovirus genotypes be effective for biological control of *Spodoptera exigua*?

Abstract

Previous studies identified distinct genotypes of *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV) that were associated with horizontal transmission (named HT-SeG25) or vertical transmission (named VT-SeAl1) in the host insect, *S. exigua* (Lepidoptera: Noctuidae). We examined the use of mixtures of occlusion bodies (OBs) of these genotypes as the basis for a virus preparation that could provide immediate pest control and establish a persistent sublethal infection in the survivors of an OB application for transgenerational pest suppression. Mixtures of HT-SeG25 + VT-SeAl1 comprising 25:75% or 75:25% of each genotype, respectively, resulted in improved OB pathogenicity in terms of concentration mortality metrics compared to OBs of VT-SeAl1 alone or similar values compared to OBs of the HT-SeG25 genotype alone. In contrast, no significant differences were observed in speed of kill or mean OB production per larva. Laboratory and greenhouse trials revealed that the prevalence of sublethal infection in adults that survived OB treatments in the larval stage increased with the proportion of VT-SeAl1 present in the inoculum, as determined by qPCR. Greenhouse trials indicated that the 75% VT-SeAl1 + 25% HT-SeG25 mixture was as effective as methoxyfenozide in preventing pest damage to pepper fruits. The potential contribution of vertically transmitted genotypes to transgenerational suppression of pest populations is discussed.

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CHAPTER V

Chemical and biological stress factors on the activation of nucleopolyhedrovirus infections in covertly infected *Spodoptera exigua*

Abstract

Following the consumption of baculovirus occlusion bodies (OBs) insects may succumb to lethal disease, but the survivors can harbor sublethal covert infections and may develop, reproduce and transmit the infection to their offspring. The use of different chemical and biological stressors was examined to determine whether they could be used to activate covert infections in populations of *Spodoptera exigua* larvae infected by the homologous nucleopolyhedrovirus (SeMNPV). Treatment of covertly infected *S. exigua* second instars with Tinopal UNPA-GX, hydroxylamine, paraquat, *Bacillus thuringiensis* var. *kurstaki* crystals, spores or mixtures of crystals + spores, or a heterologous nucleopolyhedrovirus (*Chrysodeixis chalcites* SNPV), did not result in activation of SeMNPV covert infections. Similarly, virus treatments involving permissive NPVs did not result in greater mortality in covertly-infected insects compared with the virus-free controls. In contrast 0.1% copper sulfate, 1% iron (II) sulfate and 1 mg/l sodium selenite treatments resulted in 12 – 41% lethal polyhedrosis disease in covertly infected larvae. A greenhouse trial using copper sulfate and sodium selenite as activation factors applied to covertly infected *S. exigua* larvae on sweet pepper plants resulted in very low levels of SeMNPV activation (< 3%). These results highlight the important roles of copper, iron and selenium in insect immunity and baculovirus induced disease. However, these substances seem unlikely to prove useful for activation of covert SeMNPV infections in *S. exigua* larvae under greenhouse conditions.

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CHAPTER VI

General discussion

Baculovirus transmission has been thought to occur mainly via horizontal transmission (HT), but increasing evidence of vertical transmission (VT) that involves covert infection in Lepidoptera and the subsequent transmission of the pathogen to offspring via the host's reproductive tissues (Cory, 2010; Cory, 2015). The advent of highly sensitive molecular techniques has revealed the presence of covert infections in laboratory and natural insect populations, previously only detectable by the spontaneous occurrence of disease in apparently healthy insects (Kukan, 1999). *Spodoptera 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 *Neodiprion sertifer* (Krokene et al., 2013) are species in which covert infections have been detected in field collected insects.

In Almería, *S. exigua* populations annually arrive from North Africa during the months of June to October, but this population also seems to be established as a resident species in the horticultural area due to the protection from environmental factors provided by greenhouses (Belda, 1994). The *S. exigua multiple nucleopolyhedrovirus* (SeMNPV) naturally contributes to suppress pest outbreaks (Caballero et al., 1992), although the effect of this agent is often too late to avoid crop production losses. Currently, horticultural growers control *S. exigua* damage by integrating SeMNPV-based insecticides in wider integrated pest management programs. Studies on local insect populations revealed that SeMNPV frequently produces asymptomatic infections that are vertically transmitted to the progeny (Cabodevilla et al., 2011a). Long-term data is required to understand this phenomenon and evaluate the frequency of this type of infection in natural populations.

New Generation Sequencing (NGS) technology has proven to be a powerful tool for the discovery of novel insect viruses, especially those causing cryptic infections (Liu et al., 2011; Pascual et al., 2012). Novel RNA viruses

belonging to the *Iflaviridae* were identified in the transcriptome of *S. exigua* tissues in individuals from laboratory colonies (Pascual et al., 2012), and were later characterized (Choi et al., 2012; Millan-Leiva et al., 2012), leading me to examine baculovirus and iflavirus covert infections in *S. exigua* field populations from greenhouses of Almería and their influence on the transmission of SeMNPV (**Chapter II**). In this study it was demonstrated that two iflaviruses species (*S. exigua* iflavirus-1: SeIV-1 and *S. exigua* iflavirus-2: SeIV-2) and SeMNPV persist naturally in both field-caught adults and their laboratory reared offspring, causing single or multiple covert infections. This is the first time that iflaviruses were detected and quantified in *S. exigua* field-collected adults. The prevalence of SeIV-1 (13%) and SeIV-2 (8%) in field insects was lower than that of SeMNPV (54%). For SeMNPV, more than half of field adults (54%) were positive for baculovirus DNA, a higher value than the 16% of SeMNPV viral transcripts found in those insects collected during the 2006-2007 growing season (Cabodevilla et al., 2011a). This may be due to the use of a 5-fold more sensitive technique, real-time quantitative PCR (qPCR), capable of detecting 6.8 genome copies per reaction (Virto et al., 2013). Additional reasons for the recent increase of SeMNPV prevalence could be the income of infected individuals into the local population coming from northern Africa, and more likely the effect of spraying SeMNPV-based insecticides during 2010-2011. In line with previous findings, this result shows that baculovirus asymptomatic infection is widely spread in wild populations, especially those belonging to the genus *Spodoptera* (Abul-Nasr et al., 1979; Cabodevilla et al., 2011a; Fuxa and Richter, 1991; Smits and Vlak, 1988; Swaine, 1966; Vilaplana et al., 2010), characterized by its migration behavior as part of an effective strategy for viral dispersal (Fuxa, 2004; Vilaplana et al., 2010) and the maintenance of the virus during adverse environmental conditions (Cory and Myers, 2003).

Perhaps the most novel contribution of this study was to reveal mixed infection involving two different viral families in *S. exigua*. Persistent viruses are common in mixed infections (Carrillo-Trip et al., 2015). Here, co-infections caused by baculovirus and iflavirus were detected at a low prevalence (less than 9% of multiple infections) in both field-collected adults and their offspring. Interestingly, early studies already reported iflavirus-like particles contaminating *Autographa californica* NPV OB preparations (Vail et al., 1983a; Vail et al., 1983b), that

suggests a functional association between these two virus families. Similarly, *Ectropis obliqua* and *Perina nuda* iflaviruses were isolated from lepidopteran cadavers that succumbed to granulovirus (Wang et al., 2004) and nucleopolyhedrovirus (Wang et al., 1999) infections respectively. More recently, electron microscopy images and RT-PCR analysis support the hypothesis of a physical association between SeIV and SeMNPV suggesting the co-occlusion of both types of viruses in the same OB (Jakubowska et al., 2016).

The exploitation of a common host allows virus-virus interactions including immunological relations that could affect the host response. Interactions between co-infecting microorganisms are invariably complex and their consequences unpredictable. Mutualistic host-pathogen associations include that of a novel densovirus infecting *Helicoverpa armigera* larvae that appears to confer resistance against baculovirus and *Bacillus thuringiensis* infections (Xu et al., 2014). On the other hand, *Wolbachia* covert infections increased susceptibility to baculovirus super-infection in *S. exempta* larvae (Graham et al., 2012). Whether the iflavirus-baculovirus association could have implications for the biosafety or efficiency of baculovirus-based insecticides is a key point that needs to be addressed. Initial bioassays showed that SeMNPV pathogenicity diminish when OBs were contaminated with SeIV (Jakubowska et al., 2016). Current laboratory bioassays are being performed to determine how SeIV covert infections might affect host responses to SeMNPV infection and the insecticidal characteristics of iflavirus-contaminated OBs.

S. exigua field-caught males and females were infected by iflavirus and baculovirus at a similar frequency, however the fact that apparently healthy females produced infected individuals in the progeny was intriguing. The contribution of the male linage to VT was investigated in **Chapter III** in order to elucidate the gender effect on transgenerational transmission. I observed that sublethally infected male and female adults were capable of transmitting the infection to descendants, although infected females were twice as efficient as males in vertical transmission. Similar experiments crossing infected and uninfected parental lines carried out in various NPV-host systems demonstrated that both sexes are involved in VT (Khurad et al., 2004; Vilaplana et al., 2008). Moreover viral particles and viral transcripts in *Bombyx mori* (Khurad et al., 2004) and *Plodia interpunctella* (Burden et al., 2002) gonads, respectively (of both males

and females), confirmed that baculoviruses are capable of being transmitted transovarially. In a similar way, the *Drosophila* sigma virus transmission rates are greater in females than males of *D. obscura* and *D. affinis* (Fleuriet, 1988; Longdon et al., 2011), despite the fact that transmission occurs through both eggs and sperm. Egg surface decontamination has been used to differentiate between transovarial and transovum pathways (see Kukan, 1999). After soaking eggs in a sodium hypochlorite solution, I found similar values of transgenerational transmission through decontaminated and non-decontaminated eggs, supporting the idea of transovarial transmission. Nevertheless in natural habitats it is likely that both pathways contribute to VT (Cory and Myers, 2003).

Interestingly, a positive relationship was observed in the offspring between the proportion of infected insects and their viral load. As such, the higher the prevalence of VT, the higher the virus titers found in the host insect. Unexpectedly, as in **Chapter IV**, very few insects that were not exposed to virus proved positive by qPCR analysis, suggesting low level of infection in the presumed virus-free population. With the use of highly sensitive molecular techniques, asymptomatic infections have been reported in apparently healthy Lepidopteran insect colonies, underlining the difficulty of maintaining a population totally free of virus infections (Burden et al., 2003; Hughes et al., 1993; Hughes et al., 1997; Vilaplana et al., 2010). In fact, SeMNPV covert infections are detectable throughout all developmental stages of the host, despite noticeable viral load fluctuation (Murillo et al., 2011), as also observed in SpexNPV in its homologous host (Graham et al., 2015). It is interesting to consider that insects which do not die after baculovirus treatment can harbour covert infections that are transmitted to the progeny, and that might produce sublethal effects on the host. Adverse effects such as reduced developmental rate (Cabodevilla et al., 2011b; Gothama et al., 1995; Milks et al., 1998), reduced reproductive capacity (Cabodevilla et al., 2011b; Milks et al., 1998; Patil et al., 1989), or increasing susceptibility to superinfection (Cabodevilla et al., 2011a; Cabodevilla et al., 2011b), have been reported in insect survivors of a baculovirus treatments. As covert infections can persist for several generations within the host (Burden et al., 2003; Cabodevilla et al., 2011b; Vilaplana et al., 2010), virus transgenerational effects could be considered from the perspective of designing new pest control strategies.

For this reason, after studying the natural occurrence of covert infections and their efficient transmission in *S. exigua* insects, the usefulness of this type of infection was evaluated as an alternative to SeMNPV inundative-applications that are performed in the Almería region (**Chapters IV and V**). The addition of VT genotypes to baculovirus-based insecticides might result in an improvement in the technical product for field applications. The working hypothesis (**Chapter IV**) was that in OB mixtures, HT genotypes would confer traits for rapid pest suppression, whereas VT genotypes favour the establishment of covert infections for transgenerational transmission with medium or long term effects on the pest population. Based on the existence of SeMNPV genotypes associated with different transmission routes (Cabodevilla et al., 2011a), and considering that mixtures of genotypes can produce unpredictable responses (Hodgson et al., 2001; Simon et al., 2006), I aimed to select a mixture of OBs comprising VT and HT genotypes which encompassed useful insecticidal properties and the ability to produce covert infections (**Chapter IV**). According to previous results (Cabodevilla et al., 2011a), I observed that the HT genotype was three-fold more pathogenic than the VT single genotypic strain, a difference attributable to genomic differences in the *Se4* and *Se5* genes (Serrano et al., 2015; Theze et al., 2014). Interestingly, OBs mixtures comprising either 25 or 75% of the HT genotype were more pathogenic than the VT genotype and equally pathogenic as the HT genotype alone. However, the VT genotype was the most effective in producing covert infections, followed by the three OBs mixtures which generated intermediate values and the HT genotype which was the least efficient. These results support the hypothesis that strains that are vertically transmitted are less virulent than those that only comprise HT genotypes (Lipsitch et al., 1995). Covert infections in field trials showed the same trend as in laboratory experiments, although the results of all treatments were slightly lower than those obtained in the laboratory, probably because larval feeding behaviour and the acquisition of a lethal infection could not be controlled, or the distribution of the OBs on the plant surface was not completely homogeneous and also due to other environmental factors that modulate host responses to virus infection (**Chapter V**). Nevertheless, the high prevalence of covert infection following application of OBs mixtures on plants is suggestive of a long-term response in the insect population, but further

field trials are needed to determine the magnitude of this effect over multiple host generations.

So far, the selection of an active ingredient in baculovirus-based insecticides has been based on the insecticidal potential, principally OB pathogenicity and speed of kill (Arrizubieta et al., 2015; Bernal et al., 2013). However, these genotypes do not always have high transmissibility leading to long-term control (Takahashi et al., 2015), a desirable property that can be supplied by VT genotypes. In fact, the success of *Anticarsia gemmatalis* NPV in soya crops in Brazil is based on the virus' capacity to persist and be transmitted during the crop cycle, requiring only a single application of OBs (Moscardi, 1999). As such, this virus is also able to cause epizootics some years after an inoculative release (Fuxa and Richter, 1999). In this context, transgenerational transmission of pathogens gains importance, not only because of the greater virus persistence, but also for its contribution to pest control by the long-term suppression effect of spontaneous disease reactivation in sublethally infected larvae.

I also examined the susceptibility of the progeny from field-treated insects to superinfection. Despite previous laboratory assays indicating that sublethally infected *S. exigua* colonies tend to be more susceptible to the SeMNPV than virus-free populations (Cabodevilla et al., 2011a; Cabodevilla et al., 2011b), here laboratory-reared descendants from field treated lineages were equally susceptible. This discrepancy could be attributed to the lower prevalence of sublethal infections found in the adults challenged in the field (30-76%) compared to those sublethally infected in laboratory conditions (80-100%) (Cabodevilla et al., 2011b). Field assays included the two single genotypes and the mixture that comprises 75% of VT + 25% of HT genotype evaluation for their efficiency as crop protection agents in fields. As previously described in other studies for SeMNPV applications (Bianchi et al., 2000; Lasa et al., 2007; Smits et al., 1987), all viral treatments showed evidence of providing efficient crop protection, especially when *S. exigua* larvae directly attack fruits. Results in **Chapter IV** demonstrated that a mixture that comprised 75% of a VT genotype + 25% of a HT genotype was as pathogenic as the HT genotype alone, while it produced similar prevalence of covert infection as the VT genotype alone. Moreover the mixture resulted equally efficient as a chemical insecticide (methoxyfenozide) for pest control. The incorporation of VT genotypes in baculovirus-based insecticides, that used highly

pathogenic genotypes resulted in similar pest mortality as HT genotypes or even chemical-based insecticides, and may contribute to long-term suppression through transgenerational transmission effects. The possibility of inoculative instead of inundative OB releases and the incorporation of new genotypes into baculovirus-based insecticides, might also act as a preventive strategy to avoid the development of resistance, which has been reported for other baculovirus insecticides (Asser-Kaiser et al., 2007; Asser-Kaiser et al., 2011; Schmitt et al., 2013; Undorf-Spahn et al., 2012).

The mechanisms by which covert infections are triggered into lethal infections are poorly understood. In this study I tested whether covert infections could be activated under certain stressful conditions to trigger lethal infections **(Chapter V),** as fatal reactivation can account for up to 20% mortality in the offspring of field-caught females (Cabodevilla et al., 2011a). Baculovirus infections not only depend on the virulence factors of the pathogen, but also on the role of the host immune system (Jakubowska et al., 2013; Pascual et al., 2012). Some essential trace elements for the insect immune system (Chaturvedi et al., 2004) were evaluated as stress factors to trigger lethal polyhedrosis diseases. In particular, under laboratory conditions, 0.1% copper sulfate, 1% iron (II) sulfate and 1 mg/l (1 ppm) sodium selenite triggered lethal NPV infections (12, 15 and 41% mortality, respectively) in covertly infected *S. exigua* larvae. These trace elements, at an appropriate concentration, are required to maintain immune system function, preventing not only oxidative stress but also viral mutations which could increase viral pathogenicity (Chaturvedi et al., 2004). Alterations in the copper and iron levels were found in the hemolymph during the development of NPV infected *Heliothis virescens* larvae (Popham et al., 2012a; Popham et al., 2012b). Previously, copper sulfate had been reported as an NPV activation factor in covertly infected *Lymantria dispar* larvae (Ilyinykh et al., 2004). Apart from chemical compounds, heterologous viruses were tested without a notable activation response, whilst several studies have reported virus activation following cross inoculation with heterologous viruses (Cooper et al., 2003; Fuxa and Richter, 1992; Hughes et al., 1993; Kouassi et al., 2009). In this regard, the prevalence of virus activation might depend on the virus species and its genotype, or the dosage required to initiate an infection.

Unfortunately, I failed to induce high rates of NPV infections in greenhouse trials possibly because of the behaviour of the larvae in these conditions, or due to biotic and abiotic factors. Host plant chemistry (Shikano et al., 2010), insect nutrition (Ojala et al., 2005; Vogelweith et al., 2011), or insect density (Reeson et al., 1998; Wilson and Graham, 2015), among others are factors that influence the host vigor and susceptibility to NPV infections, thereby modulating viral activation in covertly infected larvae. Obviously, plant crops and semi-synthetic diet differ in their nutritional content that may also alter the insect response after being challenged by baculovirus (Lee et al., 2006). Also host density can affect the susceptibility and immune response of the host, since *Spodoptera littoralis* and *S. exempta* larvae reared gregariously have been described as more resistant to NPV infections than those reared individually (Reeson et al., 1998; Wilson and Graham, 2015). Therefore, when I tried to extrapolate laboratory results to field conditions I had to be aware that there were a number of factors that could affect to the outcome of the experiment. Probably for this reason trace metals were capable of inducing NPV lethal disease in laboratory insects but not under field conditions.

To conclude, in this thesis covert infections and vertical transmission of SeMNPV were observed and mainly occurred transovarially through females. Covert infection and transmission to offspring were common in natural *S. exigua* populations and in insect survivors of a sublethal dose of OBs. These findings open the door to design novel pest control strategies that maximize the effectiveness of baculovirus-based insecticide applications. Novel strategies have been proposed based on the inclusion of VT genotypes into the active ingredient of baculovirus-based insecticides and by the identification of chemical factors that triggered lethal disease in covertly infected larvae in order to initiate viral epizootics.

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CONCLUSIONES

1. Las poblaciones de *Spodoptera exigua* naturales de los invernaderos de Almería albergan infecciones encubiertas del SeMNPV, SeIV-1 y SeIV-2, habiéndose detectado el primero de ellos en una proporción de adultos significativamente mayor (54%) que la de los iflavirus 1 y 2 (13 y 8% respectivamente). Además la prevalencia de cualquiera de estos tres virus no varía en relación con el sexo de los insectos.

2. Las infecciones encubiertas producidas tanto por el SeMNPV como por ambos iflavirus en adultos de *S. exigua* capturados en campo se transmiten verticalmente a la descendencia, aunque el SeIV-1 (39%) lo hizo con una frecuencia significativamente mayor que el SeMNPV (21%) y el SeIV-2 (19%). Los tres virus fueron capaces de transmitirse tanto por hembras supuestamente libres de virus como por aquellas otras positivas para el SeMNPV.

3. Las infecciones mixtas causadas por baculovirus e iflavirus son poco frecuentes en poblaciones naturales de *S. exigua* originarias de los invernaderos de Almería, así como en su descendencia. No obstante, se observan todas las combinaciones posibles de infecciones mixtas por dos o tres virus que inciden tanto en los adultos de campo como en su descendencia.

4. Un elevado porcentaje de los adultos de *S. exigua* que sobrevivieron a una dosis del SeMNPV adquirieron una infección encubierta, y tanto los machos como las hembras fueron capaces de transmitir dicha infección a su descendencia; no obstante, la eficiencia de transmisión por las hembras fue doble que la de los machos. Además, la distribución del virus en la progenie no está sesgada por el género, siendo igual de probable infectar machos o hembras.

5. La descontaminación superficial de los huevos no afectó significativamente a los niveles de transmisión viral a la descendencia, lo

cual indica que la principal vía de transmisión del SeMNPV es intraovum más que transovum.

6. En la progenie de individuos subletalmente infectados (F_1), se detectó una correlación positiva entre la carga viral que porta cada insecto y el porcentaje de individuos infectados del grupo parental del que provienen, es decir, cuantos más insectos infectados hay en un grupo, más carga viral albergan dichos individuos.

7. Las mezclas de OBs de genotipos asociados a la transmisión horizontal (TH) y vertical (TV) fueron tan patogénicas (concentración letal media) como el genotipo de TH sólo, siendo a su vez las mezclas en las proporciones 75:25 y 25:75 significativamente más patogénicas que el genotipo de TV. Sin embargo, no se encontraron diferencias significativas en cuanto a virulencia (tiempo medio de mortalidad) y productividad (OBs/larva) entre los genotipos de TV, TH o alguna de sus mezclas.

8. En condiciones de laboratorio, la capacidad de producir infecciones encubiertas en los individuos de *S. exigua* supervivientes a una dosis subletal del virus, fue significativamente mayor utilizando el genotipo de TV (90%) que el de TH (45%), obteniéndose valores intermedios para cualquiera de las mezclas de OBs de dichos genotipos.

9. En condiciones de invernadero, la prevalencia de infecciones encubiertas en adultos supervivientes a cualquiera de las aplicaciones virales fue menor que la obtenida en condiciones de laboratorio. El nivel de infecciones encubiertas sigue una tendencia ascendente proporcional a la cantidad de genotipo de TV que contiene la mezcla. Esto sugiere, que la utilización de combinaciones de genotipos de TV y TH en una adecuada proporción, puede mantener las características insecticidas de la materia activa y mejorar la capacidad de producir infecciones encubiertas en los individuos que sobreviven a los tratamientos de bioinsecticidas basados en baculovirus.

10. La mezcla de OBs de genotipos de TV y TH en la proporción 75:25% pulverizada sobre plantas de pimiento dulce a una concentración de 5×10^8 OBs/l, fue tan eficaz como el metoxifenocida reduciendo el porcentaje

de frutos dañados. Por tanto, la utilización de una mezcla adecuada de genotipos (75TV:25HV), además de ser eficaz para combatir las plagas causadas por *S. exigua*, puede contribuir a reducir el número de aplicaciones y las concentraciones necesarias para un control satisfactorio de la plaga, aunque esto requeriría ensayos de campo adicionales.

11. En condiciones de laboratorio, la aplicación de sulfato de cobre (0,1%), sulfato de hierro (1%) y selenito de sodio (1ppm) sobre larvas del segundo estadio de *S. exigua* que mantienen una infección encubierta del SeMNPV, produjo una activación del virus de 15, 12 y 41% respectivamente. Sin embargo, en condiciones de invernadero, la activación del virus fue mucho menor y varió entre un 1 y 3%, por lo que dichas aplicaciones no serían efectivas para reactivar infecciones encubiertas bajo las condiciones de invernadero descritas en este trabajo.

CONCLUSIONS

1. Natural populations of *Spodoptera exigua* from Almerian greenhouses harbour covert infections produced by SeMNPV, SeIV-1 and SeIV-2, the former was detected in a significantly higher proportion of adults (54%) than the other two viruses (13 and 8%, respectively). Furthermore, the prevalence of three viruses did not vary according to host gender.

2. Covert infections produced by SeMNPV, as well as SeIV-1 and SeIV-2, are transmitted vertically from field-caught adults of Almería to their offspring, although SeIV-1 transmission (39%) was more prevalent than that produced by SeMNPV (21%) or SeIV-2 (19%). The three viruses could be transmitted through both healthy and SeMNPV sublethally infected females.

3. Mixed infections caused by both baculovirus and iflavirus occurred in natural *S. exigua* populations from Almerían greenhouses and in their progeny, albeit at low prevalence. Nevertheless, all possible combinations, double and triple infections, were detectable in field adults and their offspring.

4. The prevalence of SeMNPV covert infection in adult survivors to a sublethal dose reached very high prevalence. Vertical transmission was observed when male or female parents harbored a sublethal infection, but female-mediated transmission was twice as efficient as that of males. Both male and female offspring were infected by their parents in similar proportions.

5. Transgenerational transmission occurred principally via the transovarial route rather than via transovum transmission, as egg surface decontamination had no significant effect on the prevalence of transmission to the offspring.

6. A positive relationship was detected between the proportion of infected adults in the offspring produced by each mating group and their viral load,

suggesting that adults that transmit the virus to a high proportion of their progeny tend to transmit greater amounts of viral DNA.

7. OBs mixed populations involving horizontally and vertically transmitted genotypes (HT and VT respectively) were as pathogenic (mean lethal concentration) as the HT genotype alone, whereas OB mixtures comprising 25:75% of each genotype and vice versa result in improved OB pathogenicity compared to the VT genotype. However, no significant differences were observed in speed of kill or OB production per larva between genotypes and their mixtures.

8. In laboratory conditions, the ability to produce covert infections in *S. exigua* survivors of a sublethal dose of OBs was significantly higher using the VT genotype (90%) than the HT genotype (45%), whereas intermediate values of covert infections were observed using OB mixtures as inoculum.

9. Under greenhouse conditions, the prevalence of covert infections in adult survivors of virus application was lower than that obtained under laboratory conditions. Covert infection increased with the proportion of the VT genotype in the inoculum mixture. This suggests that the use of appropriate combinations of VT and HT genotypes could maintain the insecticidal properties of the active ingredient and improve the prevalence of covert infections in insect survivors of baculovirus applications to crops.

10. Greenhouse applications of 5 $\times 10^8$ OBs/l comprising 75% VT and 25% HT genotypes was as effective as a methoxyfenozide treatment in preventing pest damage to sweet pepper fruits. Therefore, the use of the 75VT:25HT mixture was an effective crop protection method to control *S. exigua* and might contribute to extending the interval between applications of this product, although this requires additional field testing.

11. In laboratory conditions, physiological stressors such as 0.1% copper sulfate, 1% iron sulfate and 1 ppm sodium selenite triggered covert virus infections into lethal polyhedrosis disease in 15, 12, and 41% of second instar larvae, respectively. However, when these compounds were tested under greenhouse conditions, little virus-induced mortality was observed

(1-3%). Consequently, these substances were not effective at activating covert virus infection under greenhouse conditions described in this assay.

LIST OF PUBLICATIONS

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Virto, C., Navarro, D., Téllez, M.M., Herrero, S., Williams, T., Murillo, R., Caballero, P., 2013. Natural populations of *Spodoptera exigua* are infected by multiple viruses: Implications for the production and use of virus insecticides. IOBC/WPRS Bulletin. 90, 175-177.

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Virto, C., Navarro, D., Téllez, M.M., Murillo, R., Williams, T., Caballero, P., 2013. An examination of stress-related activation of SeMNPV in covertly infected *Spodoptera exigua.* IOBC/WPRS Bulletin. 90, 203-205.

Virto, C., Williams, T., Navarro, D., Téllez, M.M., Murillo, R., Caballero, P., 2016. An examination of stress-related activation of SeMNPV in covertly infected *Spodoptera exigua*. Journal of Applied Entomology. Accepted. DOI: 10.1111/jen.12349

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The beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae) is an important pest of pepper crops in Almería greenhouses. Recently a baculovirus-based insecticide, which provides better crop protection than conventional chemical insecticides, has been developed to control this pest. However, so far applications of baculovirus-based insecticides are almost invariably based on inundative releases, similarly to chemical products applications. The study of viral covert infections, vertical transmission and their impact on successive host generations shed some light on the basis of novel control strategies to maximize the effectiveness of field applications by improving the virus long-term effect on the host.

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