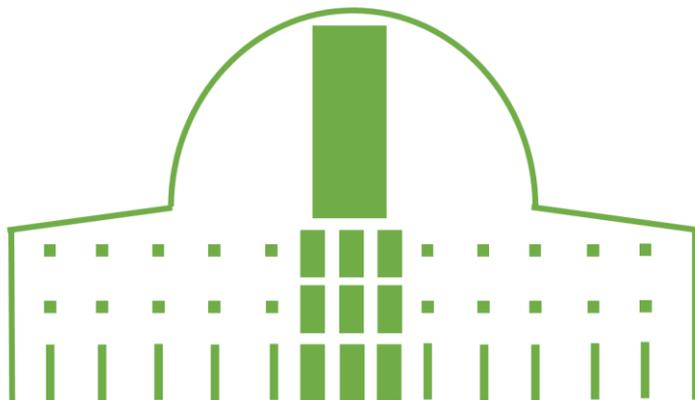


# Interference of iflavirus in SeMNPV as a biological control agent

ARKAITZ CARBALLO PALOS  
Pamplona, 2017







Departamento de Producción Agraria  
Universidad Pública de Navarra



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ARKAITZ CARBALLO PALOS

Pamplona, 2017

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Instituto de Agrobiotecnología  
Agrobioteknologiako Institutua





Memoria presentada por

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Para optar al grado de Doctor por la Universidad Pública de Navarra

**Interference of iflavirus in SeMNPV as a  
biological control agent**

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## RESUMEN

La rosquilla verde, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), es una especie polífaga ampliamente distribuida por todas las zonas templadas del mundo, incluyendo España, donde se ya ha establecido, causando grandes pérdidas económicas en cultivos hortícolas de invernadero. Como alternativa al control químico, el nucleopolidrovirus múltiple de *Spodoptera exigua* (SeMNPV, *Baculoviridae*) se ha propuesto, por su especificidad y eficiencia, como una alternativa preferente para combatir las plagas de *S. exigua* en el ámbito de la producción integrada o biológica. La aplicación del virus en campo no solo mata a un elevado porcentaje de las larvas, sino que las que sobreviven al tratamiento adquieren una infección subletal que es transmitida a los individuos de las siguientes generaciones favoreciendo así el control mediante SeMNPV de estas poblaciones de *S. exigua*.

Las infecciones encubiertas son un fenómeno muy habitual en las poblaciones naturales de insectos. De hecho, en las poblaciones de campo de *S. exigua*, se ha detectado un amplio complejo de virus mediante el uso de nuevas técnicas de secuenciación (Next-Generation Sequencing, NGS). Concretamente, en las poblaciones almerienses de *S. exigua* se han identificado dos nuevos virus de RNA de la familia *Flaviviridae*: iflavivirus 1 (SeIV-1) e iflavivirus 2 (SeIV-2). Ambos iflavivirus se encuentran con relativa frecuencia produciendo infecciones mixtas con el SeMNPV. Aunque se sabe que estos virus no producen infecciones letales en los insectos, se desconoce los efectos que estas tienen sobre el insecto huésped y como interfieren sobre la capacidad insecticida del SeMNPV como agente de control biológico. El objetivo de esta tesis ha sido determinar algunos aspectos de las interacciones de los iflavivirus SeIV-1 y SeIV-2, que infectan naturalmente a poblaciones de *S. exigua*, con el SeMNPV, que ha sido desarrollado como bioinsecticida para combatir las plagas causadas por *S. exigua*.

En primer lugar, se estudió la prevalencia y abundancia de SeIV-1 y SeIV-2 en adultos de *S. exigua* de diferentes orígenes geográficos, para establecer su importancia en poblaciones naturales y experimentales de la plaga. Se determinó que ambos iflavivirus infectaban de manera persistente a seis poblaciones de *S. exigua* de diversos orígenes establecidas en cautividad, aunque con variaciones en la abundancia relativa de ambos iflavivirus según su procedencia.

En estudios previos se había observado que las infecciones letales del SeMNPV en huéspedes infectados por SeIV generaban OB (cuerpos de oclusión) con iflavivirus asociados. Esta asociación mejora la estabilidad del iflavivirus fuera del huésped y su transmisibilidad e infectividad. En cambio, las propiedades insecticidas de los OBs asociados a SeIV se vieron reducidas. En concreto, se observó que la presencia de SeIV disminuye la patogenicidad ( $CL_{50}$ ) de OBs de SeMNPV, aunque no hubo variaciones

significativas en el tiempo letal del virus (TMM) o en la producción de OB por larva en el momento de su muerte.

En larvas coinfectadas con el SeMNPV y suspensiones de iflavirus enriquecidas en SelV-1, SelV-2 o una mezcla de ambos, se han estudiado aspectos como la patogenicidad, tiempo letal, producción de OBs tras la muerte de la larva y la prevalencia del SeMNPV en adultos supervivientes a la infección. La concentración letal media ( $CL_{50}$ ) del SeMNPV disminuyó en presencia de SelV, aunque el tiempo medio de mortalidad no resultó afectado. La producción de OBs fue significativamente inferior en los individuos coinfectados. En larvas de *S. exigua* del cuarto estadio coinfectadas con SeMNPV y SelV se observó una reducción del incremento de peso explicando así la diferencia de producción de OBs por larva. Por último, la prevalencia de SeMNPV no se vio modificada por una coinoculación con iflavirus, pero la carga viral del SeMNPV aumentaba con la presencia de SelV-2 con respecto al control y a SelV-1.

Los OBs producidos en larvas de *S. exigua* coinfectadas por SeMNPV e iflavirus presentaban características físicas diferenciales que nos permitió separar dos subpoblaciones de OBs en función del número de SelV-1 asociados a los OBs. Estas dos subpoblaciones de OBs mostraban distinta densidad específica por lo que fueron separados en un gradiente de sacarosa. OBs con menor densidad específica presentaban un menor número medio de genomas por OB del SeMNPV debido a una mayor presencia de genomas de SelV-1. Aunque el número de ODVs por OB no fue significativamente distinto en ambas subpoblaciones de OBs, si se pudo demostrar que el número medio de nucleocápsidas por ODV fue menor en los OBs que llevaban asociados un mayor número de genomas de SelV-1. Este resultado está en consonancia con la baja infectividad que presentan los OBs asociados a iflavirus detectada anteriormente.

Por último, y debido a la importancia de iflavirus en las poblaciones de *S. exigua*, se estudió como afectaba la infección por iflavirus a algunas de las características biológicas de *S. exigua*. Se comprobó que las infecciones del SelV-1 afectaron negativamente a la ganancia de peso de las larvas y pupa y la supervivencia del adulto, comparado con insectos no tratados. Sin embargo, la inoculación del SelV-2 no produjo variaciones en ninguno de los parámetros estudiados. El análisis de la evolución de la infección por SelV a lo largo del desarrollo larvario, de pupa y adulto del huésped reveló aumentos importantes en la carga del SelV-1, mientras que el SelV-2 se mantuvo siempre a niveles bajos (basales). Las larvas de *S. exigua* con infecciones persistentes por SelV-1, naturales o inducidas, presentaron una mayor susceptibilidad al SeMNPV. El valor de la  $CL_{50}$  del SeMNPV para las larvas con infección persistente por SelV-1 fue más de 4 veces menor que las larvas libres de SelV-1, mientras que el TMM se redujo en 12 horas.

A parte de estos efectos positivos, las infecciones persistentes de SelV-1 también pueden afectar negativamente a ciertas características del SeMNPV como son la

capacidad insecticida de los OBs asociados a SeIV y la transmisión horizontal del SeMNPV en el medio. La asociación de los iflavirus puede afectar a la seguridad del SeMNPV como agente de control biológico ya que estos iflavirus guardan una estrecha relación con otros virus de RNA patógenos para humanos (hepatitis, polio, etc.). Todo ello nos lleva a concluir que las infecciones de iflavirus en las poblaciones de *S. exigua* pueden ser un inconveniente para la bioseguridad y para la producción a gran escala de bioinsecticidas, que actualmente se basa en el uso de líneas de insectos vivos los cuales deberían estar libres de infecciones.



## SUMMARY

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is a polyphagous species widely distributed in all template areas of the world, including Spain, where it has been established causing great economic losses in horticultural greenhouse crops. As an alternative to chemicals, *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV, *Baculoviridae*) has been proclaimed, due to its specificity and efficiency, as a preferential alternative against *S. exigua* pests taking part in integrated or biological production. Virus field application not only kills a high percentage of larvae, but those that survive to the treatment acquire a sublethal infection that is transmitted to the individuals of the following generations supporting the control by SeMNPV of these populations of *S. exigua*.

Covert infections are a common phenomenon in natural insect populations. Specifically, in *S. exigua* field populations, a large virus complex has been detected using new sequencing techniques (Next-Generation Sequencing, NGS). Specifically, in the Almerian *S. exigua* populations two novel RNA viruses of the *Iflaviridae* family have been identified: iflavirus 1 (SeIV-1) and iflavirus 2 (SeIV-2). Both iflaviruses are relatively frequently producing mixed infections with the SeMNPV. Although it is known that these viruses do not produce lethal infections in insects, the effects they have on the insect host and how they interfere with the SeMNPV insecticidal capacity as a biological control agent are still unknown. The objective of this thesis has been to determine some features of the interactions of the iflavirus SeIV-1 and SeIV-2, which naturally infect populations of *S. exigua*, with the SeMNPV, which has been developed as a bioinsecticide to combat pests caused by *S. exigua*.

Firstly, the prevalence and abundance of SeIV-1 and SeIV-2 in adults of *S. exigua* from different geographical origins were studied to establish their importance in natural or experimental pest populations. It was concluded that both RNA viruses persistently infected six populations of captivity *S. exigua* from diverse origins, with variations in the relative abundance of both iflaviruses according to their origin.

In previous studies, it has been observed that lethal infections produced by SeMNPV in SeIV infected host generated OBs (occlusion bodies) with associated iflavirus. This association improves the iflavirus stability outside the host and its transmissibility and infectivity. However, the SeIV associated OBs insecticide properties were reduced. Specifically, a decrease in the SeIV presence of the pathogenicity (LC<sub>50</sub>) of SeMNPV OBs was observed. Although no significant variations of the lethal time of the virus (MTD) or the OB production per larva after death were detected.

In co-infected larvae with SeMNPV and enrichment suspensions in SeIV-1, SeIV-2 and a mix of both, aspects such as the pathogenicity, lethal time, OB production after larval

death an SeMNPV prevalence in infected survival adults were studied. The SeMNPV mean lethal dose ( $CL_{50}$ ) was decreased in presence of SelV, however, no effect on mean time to death was observed. The OB production was significant decreased in co-infected individuals. Fourth instar co-infected with SeMNPV and SelV *S. exigua* larvae present a weight gain reduction explaining the difference in production of OBs per larva. Lastly, the SeMNPV prevalence was not altered by a co-inoculation with iflavirus, but SeMNPV viral loads were increased in presence of SelV-2 regarding control and SelV-1.

OBs produced in SeMNPV and iflavirus co-infected *S. exigua* larvae showed differential physical characteristics that allowed us to separate two OBs subpopulations due to the number of SelV-1 associated with the OBs. These two OBs subpopulations presented different specific density were separated into a sucrose gradient. Lower specific density OBs showed lower mean genomes per SeMNPV OBs number due to a higher presence of SelV-1 genomes. Although ODV per OB number did not significantly differ in both OBs subpopulations, we have shown that the average number of nucleocapsids per ODV was lower in higher number of SelV-1 genomes associated OBs. These results are in consequence with the lower infectivity of the iflavirus associated OBs previously detected.

Finally, due to the importance of iflavirus in the populations of *S. exigua*, the effect of the iflavirus infection over some of the *S. exigua* biological characteristics was studied. The SelV-1 infections negatively affect the larval and pupal weight gain and the adult survival, compared to mock-insects. However, no variations in all the studied parameters were observed in a SelV-2 inoculation. The SelV evolution analysis during the host larval, pupal and adult development showed important increases in SelV-1 loads, while lower levels were always detected for SelV-2. Natural or induced persistent SelV-1 infections on *S. exigua* larvae exposed an increase on SeMNPV susceptibility. SeMNPV  $LC_{50}$  value for SelV-1 persistent infection larvae was 4-fold lower than SelV-1 free larvae, while MTD was reduced in 12 hours.

Despite these positive effects, the SelV-1 persistent infections also could negatively affect some SeMNPV characteristics such SelV associated OBs insecticide capacity and the SeMNPV horizontal transmission in the field. The iflavirus association could affect the SeMNPV security as a biological control agent since these iflavirus are closely related to other human pathogenic RNA viruses (hepatitis, polio, etc.). Altogether leads us to conclude that iflavirus infections in *S. exigua* populations could be a disadvantage for biosecurity and for the bioinsecticide large-scale production, which is currently based on the use of live insect lines, which should be infections free.

## CHAPTER I

# Introduction

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## 1. GENERAL INTRODUCTION

Over the last decade crop protection has undergone a remarkable shift on the control methods and the principles they are based on, mainly due the implementation of the European Pesticides Regulation (CEE 1107/2009) concerning the use of pesticides. This measure seeks to avoid the negative effect on human health of pesticides, along with minimizing their impact on the environment. Therefore, the use of harmful chemical substances has been limited or banned, and replaced by environmental friendly ingredients, whilst alternative control means are encouraged to be implemented in the framework of the Integrated Pest Management (IPM) programs (1). In this sense, in Spain, the RD 951/2014 was enforced to implement European requirements, forcing growers into environmental friendly methods. Among them, biopesticides are considered to pose lower risks than synthetic pesticides, hence the increasing interest to include them in IPM programs.

In the horticultural area of Almeria, in southern Spain, *Spodoptera exigua*, (Lepidoptera: Noctuidae), known as *gardama*, is one of the major pests on sweet pepper, among others vegetable crops. To control outbreaks of *S. exigua* larvae, chemical pesticides were used in the past, leading to important ecological and economical problems including: 1) the development of resistance in *S. exigua* populations to a variety of substances (2-4), 2) the presence of toxic residues on the commodities leading to commercial ban of products exceeding the established maximum residue levels (5), and 3) the negative effects on non-target organisms, especially beneficial insects such as predators and parasitoids (6, 7). In this context, increasing number of Almerian growers have embraced biological control of pests and organic production systems have been widely adopted over the last decade. Among other entomopathogens, the Microbial Insecticides Research group (UPNa-CSIC) has deeply studied the *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) (*Baculoviridae*; *Alphabaculovirus*) over the past 20 years. Studies conducted on the SeMNPV pesticide properties, eventually succeeded to develop an effective biological tool against this pest to be used by the growers (8). The SeMNPV nowadays constitutes the active ingredient of three commercial bioinsecticides available and registered against *S. exigua*, namely Spod-X® (Certis), Spexit® (Andermatt Biocontrol), and Virex® (BIOCOLOR).

Lepidopteran natural populations frequently have to deal with a large number of pathogens and covert infections are almost ubiquitous (9). *S. exigua* field populations are not an exception and recent studies have revealed high levels of prevalence of SeMNPV covert infections (10). Moreover, thank to next-generation sequencing (NGS) techniques, the transcriptome analyses of *S. exigua* larvae revealed the presence of novel RNA viruses (11). Two species of *Iflaviridae* family described as SelV-1 (12) and SelV-2 (13) present special interest. These iflaviruses were readily detected both in laboratory *S. exigua* colonies (14) and field populations (15). Previous works have shown that although these viruses do not have a visible pathological effect on the insects, they can replicate and be persistently transmitted to the offspring (15). Iflavirus persistent infections are frequently part of mixed infections and the symptoms and severity depend on simultaneous infections caused by other pathogens sharing the same host (16), and on their direct or indirect virus-virus interactions (17). In this context, SelV persistent infections would affect the outcome of SeMNPV infection and the insecticidal properties of this virus.

The aim of this thesis is to study the interference of recently discovered *Spodoptera exigua* iflavirus (SelV) in the bioinsecticidal properties of *Spodoptera exigua* *Alphabaculovirus* (SeMNPV) as a biological control agent. These studies have been specifically focused on: i) the incidence of iflavirus infections in *S. exigua* laboratory cultures, ii) the insecticidal properties of the SeMNPV occlusion bodies (OBs) associated with SelV-1, iii) the effect of SelV-1/SelV-2, simultaneously co-inoculated, on the insecticidal properties of SeMNPV, iv) the physical association of iflavirus and SeMNPV OBs and its consequences on OB conformation and structure, and v) the fitness cost of a covert infection of SelV in a *S. exigua* population and the effect on the development of subsequent infection by the SeMNPV.

## **2. *Spodoptera exigua***

### **2.1 Geographical distribution and host plants**

The beet armyworm, *S. exigua*, is a migratory species that originates from southeast Asia and it is currently distributed worldwide in all temperate, tropical and subtropical climate areas (18). *S. exigua* presence has been reported to some

extent across five continents: America, Asia, Europe, Australia and Africa (19) (Figure 1).



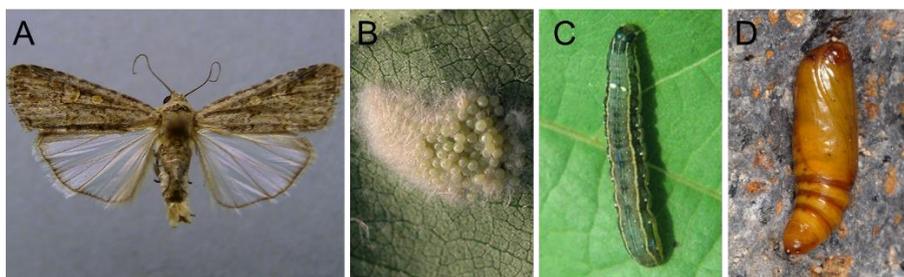
**Figure 1:** Geographical distribution of *Spodoptera exigua* in the world. Green dots represent locations where *S. exigua* presence has been reported. Adapted from CABI (19).

Due to the lack of diapause in this species, temperatures mainly regulate population settlement, so that cold winter prevents development in northern Europe, while permanent presence in warmer regions such as southern Spain is reported. Atlantic air streams facilitate adult migration to the north (20), where *S. exigua* can survive in protected environments such as greenhouses in The Netherlands (21, 22). In Almería, Spain, it has been established as a permanent species, detectable year-round (23).

*S. exigua* is a highly polyphagous species hosting more than 60 plant species from 23 different families (24). The beet armyworm outbreaks result in damage of economically important vegetables crops such as cucumber, sweet pepper, courgette, beans, watermelon, and open-field crops like sunflower or alfalfa (25), as well as ornamental and non-commercial plants (23). The injury is produced by the larval stage that feed on leaves, fruits and stems producing large economic losses. In southern Spain, *S. exigua* has been considered a key pest in sweet pepper for decades (26).

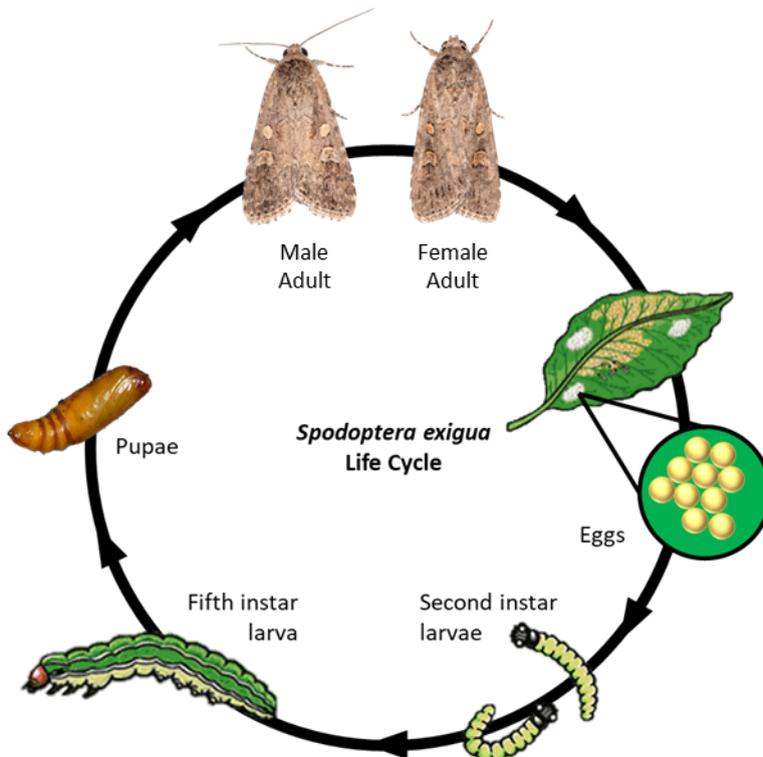
## 2.2 Morphology and biology

*S. exigua* adults are medium-sized moths with a 25-30 mm wingspan, brownish to grey forewings and semi-transparent hindwings crossed by dark veins (Figure 2A) (18). The spherical eggs ( $\varnothing = 0.35\text{-}0.37$  mm) are laid in clusters, often covered by female scales. Their color varies from yellowish-white to greenish and dark brown, becoming black shortly before hatching (27) (Figure 2B). The larval first two instars are pale green or yellow and feed gregariously on leaves. Third instars acquire light stripes and fourth instars turn dark green or yellowish green with dark stripes running laterally and dorsally. 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 2C). At this point the larvae reach up to 3.6 cm long (18, 28).



**Figure 2:** Morphology of the four development stages of the *S. exigua*: A, adult with open wings. B, egg masses. C, fifth larval instar. D, pupa.

Normally, five larval instars develop, even though additional instars have been reported under certain conditions. Pupation occurs in the soil, and takes between six and ten days at a 30 and 25 °C, respectively. Pupae are reddish-brown and 15-20 mm long (18) (Figure 2D). Pupal sexual dimorphism is easily distinguishable by observing the sternum of the last two abdominal segments. Females may be distinguished from males by the presence of a longitudinal groove on the ventral surface of the penultimate segment, whereas males possess two circular notches on the last segment.



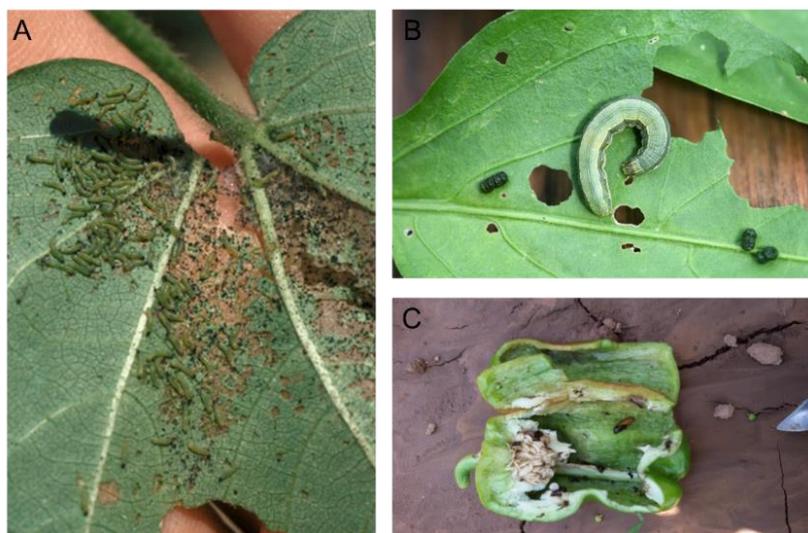
**Figure 3:** *Spodoptera exigua* life cycle scheme: egg-masses are laid on the underside of leaves. After larval hatching, larvae pass generally through five instars to reach pupation, that occurs in the soil. After six or seven days, adult emerge, mate and reproduce, starting a new generation.

*S. exigua* life cycle development is temperature-dependent and thus climate is the main factor modeling the number of generations per year. Gravid females laid a range of 600-1700 eggs (29), in clusters of 30-200 egg, during the next two to three days after mating. At 25 to 30 °C, the eggs mature in between 2-3 days, whilst larval development takes 8.5 days at 33 °C and 120.5 days at 15 °C respectively (30). Extremely low (12 °C) and high (36 °C) temperatures hinder this insect development. Under favorable conditions (30 °C), the entire cycle can be accomplished in as few as 20 days (30). The number of cycles per year varies according to the geographical region and the host plants. A maximum of eleven generations per year have been described in China (27). In southern Spain, three to four generations per year are estimated to occur in outdoor crops. Greenhouse

conditions involving low competition for food, high nutritional quality, a limited number of natural enemies and high temperatures and relative humidity, result in up to seven or eight generations per year (23). As a migratory species, adults have a high capacity to travel large distances of up to 3500 km in only 9 or 11 days (31).

### 2.3 Ecology and damage

*S. exigua* is responsible of countless economic losses caused by larval feeding behavior, mainly defoliation and fruit perforation. First instars feed in groups on the underside of the leaves reducing the photosynthetic area by removing leaf parenchyma (Figure 4A). As they grow, larvae consume complete leaf surfaces causing skeletonized leaves and severe defoliation (Figure 4B). Third instars disperse and become solitary and fourth and fifth instars move to the top of the plant affecting plant canopy and renewable foliage. Also, direct damage has been observed during high infestations, as caterpillars bore holes and facilitate entry of microorganism that cause fruit rotting, leading to severe reductions of crop commercial value (32) (Figure 4C).



**Figure 4:** Feeding damage produced by *Spodoptera exigua* on pepper crops. A, first instars larvae produce superficial damage on leaves. B, defoliation produced by last instar larva and, C, damage produced by consumption of the inner part of the fruits.

In southern Spain greenhouses, covering a production surface area of 29,500 ha (this figure can be increased to 44,000 ha/year if double cultivation cycles are considered), mainly sweet pepper, exceeded 2.6 million Tn in the 2014-15 season, of which 1.9 Tn worth 1,8 million € were exported. The economic relevance of the beet armyworm control in this area was estimated at 6 million €, the cost of pesticides against this pest (33).

## 2.4 Control methods

*S. exigua* is considered one of the most important lepidopteran pests of Almeria greenhouses. Over decades, the main method to control this pest outbreaks has been by spraying synthetic pesticides (34, 35). However, the continuous use of chemical control, has led to economic and environmental problems such as the presence of high levels of insecticidal residues in commercial vegetables (36) or the negative impact to non-target insect species populations including their natural enemies (8). In addition, widely field-sprayed chemical insecticides have resulted in high rates of resistance development in target pests over the years (2, 3, 37). Over the last 50 years, *S. exigua* larvae have been reported resistant to 39 active ingredients belonging to different groups of insecticides such as abamectin, cypermethrin, endosulfan or spinosad (35, 37-39), and also to more recently developed biotechnical insecticides in the insect growth regulators (IGR) group, like methocfenozide, glugenuxoron or lugernuron (40, 41). To overcome these drawbacks a so called *green revolution* was conducted by the farmers of the Almerian greenhouses, who adopted a completely different control strategy by successfully introducing predators, parasitoids and entomopathogens against the major pests, along with the improved management of crops (42). Such efforts resulted in a drastic increase of organic production, that raised up from 1,400 hectares in 2007 to 26,372 in 2014 (43, 44). Today, the enforcement of European policies in the use of pesticides has driven growers to the implementation of Integrated Pest Management (IPM) and the sustainable use of pesticides (1).

One of the alternatives to organic insecticides involve the use of synthetic sexual pheromones to interfere with *S. exigua* reproductive rates through males capture and mating disruption (43, 45). Several compounds, identified as female pheromones have been tested under field conditions to massively catch males (46,

47). 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 (48, 49).

Despite being useful for monitoring, sex pheromones are applied at low density populations in IPM programs (43). Egg masses and young larvae were reduced to 6 and 1% after Z9E12-14:OAc and Z9-14:OAc blend application in field conditions (46), in contrast, in high density populations, pheromones are ineffective to pest control and additional control methods are required (50).

On the other hand, natural enemies act as insect mortality factors and are one of the most powerful sources of pest control. Among natural enemies, several predators, parasitoids and entomopathogens (fungi, bacteria and viruses) can be found. These natural enemies are important mortality factors in open field crops (51). However, predators and parasitoids, although generally beneficial as natural control factors, have not been commercialized as biological control agents (22). Predator species affecting *S. exigua* are diverse. From eggs to early instars, *S. exigua* is a prey for bugs such as *Lygus hesperus*, *Nabis americanoferus* (Hemiptera: Nabidae) and *Orius tristicolor* (Hemiptera: Anthocoridae), and beetles such as *Collops vittatus* (Coleoptera: Melyridae) and *Hippodamia convergens* (Coleoptera: Coccinellidae) (52). For the control of *S. exigua*, there is few information on the application of predators and parasitoids as biological control agents.

Different parasitoid species can attack different *S. exigua* life stages. The endoparasitoid *Chelonus insularis* (Hymenoptera: Braconidae) is the most abundant parasitoid of eggs and young larvae (53, 54) whereas some parasitoids of larvae such as *Hyposoter exiguae*, *Pristomerus spinator* (Hymenoptera: Ichnemonidae) and *Microplitis pallidipes* (Hymenoptera; Braconidae) (55) 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: Ichneumonidae) and the tachinid *Gonia bimaculata* (Diptera: Tachinidae), which produce considerable levels of mortality in non-treated crops outside greenhouses (53, 56). Because of their

specificity, biological control with parasitoids has been a success with other pest species. One example in the Mediterranean area is *Trichogramma achaeae* (Tricotop®) against *Tuta absoluta*. However, not many parasitoids have been commercialized to date.

Over the last 15 years, entomopathogens have been commercialized as biological control agents for pest control (57), since a wide variety of entomopathogenic species cause diseases in *S. exigua* populations. The fungi *Beauveria bassiana*, *Metarrhizium anisopliae* (Hypocreales: Clavicipitaceae), *Nomurea rileyi* (Moniliales: Moniliaceae) (58, 59); and the nematode *Steinernema carpocapsae* (Nematoda: Steinernematidae) have been reported among the most relevant (60). However their application is very limited due to the high temperatures and the lack of moisture in the greenhouse crops of southern Spain (36). One of the most used commercialized fungus as biological control agent is *B. bassiana*, under several trade names (Mycotrol®, Organiguard®, etc). Also several strains of *Bacillus thuringiensis* (Bt), are known to infect and kill *S. exigua* larvae (61, 62), but the emergence of Bt-resistant strains (63) highlight the search for pathogens based on different modes of actions for bioinsecticide development. Some studies, despite the complexity, consider the integrated use of two or more biocontrol agents against one or multiple hosts (64).

The *S. exigua* multiple nucleopolyhedrovirus (SeMNPV) has been widely reported as one of the most effective *S. exigua* control agents (21, 65-67). SeMNPV naturally cause epizootics that can reduce *S. exigua* population levels down to 80% (68), making this one of the most pathogenic and virulent baculoviruses described so far. Combination of three SeMNPV genotype variants have been used as the active ingredient of the first bioinsecticide based on a native baculoviruses registered in Spain (8).

### 3. BACULOVIRUSES

#### 3.1 Morphology and taxonomy

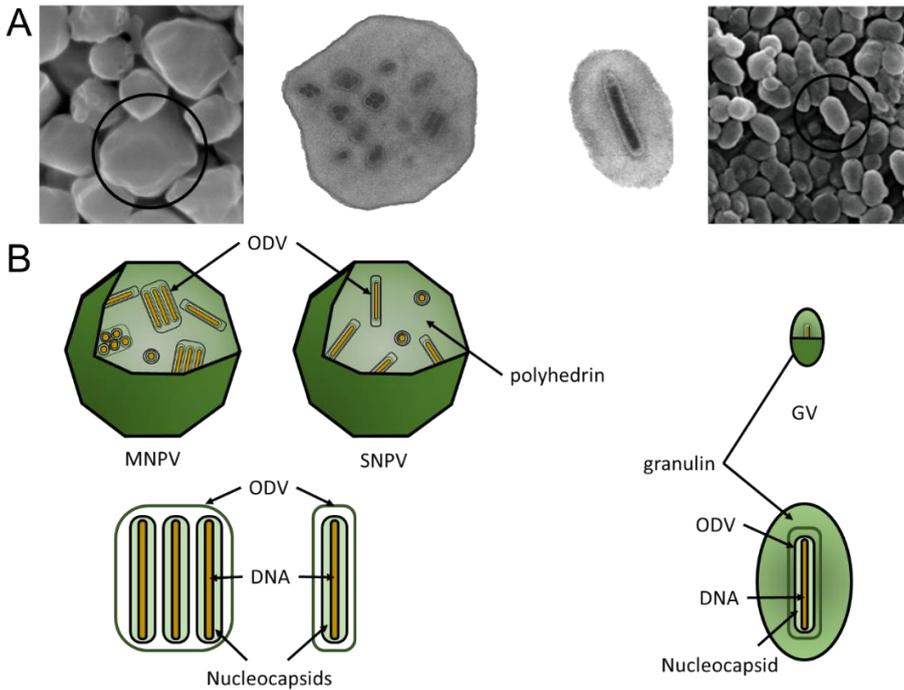
Baculoviruses are arthropod-specific viruses nearly found ubiquitous in the environment that have been isolated from more than 600 insect species including the orders Lepidoptera, Hymenoptera and Diptera (69, 70). They are DNA

viruses with double-stranded, circular, supercoiled genomes ranging from 80 to 180 kbp in size (71). Currently, baculoviruses are mainly known as biological control agents for insect pests (72) and as gene expression vectors of proteins of medical interest and for gene therapy (73).

Current classification of the group is based on morphological and biological characteristics. Thus, the family *Baculoviridae* is divided into the following four genera: 1) *Alphabaculovirus* (lepidopteran-specific nucleopolyhedroviruses), 2) *Betabaculovirus* (lepidopteran-specific granuloviruses), 3) *Gammabaculovirus* (hymenopteran-specific nucleopolyhedroviruses) and 4) *Deltabaculovirus* (dipteran-specific nucleopolyhedroviruses) (74, 75). A former and widely used classification based on morphological characteristics used to split the group into two genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Figure 5B). While NPVs contain several occlusion derived virions (ODVs) than produce large polyhedron-shaped structures with a diameter of 0.5 to 15  $\mu\text{m}$  and may contain either single (SNPV) or multiple (MNPV) nucleocapsids (Figure 5B), depending on the species, GVs have just a single-nucleocapsid per ODV, granular-shaped structure and 150 nm in diameter and 400-600 nm in length. The protein matrix is made of polyhedrin for the genera *Alphabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus* (NPVs) and granulins for the genus *Betabaculovirus* (GVs). *Alphabaculovirus* is divided in two groups depending on the BV envelope fusion protein, group I and II present protein GP64 and F protein, respectively (76). So far, the International Committee of Taxonomy of Virus (ICTV) has recognized 49 species belonging to the *Baculoviridae* family, of which 32 are *Alphabaculovirus*, 14 are *Betabaculovirus*, 2 are *Gammabaculovirus* and 1 is *Deltabaculovirus* (77). The SeMNPV belongs to group II. Even though a virus may infect several insect species, all baculoviruses are named after the host from which they were first isolated and this nomenclature has remained unchanged.

The *Alphabaculoviruses* produce OBs, highly stable in the environment outside the host and responsible for the virus horizontal transmission (78). The OBs contain virions or ODVs, which consist of a protein-lipid membrane packing a number of nucleocapsids of 40-60 nm in diameter and 230-385 nm in length (Figure 5A) (79-82). ODVs are responsible for initiating the primary infection at the midgut epithelial cells. Their envelope is produced *de novo* at the nucleus of the infected

cells (83). BVs are single-nucleocapsid virions that acquire the cytoplasmic membrane of the host cells during cell-to-cell infection placing virus proteins in the lipoprotein envelope such as GP64 (group I) or F protein (group II) that allows their budding and entry into a new host cell (84-88).

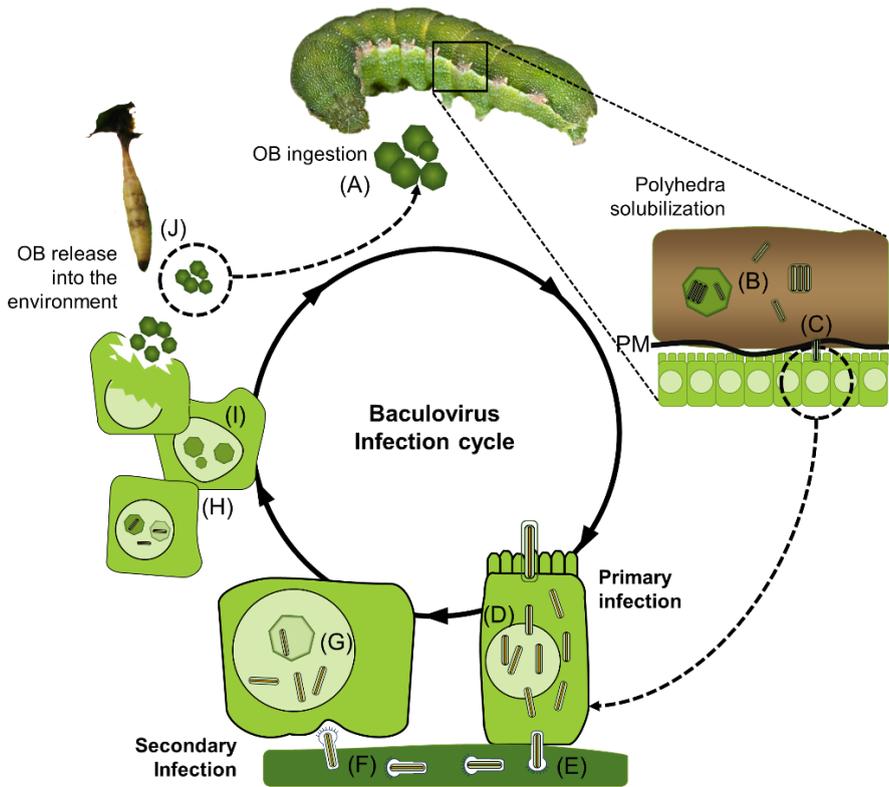


**Figure 5:** Occlusion bodies (OB) of former groups of *Nucleopolyhedrovirus* (NPV), *Granulovirus* (GV) in electron microscope images, A, and schematic representation, B, of morphology and structure of multiple (MNPV) and single (SNPV) occlusion derived virions (ODV). Virions contain single or multiple nucleocapsids where DNA is packed.

### 3.2 Infection cycle

The *Alphabaculovirus* infection cycle starts when the insects ingest OB-contaminated leaves (Figure 6A). Two different virions coexist during the infection cycle: ODVs, responsible for primary infection at the midgut, and BVs, responsible for secondary infection, occurring into the hemocoel. Both phenotypes contain identical genetic information and similar nucleocapsid structure (78). In the midgut, OBs are dissolved in the alkaline pH and the ODVs are released (75) (Figure 6B).

Then, ODVs cross through the peritrophic membrane (PM), a non-cellular matrix composed by chitin and glycoproteins (Figure 6C) and fuse to the midgut epithelial cell membrane allowing nucleocapsids to enter into the cell cytoplasm (Figure 6C). Nucleocapsids are transported to the nuclear membrane through actin filaments (89, 90) and then pass through the nuclear pores into the nucleus (Figure 6D) (91).



**Figure 6:** During primary infection, larvae feed on OB-contaminated leaves (A). Basic pH solubilizes OBs in the insect midgut (B). ODVs are released and pass through the peritrophic membrane (C) fusing with the microvilli of midgut epithelial cells. At the nucleus, nucleocapsids release the viral genome to initiate replication (D). Progeny nucleocapsids assemble and bud through the basal membrane (E) acquiring an envelope containing GP64 or F fusion protein present in the virus-modified cell membrane. Budded virions (BVs) then disperse through the hemolymph (F), enter new cells by endocytosis and replicate in the nucleus (G). Newly assembled nucleocapsids may be enveloped to form ODVs that are occluded into OBs (H). OBs accumulate in the nucleus (I) and eventually OB storage lead to membrane rupture and cell tissue disruption. Upon death, larvae hang from the upper leaves (J), tegument breaks and OBs are released, contaminating host plants to initiate a horizontal transmission infection cycle (A).

DNA replication starts and new assembly BVs are formed and then they infect the surface cells of the tracheal system (Figure 6E) which serve to disperse the infection through the susceptible organs and tissues of the hemocoel cavity (Figure 6G) (92-94). Eventually, some nucleocapsids may bypass the nuclear replication process and continue directly to the basal side of the cell to spread the infection (95). In the last stage of the infection, at the nucleus of infected cells, new ODVs are produced at the same time than polyhedrin leading to the formation of complete OB particles (Figure 6H) (96, 97). There is a membrane rupture and OBs are released into the hemocoel (Figure 6I). A few days after infection, hosts display visible infection symptoms, such as less activity and darkest tegument color (98). Lastly, virus infection induces larvae to climb to upper parts of the plants (99-101), a behavior that improves viral dispersal into the environment along with cadaver liquefaction (Figure 6J), which is due to viral chitinase and cathepsin activity (102).

### 3.3 Genome organization

The genome of the baculovirus consists of a unique and circular double-stranded DNA molecule varying from 80 to 180 kb depending on the species and the genotypic variant (71). The whole genome contains between 89 to 183 genes distributed in both strains and orientations (103). *Deltabaculovirus* and *Gammabaculovirus* have shorter genomes. The non-coding regions represent less than 10% of the genome comprising promoter and homologous regions (hrs). The hrs are repeated regions that have been suggested to be involved in origins of the replication in some baculoviruses (104, 105).

Genes from the 62 sequenced lepidopteran specific baculovirus genomes are functionally classified into five categories: transcription, replication, structural proteins, auxiliary proteins and proteins of unknown function (70). Due to the large genetic diversity of baculoviruses, only 29 core genes have been identified in all species (106, 107). They are involved in essential biological functions, such as DNA replication (*DNApol*, *helicase*, *lef-1*, etc), transcription (*p47*, *lef-4*, *lef-8*, etc), assembly and packaging (*vlf-1*, *vp91*, etc) and genes essential for oral infectivity (*p74*, *pif-2*, *pif-3*, etc) (71, 106, 108). Since they are conserved in all baculovirus genomes sequenced to date, the comparison of these genes is a highly suitable tool for phylogenetic studies (70). A lack of one of these core genes prevents the

baculovirus infectious cycle. On the contrary, not all the non-essential genes are necessary for the infection cycle. Examples of these genes are anti-apoptotic genes (*p35* and *p49*) (109, 110), enhancins (111) and glycoproteins (*gp64*) (86). Baculoviruses differ considerably in insecticidal properties like host range, pathogenicity, virulence and ecological fitness, and this may be attributed, at least in part, to their complex of auxiliary genes. Moreover, baculoviruses carry genes homologous to those of other organisms with eukaryotic or prokaryotic cells and to those of other viral families (112). Their origin and acquisition mechanisms are now being investigated. Recombination events and transposon insertions appear to play a role in the uptake of new genes from co-infecting viruses or from the insect host (113). Baculoviruses might be considered as a rich source of genes possibly acquired from many different organisms over a long period of evolutionary history. Phylogenetic analyses based on sequenced baculovirus genomes have revealed that, throughout an evolutionary process, baculoviruses have gained features to infect a wider range of cell types and become more independent from the host cell machinery.

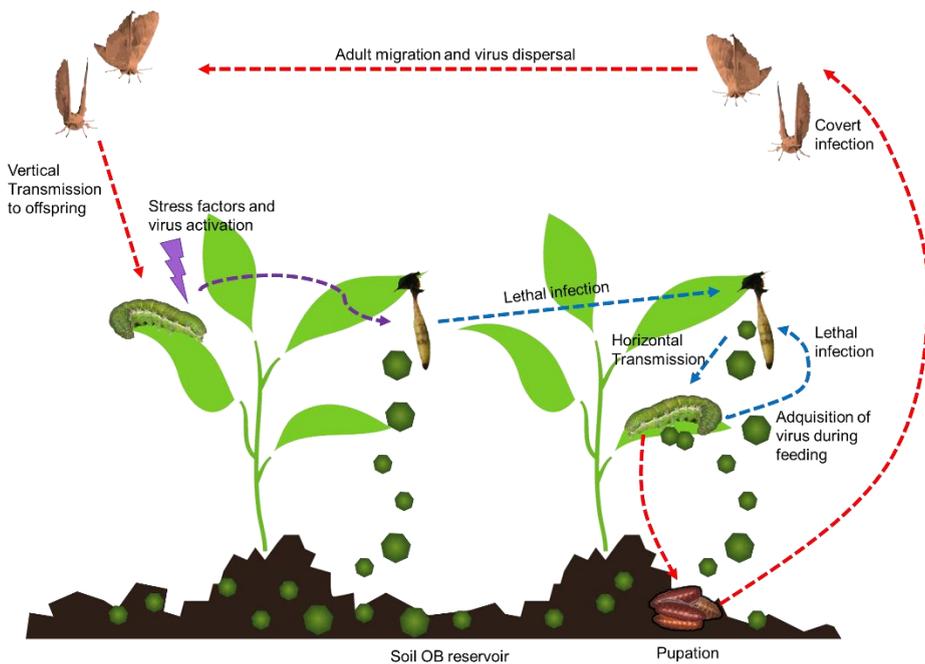
### 3.4 Baculovirus transmission

Anderson and May (114) define pathogen transmission as the process by which a pathogen passes from an infected to a healthy host. The baculovirus mode of transmission was thought to be mainly horizontal for a long time. However, it is well accepted now that baculovirus transmission comprises two routes: vertical transmission (VT), occurring from the parents to their offspring, and horizontal transmission (HT) between individuals sharing the same habitat (115). Nevertheless, the relevance of vertical transmission studies lead to a model comprising a combination of both routes (Figure 7), thereby the virus might use either one depending on the probability of success driven by environmental conditions (116).

Horizontal transmission is mediated by OBs, and occurs when an infected insect dies and high quantities of OBs are released into the environment, which can be ingested by susceptible host and start a new infectious cycle. In this case, OBs can reach a new host throughout several routes including contaminated soil, feces

or regurgitation of diseased larvae (117, 118), cannibalism of infected individuals (119, 120) or after release of baculovirus-based insecticides (32).

Other means of horizontal viral dispersion involve interaction with other species such as predators and parasitoids. For instance, insectivorous birds have been reported capable to act as dispersion vectors, since viable OBs were found in avian feces shortly after the ingestion (121). The parasitoid wasp can act as a vector transmitting the virus directly from an infected to a healthy insect. Parasitoid females that develop in an infected host have demonstrated to be able to transmit infective virus doses (55). Finally, a steadily increasing manner to cause horizontal transmission is by using baculovirus-based bioinsecticides (26, 72).



**Figure 7:** Baculovirus transmission routes, mode of infection and dispersal pathways in the environment. After larvae ingest OBs, either lethal (blue arrows) 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, thereby producing one or more infection cycles. The virus is dispersed by biotic or abiotic agents to or from the main reservoir, the soil. Alternatively a sublethally-infected adult may pass the virus to the offspring which eventually can disperse over long distances via migration. Vertical transmission might occur throughout generations until a triggering factor switches the covert form to an overt infection (purple arrows). Adapted from Virto et al. (33).

Horizontal transmission efficacy relies on OB persistency in the environment, since biotic and abiotic factors can negatively affect virus stability (117). Baculoviruses adopt the so called “sit-and-wait” transmission strategy, thereby spatial components are involved in the process. Their ability to retain infectivity during periods in the environment varies widely depending on the ecosystem and the presence of the OB structure. The effects of the most influential abiotic factors (UV radiation, seasonal temperature, precipitation and pH) have been mainly studied for OB persistence (122) and, coupled with host population abundance, determine the probability of OB transmission success.

During the middle stages of infection, host behavior are modified. A study carried out to quantify this behavior showed that *Mamestra brassicae* infected larvae climbed five more times further to the upper leaves of the plant than healthy counterparts (99, 123). After lethal infections, the death of the host is followed by a massive release of virus particles.

In favorable conditions, horizontal transmission might reach a large number of individuals in a limited distance (117). However, vertical transmission may play a relevant role in long distance dispersion favored by migratory host behavior. Insects that become infected during the larval stage by the ingestion of OB sublethal doses might develop an adult covert infection (124, 125). Although the adult stage is not susceptible to develop fatal infections, covertly infected individuals performing long flights may result in the colonization of new areas where the disease is likely spread into the host populations by interbreeding and offspring transmission (10).

The viral pass from adults to the progeny comprises two pathways: transovum, when viral particles that superficially contaminate the egg gain entry into neonates when consumed along with the chorion during hatching (126), or transovarial, when the virus particles pass internally through the infected embryo (127, 128). Superficial decontamination experiments allowed distinguishing between both routes, that depends on virus-host system. For instance, the transovarial pathway has been reported for several virus-host systems such as *S. exigua* (129), *Lymantria dispar* (130), *Bombyx mori* (124) and *Spodoptera exempta* (131).

Natural insect populations exhibit large density fluctuations driven by climate conditions, food availability or the action of a number of biotic factors. In periods of low host densities, vertical transmission is favored, given the decreasing rates of host-virus encounters (116). Transgenerational transmission result in a strategy for long term persistence that might influence the host population dynamics (10, 131, 132).

### 3.5 Baculovirus covert infections

Covert infections, also known as unapparent, occult, asymptomatic or sublethal infections are non-lethal infections involving sophisticated interactions between pathogen and host in which the virus avoids clearance by different strategies and remains in the host for long periods of time with low biological cost for the host (133). Despite of being latent for a long time, the virus remains fully competent to be reactivated and develop an overt lethal infection (141, 229). The use of PCR-based techniques revealed the existence of covert infections in apparently healthy individuals (10, 15, 134-137), and has made the quantification of viral loads possible, providing limits of detection as low as 5-7 genomes per reaction (138, 139). Both, field populations (140, 141) and laboratory insect cultures (134, 141) were found to harbor long-term covert infections by this means. Also, covert infection have been detectable across all developmental stages of the host (142, 143).

Sublethal infections were successfully established in laboratory conditions by treating larval individuals with sublethal viral doses (10, 144). By doing so, detrimental effects on host development has been related with the pathological effect of virus for several species including *S. exigua* (10) and *M. brassicae* (145). The fitness cost include reduction in development rates (21, 131, 146), lower larval and pupal weight gain, and extended larval development time and pre-oviposition period. Also, reproduction traits were negatively affected as fecundity and fertility, are reduced, for instance egg laying was reduced down to 50% (147). Notably, covertly infected females delayed oviposition 24 h respect to healthy counterparts, giving the possibility of dispersion of the infection to longer distant places (131, 148).

Covert infections could be classified in latent or persistent according to viral transcriptional activity. During latent infections, viral genes are down regulated

and viral transcripts remain undetectable. In persistent infections, several viral genes are expressed and viral particles produced (149). Molecular techniques such as PCR or RT-PCR allow to tentatively differentiate between latent or persistent infections (150). In a persistent infection, there is a constant and low-level production of viral particles in infected cells. Pathological effects may also be observed that can be costly to host fitness.

Virus strategies to achieve covert infections include selection of cell type for the maintenance of viral genomes, the modulation of viral gene expression, and the avoidance of clearance by the host immune system (138). For example, high prevalence of SpexNPV in legs and wings of *Spodoptera exempta* (Lepidoptera: Noctuidae), suggest that these tissues might act as a reservoir to avoid the haemolymph immune system-associated traits (133). The molecular basis of covert infection might likely reside on host-virus interactions through the action of microRNA. Recent findings on the *Helicoverpa zea* HzNV-1 nudivirus (family *Nudiviridae*, a non-occluded close family to baculoviruses), described novel mechanism where viral microRNA was responsible of latency establishment (151). In other studies carried out in AcMNPV infected cells, small interference mechanism RNA (siRNAs) produce the transcriptional inhibition of host genes involved in host defenses (9). There are also examples of baculovirus miRNAs that target host genes (133). These findings and further studies will contribute to improve the understanding of the molecular basis of covert infections.

The high prevalence of covert infections in field lepidopteran populations (10, 140) suggest an effect on host dynamics, and eventually, the possibility of an epizootic development. The spontaneous occurrence of baculovirus deaths in apparently healthy insect colonies has motivated the study of triggering factors, providing an evidence that these types of infections are capable of reactivation (152). Stress factors such as overcrowded rearing conditions (138), marked changes in temperature or relative humidity (153, 154), changes in diet components (139), chemical stress (155-157), parasitism and infections by a second pathogen (142, 157) are mentioned in the literature, but not clear conclusions can be drawn in all cases. Some successful activating factors under laboratory conditions have been studied with the aim of field applications for *S. exigua* control (157). The field-spray of chemicals such as selenite sulfate, copper sulfate and iron sulfate that had

triggered 12-41% sublethal infections in treated larvae under laboratory conditions, resulted in far lower rates of NPV-mortality under field conditions.

The challenge of second infections (superinfection) is the most consistent triggering factor of covert infections (158, 159). Same virus genotypes, heterologous baculoviruses or even different host biotypes could reactivate covert infections. A *Spodoptera litura* NPV (SpltNPV) covert infection was activated by a heterologous *Mythimna separata* NPV (MyseNPV) (159). In *S. exigua*, covertly infected larvae were twice as susceptible to lethal superinfection as healthy conspecifics, when challenged with the same nucleopolyhedrovirus (SeMNPV) (147). Furthermore, a *S. exigua* colony derived from field collected adults that were covertly infected was over three-fold more susceptible to lethal infection by SeMNPV than a virus-free laboratory colony (10), indicating non protective effects from covert infection. However, not all the superinfections produce a detrimental effect on insect hosts. Covert infections exert different responses to new infection compared to non-covertly infected individuals. No common trends can be outlined and the responses widely varied depending on the host-pathogen system. For example, the vertically transmitted bacterial symbiont, *Wolbachia*, can provide protection to hosts against infection by a small RNA virus (*Dicistroviridae*) (160). However, the presence of this bacteria in *S. exempta* natural populations remarkably increases larval susceptibility to *S. exempta* NPV (161). For insect-baculovirus pathosystems, the potential benefits of covert infection on the probability of infection by additional pathogens are not clear. Infection of *Helicoverpa armigera* by a densovirus (*Parvoviridae*) appears to protect it against a second infection by baculoviruses or the bacterial pathogen *Bacillus thuringiensis* (162).

### 3.6 *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV)

Firstly isolated by Steinhaus in 1950 (163) and described by Hunter and Hall (164), SeMNPV strains are very numerous and from many different origins worldwide, including California (165), Florida (166), Egypt and The Netherlands (167), Japan (168), Thailand (169) and Spain (15, 68, 147, 170). Different geographical isolates comprise mixtures of different genotypes that can be detected readily even between closely related variants from a single isolate (166). Bioassays have revealed the variability of biological traits related to insecticidal properties,

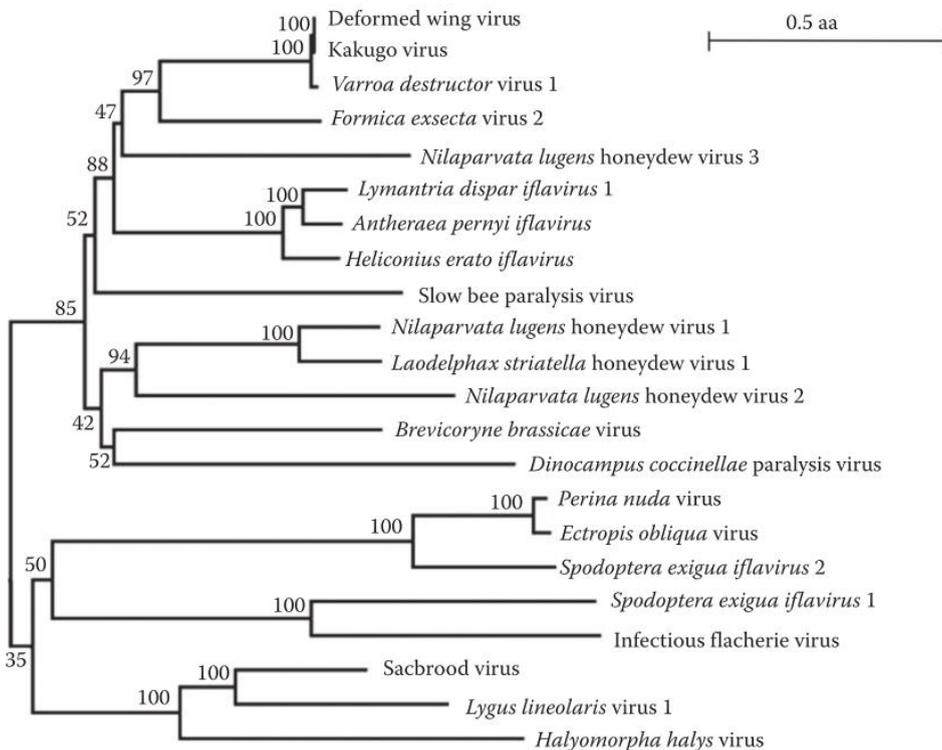
such as pathogenicity, virulence, and OB production upon larval death (171). SeMNPV exceptional features as a biocontrol agent including full specificity (68), high virulence and the fact that can be produced on a large scale at a reasonable cost (26), postulated this virus as a firm candidate for biopesticide development to tackle economic losses due to *S. exigua* in pepper crops of Almerian greenhouses (8). Currently, SeMNPV-based products are commercialized in different countries including Spain, where the trade-names Spod-X<sup>®</sup> (CERTIS,USA), Virex<sup>®</sup> (BIOCOLOR, S.L. Spain), Spexit<sup>®</sup> (Andermatt-Biocontrol, Switzerland) are available in the phytosanitary market. These products differed in the origin of the strains they are based on, that confer unique insecticidal properties (172). Namely, Virex<sup>®</sup> comprises three different SeMNPV genotypes selected from a large number of isolates collected from Almerian greenhouses, a mixture with improved bioinsecticidal characteristics (pathogenicity and virulence) over the natural SeMNPV populations against local *S. exigua* biotypes. Field studies demonstrated that Virex<sup>®</sup> applications in greenhouse sweet pepper crops provided efficient control of the pest better than chemical insecticides (32). Recent studies on SeMNPV vertical transmission and their application on biological control, suggest the possibility of using vertically transmitted genotypes to promote covert infections and reactivation in field application approaches (157). The double transmission means of SeMNPV, combining horizontal and vertical (covert infections) routes, is potentially a novel aspect to be considered to improve the baculovirus-based application efficiency in crops. The introduction of SeMNPV vertically transmitted genotypes (10) in the active ingredient might favor the implementation of inoculative strategies for field applications, favoring the sustainable use of such means to control *S. exigua* pest populations.

## 4. IFLAVIRUS

### 4.1 Taxonomy and nomenclature

The *Iflaviridae* family was established in 2008 (173) and classified under the order *Picornavirales* (174). Currently, this family comprises fourteen assigned species isolated from insects, all of them in the unique *Iflavirus* genus (175, 176). However, numerous putative member may be considered as belonging to the genus *Iflavirus* according to the genome organization. First, only economically important

insect species of iflavivirus were described such as the ones causing disease in honeybees, *Apis mellifera* (167), or silkworms, *Bombyx mori* (177), potentially as etiological agents. However, the number of family members is likely to be quickly increased as NGS technology in insect transcriptome analysis identifies new species (166). Iflaviruses are genetically diverse. Phylogenetic analyses showed (Figure 8) that the viruses of different clades have as low as 20 to 30% amino acid (aa) identity. Yet this identity can be as high as 82% to 87% for nucleotide (nt) and aa similarity, between close evolved species, such as *Perina nuda* virus (PnV) and *Ectropis obliqua* virus (EoV), respectively.



**Figure 8:** Phylogram of the recognized and putative members of the genus *Iflavivirus*. Full-length polyprotein sequences were used. The tree was produced using the neighbor-joining method and evaluated with bootstrap analysis, 1000 replicates, percentage of boot-strap support of each branch is indicated. Branch length indicates evolutionary distance; scale bar shows 0.5 amino acid substitutions per site. Adapted from Fannon and Ryabov, 2016 (177).

Firstly discovered in the apiculture sector (*Apis mellifera*) (178) and in silk production (*Bombyx mori*) (179), iflaviruses have been described in insects belonging to the orders Lepidoptera, Hymenoptera and Hemiptera and also in parasitic mites (Acarina). In honeybees, sacbrood disease is an example of fatal iflavivirus infection. Fluid accumulates under the skin and larvae become a fluid sac, then turn dark brown and finally die (180). Sacbrood virus (SBV) isometric particles were first characterized in 1964 by electron microscopy (181). In silkworms, an infective phenotype was observed that consists of a strong diarrhea that leads to host flaccid body and brownish color. The name *Infectious Flacherie Virus* (IFV) came after viral symptoms and ended up in i-fla-virus. IFV was the first iflavivirus whose complete genome sequence was published (182), but more complete genomes of iflaviruses have been generated over time (Table 1).

**Table 1:** Members of the genus iflavivirus with full-length genome sequences known.

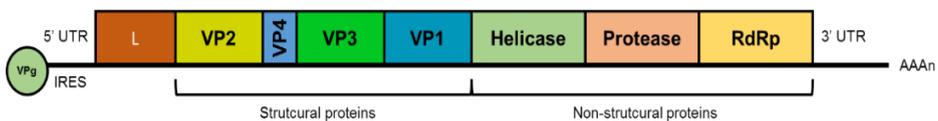
Isolate name (Acronym)	GenBank Accession Number	Host Common Order	Ref.
Infectious Flacherie Virus (IFV)	NC_003781	<i>Lepidoptera</i>	(182)
<i>Perina nuda</i> virus (PnV)	NC_003113	<i>Lepidoptera</i>	(183)
<i>Ectropis obliqua</i> virus (EoV)	NC_005092	<i>Lepidoptera</i>	(184)
Deformed wing virus (DWV)	NC_004830	<i>Hymenoptera</i>	(185)
<i>Varroa destructor</i> virus 1 (VDV-1)	NC_006494	<i>Hymenoptera</i>	(186)
Sacbrood virus (SBV)	NC_002066	<i>Hymenoptera</i>	(187)
<i>Formica exsecta</i> virus 2 (Fex2)	KF500002	<i>Hymenoptera</i>	(188)
<i>Nilaparvata lugens</i> honeydew virus 1 (NLHV-1)	AB766259	<i>Hemiptera</i>	(189)
<i>Nilaparvata lugens</i> honeydew virus 2 (NLHV-2)	NC_021566	<i>Hemiptera</i>	(189)
<i>Lymantria dispar</i> iflavivirus 1 (LdIV1)	KJ629170	<i>Lepidoptera</i>	(190)
<i>Antheraea pernyi</i> iflavivirus (HeIV)	KF751885	<i>Lepidoptera</i>	(191)
<i>Heliconius erato</i> iflavivirus (HeIV)	KJ679438	<i>Lepidoptera</i>	(192)
Slow bee paralysis virus (SBPV)	NC_014137	<i>Hymenoptera</i>	(193)
<i>Brevicoryne brassicae</i> virus (BrBV)	NC_009530	<i>Hemiptera</i>	(194)
<i>Spodoptera exigua</i> iflavivirus 1 (SelV-1)	NC_016405	<i>Lepidoptera</i>	(12)
<i>Spodoptera exigua</i> iflavivirus 2 (SelV-2)	JN870848	<i>Lepidoptera</i>	(13)
<i>Lygus lineolaris</i> virus 1 (LyLV-1)	JF720348	<i>Hemiptera</i>	(195)

Adapted from Fannon and Ryabov, 2016 (177).

Iflavirus nomenclature has been modified overtime according to taxonomical criteria. In the past the term “insect picorna-like” viruses was used to identify several virus species current members of the genus *Iflavirus* or the family *Dicistroviridae*, due to the similarity to picornaviruses (single-stranded linear RNA viruses with positive polarity), infectious to vertebrates. However, this term does not refer to a definite taxonomic category and should be avoided. The restructuration of the virus family names led to the modification of virus names. For example, former *Ectropis oblicua* picorna-like virus (EoPV) has been turned to *Ectropis oblicua* virus (EoV). The addition of “iflavirus” to the name has been adopted for the more recently classified species (Table 1).

## 4.2 Genome Organization

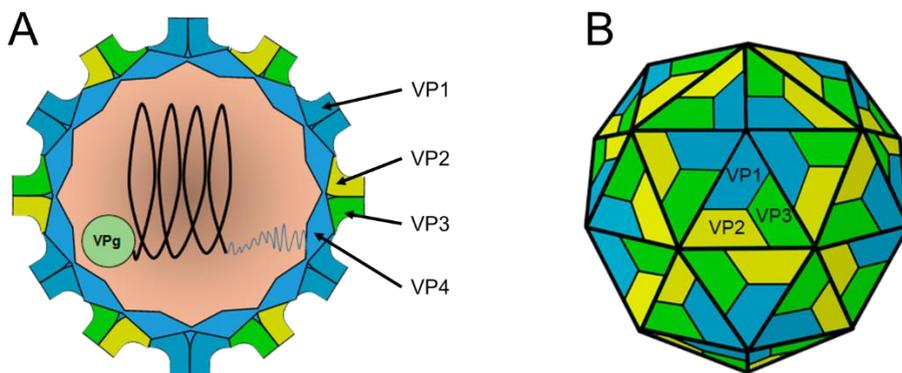
The iflavirus capsid harbors a single molecule of linear single-stranded positive RNA, (+)ssRNA, which is non-segmented and 8.6 to 10.3 kb long (196) (Figure 9). At the 5' terminus, the RNA virus possess a genome-linked viral protein (VPg), followed by a non-translated region (NTR) of 156 to 178 nucleotides, shorter than other picornaviruses, bearing the replication signals and the internal ribosomal entry site (IRES). A single open reading frame (ORF) follows. It codes for a large polyprotein, ranging in size from 2,800 to 3,200 amino acids (aa), with the structural proteins and the nonstructural proteins encoded by the 5'- and 3'-parts of the ORF, respectively (Figure 9). At the 3' end, a short NTR terminus with a polyA tail of 30 to 650 nucleotides is found. Generally, a leader protein (L) fused to the N terminus of the polyprotein is present. It differs considerably in function and length in different iflaviruses (197).



**Figure 9:** Iflavirus genome organization. VP1-VP4 are the structural proteins while Helicase, Protease and RdRp belong to non-structural proteins. VPg, leader protein (L), IRES and PolyA tail are also represented.

### 4.3 Viral capsid

The iflavirus viral capsid consists in an icosahedral symmetry structure (27 to 30 nm in diameter) tightly packaged in the host cell cytoplasm and composed of 60 heterotrimers called protomers or capsomers, each one of them formed by the viral structural proteins VP1, VP2, VP3 and VP4 (Figure 10). While VP1-VP3 assemble on the external part of the capsid, several smaller VP4s molecules are located inside the virion (198), but little is known about them. The protomers build 12 pentamers that give rise to the viral capsid (199). The capsid structure is type  $T = 1$  or pseudo  $T = 3$  (198), similar to animal (foot-and-mouth disease virus) and human (poliovirus) infectious RNA viruses (200). Empty viral capsids are commonly found (201).



**Figure 10:** Internal (A) and external (B) scheme of the iflavirus viral capsid. Genome and VPg (virion genome linked protein) are inside the capsid. The capsid is formed by VP1-VP4 proteins. While VP1, VP2, and VP3 externally conform the capsid, VP4 protein is in the inner side.

Mature VP1-VP3 proteins, derived from the polyprotein processing, have an 8-stranded  $\beta$ -barrel domain described for coat protein molecules (174). The structure is similar to mammalian picornaviruses (202) but, unlike mammalian enteroviruses, which have a ‘Rossmann canyon’ around the 5-fold axis containing the binding host cell receptor (203, 204), iflaviruses present 2 nm diameter holes in the middle of each of the five protomer units (198, 200, 205).

## 4.4 Non-structural proteins

In addition to the capsid proteins, iflavivirus RNA transcribes three non-structural proteins: the helicase, the protease and the RNA-dependent RNA polymerase (RdRp).

### 4.4.1 *Helicase*

The helicase belongs to the helicase superfamily III (174, 206, 207), which is characterized by three sequence motifs: A, B and C. Motif A is characterized by the canonical sequence GxxGxGK(S/T), motif B has the (Y/F/W)2X5QX5(Q/D)D sequence and, motif C is less well conserved. Mainly, the function of the iflavivirus helicase is involved in duplex unwinding during viral replication, and could also participate in translocation (208). Domain A and B could be involved in NTP-binding, as in the poliovirus membrane-associated helicase (209).

### 4.4.2 *Protease*

Iflavivirus chymotrypsin-related cysteine proteases (3C proteases) are encoded for processing polyprotein precursors (210). They have a catalytic cysteine extremely conserved and is followed by a conserved GXH motif. Protease functions as an autoprotease, cleaving itself from the polyprotein precursor and aiding in the cleavage of the rest of the proteins (210, 211).

### 4.4.3 *RdRp*

The RNA-dependent RNA polymerase is the best conserved region to compare RNA viruses and it is the best spot for phylogenetic studies (212). It belongs to the RNA polymerase superfamily I (213). From the eight sequence motifs described (214), three of them were well conserved and recognized: Motif I (KD(E/T), motif V (SGxxxTxxx(S/T)) and motif VI (YGDD). As found in other viruses (215), motifs V and VI may involve the catalytic center. RdRp function is involved in the synthesis of both plus- and minus-strand viral RNA and also includes RNA binding, NTP binding and RNA strand displacement elongation (216).

## 4.5 Infectious cycle and virus replication

The iflavivirus infectious cycle starts when the virus binds to a host cell surface receptor. This interaction leads to virus internalization. Once inside, low pH

destabilizes capsid protein contact, creating pores and releasing the viral genome into the cytoplasm of the host infected cells (217, 218). An association between the capsids and vesicular structures of the cell has been observed (219) giving a base for virus replication (220).

Viral RNA works like messenger RNA, translating the polyprotein by the host cells ribosomes. RdRp starts transcribing viral (+)ssRNA into the complementary strain (-)RNA and giving place to a dsRNA (221), a replication intermediate that acts as a template genome replication. An internal ribosomal entry site (IRES) in the 5' region is needed for the cap-independent translation (222-224). The VPg 22 to 25 aa polypeptide attached at the 5' end through a phosphodiester bond (225) serves most likely as a primer to initiate RNA polymerization by RdRp (226, 227). Generally, replication occurs in the cytoplasm and takes 2 to 3 hours to remodel it for its own benefit. Iflavivirus induce a change in the host membrane permeability and production of membranous structures, implicating viral and host proteins, where replication takes place (220, 228).

Polyprotein processing conducted by proteases follows the proteomic cleavage, that generates VP0, VP1 and VP3 (197). A pentamer with these three protomers is assembled. Encapsidation is not clearly known. Two models are suggested: i) RNA is inserted into pre-formed empty capsids or, ii) pentameres condense around DNA to form the procapsid structure. Finally, a VP0 cleavage takes place into VP2 and VP4 to capsid lock leading to a stable state prior to spread infection into other host cells (229).

#### 4.6 Pathology

Iflavivirus was deeply described due to the acute symptoms that the silkworm *Bombyx mori* shows in silk industry produced by the Infectious Flacherie Virus (IFV) (179). The midgut epithelium is primarily infected followed by a densely packed cell bunches formation, organelles trail off and finally cells release their content to the gut lumen (230). Secondary infections in neighboring cells by phagocytosis finally lead to a lethal diarrhea. A silkworm crop IFV infection was severe and fast and produced an estimated 27-35% losses (231).

There are many examples of iflaviruses that give rise to clear infectious phenotypes, diseases, deformations and even death. The deformed wing virus (DWV), responsible for economically important losses in honeybee colonies (*Apis mellifera*) (232, 233), does not cause insect death but produces deformed wings, paralysis, altered abdominal shape, reduced life span and learning deficits (185, 234). The Kakugo virus (KV) primarily infects honeybee neural tissues causing aggressive patterns (235). The Sacbrood virus (SBV), associated with the vector *Varroa destructor* mite infestations (236, 237), primarily courses asymptotically, but when larvae become more vulnerable to other infections, it can block larval pupating and eventually cause larval death (181, 238). As a vector, *V. destructor* has an important role and may take part in virus replication (239). Iflaviruses could also play an important role in mixed infections, swinging other viral infections to produce antagonistic, synergistic or mutualistic effects (see section 5).

However, as occurs in baculovirus covert infections (142, 240), the majority of iflaviruses could asymptotically infect insects or may have not clear effects on host phenotypes. Due to the development of NGS technologies (241), a large number of new asymptomatic iflavivirus species have been discovered (Table 1) related to covert asymptomatic infections. This is the case of the *Spodoptera exigua* iflavivirus 1 (SeIV-1) (12), *Spodoptera exigua* iflavivirus 2 (SeIV-2) (12, 13), and the *Lymantria dispar* iflavivirus 1 (LdIV1) recently discovered, which cause a persistent covert infection in their homologous host (190). Also other iflavivirus species like Acute bee paralysis virus (ABPV) or Kashmir bee virus (KBV) were detected in honeybees in the absence of disease symptoms (180).

Tissue tropism is an important issue in iflavivirus pathology. Primary infection sites may vary. For example, for Infectious Flacherie Virus (IFV), globet cells of the midgut epithelium are the primary targets, suggesting an oral infection. In *S. exigua*, SeIV-1 and SeIV-2 also midgut and fat body abundance are higher than in other tissues (14). In honeybees, Deformed wing virus (DWV) have been detected in head, thorax and abdomen whereas Kakugo virus (KV) exhibits a strong neurotrophism (235). As a consequence of virus tissue tropism, viruses may course symptomatic or asymptomatic infection (242). In fact, even the same virus causes either symptomatic or asymptomatic infections depending on transmission route (177).

Generally, acute symptomatic horizontally infections and covert vertically-transmitted infections produce different host effects. A covert infection establishment usually involves the host immune system response. The molecular basis resides in the regulation of host-virus interactions through the action of microRNAs (miRNA), small non-coding, hairpin RNAs produced by both virus and host to regulate transcription and translation (243). The host immune system produces interfering RNAs (siRNAs) to obstruct expression of viral genes (9) while viruses produce their own miRNAs to interfere with host responses to their own advantage (244). Concerning iflaviruses, Nayak et al. (245) demonstrate the dual effect between *Drosophila melanogaster* C virus (DCV) and cricket paralysis virus (CrPV) in *Drosophila*. DCV binds to dsRNA and prevents processing by Dicer 2 while CrPV inhibits the activity of the endonuclease Argonaute 2 (Ago2). CrPV induces substantial mortality in flies whereas DCV establishes a symbiotic covert infection without appreciable mortality. In another study, the LdIV1 was induced by a viral suppressor of RNA silencing in cell culture, suggesting that acute infections are restricted by RNA interference (RNAi) (190).

#### 4.7 Transmission

Iflaviruses are transmitted both horizontally and vertically. The route of transmission has been in relation to the degree of symptom development and the accumulation of viral loads in the individuals. Generally, vertically transmitted iflaviruses do not accumulate to high levels, neither produce any symptoms. Iflaviruses that are transmitted horizontally often cause symptomatic infections with highly pronounced symptoms and eventually host death (177).

Asymptomatic infections are vertically transmitted to offspring allowing the virus to persist in the population. Deformed wing virus is transmitted from queens to both drones or workers (246) by infected semen or covertly infected queens, even though only present in the ovaries (247). Also, inbreeding results in a vertical transmission of SeIV in *Spodoptera exigua* (15). However, the most likely pathway seems to be horizontal transmission, generally via food contamination by frass deposition of infected individuals, which is favored by rearing conditions of laboratory insect cultures (14, 236). In occasion, vectors, such as the mite *Varroa destructor* (248) or *Tropilaelaps mercedesae* (249) are involved in horizontal

transmission of different iflaviruses to *Apis mellifera*. Notably, DWV *Varroa*-vectored infections dramatically amplified the virus virulence leading to high rates of casualties in bee colonies (250). Another example of dual transmission is the *Helicoverpa armigera* iflavirus (HaIV), whose horizontally transmission efficiency is dose-dependent, and low rates of transmission are found by infected parents to offspring. In this case superficial egg decontamination result in decreased HaIV prevalence, suggesting the course of transovum transmission (251).

#### 4.8 Iflavirus-based bioinsecticides

One of the main goals of Insect Pathology is the discovery of suitable pathogens for the development of microbial control agents. The arrival of massive sequencing techniques revealed growing numbers of arthropod-infecting RNA viruses that could potentially be used as biological control agents. In particular, RNA viruses are the most prevalent found in insect-pests species.

However, the use of RNA viruses as microbial control agents rises major concerns due to the taxonomic similarities in the order *Picornavirales* between RNAviruses infecting insects and RNA viruses causing important human diseases, such as Hand, foot and mouth disease (HFMD) (252), rhinovirus, hepatitis or polio (253-256), among others. The potential exchange of genetic material is not easily measurable but legitimates biosecurity concerns. In fact, early studies indicated certain levels of cell immunology response in mammal cells to RNA insect viruses (253-255), and more recently the possibility of host range expansion of RNA insect viruses leading to replication in human cells (257). The high homology-dependent recombination capacity of these viruses is well known (258). In fact, viruses such as Acute bee paralysis virus (ABPV) or Kashmir bee virus (KBV) are considered closely related and reported with common origins (193). Likewise, recent recombinant events between DWV and KV leading to novel genomes with mixed characteristics were reported (259). Thus, the high rate of mutation and genetic exchange might drive crucial modification of host range, completely undesirable to prevent lack of efficacy of bioinsecticides based on RNA viruses and the impact on non-target species.

Lastly, the lack of reliable cell lines for large-scale RNA virus production is a serious drawback that remains unsolved. Only at the laboratory level, a slight

production based on transcript synthesis has been achieved (260). Alternatively, other approaches for large scale production of iflaviruses in large quantities are currently being under study, including the use of recombinant baculoviruses as expression vector systems. For example, the production of large copies of the dicistrovirus *Rhopalsiphum padi* virus (RhPV) was possible in a baculovirus expression system, allowing the possibility of development of a bioinsecticide against the aphid *R. padi* (261).

Altogether, major drawbacks exist on iflavirus bioinsecticide development that explain the lack of serious studies conducted so far. The production technology needs a significant advance until RNA viruses can be perceived commercially profitable and biosecure as biocontrol agents. For instances, another approach that would overcome field application concerns, is the use of RNA viruses as protein expression vectors for developing genetically engineered crop plants resistant to pest damage (16).

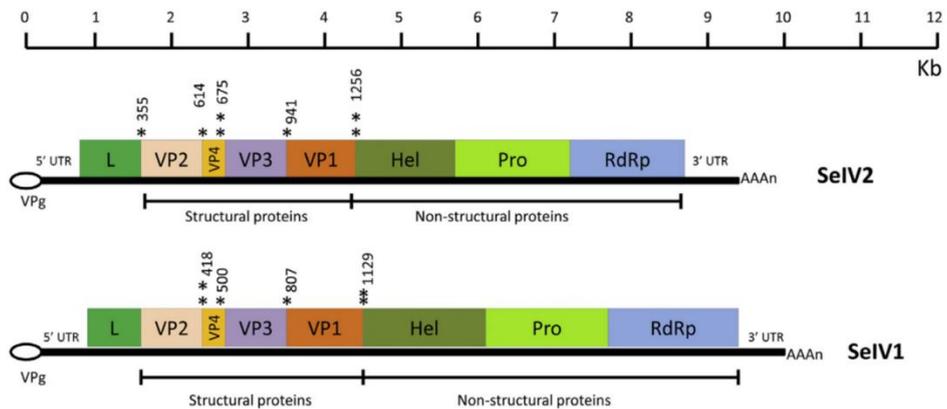
#### **4.9 *Spodoptera exigua* iflavirus (SeIV)**

Sequence analysis of *S. exigua* tissue transcriptome led to the discovery of two iflaviruses classified as SeIV-1 (12) and SeIV-2 (13) among other RNA virus species (241). Besides being highly prevalent in insect cultures, these iflaviruses were detected in natural population of *S. exigua* (129) in single, double or triple combinations.

Both species share the common genome organization of iflaviruses with slight differences between them. Iflavirus 1 is 10,347 nucleotides long (excluding the poly(A) tail). As with other iflaviruses, sequence is A/U rich (more than 60% of A/U), while 5'UTR and 3'UTR are 344 and 334 nucleotides long, respectively. A hypothetical polyprotein of 3,222 aa has structural proteins at the N-terminal part while, at the C-terminal, non-structural proteins are located. A ribosomal skipping signal motif was found at position 418 (12). The SeIV-2 genome is 9501 nt in length (excluding poly(A) tail). Likewise, it is A/U rich with 391 nt for the 5'UTR, with IRES activity, and with 77 nt for 3'UTR. Also, it has a 3010 aa polyprotein with structural and non-structural proteins organized in the same way as SeIV-1 and other iflaviruses (13).

Comparisons at a genomic level point out major differences between both species. Phylogenetic studies each virus clade in different clusters of a phylogenetic tree representing the seventeen species sequenced so far (Figure 8). That is, while SelV-1 belongs to the group of Infectious Flacherie Virus (IFV), SelV-2 forms another clade with *Perina nuda* virus (PnV) and *Ectropis obliqua* virus (EoV). Both share low rates of similarity, with 40% nt identity and 27% aa similarity (14).

Biological studies indicate tissue tropism of the SelV iflavirus, as they are more abundant in gut tissue than fat body or hemolymph during the larval stage. The relative abundance of viral loads increases with larval stage suggesting viral replication during host development (14), despite non-specific symptoms of the infection can be detected.



**Figure 11:** Schematic representation of the genome organization for iflaviruses 1 and 2. Structural proteins: VP1, VP2, VP3 and VP4. Non-structural (functional) proteins: Hel-helicase, Pro-protease and RdRp-RNA dependent RNA polymerase; \* predicted protease cleavage sites of capsid proteins, VPg a small < 5 kDa virus protein 50 bound; L-leader peptide; 5' and 3'UTR-untranslated regions; AAAn-poly(A) tail. All genomes are drawn proportionally, and the nucleotide kilobase scale is included on top. Homologous proteins are marked with the same color (14).

## 5. INTERSPECIFIC VIRUS INTERACTIONS

Interactions between viruses infecting a common host are an expectable phenomenon (262, 263) that can occur as a result of a consecutive (superinfection) or a simultaneous infection with two or more different viruses (co-infection) (264-266). Interspecific virus interactions in host infections have been explained to occur

at three different levels: (i) direct interactions of viral genes and proteins, (ii) indirect interactions due to environmental consequences of the host infection, and (iii) immunological interactions due to the role of the host immune system (17). These interactions can lead to different scenarios in which both viruses can benefit from the interaction having a positive effect on their fitness (267), or alternatively a situation of mutual exclusion (266) or the exclusion of a second infection by the first pathogen (268).

There is a limited number of studies on insect mixed infections involving baculoviruses and relatively distant viral species (196), that examine the viral fitness. For instance, *Adoxophyes honmai* nucleopolyhedrovirus (AdhoNPV) and the *A. honmai* entomopoxvirus (AdhoEPV). A co-inoculation on fourth instar *A. honmai* showed a decrease of AdhoEPV OB production, whereas no differences on AdhoNPV production was observed. Contrary, a superinfection of AdhoNPV in fourth instar larvae over previously inoculated larvae (during the first instar) with the same virus produces a decrease on AdhoNPV OB production. Thus, depending on the sequence timing of infection, production is negatively affected on both viruses (269).

Morris et al. (270) suggested extreme caution for *in vivo* baculovirus production, as RNA viruses were found associated with *Autographa californica* Nucleopolyhedrovirus (AcMNPV) in *A. californica* colonies, and consequently the spreading of an unknown RNA virus might occur. In another case, an interaction between the baculovirus *Perina nuda* nucleopolyhedrovirus (PenuNPV) and the iflavirus PnPV exert an antagonistic effect on the systemic PenuNPV infection in the ficus transparent wing moth, *P. nuda*, larvae (271, 272). On the contrary, another RNA virus known as densovirus, can assist the host against biological control agents. In *Helicoverpa armigera* field populations the densovirus HaDENV-1 increased host resistance to both biological agents HaNPV and *Bacillus thuringiensis* (162).

Multiple infections are more frequent than thought before, but interaction studies might involve a high level of complexity not always approachable. As a matter of fact, multiple infection by baculoviruses and iflaviruses affect *S. exigua* both in field (15) and laboratory populations (14). Further studies of baculovirus-

iflavirus multiple interaction may lead to elucidate the antagonistic or synergistic interaction between both viruses, and whether these interactions might affect the insecticidal properties of SeMNPV.

## 6. AIMS OF THE THESIS

In the present thesis, we studied the interaction between iflaviruses (SeIV-1 and SeIV-2) and the SeMNPV on a *Spodoptera exigua* population. The study is focused on the effect of both iflavirus species on the insecticidal properties of the SeMNPV through different approaches including direct virus-virus interaction or indirectly when the host fitness is affected.

In **Chapter II**, the characterization of RNA virus population through transcriptome analysis in *S. exigua* including SeIV-1 and SeIV-2 and a novel *Spodoptera exigua* nora virus (SeNV) is addressed. We determined the prevalence of iflavirus infections in *S. exigua* laboratory cultures of different geographical regions and quantified iflavirus covert infections through host generations. The variation of relative abundance of SeIV-1 and SeIV-2 allowed to purify mixed population to initiate further biological studies.

During subsequent transcriptomic studies, the SeIV-1 was observed in the offspring of insects that survived following inoculation with SeMNPV OB. The tracking of the iflavirus origin suggested an association of this virus with the occlusion bodies of the baculovirus. In **Chapter III**, the effect of this association on the stability and infectivity of both viruses was determined. A reduction in baculovirus pathogenicity, without affecting its infectivity and productivity, was observed when associated with the iflavirus. In contrast, viral association increased the infectivity of the iflavirus and its resistance to ultraviolet radiation and high temperature, two of the main factors affecting virus stability in the field.

**Chapter IV** is focused in the study of the interaction of iflaviruses and baculoviruses after simultaneous co-infection in the same host. Specifically, we evaluated the effect of iflavirus co-infection on the insecticidal characteristics of SeMNPV occlusion bodies (OBs). Notably, the iflavirus co-inoculation increased the SeMNPV induced mortality, but also altered baculovirus transmission traits by

reducing OB production upon larval death and viral loads in adults covertly infected by the NPV.

In **Chapter V**, the physical association of SeIV and SeMNPV OBs generated upon co-inoculation in larvae is further examined. By using molecular techniques, the conformation and structure of generated OBs and ODVs, were found to be affected by co-inoculation, compared with OBs multiplied in iflavirus-free larvae. These differences were related to the reduction in the pathogenicity observed for OB-associated SeIV.

The relatively high abundance of iflaviruses in natural populations and insectary cultures, drove us to a final approach. **Chapter VI** is focused on the study of the pathological effects of iflaviruses on host fitness and on the insecticidal properties of SeMNPV when a subsequent infection (superinfection) was induced. SeIV-1 negatively affected developmental parameters, whilst SeIV-2 did not register measurable changes. Viral loads of SeIV-1 significantly increased by contamination of egg masses, but SeIV-2 was maintained at basal levels. Regarding SeMNPV insecticidal properties, in a population of insects with a persistent infection of SeIV-1, LC<sub>50</sub> and speed of kill decreased, improving the infectivity properties of the baculovirus as a biological control agent.

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## CHAPTER II

# Simultaneous occurrence of covert infections with small RNA virus in the lepidopteran *Spodoptera exigua*

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### ABSTRACT

Viral covert infections in invertebrates have been traditionally attributed to sublethal infections that were not able to establish an acute infection. Recent studies are revealing that, although true for some viruses, other viruses may follow the strategy of establishing covert or persistent infections without producing the death of the host. Recently, and due to the revolution in the sequencing technologies, a large number of viruses causing covert infections in all type of hosts have been identified.

The beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae) is a worldwide pest that causes significant losses to agricultural and ornamental plant industries. In a previous project, we used NGS to obtain a comprehensive transcriptome of the larval stage, revealing the presence of an important number of unigenes belonging to novel RNA viruses, most of them from the order *Picornavirales*. In order to characterize *S. exigua* viral complex, in this work we have completed the genomic sequences of two picorna-like viruses, and compared them to a SeIV-1, a member of *Iflaviridae* previously described by our group. We performed additional studies to determine virus morphology, horizontal transmission, tissue and life stage distribution and abundance in the hosts. We discuss the role of virus persistent infections on insect populations.

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## 1. INTRODUCTION

Insects – both beneficial and pest – are hosts for DNA and RNA viruses. RNA viruses identified in insects, often referred to as picorna-like viruses, belong to more than 10 families, some of which are already assigned to two virus orders, *Picornavirales* and *Nidovirales*. Among RNA viruses, those with single-stranded positive RNA (ss(+)RNA) form the most abundant class (1). ss(+)RNA viruses which infect insects belong to at least five families: *Dicistroviridae*, *Iflaviridae*, *Nodaviridae*, *Flaviviridae*, *Alphatetraviridae* (2), of which *Dicistroviridae*, *Iflaviridae* and *Picornaviridae* were included in a recently accepted virus order *Picornavirales* (3).

The revolution in DNA sequencing technologies have resulted in an outburst of new genome sequence information, including viral genomes (4). Vast quantities of viral sequences are identified in environmental samples by metagenomic sequencing (5-7).

In case of insect viruses, the new discoveries often result from sequences from insect cDNA libraries or RNAseq projects (8-11). Significant increase in viral sequence quantities specially applies to RNA viruses for two reasons: (i) they often do not cause acute infections but rather persistent non-symptomatic infections, and thus in the past did not attract research interest by showing obvious visible pathologies in insects; (ii) RNA isolation methods for mRNA extraction favor targeting in majority polyadenylated RNA viral genomes.

In the course of transcriptional studies on larvae from a highly dispersive polyphagous pest of vegetable, field and flower crops, *Spodoptera exigua* (Lepidoptera: Noctuidae) we identified several unigenes with homology to at least three different picorna-like viruses (10). Based on the sequence features two of these viruses are members of *Iflaviridae* and the third virus shows homology to *Drosophila* Nora virus. All three RNA viruses are present in our laboratory *S. exigua* populations in a persistent manner, without inducing apparent disease symptoms.

Pathogenicity of picorna-like viruses' infections can vary broadly from lethal to persistent commensal infections (9). Persistent infection means co-existing of host and its pathogen in an equilibrium where virus transmission is assured and there is low or absent fitness cost to the host (12, 13). In insect

virology, the terms covert and overt are widely used to categorize infections (14). Covert infections develop clear disease symptoms, whilst covert infections are characterized by the absence of disease symptoms. Studies on baculovirus covert infections revealed that they can be reactivated to the lethal form (15) and they can be transmitted vertically (16). RNA viruses have been reported to persist in insect host populations as covert infections (17, 18). It is being investigated how this persistence is maintained. Recent studies in *Drosophila* indicate that persistent infection with *Nora virus* (ss + RNA) is not affected by fly immune pathways – including RNAi machinery-habitually engaged in RNA virus infections (19). Van Mierlo et al. (20) suggest that dynamic interactions between antiviral RNAi response and viral counter-defense determine viral persistence, and that suppression of host RNAi machinery is not the main determinant of viral pathogenicity. We previously reported the genomic sequence of one of the RNA viruses from *S. exigua*, *S. exigua* iflavivirus 1, SelV-1 (21). Here, we report the complete genomic sequence of two additional small RNA viruses found in *S. exigua* larvae. The aim of this study is to provide a comprehensive description of the small RNA viral complex of *S. exigua*, by studying their genomic features, developmental stage and tissue distribution, a relative abundance in different laboratory insect populations, as well as testing their transmission and ability to infect *S. exigua* larvae.

## 2. MATERIAL AND METHODS

### 2.1 Insects

*S. exigua* insects were used in the experiments. Detection, sequencing and tissue distribution were performed in ALM, XenR and FRA colonies. ALM laboratory colony was established from insects collected in southern Spain (22). XenR colony originated from the insects collected in the fields of Prattville, AL (USA) and selected for resistance to *Bacillus thuringiensis* (22). FRA colony was kindly provided by M. López-Ferber (INRA, St Christol les Alés, France). Abundance of RNA viruses was investigated in different laboratory colonies, CRT, MEX and SUI colonies. CRT colony was kindly provided by P. Bielza (Universidad Politécnica de Cartagena, Spain). MEX colony was provided by T. Williams

(INECOL, Coatepec, Mexico). SUI insects were provided by Andermatt Biocontrol AG (Grossdietwil, Switzerland).

## 2.2 Sequence determination and assembly (RACE and NGS)

A few contigs of each of the RNA viruses were detected in a *S. exigua* transcriptome sequencing project described elsewhere (10). Genome sequence completion of SeIV-1 is described in Millán-Leiva et al. (21). For *S. exigua* iflavivirus 2 (SeIV-2) and *S. exigua* Nora virus (SeNV), appropriate primers sets were designed to close the expected gaps (Table 1). Rapid amplification of cDNA ends (RACE) was employed to reach the end of the sequences at 5' and 3'UTR as described in Millán-Leiva et al. (21). At least two clones for each PCR product were sequenced. Paired-end Hiseq2500 Illumina reads (Illumina Inc.), obtained from XenR and FRA larvae employed elsewhere (23), were mapped to the genome sequences and used to confirm obtained genomes and for the final assembly of the consensus sequences.

**Table 1:** Primer sets used in the study.

Name	Sequence	Aim	Position in the genome
ME1	CACTGAACCTGCCGCTTGATTCG TAG	Complete SeIV-2 sequence	1659-1684
ME2	GCAAGAACGGCTATGAAGAGTGT TGGTA	SeIV-2 5' RACE	2027-2055
ME3	TCACAGCCAGCACAATCAGG	Complete SeIV-2 sequence	1805-1824
ME4	CCGTAGAGGAGTGGAAGTCATC	Complete SeIV-2 sequence	3268-3289
ME5	GGGTATGTTAGCATCTATGTGGA G	Complete SeIV-2 sequence	3035-3058
ME6	ATGTTACCAAGACCGCAACC	Complete SeIV-2 sequence	5557-5576
ME7	CATTTGGGTGGAGAAGTATGGTA AAGTTGG	Complete SeIV-2 sequence	9277-9306
ME8	GACTGTATGGCGTCTATTGGCAT CACC	Complete SeIV-2 sequence	8960-8986
ME11	TTGTTGACAGCACTGAGGGTCTA CTTCG	Complete SeIV-2 sequence	2364-2391
ME12	CTACCATACACTCTTCATAGCCGT TCTTGC	Complete SeIV-2 sequence	2026-2055
ME13	CGAAATAGTAGAAGCCGCAGT	SeIV-2 5' RACE	2276-2296
ME56	GATGGTATTGTGCTGGTCAGACG ACG	Complete SeNLV sequence	4897-4922
ME57	GCATCCATGTGTCTTCTTGGT	Complete SeNLV sequence	5438-5458
ME58	CTGCTGCGCACTACCAGAGCC	Complete SeNLV sequence	7868-7888

**Table 1:** Continued.

Name	Sequence	Aim	Position in the genome
AN44	GATTTTCAAGTCCTAACATACTA CCGAC	SeNLV 5'RACE	1945-1973
AN45	CTGCTGTAGCGACGAGTGAACA CAATC	SeNLV 5'RACE	2056-2083
AN46	CAGCAGTTGTCGTCGTG	Complete SeNLV sequence	4025-4041
AN47	GGTGGCTGTTGATGCTAGT	Complete SeNLV sequence	964-982
AN48	GTACGGCATGGAATACTCGCAC	Complete SeNLV sequence	6115-6136
AN49	CCGAGCACATCACGAGCG	Complete SeNLV sequence	6390-6407
AN50	GTGGCTATCACTGGTCGTTCC	Complete SeNLV sequence	6541-6561
AN51	GCAGAATCACTGTTAGAGGC	Complete SeNLV sequence	6886-6905
AN52	GTAACACAACACCACACTGTAG CA	Complete SeNLV sequence	8649-8673
AN53	CTTGAATTTCTCCTGATGTGTCTG TC	Complete SeNLV sequence	8762-8787
AN54	GCGTACCAGATGACCTAACGGAC GAAGT	SeNLV 3'RACE	10015-10042
AN55	CTGCTCGTGCCACACCAACAATC C	SeNLV 3'RACE	10408-10431
AN30	TGTGAAGTTAGACACGCATGGAA	SeIV-1 qPCR	9743-9765
AN31	CGACTTGTGCTACTCTTTCATCA A	SeIV-1 qPCR	9816-9840
A184	CCGCTCGCTTATTGAAACGT	SeIV-2 qPCR	1323-1342
A185	CATGAGACAGCTGGAATTGGAA	SeIV-2 qPCR	1380-1401
AN-42	GGTTAAGATTAGATCACGCTATT GGT	SeNLV qPCR	6202-6228
AN-43	CATGCTTCAGATGTTTGACAATCT C	SeNLV qPCR	6256-6280

### 2.3 Phylogenetic analysis

The analysis included most of the members of the *Iflaviridae* family, a few viruses from both genera of *Dicistroviridae* and the unclassified *Drosophila Nora virus*. Multiple sequence alignment of the predicted conserved domains of the RNA-dependent RNA polymerase (RdRp) and helicase amino acid sequences available in the GenBank were performed using PRALINE (24) and COBALT (25) softwares, with a Blosum62 matrix and a homology-extended strategy (PSI-BLAST) with 3 interaction for the PRALINE software, and the RPS-BLAST for COBALT, for the best adjustment in the alignment. The best fitting model of

molecular evolution was selected using Treefinder (26). Bayesian phylogenetic analyses were performed with the predicted RdRp and helicase domain separately using BEAST v1.7.5 (27) with the Dayhoff + G1 model, with a chain length of 10 mln generations and sampling every 1000 trees, in order to establish convergence for all parameters. The BEAST outputs were analyzed using TRACER v1.5 ([tree.bio.ed.ac.uk/software/tracer](http://tree.bio.ed.ac.uk/software/tracer)). The sample of the trees was summarized into the maximum clade credibility (MCC) phylogeny using TREEANNOTATOR v1.7.0 ([beast.bio.ed.ac.uk/TreeAnnotator](http://beast.bio.ed.ac.uk/TreeAnnotator)), discarding the first 25% of sampled trees as burn-in. The Bayesian analyses were performed with JTT, Blosum62 and MIX models resulting in the same tree structure, confirming the final trees' structure.

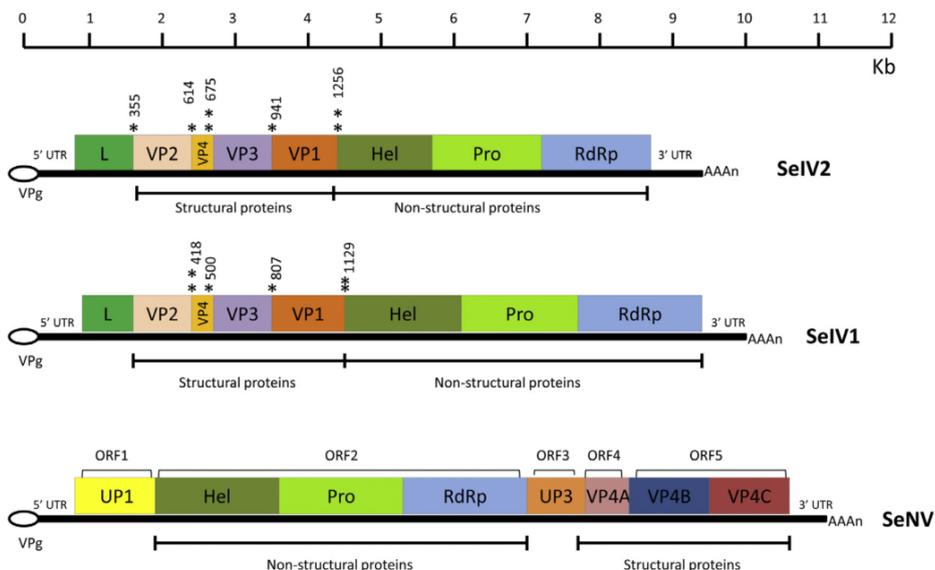
## 2.4 Detection of small RNA viruses

RNA viruses' presence and abundance were tested by detection of viral RNA using reverse transcription quantitative real-time polymerase-chain reaction (RT-qPCR). For this purpose, total RNA was isolated using RNAzol reagent (MRC Inc., Cincinnati, OH) according to the manufacturer's protocol. One  $\mu\text{g}$  of each RNA was DNase treated (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA using specific primers (Table 1) and SuperScript II Reverse Transcriptase from Invitrogen (Carlsbad, CA). RT-qPCR was carried out in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). All reactions were performed using SYBR Premix Ex Taq from Takara Bio Inc. (Otsu Shiga, Japan) in a total reaction volume of 25  $\mu\text{l}$ . Forward and reverse primers were designed using Primer Express software (Applied Biosystems, Foster City, CA), and their efficiencies were calculated to be 107%, 95% and 100% for SeIV-1, SeIV-2 and SeNV primer sets, respectively (Figure 1). Primers were added to a final concentration of 0.3  $\mu\text{M}$ . Ct values for the three viruses were normalized to the Ct values of the ATP synthase gene, previously shown to have similar expression levels in different *S. exigua* tissues (28). When the Ct value of  $\geq 32$  from the qPCR with a non-diluted cDNA prepared from 1  $\mu\text{g}$  of total RNA as a template was obtained, the larvae were considered virus-free.

## 2.5 Testing RNA virus transmission in virus-free insects

Virus transmission was determined in XenR virus-free insects, placed in the insect chamber together with *S. exigua* insects that carry RNA viruses. Larvae

that emerged from the virus-free eggs were named generation F0, and were confirmed virus-free by RT-qPCR. For each subsequent generation (F1–F3) second instar larvae were collected to determine virus presence and abundance using RT-qPCR, as described above. cDNA for virus detection has been synthesized using oligo(dT) primers.



**Figure 1:** Schematic representation of the genome organization for the three *S. exigua* picorna-like viruses. Structural proteins: VP1, VP2, VP3 and VP4. UP1 and UP3 are proteins in SeNV of unknown functions. Non-structural (functional) proteins: Hel-helicase, Pro-protease and RdRp-RNA dependent RNA polymerase; \* predicted protease cleavage sites of capsid proteins, VPg a small < 5 kDa virus protein 5' bound; L-leader peptide; 5' and 3'UTR-untranslated regions; AAAn-poly(A) tail. All genomes are drawn proportionally, and the nucleotide kilobase scale is included on top. Homologous proteins are marked with the same colour.

## 2.6 RT-qPCR for developmental stage and tissue distribution

The presence and abundance of RNA viruses in various developmental stages and larval tissues was determined by RT-qPCR, as described above. cDNA was synthesized using oligo (dT) primers. For testing the presence of the virus in different developmental stages, *S. exigua* SUI eggs, larvae L2, L3 and L5, as well as pupae and adults, males and females, were collected and homogenized for total RNA extraction. A pool of five individuals was used for each replicate and the experiment was repeated at least three times. For testing the presence of the virus in different tissues, last instar larvae were dissected and the midguts, fat

bodies and hemocytes were collected for total RNA extraction. A pool of at least five larvae was used for each replicate, and the experiment was repeated three times.

## 2.7 Virus purification and electron microscopy

Viral particles were purified from guts of fourth and fifth instar larvae. Approximately one hundred guts were used for one purification procedure. Collected guts were pooled, lyophilized and subsequently homogenized in 4 ml TE buffer (10 mM Tris–HCl pH 8–8.6, 1 mM EDTA, 0.06% SDS), and filtered through two layers of cheesecloth. One volume of chloroform was added to the filtrate, and the resulting solution was incubated 10 min at 4 °C with shaking. The filtrate was centrifuged 5 min at  $10,000 \times g$  and the upper water phase was collected, and loaded on caesium chloride discontinuous gradient. Tubes were centrifuged 3 h at  $100,000 \times g$  at 4 °C (Beckman) and the band between 1.2 g/ml and 1.5 g/ml CsCl was collected. The density of the collected band was adjusted to 1.35 g/ml, and a second centrifugation in CsCl gradient was performed for 16 h at  $100,000 \times g$  at 4 °C. The band of a density 1.3 g/ml corresponding to the viruses was collected and the viral particles were precipitated using 10% PEG. The resulting precipitate was resuspended in 200  $\mu$ l of water and stored at 4 °C for microscopy. Due to nearly identical sizes, particles of all three virus species were inseparable using described gradient centrifugations.

Purified virus particles were prepared for transmission electron microscopy by negative staining as follows. A drop of purified virus was placed on a carbon-coated grid and subsequently a drop of aqueous solution of 2% phosphotungstate (PTA) was placed on the grid. The preparation was allowed to dry before examination in JEOL JEM-1010 with transmission of 100 kV. The samples were photographed with digital camera AMT-RX80 at a magnification of 60,000 $\times$  and visualized with the image acquisition software.

## 2.8 Viral abundance in the laboratory insect colonies

*S. exigua* laboratory cultures ALM, CRT, MEX and SUI were tested for the RNA viruses' presence and abundance. Briefly,  $\pm 30$  adults were individually dissected and the abdomen was used for extraction of total RNA using TRIZOL reagent (Invitrogene, Paisley, UK) according to the manufacturer's protocol. 0.5 –

1 µg of the total RNA was DNase treated (Promega, Madison, WI) and reverse transcribed to cDNA, using oligo(dT) primers and the ImProm-II reverse transcriptase (Promega, Madison, WI). Presence and abundance of RNA viruses was determined using RT-qPCR in a Real Time GFX96 Detection System (Bio-Rad, Hercules, CA) in 96-well reaction plates. All reactions were performed using iQ SYBR Green Supermix (Bio-Rad) in a total volume of 12.5 µl. Forward and reverse primers were designed and added as described above. Ct values for the three viruses were normalized to the Ct values of the ATP synthase gene, as described above. When the Ct value of  $\geq 32$  from the RT-qPCR with a non-diluted cDNA prepared from 1 µg of total RNA as a template was obtained, the sample was considered virus-free. Mean average ( $\pm$ SD) was estimated to compare the prevalence of each virus in the populations.

### 3. RESULTS

#### 3.1 Genome organization

SeIV-1 genome has been previously characterized by our group (21). Genomic sequences of SeIV-2 and SeNV were completed and compared. Monopartite positive-sense single-stranded RNA genomes of SeIV-1 and SeIV-2 have a length of 10,340 and 9,504 nt excluding poly(A) tails, respectively (GenBank accession numbers JN091707 and KJ186788, respectively). The sequences are A/U rich, 56% and 61%, for SeIV-1 and SeIV-2, respectively. 5'UTR was predicted to be 344 and 391 nt long, and 3'UTR is 334 and 76 nt for SeIV-1 and SeIV-2, respectively. Monocistronic genomes comprise one large uninterrupted ORF, that translates into 3222 aa and 3012 aa polyproteins, respectively. The polyproteins of the two viruses show a 27% similarity at aa level. Structural and functional proteins and their cleavage sites were identified based on the homology to other iflaviruses. N-terminal site is composed of structural proteins VP1-VP4, headed by a leader peptide (L), while C terminal part contains three functional proteins: helicase, protease and RdRp (Figure 1).

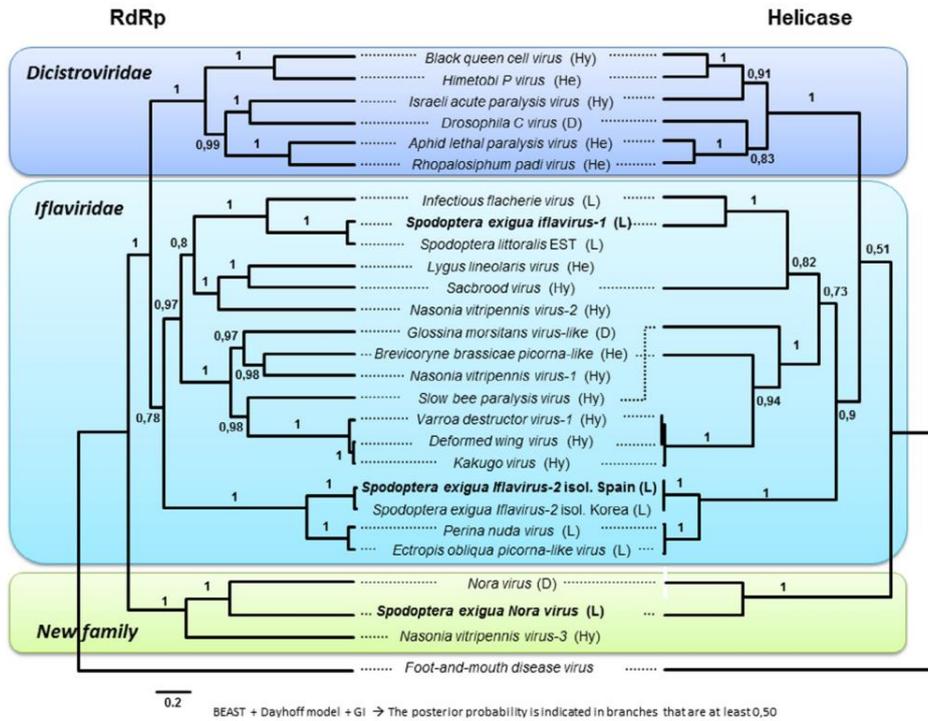
SeNV virus genome structure is very distinct from SeIV-1 and SeIV-2 genomes. It is composed of 11,056 nt long polycistronic ss(+) RNA genome containing five ORFs (GenBank accession number KJ186789). In contrast to the *S. exigua* iflavivirus genomes, N terminal part of SeNV genome is composed of non-

structural proteins, while C terminus contains structural proteins. Helicase, protease and RdRp constitute one ORF 2 (Figure 1). ORF 4 and ORF 5 encode structural proteins, with homology to *Drosophila Nora virus* VP4 capsid protein. ORF 1 and ORF 3, positioned along the genome similarly to corresponding ORFs of the *Drosophila Nora virus*; they may be related to *Drosophila Nora virus*' ORF 1 and ORF 3. Most SeNV ORFs overlap (2, 3, 4 and 5).

### 3.2 Phylogenetic analysis

Two proteins encoded in all *Picornavirales* show sufficient sequence conservation to investigate phylogenetic relationship. Helicase and RdRp were used to examine phylogenies of *S. exigua* RNA viruses with other known small RNA viruses. SeIV-1 and SeIV-2 clearly belong to the family *Iflaviridae* in the order *Picornavirales* (Figure 2). SeIV-1 groups with the iflavivirus type species, Infectious flacherie virus (IFV), while SeIV-2 groups with *Perina nuda virus* and *Ectropis obliqua* picorna-like virus, also belonging to *Iflaviridae*. *Iflaviridae* is a recently established virus family (29) that contains so far one genus, *Iflavirus*, with IFV, being the type species and the family name provider. Seven virus species belong to this genus as for 9th International Committee on Taxonomy of Viruses (ICTV) report (30). Despite being members of the same family, SeIV-1 and SeIV-2 fall in two distinct branches being formed by the members of *Iflaviridae*. They show 40% nt overall identity and a 27% similarity at aa level. SeIV-1 is most closely related to IFV as well as to a putative *Spodoptera frugiperda* iflavivirus, for which only EST with similarity to picorna-like virus is available in the GenBank. SeIV-2 is closely related to lepidoptera-infecting viruses such as *Ectropis obliqua virus* (EoV) and *Perina nuda virus* (PnV), and according to its sequence homology it could be considered a different isolate from the recently characterized SeIV-2 isolate from Korea (31). Helicase and RdRp phylogenies show that iflaviruses form two independent branches and SeIV-1 and SeIV-2 belong to separate branches.

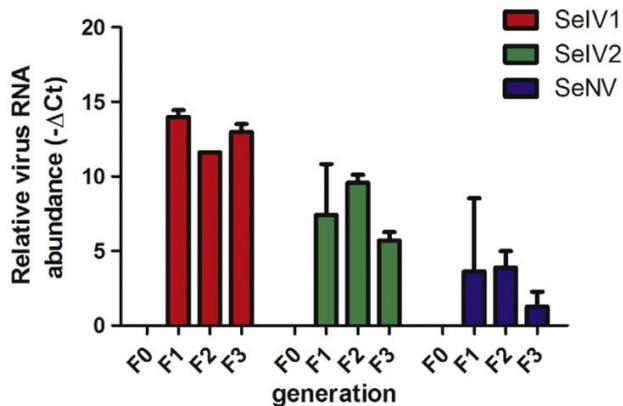
SeNV based on its sequence does not belong to any established virus family and it neither belongs to the order *Picornavirales* as defined by Le Gall et al. (3). It is closely related to the *Drosophila Nora virus* and *Nasonia vitripennis virus 3* (Figure 2). These three viruses possibly form a new viral family in the order *Picornavirales*.



**Figure 2:** Phylogenetic trees based on Bayesian analysis of the conserved amino acid sequences containing domains I to VIII of the putative RdRp, and A to C of helicase from different members of the order *Picornavirales*. Amino acid sequences were aligned using COBALT and PRALINE software. Phylogenetic relationships were reconstructed using BEAST software with a Dayhoff + G1 model. Posterior probability of at least 0.5 is indicated in the branches. The scale bar indicates an evolutionary distance of 0.2 amino acid substitutions per position in the sequence. The order of the host species is shown between brackets (Hy: Hymenoptera, He: Heteroptera, D: Diptera, L: Lepidoptera, Or: Orthoptera). GenBank accession numbers of the sequences employed in the analysis are: Aphid lethal paralysis virus (AF536531.1), Black queen cell virus (AF183905.1), *Brevicoryne brassicae* picorna-like virus (EF517277.1), Deformed wing virus (AJ489744.2), *Drosophila* C virus (AF014388.1), *Ectropis obliqua* picorna-like virus (AY365064.1), Foot-and-mouth disease virus (AAF09193.1), *Glossina morsitans* virus-like (EZ407281.1), *Himetobi P* virus (AB017037.1), *Infectious flacherie* virus (AB000906.1), *Israeli acute paralysis virus* (EF219380.1), Kakugo virus (AB070959.1), *Lygus lineolaris* virus (JF720348.1), *Nasonia vitripennis* virus 1 (FJ790486.1), *Nasonia vitripennis* virus 2 (FJ790487.1), *Nasonia vitripennis* virus 3 (FJ790488.1), Nora virus (GQ257737.1), *Perina nuda* virus (AF323747.1), *Rhopalosiphum padi* virus (AF022937.1), Sacbrood virus (AF092924.1), Slow bee paralysis virus (EU035616.1), *Spodoptera exigua* iflavirus 1 (JN091707.1), *Spodoptera exigua* iflavirus 2 isolate Korea (JN870848.1), *Spodoptera exigua* iflavirus 2 isolate Spain (KJ186788), *Spodoptera exigua* Nora virus (KJ186789), *Spodoptera littoralis* EST (FQ021401.1), *Varroa destructor* virus 1 (AY251269.2).

### 3.3 RNA viruses' horizontal transmission in virus-free insects

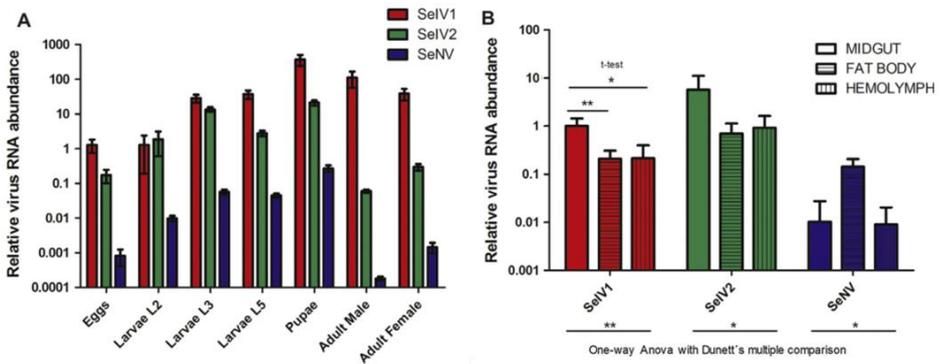
We have tested viral horizontal transmission in virus-free insects from the colony Xen-R. First, Xen-R colony was confirmed virus-free by RT-qPCR, and subsequently L2 larvae from this colony have been placed in the insect chamber containing *S. exigua* RNA viruses-positive colonies. Already in the first generation (F1) after being placed in the virus-positive insect chamber, the insect from Xen-R colony showed presence of the three RNA viruses (Figure 3).



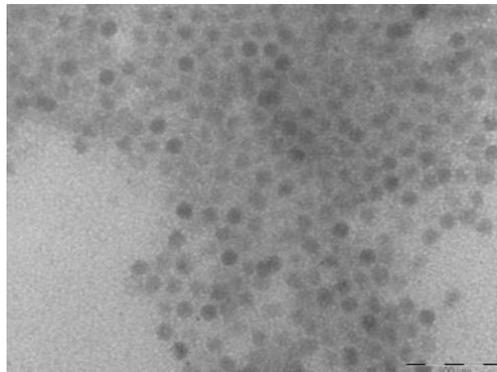
**Figure 3:** Relative abundance of *S. exigua* picorna-like viral RNA in four generations (F0–F3). Virus-free generation F0 was introduced to the insect chamber together with insects carrying picorna-like viruses.

### 3.4 Viral distribution in different developmental stages and tissues of *S. exigua*

Presence and relative abundance of the *S. exigua* RNA viruses was estimated by RT-qPCR detection of viral RNA in the eggs, larvae, pupae and adults of *S. exigua*. All three viruses were detected in every developmental stage examined, however with different ratios (Figure 4A). Surprisingly to us the viruses were most abundant in pupae stage. The increase in virus load is observed with the increase of insect developmental stage, being indicative of viral replication.



**Figure 4:** Relative abundance of *S. exigua* picorna-like viral RNA in different developmental stages and tissues assessed by RT-qPCR analysis: (A) in *S. exigua* eggs, larvae, pupae and adults. SelV-1 expression in eggs was set to 1 and the rest of samples compared to the expression level in this sample. (B) In midgut (MG), fat body (FB) and haemolymph (H) assessed by RT-qPCR analysis. SelV-1 expression in MG was set to 1 and all the rest of samples compared to the expression level in this sample. RT-qPCR primers were previously checked for efficiency to enable quantitative comparison between viruses, and they were found similar. Expression levels of each virus in each tissue were tested by t-test (each pair) and with one way ANOVA (each virus). \**p*-value < 0.05, \*\**p*-value < 0.01.



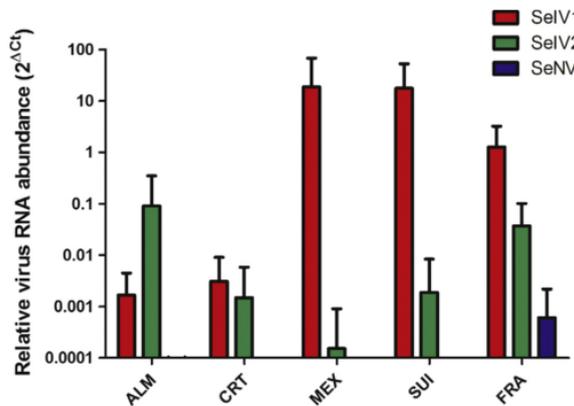
**Figure 5:** Electron micrograph of negatively stained *S. exigua* picorna-like virus particles purified by CsCl gradient centrifugation. The scale bar indicates 100 nm.

RNA viruses were also detected in three larval tissues; midgut, fat body and hemocytes. All three viruses were detected in all tested tissues (Figure 4B). SelV-1 and SelV-2 showed a very similar tropism. As previously described, SelV-1 was mainly present in the larval midgut. Similarly, SelV-2 showed tendency of midgut tropism. In contrast to the two iflaviruses, no clear enrichment of SelNV was

detected in any of the tested tissues, which suggests its homogeneous distribution in the larval body. Comparable efficiencies of the three primer sets used for the detection of the RNA viruses in RT-qPCR allowed us an estimation of the relative abundance of the viruses in the studied tissues. Overall abundance was similar for SeIV-1 and SeIV-2 and radically lower for SeNV.

### 3.5 Virion structure

*S. exigua* RNA virus particles migrated to the density of 1.3 g/ml in the gradient centrifugation. During the studies development, all examined insects were positive for at least two of the three reported RNA viruses and we were not able to separate the three viruses in our centrifugation conditions. Electron microscopy observation of the negatively stained purified viruses revealed structures of 25–30 nm of the diameter (Figure 5) which likely represent a mixture of the three RNA viruses.



**Figure 6:** Abundance of *S. exigua* picorna-like viral RNA in larvae from different laboratory populations.

### 3.6 Abundance of the RNA viruses in *S. exigua* laboratory colonies

Given the high variability in virus abundance found among the different samples, we decided to estimate RNA viruses' abundance *S. exigua* from four geographically distant colonies. In all the studied colonies SeIV-1 and SeIV-2 were in strong majority to SeNV, and were always detected together, however in distinct

proportions (Figure 6). MEX and SUI colonies showed higher abundance of SeIV-1 than SeIV-2. ALM showed higher prevalence of SeIV-2 (100-fold) compared to SeIV-1. CRT colony had similar quantities of both iflaviruses. Only small quantities of SeNV detected in few *S. exigua* insects suggest a low general prevalence of this virus in *S. exigua* populations.

#### 4. DISCUSSION

RNA viruses comprise a large group of infectious viruses inducing diseases in plants, vertebrates and invertebrates. The advent of genomics has accelerated novel virus discoveries in all type of hosts, including insects (32, 33). Here we present a viral complex composed of three small RNA viruses that simultaneously infect larvae from the pest *S. exigua*.

Sequences with homology to the three RNA viruses were identified in the *S. exigua* transcriptome (10). SeIV-1 has been previously described (21). All of the identified viruses are small RNA viruses. Two of them, SeIV-1 and SeIV-2, belong to the order *Picornavirales* (3) and clearly fall within the family *Iflaviridae*, as deduced from the phylogenetic analyses of two conserved functional domains, helicase and RdRp, as well as from the genome organization. There is so far only one genus, *Iflavirus* within the family *Iflaviridae*, comprised of seven virus species according to the last ICTV report: type species Infectious flacherie virus (IFV), Deformed wing virus (DWV), *Ectropis obliqua* virus (EoV), *Perina nuda* virus (PnV), Sacbrood virus (SBV), Slow bee paralysis virus (SBPV) and *Varroa destructor* virus 1 (VDV-1) (30) and few more iflavirus-like genome sequences characterized up to date. Our phylogenetic analysis has revealed that the members of *Iflaviridae* family form two main phylogenetic branches and each branch contains a different virus infecting *S. exigua*. We believe that the outburst of new iflavirus sequences will result in creating new genera within the *Iflaviridae* family including Lepidoptera-infecting iflaviruses. According to our results, there are two well separated groups within *Iflaviridae*; the viruses that group with type species IFV, and a second group including to date only three viruses, SeIV-2, EoV and PnV (Figure 2).

SeNV differs radically from SeIV-1 and SeIV-2. It has a ss(+)RNA polycistronic genome with the homology to *Drosophila* Nora virus genome. Despite

of this homology, SeNV has one additional ORF in comparison to *Drosophila* Nora virus (ORF 5). ORF 2 encodes picorna-like virus core of functional proteins: helicase, protease and RdRp. ORFs 4 and 5 are homologous to ORF 4 from *Drosophila* Nora virus, and encode capsid proteins. Similarly, ORF 3 encodes most likely a capsid protein, which in *Drosophila* Nora virus is necessary for fecal–oral transmission (34). ORF 1 in *Drosophila* Nora virus was shown to suppress RNAi (20). It is very likely that ORF 1 will hold similar function in SeNV. As in *Drosophila* Nora virus some ORFs overlap (ORF2, ORF3, ORF4 and ORF5), suggesting a ribosomal frameshifting during translation (35). In contrast to *Drosophila* Nora virus, which contains 85 nt – long non-coding RNA between ORF3 and ORF4 (possible IRES), the SeNV genome lacks non-coding RNA. Viruses with similarity to *Drosophila* Nora virus are taxonomically unassigned. A third virus that belongs to the same branch is *Nasonia vitripennis* virus 3 (9). According to the phylogenetic analyses these three viruses will most likely define a new family of invertebrate viruses within the order *Picornavirales*, independent from the *Dicistroviridae* and *Iflaviridae* families.

We have never observed any clear pathology attributed to the infections with *S. exigua* RNA viruses. Iflaviruses show a broad spectrum of pathological effects on their host: from non-symptomatic infections, such as *Nasonia vitripennis* virus 1 and 2, to fatal infections such as sacbrood disease (36). The *Iflavirus* type-species, IFV was shown to attacks goblet cells of the insect midgut, which fall off to the midgut lumen (37). A disease called flacherie in *Bombyx mori* is however often caused by mix infections of IFV with densoviruses, and it is thus hard to attribute symptoms to a particular virus (38). Though SeIV-1 is closely related to IFV their pathologies seem to differ considerably, since we have not found any visible symptoms of SeIV-1 infection. It was demonstrated for DWV that a titer of  $10^{10}$ – $10^{12}$  in mites is needed to cause overt infection in bees and mites while titers lower than  $10^8$  would cause asymptomatic infections (39). Currently we do not know if pathogenic symptoms were not observed because in our conditions *S. exigua* iflaviruses never reached a threshold titer high enough to lead to an acute infection with clear pathological symptoms or if those viruses are simply persistent commensals of *S. exigua* larvae.

Persistent infections are common in the viral world and many mechanisms of persistence have been proposed (40). Recently, in the field of

insect virology, studies on *Drosophila* Nora virus revealed that the persistence was achieved by dual action of cellular reverse transcriptase and RNAi pathway (12). In our case, we observe persistent infection of a complex of picorna-like viruses in *S. exigua* colonies. The impact of persistent infections on the fitness of the insects may have different outcomes. Persistent infection overcomes and weakens host immune defences (11), which may facilitate establishment of a different pathogen, causing disease or death of the host, as observed in PnV and PenuNPV co-infections. The effects of *S. exigua* persistent infection with RNA viruses on its fitness and susceptibility to other pathogens still have to be addressed.

Testing for the presence and abundance of the RNA viruses in the different tissues showed that iflaviruses are more abundant in the gut tissue, whereas SeNV does not show any specific tissue tropism. While higher abundance of iflaviruses in the gut is in concordance with the other iflaviruses tissue distribution, the homogenous distribution of SeNV or even slight dominance in the fat body tissue is in contrast to distribution of *Drosophila* Nora virus in fly tissues. Habayeb et al. (19) found the majority of the Nora virus in the adult's gut and almost no virus in the fat body. These differences in the tissue distribution may be due to using different life stage (larval vs adult), but also different insect order and virus species itself.

We have detected all three RNA viruses in all stages of *S. exigua*, eggs, larvae, pupae and adults in the laboratory colonies. Viruses were detected in surface-sterilized eggs, which suggest transovarian vertical transmission. In the natural conditions iflavirus prevalence was at the level of 10% in the *S. exigua* adults (41). Interestingly, the prevalence in the offspring of field captured adults increased to 60%, suggesting that some unknown factors in the laboratory conditions may trigger the replication of iflaviruses. We have shown also a very rapid transmission of *S. exigua* picorna-like viruses, in contrary to a common opinion that viruses causing persistent infections show poor horizontal transmission (42).

Invertebrate hosts are often infected with two or more viruses belonging to the same or distinct families (38, 43, 44). Co-existing pathogens may have synergistic or additive effect on each other (45-47); or in contrary, antagonistic effects may occur, like in *Helicoverpa zea* larvae carrying microsporidia and infected with HzSNPV (48). Here we report a co-infection of *S. exigua* larvae with

three RNA viruses. Interestingly, *S. exigua* viral complex composition resembles the one from the *Hymenoptera Nasonia vitripennis*, also composed of two iflaviruses and one Nora-like virus (9). Similarly to *N. vitripennis* viral complex, *S. exigua* viruses cause no apparent symptoms in the infected larvae. With the increasing number of novel RNA viruses that are being discovered in insects, it would be interesting to determine if such combinations occur randomly or they result from virus complementation or synergism.

As stated by Christian and Scotti (49), the ultimate aim in the insect virology field is development of new control agents. Although the lack of clear pathological effect associated to the RNA viruses described here discards them as main active agent for the development of new biological control agents, our results indicate that they may play an important role in the ecology of the host. RNA viruses may for example synergize the effects of other viral or bacterial entomopathogens, as shown for silkworm co-infections (50). On the other hand, it has been suggested that the picorna-like viruses can have an antagonistic effect on the systemic infection of baculovirus (51). Evaluating the consequences of covert RNA virus infections in insects is therefore crucial for designing successful control strategies of pests. Our future studies will focus on assessing the virulence of RNA viruses infecting *S. exigua*, as well as their influence on the other pathogens' infection outcomes. Since it has been suggested that RNA viruses may influence insect fitness and physiology, as well as their immune status, further research on RNA viruses – *S. exigua* interactions should shed light on the mechanisms of maintaining persistence in the insects and the impact of persistent infections on control strategies of insect pests.

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## CHAPTER III

# Iflavirus increases its infectivity and physical stability in association with baculovirus

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### ABSTRACT

Virus transmission and the prevalence of infection depend on multiple factors, including the interaction with other viral pathogens infecting the same host. In this study, active replication of an iflavirus, *Spodoptera exigua* iflavirus 1 (order *Picornavirales*) was observed in the offspring of insects that survived following inoculation with a pathogenic baculovirus, *Spodoptera exigua* multiple nucleopolyhedrovirus. Tracking the origin of the iflavirus suggested the association of this virus with the occlusion bodies of the baculovirus. Here we investigated the effect of this association on the stability and infectivity of both viruses. A reduction in baculovirus pathogenicity, without affecting its infectivity and productivity, was observed when associated with the iflavirus. In contrast, viral association increased the infectivity of the iflavirus and its resistance to ultraviolet radiation and high temperature, two of the main factors affecting virus stability in the field. In addition, electron microscopy analysis revealed the presence of particles resembling iflavirus virions inside the occlusion bodies of the baculovirus, suggesting the possible co-occlusion of both viruses. Results reported here are indicative of facultative phoresis of a virus and suggest that virus–virus interactions may be more common than currently recognized, and may be influential in the ecology of baculovirus and host populations and in consequence in the use of baculoviruses as biological insecticides.

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## 1. INTRODUCTION

Interactions between different viruses are often an inevitable consequence of multiple infection of a given host (1). Multiple infections are usually the result of consecutive infections by different viruses in the same host (known as super-infection), although simultaneous infections with two different viruses (known as co-infection) have also been described (2-4). Virus–virus interactions occur at one of three levels: (i) direct interactions of viral genes or products, (ii) environmental interactions due to alterations of the host environment as consequence of the infection, and (iii) immunological interactions that result from the interaction with the host immune system (5). Independently of the type of interaction, the effect of the virus–virus interaction on viral fitness can generate a broad range of outcomes. For example, virus–virus interactions can have a positive effect on the fitness of both viruses (6), or result in mutual exclusion (3). In contrast, non-autonomous viruses such as satellite viruses and virophages, occupy an intermediate position, as they depend on other viruses for replication and transmission, but both have negative effects on the production of their host viruses (7, 8).

Baculoviruses are large DNA viruses that infect invertebrates, particularly insects of the order Lepidoptera (9). Viruses of the genus *Alphabaculovirus*, i.e., nucleopolyhedroviruses of Lepidoptera, are used worldwide as the basis for biological insecticides and as expression vectors for the production of recombinant proteins (10, 11). The baculovirus replication cycle involves two types of virions: occlusion derived virions (ODV) are responsible for the establishment of the primary infection in insect midgut cells, whereas budded virus (BV) is responsible for the systemic spread of infection within the insect. ODVs are embedded in a polyhedral occlusion body (OB), formed mainly by polyhedrin, that is responsible for protecting ODVs in the environment. During the infection process, the OBs dissolve in the gut of the insect host with the subsequent release of the ODVs that fuse to the midgut epithelial cell membranes and release nucleocapsids that are transported to the nucleus, starting the infective process (12).

Iflaviruses (family *Iflaviridae*, genus *Iflavivirus*) are positive single-stranded RNA viruses that exclusively infect arthropods (13). Most iflaviruses produce

inapparent sublethal infections in insect hosts, although some of these viruses can result in lethal infections in silkworms (14) and honeybees (15). The number of described iflaviruses is relatively low, probably due to the lack of severe effects produced by most of these viruses (16). Only recently, the application of massive parallel sequencing methods has revealed, through the occurrence of expression sequence tags (ESTs) with homology to iflaviruses, the existence of new members of this family that were previously undetected (17-19). The *Spodoptera exigua* iflavirus 1 (SeIV-1) was the first iflavirus described in *S. exigua*. SeIV-1 has a genome of about 10 kb that codes for a 3,222 amino acid polyprotein that, following proteinase processing, results in the structural and nonstructural viral peptides (20). Despite its high infectivity, specificity and ability to replicate in *S. exigua* larvae, no clear effects on host fitness have been detected in insects infected with this virus (20, 21).

In the present study, we report that caterpillars (larvae) of the beet armyworm, *S. exigua*, the offspring of insects that survived following inoculation with an isolate of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV), had acquired a persistent sublethal infection with SeIV-1. This serendipitous result was obtained during a microarray study on the expression of insect and viral genes in insects that had survived inoculation with baculovirus. Contrary to expectations, we did not detect persistent baculovirus infection, but instead observed high expression of iflavirus genes in these larvae. The source of the iflavirus infection was traced back to the SeMNPV OB inoculum. In the present study, we present evidence that this association between the viruses results in increased environmental persistence and transmission opportunities for the iflavirus and a reduction in the pathogenicity of the baculovirus. Subsequent studies revealed that mixed infections may result from co-infection with particles of both viruses physically associated with each other.

## 2. MATERIAL AND METHODS

### 2.1 Insects and viruses

A virus-free *S. exigua* colony (SUI) was obtained from Andermatt Biocontrol AG (Grossdietwil, Switzerland) and reared in continuous culture at a constant temperature ( $25 \pm 1$  °C), relative humidity (RH;  $50\% \pm 5\%$ ), and

photoperiod (16-h/8-h light-dark cycle) on artificial diet (22) in the insectary facilities of the Universidad Pública de Navarra (UPNa, Pamplona, Spain). Insects from the UPNa colony were used to start a sister colony reared at Universitat de Valencia (UV, Valencia, Spain) using similar conditions. Insects were routinely tested for the presence of SeIV-1 and SeMNPV, and confirmed to be virus-free ahead and during each experiment.

SeIV-1 was detected and isolated from the laboratory colony of *S. exigua* (20). The SeMNPV isolate used for the establishment of covert infections was originally isolated from spontaneous infections observed in the laboratory-reared offspring of field-caught *S. exigua* females (23). For this study, a fresh stock of SeMNPV OBs was amplified *in vivo* by droplet-feeding *S. exigua* fourth instar larvae. OBs were purified from cadavers by washing in 0.01% SDS twice, once in double-distilled water and finally diluted in sterile double-distilled water. OB suspension was stored at  $-20\text{ }^{\circ}\text{C}$  until required. OBs were quantified by counting in triplicate with a Neubauer chamber.

A SeIV-1-free isolate of SeMNPV was obtained after PCR screening of the UPNa baculovirus collection. The Spanish isolate SeMNPV-SP2 was found to be negative for the presence of SeIV-1 and was selected for subsequent studies. This isolate was originally obtained from a group of cadavers collected during a baculovirus epizootic in greenhouse crops in southern Spain (24, 25). OBs had been maintained at  $-20\text{ }^{\circ}\text{C}$  since 1996.

OBs from the SeMNPV-SP2 isolate associated with SeIV-1 (SeMNPV-SeIV-1) and SeIV-free OBs (SeMNPV) were produced for biological comparison. For this, groups of 25 newly molted fourth instars were allowed to drink from a suspension of  $10^4$  OBs/ml in a solution of 10% (w/v) sucrose and 0.001% (w/v) food dye. A second batch of 25 larvae was fed with 20  $\mu\text{l}$  of an identical OB concentration mixed with 80  $\mu\text{l}$  of  $1.34 \times 10^{-1}$  ng/ $\mu\text{l}$  SeIV-1 particles ( $\sim 1.46 \times 10^7$  SeIV-1 genomes/ $\mu\text{l}$ ). Larvae were reared individually until death or pupation. OBs were purified from cadavers and tested for SeIV-1 by RT-qPCR as described below. The SeIV-1 load present in OBs produced by this method was estimated at  $18.9 \pm 2.3$  SeIV-1 genomes per OB.

## 2.2 Infection with baculovirus

To infect *S. exigua* individuals with SeMNPV, larvae from a virus-free laboratory colony were challenged per os with an estimated 50% lethal concentration of OBs (23). Briefly, a batch of 30 pre-molt *S. exigua* third instars were starved overnight, allowed to molt to the fourth instar and then allowed to drink an OB suspension containing  $9 \times 10^3$  OBs/ml during a 10-min period (26). Control insects (VF) consumed droplets that did not contain OBs. Inoculated larvae were individually placed in 25-ml plastic cups perforated for ventilation and provided with artificial diet. OB-challenged (VT) larvae that did not succumb to polyhedrosis disease were reared through to pupation at  $25 \pm 1$  °C and  $50\% \pm 5\%$  RH. According to previous experiments (23), these larvae were expected to carry a persistent baculovirus infection as the experiment was originally planned to examine the expression of baculovirus and insect genes in persistently infected larvae. We were, however, unable to confirm establishment of the persistent infection as originally planned, which led us to investigate the relationship between SeIV-1 and SeMNPV. Pupae were sexed and, once adults emerged, one male–female pair of adults was placed in a paper bag, and allowed to mate and oviposit to obtain the subsequent generation (F1). An identical procedure was applied to mock-inoculated insects that were defined as control samples. Egg masses were collected and placed in 300-ml plastic containers provided with artificial diet until larvae reached the second instar. From these containers 25 larvae were individualized and reared through to the fifth instar as described above. All procedures were performed in triplicate. Four F1 fifth-instar larvae per replicate were randomly selected and pooled for RNA extraction and microarray analysis. For each sample (VT and VF), three different pools were obtained and used for subsequent gene expression analysis.

## 2.3 Microarray design, hybridization and analysis

A 44K Agilent oligonucleotide microarray was designed to study different aspects of the interaction of *S. exigua* larvae with viral (iflavirus and baculovirus) and bacterial pathogens (27, 28). In that sense, the array mainly comprised probes representing unigenes from the *S. exigua* transcriptome (18, 27), but also included 60-mer non-overlapping tiling probes covering both strands of the SeIV-1 and SeMNPV genomes. In total, the microarray contained 167 probes covering the

positive strand of SeIV-1 and 166 probes covering the negative strand. The SeMNPV genome was represented by 2,260 tiling probes covering both virus strands. The microarray was also used to determine the expression levels of 139 open reading frames (ORFs) predicted for the SeMNPV genome (29). Two different 60-mer probes were included for each of the predicted ORFs. The probes were designed using the eArray application from Agilent.

Synchronized F1-larvae from the VT and VF groups were collected and total RNA was extracted using RNeasy reagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's protocol. To further purify RNA, an RNeasy Kit (Qiagen, Hilden) was used following the protocol provided by the manufacturer. The quality of RNA was assessed by Agilent 2100 Bioanalyzer using the EukaryoteTotal RNA Nano protocol.

Agilent One-Color Spike-in Mix was added and 600 ng of total RNA was used for cRNA (complementary RNA) synthesis. The obtained cRNA (1.65 µg) was fluorescently labeled with cyanine-3-CTP, fragmented and hybridized to *S. exigua* microarray slides following the One-Color Microarray-Based Gene Expression Analysis (Quick-Amp labelling) protocol. Microarrays were scanned using G2505B Agilent scanner and data were extracted using Agilent Feature Extraction 9.5.1 software. Spike-in transcripts are a mix of unique 55-mer probes that specifically anneal to complementary control probes on the Agilent's microarrays and were used for linear normalization performed by the Agilent Extraction 9.5.1 software. Before data analysis, hybridization quality control reports were verified as correct. RNA labelling and hybridization, as well as array scanning and data extraction were performed by the Microarray Analysis Service of Principe Felipe Research Centre (CIPF, Valencia, Spain) following standard protocols.

Data analysis was performed using Babelomics 4.3 software (<http://babelomics.bioinfo.cipf.es/>) (30). First, between-arrays normalization was performed using the quartile normalization method in Babelomics (31). Normalized arrays of the VT samples were compared to VF controls and expressed as a fold-change in gene expression or abundance. For those probes having low signal levels for one of the samples (as indicative of absence in one of the samples), fold-changes in gene expression were estimated by comparison with the overall background intensity.

## 2.4 SeIV-1 detection by RT-PCR

RT-qPCR was used to detect SeIV-1 genomic RNA in the purified preparations of SeMNPV and SeIV-1. SeMNPV occlusion bodies (OBs) as well as SeIV were purified on discontinuous sucrose gradients as described below. RNA was extracted from the samples using Tripure reagent (Roche), according to the manufacturer's protocol. RNase-free glycogen (5 µg/µl) was added as a carrier during the RNA precipitation step. Purified RNA was used for cDNA synthesis using PrimeScript RT reagent kit from Takara Bio Inc. (Otsu Shiga, Japan) following the manufacturer's protocol. RT-qPCR was carried out in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). All reactions were performed using HOT FIREPOL EvaGreen qPCR mix Plus (ROX) from Solis BioDyne (Tartu, Estonia), in a total reaction volume of 25 µl. Forward and reverse primers, designed using Primer Express software (Applied Biosystems, Foster City, CA), were added to a final concentration of 0.3 µM. These specific primers were designed to amplify a 97-bp fragment in the RNA-dependent RNA polymerase (RdRp) region from 9,743 - 9,840 nt on the genome (Forward: 5'-TGTGAAGTTAGACACGCATGGAA-3' and Reverse: 5'-CGACTTGTGCTACTCTCTTCATCAA-3'). For relative quantification of virus genomes, Ct values from the RT-qPCR were compared to the standard curves obtained for known number of copies of virus genome fragment cloned in the pGEMTeasy vector. A fragment of SeIV-1 genome was cloned into pGEMTeasy vector and the standard curve prepared from the serial dilutions of known copies of the vector DNA.

Semiquantitative RT-PCR was used to detect the negative RNA strand of SeIV-1 in larvae as well as in SeMNPV OBs. Tagged primer was used for the specific synthesis of cDNA due to the occurrence of self-priming, often observed for RNA viruses. RNA was extracted as described above. For this, 0.5 µg of RNA were used for cDNA synthesis using tagged specific primer (5' - ggatgcaggctacgtgaagatacgtgtcaacaacagaccctagcg-3', tag in lowercase, SeIV-1 specific sequence in uppercase). cDNA synthesis was performed using PrimeScript RT reagent kit from Takara Bio Inc (Otsu Shiga, Japan) following the manufacturer's protocol, at 42 °C for 30 min. 2 µl were used for subsequent PCR reaction using the following primers: forward 5' -

ggatgcaggctacgtgaagatacg-3' and reverse 5' - gcagccatgttcaacctc-3', and the following conditions: 94 °C for 5 min, annealing at 55 °C and elongation at 72 °C for 30 cycles. The resulting PCR product had a size of 1,495 bp.

## 2.5 Virus purification by gradient centrifugation

For electron microscopy, OBs from SeMNPV and SelV-1-associated SeMNPV samples were additionally purified through sucrose gradients (32). Briefly, 3 ml of  $\sim 10^8$  OB/ml suspensions were loaded onto a 30 – 60% (w/w) continuous sucrose gradient and then centrifuged at  $40,000 \times g$  for 1 h at 4 °C. The OB band was harvested by puncturing the tube with a needle and collecting the sucrose fraction in a syringe. OBs in sucrose were diluted in 2 vol. of  $1\times$  TE buffer and centrifuged at  $40,000 \times g$  for 1 h. OBs collected in the pellet were suspended in sterile double-distilled water.

SelV-1 particles were purified from *S. exigua* larvae as follows. Approximately one hundred fourth and fifth instar larvae were freeze-killed and lyophilized. The lyophilized larvae were homogenized in 0.01 M potassium phosphate pH 7.4 containing 0.45% diethyldithiocarbamic acid (DIECA) and 0.2%  $\beta$ -mercaptoethanol (2 – 5 ml of buffer per gram of larvae). The homogenate was then sonicated for 20 s and subsequently filtered through a double layer of cheesecloth and then centrifuged for 15 min at  $5,000 \times g$  to remove large debris. The supernatant was centrifuged for 2.5 h at  $82,000 \times g$  (Beckman centrifuge, S28 rotor). The pellet was resuspended in 2 ml TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffer at pH 7.3 and left overnight at 4 °C. The suspension was then applied onto 20% sucrose in TAE buffer and centrifuged 2.5 h at  $100,000 \times g$  at 4 °C. The pellet was again resuspended in a small volume of TAE buffer and left overnight at 4 °C. Next, 10 - 40% discontinuous sucrose gradients were prepared and the sample was centrifuged for 2.5 h at  $100,000 \times g$ . SelV-1 purified particles were collected from the white virus fraction.

## 2.6 Determination of SelV-1 infectivity in larvae

In order to compare the ability of SelV-1 alone and associated with SeMNPV OBs to enter and replicate in host cells, virus-free *S. exigua* fourth instar larvae were starved overnight and then orally inoculated by allowing them to drink from an OB suspension of SeMNPV-SelV-1 containing  $1 \times 10^6$  OBs/ml by the

droplet-feeding method (26). Another batch of larvae was orally inoculated with the same amount of SeIV-1 particles that were estimated to be present in the SeMNPV-SeIV-1 OBs (estimated by RT-qPCR). Both SeMNPV-SeIV-1 OBs and SeIV-1 particles used in the experiment originated from two separate preparations. At 72 hpi larval midguts were dissected and SeIV-1 loads measured by RT-qPCR as described above. Ten larvae were infected with each virus preparation and ten larvae served as controls, to confirm that the SUI colony was virus-free, and that the insects were not contaminated during the experiment. Larvae were processed individually. Ct values of SeIV-1 were normalized to Cts for the ATP synthase reference gene (33), and  $-\Delta$  Ct values compared between larvae infected with SeIV-1 alone or associated with OBs. The resulting  $-\Delta$  Ct values were compared by Mann-Whitney test, due to the presence of outliers.

## 2.7 Determination of dose mortality response

The pathogenicity of SeMNPV OBs and SeMNPV-SeIV-1 OBs was determined by the droplet-feeding method (26). Briefly, groups of 30 newly molted second instars were starved overnight and orally inoculated with one of the following OB concentrations:  $2.54 \times 10^5$ ,  $8.18 \times 10^4$ ,  $2.72 \times 10^4$ ,  $9.09 \times 10^3$  and  $3.03 \times 10^3$  OBs/ml. This range of concentrations was estimated to kill between 95% and 5% of the experimental insects. Larvae that ingested droplets within 10 min were individually transferred to a 24-well tissue culture plate. A cohort of 24 larvae was allowed to drink from an OB-free suspension as controls. Larvae were reared on a semisynthetic diet at  $25 \pm 2$  °C and mortality was recorded daily for 7 days post-inoculation. The entire bioassay was performed three times. Data were subjected to Probit regression analysis using the Polo-PC program (Le Ora Software, 2002). Lethal concentration ( $LC_{50}$ ) values and relative potencies were estimated when a parallelism test confirmed that the regressions for each treatment could be fitted with a common slope (34).

## 2.8 Determination of OB production and virulence

Groups of 30 fourth instar larvae were starved overnight and allowed to drink for 10 min from an OB suspension containing  $5 \times 10^7$  OBs/ml of either SeMNPV/SeIV-1+ or SeMNPV/SeIV-1-. Individual weight measurements were first taken immediately before inoculation. Inoculated larvae were individualized in 24-

well plates containing diet and checked daily for virus-induced mortality. Larvae were monitored every eight hours for mortality and weighed daily during a six day post-inoculation period. Virus-killed larvae were frozen at  $-20\text{ }^{\circ}\text{C}$  to avoid liquefaction. Cadavers were individually homogenized in 1 ml sterile distilled water. Each homogenate was filtered through cheesecloth to remove debris and the resulting suspension was counted in triplicate in a Neubauer chamber at  $400\times$  magnification using a phase contrast microscope. The experiment was performed six times. OB production and weight gain data were not normally distributed, and were compared by Kruskal-Wallis or Mann-Whitney test (SPSS statistics V.21, 2012).

## **2.9 Effect of UV temperature treatment on stability of SeIV-1**

The effect of environmental factors such as UV radiation and high temperature on the stability of SeIV-1, alone or in association with OBs, was indirectly estimated by genome integrity measured by RT-qPCR. To this aim, two preparations of SeIV-1 (SeMNPV OBs containing SeIV-1 or SeIV-1 alone) were purified by sucrose gradient centrifugation and then exposed to different intensities of UV-C radiation or temperature for different periods of time. The amounts of SeIV-1 that remained viable for amplification were estimated by RT-qPCR.

For the UV irradiation treatment, each of the SeIV-1 preparations was exposed to 0, 3, 6, 9 and  $12\text{ J/cm}^2$  UV-C light using a crosslinker CL-1 (Herolab), at a wavelength of 254 nm. Samples of 200  $\mu\text{l}$  of each preparation were placed in 24-well plates, to achieve a suspension depth of  $\sim 1\text{ mm}$ , and were then exposed to continuous UV-C light 30 cm below the lamp, which allowed an exact dose to be administered to each sample. At each sample time, 300  $\mu\text{l}$  of Tripure was immediately added to 150  $\mu\text{l}$  of the sample, and the samples were frozen for further RNA purification. RNA was extracted and cDNA synthesized as described above. RT-qPCR was used to detect the presence and to estimate the number of SeIV-1 genomes in the samples. The experiment was performed twice and the data were analyzed by two-way ANOVA.

For the heat treatment, each SeIV-1 preparation was incubated at  $72\text{ }^{\circ}\text{C}$  for 0, 30, 60, 360 and 1,440 min in an Eppendorf thermomixer. As before, at each sample time 300  $\mu\text{l}$  of Tripure was immediately added to 150  $\mu\text{l}$  of the sample, and then frozen until RNA purification. RNA was extracted and cDNA synthesized as

described above. RT-qPCR was used to detect the presence and estimate the SeIV-1 load in each sample. The experiment was repeated twice and the data were analyzed by two-way ANOVA. Starting quantities of SeIV-1 genomes in all samples were normalized to 100%, and the decrease in estimated quantities at each sample time calculated accordingly.

## 2.10 Electron microscopy

Scanning electron microscopy (SEM) was used to examine the presence of SeIV-1 particles on the surface of OBs. For this, OBs in suspension were fixed overnight by mixing with an equal volume of fixative (4% formaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) and then washed twice with 0.1 M phosphate buffer. Samples were then partially dehydrated with 70% ethanol, dried, placed on aluminum mounts using carbon tags, sputter-coated with gold-palladium and photographed at magnifications of 6,000 $\times$  and 25,000 $\times$  using a scanning electron microscope (Philips SEM 550).

Transmission electron microscopy (TEM) was used to examine the polyhedrin matrix within OBs. For this, OBs in suspension were fixed for 2 h at 4 °C with 1.5% glutaraldehyde. The samples were then concentrated in 0.4% agar and washed with phosphate buffer (0.2 M, pH 7.3). Samples were post-fixed with 2% osmium tetroxide (OsO<sub>4</sub>) for 2 h, dehydrated and stained for 1 h with 2% uranyl acetate. The samples were then embedded in epoxy resin and polymerized for 48 h at 60 °C. After polymerization, samples were sectioned using an ultramicrotome (Leica UC6), transferred to TEM grids and stained with lead acetate. The resulting grids were observed under an electron transmission microscope of 100 kV (JEOL JEM 1010). Different fields of each sample were photographed at a magnification of 40,000 $\times$  and visualized with image acquisition software.

## 3. RESULTS

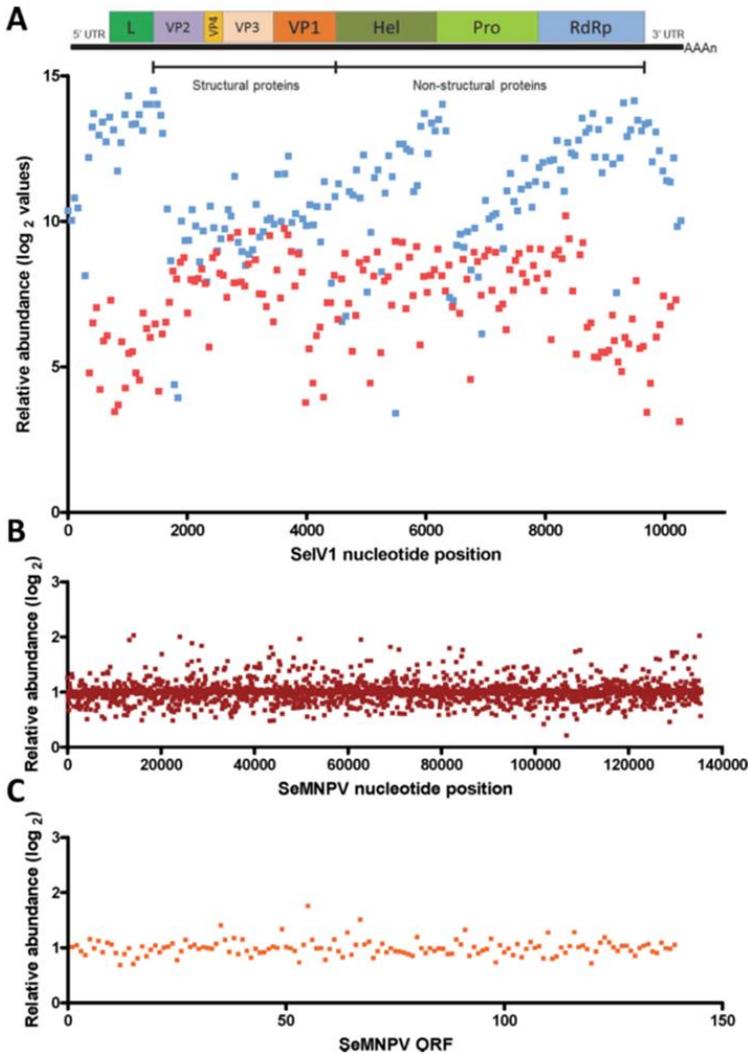
### 3.1 Detection of iflavirus in *S. exigua* larvae treated with baculovirus

Gene expression comparison between *S. exigua* larvae derived from insects previously inoculated with SeMNPV (VT), or virus-free larvae (VF), was performed using a custom-made DNA-microarray containing *S. exigua* unigene

probes; about 3,000 probes covering the complete genomes of SeMNPV and SeIV-1 (tiling probes), and the predicted ORFs from SeMNPV. Microarray comparison showed high differential expression of the probes representing the positive (average log<sub>2</sub> value of 11.1) as well as the intermediate (negative) strand (average log<sub>2</sub> values of 7.2) of the SeIV-1 genome (Figure 1A), indicating the presence and active replication of SeIV-1 in the offspring of insects that survived oral inoculation with SeMNPV OBs. In contrast, the presence of SeMNPV transcripts was not detected (ratio VT/VF equal to 1) in the VT insects (Figures 1B and 1C) which also indicates that the iflavirus was capable of autonomous replication in the absence of baculovirus transcription.

### **3.2 Influence of the virus association on iflavirus infectivity and baculovirus pathogenicity and virulence**

Semi-quantitative PCR as well as reverse transcription quantitative PCR (RT-qPCR) revealed the presence of SeIV-1 genomes in the SeMNPV OB preparation that had been purified after several centrifugation steps prior to being used to inoculate VT insects. Both positive and negative strand of the virus were detected, nevertheless the negative strand was present only in trace amounts when compared with its abundance in the larvae. This finding was indicative of a possible association between both viruses. We decided to explore the influence of such association on the insect–virus relationship of both types of virus. To determine whether iflavirus association with baculovirus favors iflavirus infectivity, the ability to establish an infection in host insects was estimated using iflavirus inoculum alone or associated with SeMNPV OBs. For this, SeIV-1-free insects were orally inoculated with a preparation of SeMNPV OBs containing SeIV-1 particles (quantified by RT-qPCR). In a side-by-side approach, a second batch of virus-free insects was inoculated with the same amount of SeIV-1 particles, quantified by qRT-PCR method described above, but in the absence of SeMNPV OBs. Three days after inoculation, insects were dissected and the abundance of SeIV-1 genomes in the midgut of the larvae, as an estimation of the SeIV-1 ability to establish a viral infection, was determined by RT-qPCR. Results revealed that the iflavirus was present in all inoculated insects, but a 2.8-fold difference in SeIV-1 load, indicated by a 1.5-fold difference in qPCR Ct values, was detected in



**Figure 1:** Abundance of viral sequences in the progeny of insects sublethally infected with SeMNPV, (virus treated, VT), measured by tiling array of the viral genomes. Values are the ratio of normalized intensity between VT and virus free (VF) samples for each of the studied probes. (A) Abundance of SelV-1 sequences and their position along the genome. Genome structure is represented at the top of the panel and aligned according to the position of the corresponding 60-mer probe. Each spot represents the relative abundance of sequences hybridizing to each probe. Abundance is reported as  $\log_2$  values, which means that a difference of 10 in  $\log_2$  values corresponds to a 1024-fold difference. Blue and red spots represent the abundance of sequences from the positive and the intermediate (negative) strand of the SelV-1, respectively. Electrophoresis panel reflects the detection of the negative strand of SelV-1 in larvae (L) and baculovirus OBs (OB) by semiquantitative RT-PCR. (B) Abundance of SeMNPV transcripts and their position along the genome. Each spot represents the relative abundance of sequences that hybridized to each 60-mer probe. (C) Expression of SeMNPV ORFs (GenBank, NC\_002169.1) in VT insects. Each ORF value represents the average of at least two probes.

insects inoculated with iflavirus associated with OBs than when inoculated alone (Figure 2A). Control insects were confirmed to be negative for both viruses.

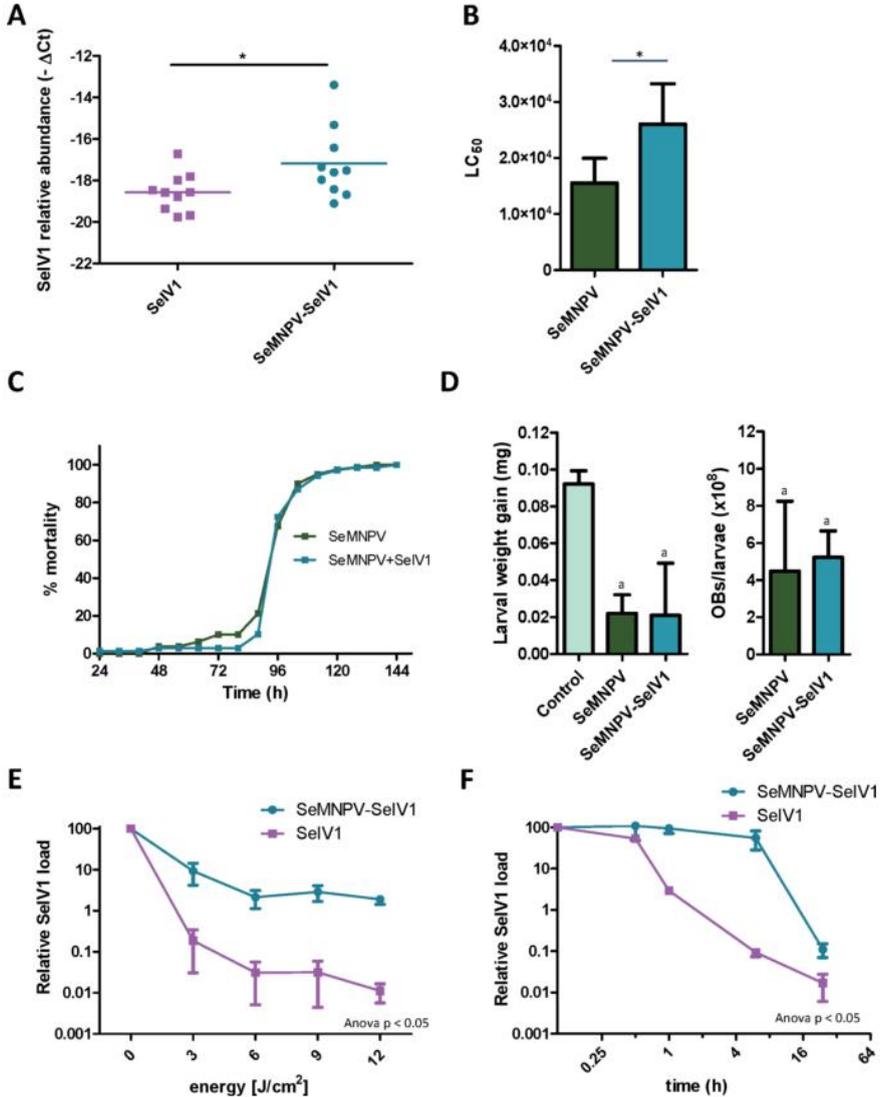
We also determined the effect of the viral association on the virulence and pathogenicity of SeMNPV OBs. In contrast to the results for iflavirus, the pathogenicity of SeMNPV OBs, estimated by peroral bioassay, was reduced by ~40% when iflavirus was associated with OBs compared to equal OB inoculum free from iflavirus (Figure 2B). However, the co-transmission of iflavirus and SeMNPV did not significantly affect the mean speed of kill of SeMNPV that varied between 98 and 102 h post-inoculation (Figure 2C). Similarly, the growth of infected host insects (Figure 2D-left), or the total OB production (Figure 2D-right), in insects inoculated by baculovirus OBs with iflavirus, did not differ significantly from that of insects inoculated with iflavirus-free OBs.

### **3.3 Influence of the virus association on iflavirus persistence in the environment**

Occlusion of baculovirus ODVs greatly improves the persistence of these virions in the environment following death of the insect host (35). If SeIV-1 is detected in association with the baculovirus occlusion body, it may be expected that such an association could contribute to increasing its stability in the environment. In agreement with such a hypothesis, experiments involving exposure to an ultraviolet light source (UV-C, 254 nm wavelength) or high temperature (72 °C) revealed that SeIV-1 particles associated with OBs maintained physical stability significantly better than naked particles. Following exposure to 3-12 J/cm<sup>2</sup> of UV-C radiation, the stability of occluded iflavirus particles, measured as the relative viral load, was approximately two orders of magnitude greater at each time point than that of naked particles (Figure 2E). Similarly, exposure of iflavirus particles to high temperature resulted in ~100-fold greater stability of OB-associated iflaviruses, following 1 or 6 h exposure, compared to naked particles (Figure 2F).

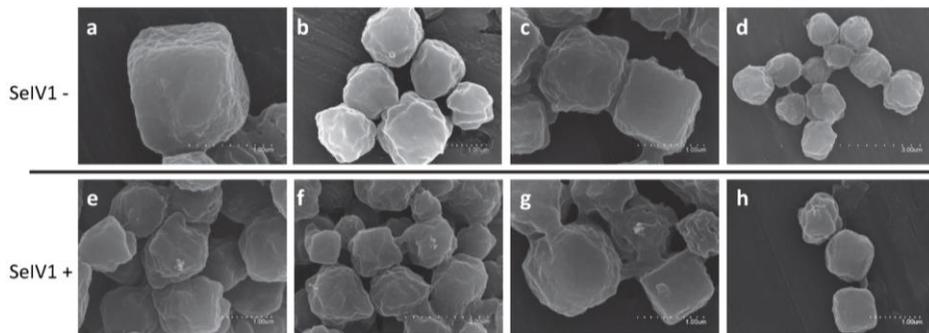
### **3.4 Detection of the iflavirus in baculovirus OBs**

RT-qPCR revealed the presence of SeIV-1 genomes in the SeMNPV OB preparation that had been purified by several centrifugation steps prior to being used to inoculate larvae. Moreover, the iflavirus particles present in the



**Figure 2:** Stability, replication and host growth effects of SelV-1 and SeMNPV in association or alone. (A) SelV-1 infection alone or associated with SeMNPV OBs after oral inoculation of *S. exigua* larvae. Asterisk indicates significant difference ( $P < 0.05$ ). (B) Pathogenicity of SeMNPV OBs alone and associated with iflavirus expressed in  $LC_{50}$ . Asterisk indicates significant difference ( $P < 0.05$ ). (C) Virulence of SeMNPV OBs alone and associated with iflavirus expressed as mean time to death (MTD). MTD values were estimated by Weibull survival analysis (36). Curves did not differ significantly ( $t$ -test,  $P = 0.08$ ). (D) Larval weight gain after oral inoculation with SeMNPV OBs alone or OBs in association with SelV-1 particles and SeMNPV ODVs (D-left). Production of OBs in *S. exigua* larvae infected with SeMNPV OBs alone or in association with SelV-1 particles (D-right). Means with the same letter are not significantly different ( $P > 0.05$ ). Relative SelV-1 load alone or associated with OBs when exposed to different doses of ultraviolet light (E) or periods of heating at 72 °C (F).

preparation were able to establish a persistent infection. This finding suggested that a physical association may exist between both viruses. The physical association between the viruses is likely to involve the localization of iflavirus particles inside or outside of OBs. To determine this, purified OBs from insects that died of SeMNPV infection in the presence of iflavirus (SeIV-1+), or the absence of iflavirus (SeIV-1-), were observed by scanning electron microscopy (SEM) (Figures 3A-3H). No differences were observed in the external appearance of each type of OB. In contrast, transmission electron microscopy (TEM) of the OBs revealed the presence of ODVs of baculovirus, each comprising 1–4 nucleocapsids (Figures 4A-4C), as well as dark spots dispersed in the matrix resembling in size and form (Figures 4D and 4E), the icosahedral particles of iflavirus embedded in the polyhedrin matrix (Figure 4F). To confirm this, the TEM procedure was repeated in an independent laboratory (Laboratory of Virology of Wageningen University, the Netherlands) to exclude the possibility of generating artifacts during sample preparation. Similarly, samples processed in the Netherlands laboratory showed the presence of dark spots resembling iflavirus particles in a certain number of OBs (Figures 4G and 4H).

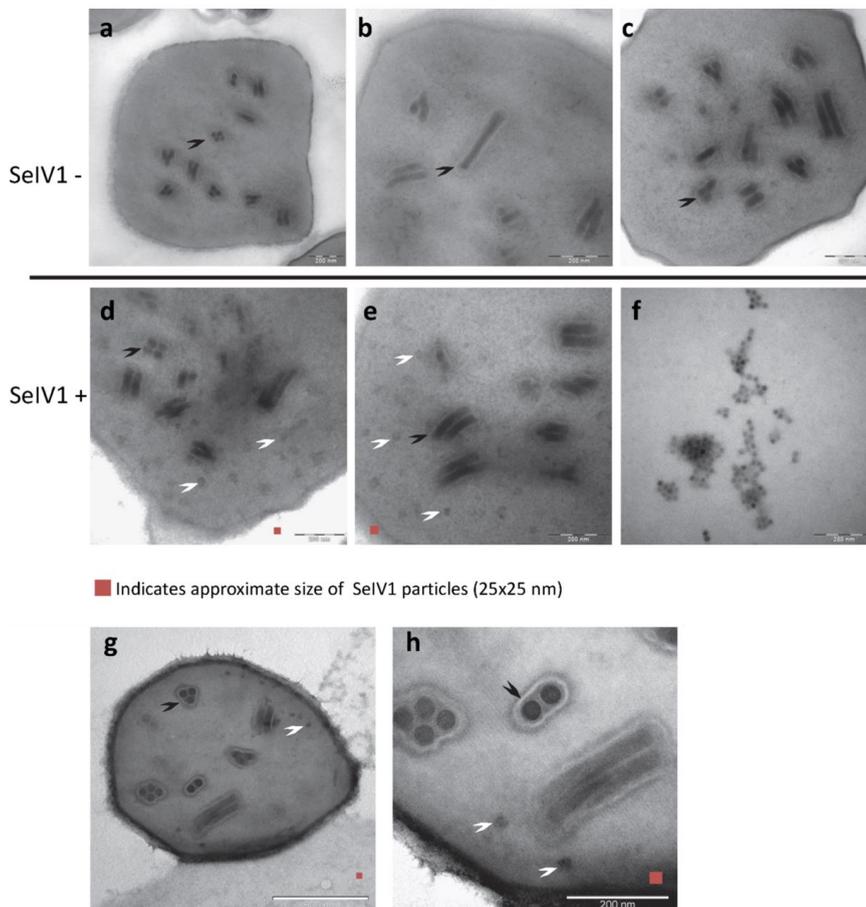


**Figure 3:** SEM images of purified SeMNPV OBs. Representative images from SeIV-1-free OBs (A–D) and SeIV-1-containing OBs (E–H).

#### 4. DISCUSSION

Traces of sequences from the SeIV-1 genome were first identified in the *S. exigua* larval transcriptome (18). Additional studies revealed that SeIV-1 can replicate, disperse through larval feces, and be horizontally transmitted with a very high efficiency but without clear pathological effects on the host (20). Horizontal

transmission in the field mainly depends on the prevalence of infected insects (as a source of inoculum), the density of susceptible hosts and on viral resistance to abiotic factors that could negatively impact the persistence of virus particles in the environment. In the present study, we obtained clear evidence that iflavivirus SeIV-1 was present in the baculovirus OBs preparation, even after extensive purification of the OBs by a series of sucrose gradient centrifugations. Moreover, this association resulted in the establishment of persistent iflavivirus infections in insects that consumed baculovirus OBs.



**Figure 4:** TEM images of purified OBs from SeMNPV and SeIV-1. Representative images from SeIV-1-free SeMNPV (A–C), SeIV-1-containing SeMNPV (D, E), and purified SeIV-1 particles (F). TEM was performed at the University of Valencia, Spain. In order to exclude the possibility of a methodological artifact, TEM of SeIV-1-containing SeMNPV was also performed at Wageningen University, The Netherlands (G, H). Some ODVs (black arrowhead) and SeIV-1-like particles (white arrowhead) are indicated. For size comparison, a red square of 25 nm per side is shown in each figure.

In certain situations, simultaneous infection with different species of baculovirus or baculovirus with other invertebrate viruses can increase the effectiveness of these pathogens as agents of biological control (37, 38). However, very few studies have focused on determining the effect of mixed infections on indicators of viral fitness. For example, mixed infection of the lepidopteran *Adoxophyes honmai* with a nucleopolyhedrovirus (AdhoNPV) and an entomopoxvirus (dsDNA virus) resulted in a reduction in the fitness of both viruses (39). In contrast, early studies on the interaction of an *Alphabaculovirus* (AcMNPV) with an unclassified RNA virus of *Trichoplusia ni* revealed that RNA virus-infected larvae had reduced growth compared to healthy insects, but with little or no significant effects on the pathogenicity or speed of kill of *Alphabaculovirus* OBs against host larvae (40). Another interaction has been recently described between viruses infecting *Helicoverpa armigera* (41). A clear negative interaction between *H. armigera* densovirus 1 (HaDNV1) and a baculovirus (HaSNPV), was observed in wild populations of this pest. Laboratory assays revealed that HaDNV1-infected insects were significantly more resistant to HaSNPV infection than non-infected insects, suggesting a mutualistic relationship between the host insect and the HaDNV1 virus (41). Similarly, a recent study on field-collected *S. exigua* insects detected simultaneous covert infections with SeMNPV and iflaviruses in around 10% of the captured adults, while about 40% of the insects were covertly infected with SeMNPV alone (42). These findings may suggest that a similar mutualistic interaction between *S. exigua* and the iflavirus exists in wild populations of this pest. In the present study, we found that the presence of the iflavirus was slightly detrimental to the baculovirus, as iflavirus-contaminated OBs were less pathogenic in healthy insects, compared to OBs that contained SeMNPV ODVs alone. In contrast, by physical association with the baculovirus, SeIV-1 particles appear capable of extending their survival in the environment and are more likely to infect the host when consumed by a susceptible insect. The last result has to be taken with caution, since the method of quantification of the IV from the two different matrices (purified IV and IV present in OBs) may be biased.

Results reported here demonstrate a possible facultative phoresis of one virus by another and suggest that the interaction of these viruses is relevant for the transmission and replication of both viruses. Iflaviruses have generally received little attention from invertebrate pathologists due to their low pathogenicity and

tendency to produce inapparent sublethal infections (20, 43). Co-infection of iflavirus with baculoviruses in field conditions has been reported recently (42). However, as the number of novel RNA viruses, including iflaviruses, increases rapidly through the use of massively parallel sequencing methods (17, 44-46) the discovery of novel interactions in natural virus populations is likely to grow accordingly. Our results suggest that virus–virus interactions may be more common than currently recognized, and may be influential in the ecology of baculovirus and host populations. In this respect attention has heavily focused on virus-pathogen interactions in honeybees due to growing concerns over colony collapse disorder (47). In another case of co-infecting microorganisms, persistent infection with *Wolbachia* has been reported to protect against infection by RNA viruses in dipterans (48), but increases mortality due to baculovirus infection in a lepidopteran (49).

In this study, although using similar conditions and viral concentrations as employed in previous experiments for the generation of persistent infections (23), we could not detect baculovirus transcription. It is possible that the presence of SeIV-1 in association with SeMNPV negatively affected the establishment of persistent infection by the baculovirus. The presence of iflavirus associated with the baculovirus is therefore likely to affect the dynamics of baculovirus transmission in natural *S. exigua* populations and could also affect the insecticidal properties of baculoviruses used as biological insecticides. Decreased pathogenicity as a result of the presence of iflavirus in association with OBs might also reduce the establishment of persistent baculovirus infections since fewer individuals are likely to become infected and viral dissemination will be limited. Moreover, these results also open the possibility of finding similar associations in other combinations of viruses of agricultural or medical importance.

In contrast to satellite and virophages, that are obligate parasites that need to be coinfecting with their counterpart viruses (50, 51), SeIV-1 is capable of acting as a facultative phoretic parasite that can exploit the OBs produced by the *Alphabaculovirus* to disperse and persist outside the host. In this sense, the *Alphabaculovirus* OB can act as a vector for iflavirus transmission. As natural populations of Lepidoptera can harbor iflaviruses in the absence of baculovirus infection, it is clear that the association is not obligatory. However, the association is clearly advantageous for the transmission of the iflavirus. Indeed, other

iflaviruses, namely *Ectropis obliqua* virus and *Perina nuda* virus, have been detected previously in mixed infections with an *Alphabaculovirus* in their respective hosts (43). Similarly, a small RNA virus was detected as a contaminant of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) preparations (52). However, in none of these cases was any physical association of the viruses determined. It is tempting to speculate that because SeIV-1 generally relies on vertical transmission to the offspring of an infected insect, the iflavirus may benefit from its association to baculovirus OBs, so that larvae that consume SeIV-1-contaminated OBs are less likely to succumb to lethal polyhedrosis disease and may survive, reproduce and vertically transmit the iflavirus infection to their offspring.

We were not able to unambiguously localize iflavirus particles to the baculovirus OBs by transmission microscopy. However, structures resembling iflavirus particles by size and shape were observed in some preparations of the OBs that were positive for the presence of SeIV-1. We believe that either whole iflavirus particles or viral RNA capable of infection is physically associated and may be occluded within the baculovirus OBs. Iflaviruses replicate in the cytoplasm whereas baculoviruses replicate in the nucleus. During infection baculovirus proteins are continuously imported from the cytoplasm into the nucleus (53, 54). SeIV-1 particles or genomic RNA may be imported into the nucleus together with baculoviral proteins and be occluded within baculovirus OBs. Alternatively, the observed SeIV-1-like particles could be the result of residual translocation of SeIV-1 virions into the cell nucleus and most of the SeIV-1 genomes detected by RT-qPCR of the OBs may be derived from naked RNA embedded in the OB matrix. We detected both positive and the negative strand of SeIV-1 RNA, with a higher abundance of the former. This is consistent with the presence of naked RNA in the OBs, as the negative strand occurs only during iflavirus replication.

The discovery of novel viruses in all types of environments has increased markedly since the development of mass sequencing technologies (55). Persistent infections are constantly being discovered in many insect species. Our studies suggest that an iflavirus may be able to employ the particles of another virus pathogen, a baculovirus, in order to increase virion persistence in the environment and as a vector to improve the likelihood of iflavirus transmission, decreasing pathogenicity of the baculovirus at the same time. Quantifying the

impact of such insect virus associations on the ecology of both pathogens and host will require detailed field studies.

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## CHAPTER IV

# Co-infection with iflaviruses influences the insecticidal properties of *Spodoptera exigua* multiple nucleopolyhedrovirus occlusion bodies: implications for the production and biosecurity of baculovirus insecticides

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### ABSTRACT

Biological insecticides based on *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) can efficiently control *S. exigua* larvae on field and greenhouse crops in many parts of the world. Spanish wild populations and laboratory colonies of *S. exigua* are infected by two iflaviruses (SeIV-1 and SeIV-2). Here we evaluated the effect of iflavivirus co-infection on the insecticidal characteristics of SeMNPV occlusion bodies (OBs). Overall, iflavivirus co-inoculation consistently reduced median lethal concentrations (LC<sub>50</sub>) for SeMNPV OBs compared to larvae infected with SeMNPV alone. However, the speed of kill of SeMNPV was similar in the presence or absence of the iflaviruses. A reduction of the weight gain (27%) associated with iflavivirus infection resulted in a 30% reduction in total OB production per larva. Adult survivors of SeMNPV OB inoculation were examined for covert infection. SeMNPV DNA was found to be present at a high prevalence in all SeIV-1 and SeIV-2 co-infection treatments. Interestingly, co-inoculation of SeMNPV with SeIV-2 alone or in mixtures with SeIV-1 resulted in a significant increase in the SeMNPV load of sublethally infected adults, suggesting a role for SeIV-2 in vertical transmission or reactivation of sublethal SeMNPV infections. In conclusion, iflaviruses are not desirable in insect colonies used for large scale baculovirus production, as they may result in diminished larval growth, reduced OB production and, depending on their host-range, potential risks to non-target Lepidoptera.

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## 1. INTRODUCTION

Recent advances in next generation sequencing have led to the discovery of a great diversity of viruses infecting insect populations (1, 2). RNA viruses belonging to the family *Iflaviridae* (order *Picornavirales*) were detected during analysis of the larval transcriptome (3) of the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae), which is a major pest of horticultural crops in tropical and subtropical and regions worldwide (4). This pest also attacks greenhouse grown vegetable and ornamental crops in temperate regions (5-7).

Iflaviruses exclusively infect arthropods (8) and although some can cause clear signs of disease in their hosts, such as the iflaviruses that infect *Bombyx mori* (9) and *Apis mellifera* (10), generally iflavivirus infections remain unnoticed until detected using molecular tools (11). Specifically, two iflaviruses were found to be prevalent in *S. exigua* natural populations in Spain, which were named *Spodoptera exigua* iflavivirus-1 (SeIV-1) (12) and *Spodoptera exigua* iflavivirus-2 (SeIV-2) (13). SeIV-1 has a 10.3 kb genome whereas SeIV-2 has a 9.4 kb genome of ssRNA. In both cases the genomes are packaged into icosahedral capsids of 27 nm diameter. The genome consists of a single ORF which is translated as a single polyprotein that is subsequently cleaved into functional and structural proteins.

Baculoviruses (*Baculoviridae*) are rod-shaped DNA viruses that are occluded in proteinaceous occlusion bodies (OBs) that have been developed as the basis for effective insecticidal products in many countries (14). The *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) has been developed for the biological control of the beet armyworm in developed and developing countries due to its high specificity, pathogenicity and virulence (15). In contrast, the use of RNA viruses as insect control agents has been limited to experimental studies (16), mainly due to their low virulence and also because of biosecurity concerns given their phylogenetic proximity to related viruses of the order *Picornavirales* that are human and animal pathogens (17).

Mixed infections comprising iflavivirus and other viruses have been reported in insects (11, 18), in conjunction with baculoviruses (19-21) and a densovirus (22). Field-caught adults of *S. exigua* were found to be infected with both SeMNPV and two iflaviruses, and their laboratory-reared offspring also

showed a high prevalence of both viruses (23, 24). Recent studies on the association between SeMNPV and iflaviruses revealed that genomes of both types of viruses were present in the OBs of SeMNPV produced in insects that were infected by both viruses. The OB structure improved the resistance of the iflavivirus to UV light and high temperatures whereas a decrease in OB pathogenicity was observed in OBs produced in co-infected insects (25).

The key variables that influence the transmission of baculoviruses include the pathogenicity of OBs, measured in terms of concentration-mortality metrics, the speed of kill and the production of progeny OBs. These characteristics also determine the effectiveness of these viruses as biological insecticides (26). As cell culture production results in rapid loss of auxiliary genes and reduced pathogenicity in insects, all baculovirus based insecticides are currently produced in lepidopteran larvae (27). Efficient baculovirus production therefore relies on a large, productive and healthy insect colony. Viruses can cause devastating epizootics of disease in insect colonies and require continuous monitoring and disinfection processes to maintain colony health (28-30). Iflaviruses are rapidly transmitted and can reach a high prevalence of infection in natural populations and laboratory insect colonies (11, 31, 32).

As iflaviruses are present in *S. exigua* populations used to produce SeMNPV as the basis for biological insecticides, we examined the consequences of coinfection with SeMNPV and SeIV-1 and SeIV-2 on the insecticidal properties of OBs produced in co-infected insects. The findings have clear relevance to the efficacy of baculovirus-based insecticides and the biosecurity and potential non-target effects of these products.

## 2. MATERIAL AND METHODS

### 2.1 Insects and virus stock

*Spodoptera exigua* virus-free insects used in the experiments were obtained from Entomotech (Almería, Spain) and Andermatt Biocontrol (Switzerland) as egg masses. Insects were reared on semi-artificial diet (33) at  $25 \pm 2$  °C,  $50 \pm 10\%$  relative humidity and 16 h:8 h light:dark photoperiod in disinfected bioclimatic chambers used exclusively for this propose.

OBs of the Spanish isolate SeMNPV-SP2 were obtained from the baculovirus collection held at the Universidad Pública de Navarra since 1996 (34). These OBs consistently proved negative for the presence of SelV-1 and SelV-2 by RT-qPCR (25). SelV-1 and SelV-2 particles were isolated from larvae previously found to harbor a persistent infection. For this, virus particles were purified from guts dissected from groups of ~200 fourth instar larvae (25). The tissues were lyophilized and homogenized in 0.01 M potassium phosphate buffer (pH 7.4) with 0.45% (w/v) diethyldithiocarbamic acid (DIECA) and 0.2% (v/v)  $\beta$ -mercaptoethanol (2.5 ml of buffer per gram of larva), sonicated for 20 seconds and filtered through two layers of cheesecloth. The filtrate was loaded on to a 10, 30 and 60% discontinuous sucrose gradient and centrifuged 6 h at  $60,000 \times g$ , 10 °C. Fraction between 30 and 60% corresponding to the virus band, was collected and RNA was extracted using RNAzol® RT (Sigma-Aldrich) following the manufacturer's protocol. Quantitative PCR (qPCR) was undertaken as described below to determine the prevalence of SelV-1 and SelV-2.

## 2.2 Effect of iflaviruses on SeMNPV pathogenicity, virulence and adult persistence

To assess the effect of iflavivirus co-infection on the insecticidal properties SeMNPV OBs, larvae were inoculated with mixtures of SeMNPV and SelV-1 or SelV-2. For this batches of pre-molt *S. exigua* second instars were starved overnight and, having molted, groups of 30 second instars were allowed to drink a suspension containing one of five OB concentrations using the droplet feeding method (35). The OB concentrations were 0 (control),  $2.54 \times 10^5$ ,  $8.18 \times 10^4$ ,  $2.72 \times 10^4$ ,  $9.09 \times 10^3$  and  $3.03 \times 10^3$  OB/ml, that were previously demonstrated to kill between 95% and 5% of inoculated larvae. Identical groups of larvae were inoculated with OBs suspensions that contained (i)  $10^9$  SelV-1 genomes/ $\mu$ l, (ii)  $10^9$  SelV-2 genomes/ $\mu$ l, or (iii) 1:1 mixture of  $5 \times 10^8$  SelV-1 and  $5 \times 10^8$  SelV-2 genomes/ $\mu$ l. Larvae that consumed the suspension within 10 minutes were individually placed in the wells of a 24-compartment plates with a piece of semi-synthetic diet and reared at  $25 \pm 1$  °C. Virus-induced mortality was recorded at 8 h intervals for 7 days post-inoculation by which time all lethally infected larvae had died. The entire bioassay was performed three times.

OB concentration-mortality results were subjected to logit regression in GLIM 4 (36). Relative potencies were estimated when a parallelism test confirmed that the regressions for each treatment could be fitted with a common slope. Time-mortality results for larvae inoculated with the highest OB concentration ( $2.54 \times 10^5$  OB/ml) were subjected to Weibull survival analysis in GLIM 4. Larval that did not die from virus infections were not included in the analysis (37). The validity of the Weibull model was determined by comparing fitted values with Kaplan–Meier survival function estimated values (38).

To determine the prevalence of SeMNPV infection in adults, the insects that survived in the treatment involving  $2.72 \times 10^4$  OB/ml were reared to the adult stage and examined by qPCR as describe bellow.

### 2.3 Effects of iflaviruses on OB production and larval weight gain

To examine the effect of iflaviruses on OB production, larvae were treated with OB suspensions with or without the presence of SeIV-1 or SeIV-2. For this, groups of 24 newly-molted second instars were inoculated with  $2.45 \times 10^5$  OB/ml (previously estimated to result in 90% mortality) and one of the following iflavirus treatments: (i)  $10^9$  SeIV-1 genomes/ $\mu$ l; (ii)  $10^9$  SeIV-2 genomes/ $\mu$ l; and (iii) a 1:1 mixture of  $5 \times 10^8$  SeIV-1 and  $5 \times 10^8$  SeIV-2 genomes/ $\mu$ l. Two additional groups of 24 insects in the second instar were inoculated with SeMNPV OBs alone or were mock-infected as controls. Larvae that consumed inoculum suspension within 10 minutes were individually placed in the wells of 24 compartment plates and reared at  $25 \pm 1$  °C, checked daily for signs of disease. The entire bioassay was performed three times. Larval weight gain, and OB production were subsequently assessed in fourth instar larvae that were co-inoculated with  $5 \times 10^7$  OB/ml and  $10^9$  SeIV-1 genomes/ $\mu$ l using the droplet-feeding method described above. This concentration of OBs was estimated to kill 95% of inoculated fourth instars. Weight measurements were taken using an electronic balance ( $\pm 1$  mg) immediately before inoculation and then at 24 h intervals during a period of 6 days. Larvae that had been inoculated in the second or fourth instar and that showed signs of the final stages of polyhedrosis disease, were placed individually in 1.5 ml microtubes and incubated at  $25 \pm 1$  °C for 4 - 8 hours until death. Virus-killed larvae were then stored at 4 °C for up to 7 days prior to OB counting. Ten cadavers per treatment and repetition were randomly selected for OB counting.

Virus-killed larvae were homogenized individually in 1 ml distilled water. OB production was estimated by counting triplicate samples of diluted OB suspension using a Neubauer hemocytometer (Hawksley, Lancing, UK) under a phase-contrast microscope at  $\times 400$  magnification. Mean OB production values were estimated and compared by t-test (SPSS v. 21.0 2012, IBM). Weight gain data were not normally distributed and therefore subjected to the Kruskal-Wallis or Mann-Whitney non-parametric test (SPSS v. 21.0 2012, IBM).

## 2.4 Total DNA and RNA extraction

For detection of covert infections, total DNA and RNA were isolate from insect tissues using the Master Pure Complete DNA and RNA Purification kit (Epicentre Biotechnologies). The abdomens of frozen adults were dissected and placed individually in a 2-ml microfuge tube with ceramic beads, 300  $\mu$ l tissue lysis solution and 1  $\mu$ l proteinase K (50 ng/ $\mu$ l). Samples were homogenized using MP FastPrep-24 tissue cell homogenizer at 4 m/s for 20 s and incubated at 65 °C for 15 min at 1,000 rpm orbital agitation. Samples were divided in two aliquots of 150  $\mu$ l each. One aliquot was used for DNA extraction and treated with 1  $\mu$ l RNase at 37 °C for 30 min. Tissue remains were pelleted by adding protein precipitation reagent (MPC), vortexed, and centrifuged at 10,000  $\times g$  for 10 min. DNA was precipitated from the supernatant with isopropanol, washed twice with 70% ethanol, and the pellet was resuspended in 20  $\mu$ l milli-Q water and stored at -20 °C. The second aliquot was used for RNA extraction. For this, protein precipitation reagent was added to the 150  $\mu$ l aliquot, centrifuged at 10,000  $\times g$  for 10 min and DNA was precipitated with isopropanol. Nucleic acid pellets were treated with RNase-free DNase buffer and 5  $\mu$ l of DNase for 30 min at 37 °C. A 200  $\mu$ l volume of 2  $\times$  T and C lysis solution was added and vortexed for 5 s followed by 200  $\mu$ l of protein precipitation reagent and vortexed 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 20  $\mu$ l DEPC water and stored at -20 °C. Blank extraction samples containing only water were processed in parallel to detect cross-contamination during the extraction process. All equipment and reagents were previously sterilized and treated with DEPC to remove RNases.

## 2.5 Virus detection and quantification

In order to quantify viral loads, qPCR and reverse transcription quantitative PCR (RT-qPCR) were performed for SeMNPV and the iflaviruses, respectively. Specific primers were used for the detection of SeMNPV DNA polymerase (23) and iflavivirus RNA-dependent RNA polymerase (RdRp) (24) sequences (Table 1). Total DNA or cDNA was used as template for amplification in order to detect SeMNPV and iflaviruses, respectively. To obtain cDNA, 1 µg of RNA was reversed transcribed to cDNA using SuperScript II Reverse Transcriptase (Promega) and Oligo (dT) primers. The reverse transcription mix consisted of 2 µl of 5× buffer (Promega), 1.2 µl MgCl<sub>2</sub> (25 mM), 0.5 µl dNTP mix (10 mM), 0.8 µl DEPC water and 1 µl ImProm-II reverse transcriptase (Promega). The mixture was added to RNA samples and incubated at 25 °C for 5 min, followed by 42 °C for 60 min and 70 °C for 15 min. qPCR based on SYBR Green fluorescence was carried out in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) in 96-well reaction plates. A 9 µl mastermix containing 5 µl SYBR, 0.5 µl of both primers (10 µM) and 3 µl water was added to 1 µl of DNA or cDNA template. For the construction of standard curves, the PCR products generated with specific primers for SeIV-1 and SeIV-2 were cloned into a pGEM®-T Easy cloning vector (Promega). Plasmid DNA was quantified using a spectrophotometer (Eppendorf BioPhotometer Plus) and two replicate samples were subjected to eight-fold serial dilutions in sterile MilliQ water (from 10<sup>-1</sup> to 10<sup>-7</sup> ng/µl) and were amplified as standards.

The qPCR protocol consisted in an initial denaturation at 95 °C for 3 min, followed by 45 amplification cycles of 95 °C for 10 s, 62 °C for 30 s, and finally a melting curve stage of 65 °C to 95 °C every 0.5 °C for 5 s. Data were acquired and analyzed using Bio-Rad CFX Manager 3.1 software (Bio-Rad). The regression characteristics of the standard curves were R<sup>2</sup> = 0.99 in all cases and slopes of -3.27, -3.56 and -3.30 for SeMNPV, SeIV-1 and SeIV-2, respectively, with an efficiency between 90 and 110% (39). The last standard concentration, 10<sup>-7</sup> ng/µl, represented the limit of detection and showed correct amplification curves and the expected melting temperatures of 83.5, 77.0 and 79.5°C for SeMNPV, SeIV-1 and SeIV-2, respectively. As a result, higher Ct values were considered to be virus-free samples. All values were normalized using a non-differentially

expressed ATP-synthase subunit C gene quantification (Table 1), following standard procedures previously described for iflaviruses (25).

**Table 1:** Primer sequences and description of the gene region targeted.

Primer	Sequence	Description
qDNApol	F: 5'-CCGCTCGCCAACACTACATTAC-3' R: 5'-GAATCCGTGTGCGCGTATATC-3'	Amplifies 149-bp region within the SeMNPV DNA polymerase gene (40).
SeIV-1q	F: 5'- TGTGAAGTTAGACACGCATGGAA-3' R: 5'-CGACTTGTGCTACTCTTTCATCAA-3'	Amplifies a 97-bp in the RNA-dependent RNA polymerase (RdRp) region from SeIV-1 (12).
SeIV-2q	F: 5'-CCGCTCGCTTATTGAAACGT-3' R: 5'-CATGAGACAGCTGGAATTGGAA-3'	Amplifies a 78-bp in the RNA-dependent RNA polymerase (RdRp) region from SeIV-2 (13).
qATP-synthase	F: 5'-GTTGCTGGTCTGGTGGGATT-3' R: 5'-AGGCCTCAGACACCATTGAAA-3'	Amplifies a 72-bp in ATP-synthase subunit C gene from <i>S. exigua</i> (25).

### 3. RESULTS

#### 3.1 Effect of iflavirus co-inoculation on SeMNPV insecticidal properties

In all cases, mortality increased significantly with  $\log_e$  [OB concentration] ( $F_{1,20}=108$ ,  $P < 0.001$ ). Overall, mixtures of SeMNPV and iflaviruses resulted in significantly increased OB pathogenicity compared to SeMNPV OB inoculum alone. The  $LC_{50}$  values were 2.4 to 3.9-fold lower in iflavirus treatments compared to SeMNPV OBs alone (Table 2).

**Table 2:**  $LC_{50}$  values, relative potencies and mean time to death (MTD). For *S. exigua* second instars inoculated with SeMNPV OBs alone or co-infected with SeMNPV OBs and iflavirus (SeIV-1, SeIV-2 or SeIV-1+SeIV-2).

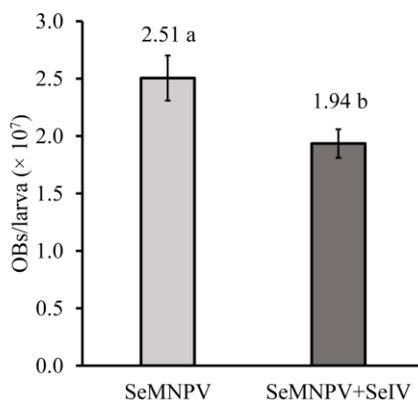
Treatment	$LC_{50}$ ( $\times 10^3$ OBs/ml)	Relative potency	95% Fiducial limits		P	MTD (h)	95% Fiducial limits	
			Low	High			Low	High
SeMNPV alone	14.4	1	10.1	20.3	-	94.9ab	92.8	97.1
SeMNPV+SeIV-1	6.1	2.4	3.4	9.1	<0.05	94.4ab	92.1	96.8
SeMNPV+SeIV-2	3.7	3.9	1.3	6.5	<0.05	92.4a	90.4	94.5
SeMNPV+SeIV-1+SeIV-2	5.1	2.8	3.1	7.4	<0.05	97.1b	95	99.3

Mean time to death values of the different SeMNPV + iflavirus treatments ranged from 92.4 to 97.1 hours post inoculation (hpi), compared to 94.9 hpi for larvae inoculated with SeMNPV OBs alone (Table 2). MTD values only differed significantly between the SeMNPV + SeIV-2 and SeMNPV + SeIV-1 +

SeIV-2 treatments ( $t$ -test=3.679;  $df = 332$ ;  $P < 0.001$ ). No mortality was registered in mock-infected larvae or in those larvae infected only with SeIV-1 or SeIV-2, or their mixture. All larvae that succumbed to SeMNPV infections showed the characteristic signs of polyhedrosis disease that was confirmed by microscopic observation of OBs.

### 3.2 OB Production and weight gain

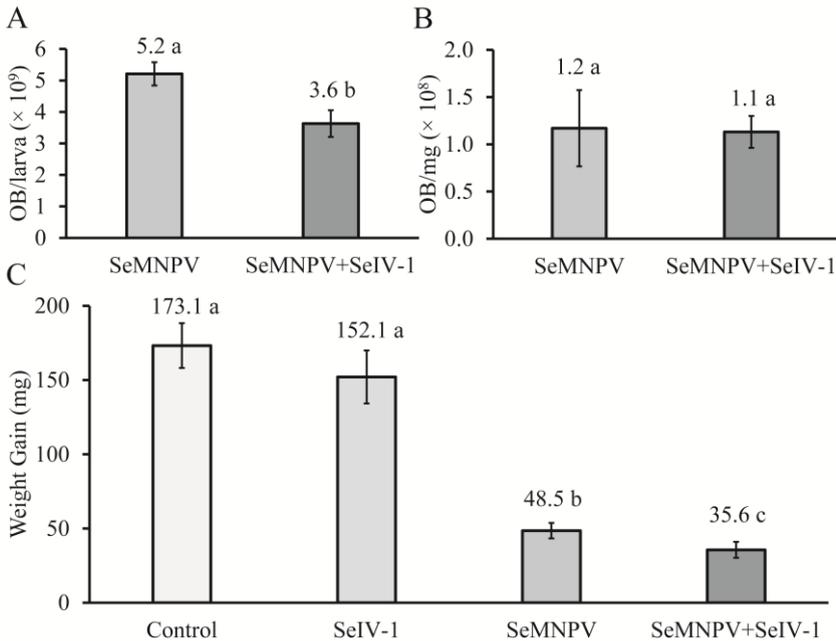
Co-infection with iflavivirus significantly reduced OB production in second instar larvae treated with SeMNPV (ANOVA,  $F_{3,116}=4.008$ ;  $P < 0.001$ ). The mean production of OBs in each SeMNPV-killed insect was similar in treatments involving SeIV-1 ( $1.89 \times 10^7 \pm 1.96 \times 10^6$ ), SeIV-2 ( $2.03 \times 10^7 \pm 2.06 \times 10^6$ ) or the mixture of SeIV-1 + SeIV-2 ( $1.89 \times 10^7 \pm 2.10 \times 10^6$ ) (ANOVA,  $F_{2,87}=2.723$ ;  $P = 0.07$ ), so the results of these treatments were pooled for comparison of treatments with and without iflavivirus. Overall, OB production per larva was reduced by 23% in larvae infected by SeMNPV + iflaviruses compared to larvae inoculated with SeMNPV OBs alone ( $t$ -test=2.335;  $df=118$ ;  $P = 0.01$ ) (Figure 1).



**Figure 1:** OB production in second instar larvae infected with SeMNPV alone or co-infected with iflaviruses. Iflavivirus treatments did not differ significantly and were pooled for analysis. Mean values followed by different letters indicate significant differences ( $t$ -test=2.335;  $df=118$ ;  $P = 0.01$ ). Bars indicate the standard error.

In order to determine whether the effects of iflavivirus on OB production were related to larval growth, fourth instar larvae were subjected to one of two treatments: SeMNPV OBs alone and SeMNPV OBs + SeIV-1. Larval weights from

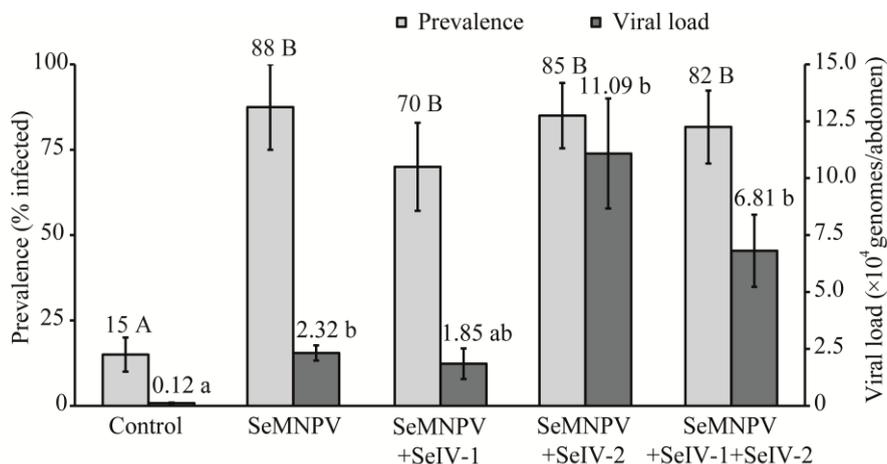
30 larvae per repetition and treatment was registered over time. For larval weight, two additional treatments, SeIV-1 alone and mock-infected larvae (control) were used. OB production in fourth instars differed significantly between treatments ( $F_{1,133}=2.23$ ;  $P < 0.001$ ), with a 30% reduction in the production of OBs in insects infected by SeMNPV + SeIV-1 compared to larvae infected by SeMNPV alone (Figure 2A). However, OB production in relation to host weight (OB/mg body weight) was similar with or without SeIV-1 co-infection ( $F_{1,129}=2.075$ ;  $P = 0.152$ ) (Figure 2B). These results can be explained by the significantly lower weight gain of larvae (27% reduction) when co-infected with SeMNPV and iflaviruses compared to larvae infected by SeMNPV alone (Kruskal-Wallis;  $\chi^2_{82.03}$ ,  $df = 3$ ;  $P < 0.001$ ) (Figure 2C).



**Figure 2:** OB production and weight gain for fourth instar larvae treated with SeMNPV or SeMNPV + SeIV-1. Mean OB production (A), mean OB production per larval weight (mg) (B), and median weight gain of fourth instar larvae treated with water (control), SeIV-1, SeMNPV and SeMNPV + SeIV-1 (C). Columns headed by values with different letters indicate significant differences (Kruskal-Wallis;  $P < 0.05$ ). Bars indicate the standard error (A, B) or the interquartile range (C).

### 3.3 Detection and quantification of viral loads in sublethal SeMNPV and iflavirus infections

Of the second instar larvae treated with a concentration of  $9.09 \times 10^3$  OBs/ml, 30 to 70% died of lethal polyhedrosis disease. The presence of sublethal infections was estimated by qPCR in adult survivors of this treatment. The prevalence of sublethal infection by SeMNPV was similar between larvae treated with SeMNPV with or without SeIV-1 or SeIV-2 and ranged from 70 to 88% (GLM;  $P < 0.001$ ) (Figure 3, pale shaded columns). Unexpectedly, low-levels of SeMNPV were detected in the control groups, probably due to the presence of a low-level persistent infection in the insect colony, as observed in a previous study (40). Co-inoculation of SeMNPV+SeIV-2, or a mixture of SeIV-1 + SeIV-2, resulted in a significant increase in the SeMNPV load, which was over three-fold higher than SeMNPV loads registered in insects treated with SeMNPV + SeIV-1 or SeMNPV alone. A similar response was registered for the group inoculated with SeMNPV + SeIV-1 + SeIV-2 (Kruskal-Wallis;  $\chi^2=23.67$ ;  $df=4$ ;  $P < 0.001$ ) (Figure 3, dark columns).



**Figure 3.** Prevalence of covert infection and viral load of SeMNPV in adults. Mean prevalence of covert infection by SeMNPV in adult survivors to a virus challenge (pale gray columns) and the viral load (dark shaded columns) of SeMNPV-positive adults by qPCR. Mean prevalence values followed by different upper case letters indicate significant differences between viral treatments (GLM;  $P < 0.05$ ). Error bars indicate SE. Median values of viral load followed by different lower case letters differ significantly (Kruskal-Wallis;  $P < 0.05$ ). Error bars indicate interquartile range (viral load).

#### 4. DISCUSSION

The effectiveness of baculovirus-based insecticides relies on a diversity of virus-related and environmental factors (14), but also on the susceptibility of the pest population to baculovirus applications, which can be altered by cryptic infections involving other types of viruses (23, 41). Here we demonstrated that the insecticidal properties of SeMNPV OBs were altered when the baculovirus replicated in hosts that were also infected by the iflaviruses SeIV-1 or SeIV-2. Previous studies indicated that these iflaviruses could be present in 15% of field populations of *S. exigua* in southern Spain, in which 8.3% of individuals were simultaneously infected by both iflaviruses and SeMNPV (23).

As lethal disease, has not been observed in insects infected by SeIV-1 or SeIV-2 to date, it appears that the co-inoculation of the iflaviruses increases the lethality of the SeMNPV infection. Similar effects on the mortality response have been observed following co-inoculation of mixtures of pathogens in lepidopteran larvae. For instance, the mixture of *Bacillus thuringiensis* (Bt) and *Helicoverpa armigera* nucleopolyhedrovirus (HaNPV) resulted in potentiation or additive effects on the mortality of *Plutella xylostella* that depended on the concentration of the bacteria in the mixture (42). The gut microbiota of the host can also modulate baculovirus pathogenicity and speed of kill (43), as bacterial virulence factors seem to contribute to the success of viral infections in the insect midgut. Persistent infection by the endosymbiont *Wolbachia* also resulted in increased susceptibility to lethal nucleopolyhedrovirus infection in *Spodoptera exempta* (44). These findings contrast with those involving persistent infection of *Helicoverpa armigera* larvae by a densovirus that provided resistance against both Bt and HaNPV infection (45). Interestingly, densovirus infected individuals of *H. armigera* developed faster and had higher fecundity than healthy conspecifics (45), whereas in the present study, we observed lower weight gain in iflavivirus infected larvae. This resulted in a reduced weight at death and a corresponding reduction in total OB production per larva compared to larvae infected with SeMNPV alone. The positive relationship between larval body weight and OB production is well established (46). Our results also confirm previous findings on the reduced growth of iflavivirus infected *S. exigua* larvae (24).

In a previous study on the interaction of SeMNPV OBs and SeIV-1, we observed that the inoculation of *S. exigua* larvae by SeIV-1-contaminated OBs resulted in a significant reduction in OB pathogenicity compared to OBs that were not contaminated by iflavirus, whereas OB production per larva and the weight gain of insects infected by SeMNPV were not significantly affected by the presence of SeIV-1 particles in the inocula (25). It is difficult to compare these studies directly due to differences in the amounts of iflavirus present and the nature of the mixed virus inocula. Specifically, in our previous study insects were infected by both SeIV-1 and SeMNPV which resulted in the production of progeny SeIV-1 particles that were intimately associated with the polyhedrin matrix of OBs. The average ( $\pm$ SE) number of SeIV-1 particles per OB was estimated at  $18.9 \pm 2.3$  particles/OB (25). In contrast, in the present study OBs and iflavirus particles were inoculated in mixtures in which the concentration of iflavirus remained fixed ( $10^9$  particles/ $\mu$ l) and the concentration of SeMNPV OBs varied. As such, the ratio of iflavirus particles to OBs varied from  $4 \times 10^6$  to  $3.3 \times 10^7$  SeIV particles per OB in the inocula consumed by second instar larvae, compared to  $2 \times 10^4$  SeIV-1 particles per OB in the inoculum fed to fourth instar larvae. As a result, the differences in larval weight gain and OB production are likely to be due to the higher concentrations of iflavirus particles present in inocula mixtures with OBs used in the present study compared with our previous study (25).

Similarly, the apparent contradiction between the present study that detected increased SeMNPV OB pathogenicity in mixtures with iflavirus, and our previous study in which SeMNPV OB pathogenicity was reduced when in association with iflavirus, may have been due to the higher quantity of iflavirus particles per OB in the inocula used to infect insects in the present study, or related to the physical association of iflavirus with OBs in the previous study (25). Alternatively, OBs may have undergone modifications during assembly in cells co-infected by iflaviruses, which affected the physical features or number or infectivity of occlusion derived virions occluded within each OB (25). These issues can only be resolved through future studies.

The concentration of iflavirus used in the present study may initially appear unrealistically high. However, recent studies on an iflavirus of *Helicoverpa armigera* revealed the presence of up to  $10^6$  iflavirus genomes/ $\mu$ g of larval feces (47). This was sufficient to assure the horizontal transmission of the iflavirus to

conspecific larvae. Moreover, as quantitative studies have suggested that nucleopolyhedrovirus OBs are usually present at low densities on plant foliage in natural and agricultural ecosystems (48) small numbers of OBs may be consumed together with large quantities of iflavirus particles released from iflavirus-infected insects feeding on the same plant, although this has not been demonstrated empirically in field or laboratory studies.

In studies performed prior to the advent of molecular techniques, ELISA was used to detect a picorna-like virus that severely stunted the growth of *Trichoplusia ni* larvae, although no virus-specific mortality was observed (19, 20). Mixed infections of this virus with *Autographa californica* multiple nucleopolyhedrovirus (AMNPV) resulted in contamination of progeny OBs with the picorna-like virus (20). The molecular mechanisms by which iflaviruses and baculoviruses interact are unclear, more so because SeMNPV replicates in the cell nucleus, whereas iflaviruses replicate in the cytoplasm (11).

Recent advances in recognizing the regulatory role of host and viral microRNAs (miRNA) in virus pathogenesis and virulence are likely to greatly improve our understanding of the molecular mechanisms that modulate insect host-virus interactions (49). Virus-encoded miRNAs have been characterized in nucleopolyhedrovirus (50, 51) and RNA virus infections (52), in addition to a suite of miRNA-mediated antiviral and apoptotic responses by host cells (53-55). The mechanisms by which SeMNPV or iflaviruses may be able to modulate the innate immune responses of the host through miRNA-mediated suppression strategies are presently unclear although both sublethal infection and interspecific virus interference are likely to be regulated to some degree through such mechanisms (55, 56).

SeMNPV infections are transmitted both horizontally from infected to healthy larvae and vertically from parents to offspring (57). The persistence of the virus in sublethally infected adults may also contribute to transgenerational pest suppression if a portion of the sublethal infections are reactivated to cause lethal disease (58, 59). In contrast, the transmission of iflaviruses is believed to be mainly horizontal (11). However, we previously observed that SeIV-1 and SeIV-2 were efficiently transmitted from field-caught infected adults to their offspring in the laboratory (23). Both the prevalence of sublethal infection by SeMNPV and viral load (number of copies of SeMNPV genomes) were influenced by the presence of

iflaviruses in adult insects. Notably, the abundance of SeMNPV genomes in insects from treatments involving SeIV-2 (SeMNPV + SeIV-2 and SeMNPV + SeIV-1 + SeIV-2) were several folds higher than SeMNPV loads in insects from treatments involving SeMNPV alone or SeMNPV + SeIV-1. High loads of nucleopolyhedrovirus may promote transmission either vertically to offspring (40) or horizontally through the activation of sublethal infections (59). In either case, once established, the intimate association between iflavirus and SeMNPV would likely be maintained, either through physical association of iflavirus particles and OBs or by dual infection of host offspring through simultaneous vertical transmission by both viruses (25).

Cryptic infections such as those caused by iflaviruses could modulate the performance of SeMNPV-based insecticides. As both types of viruses occur in natural populations of *S. exigua* nucleopolyhedrovirus-iflavirus interactions are likely to be common in nature. Iflaviruses are not desirable in insect colonies used for large scale production, as they may result in diminished larval growth, reduced OB production and, depending on their host-range, potential risks to non-target Lepidoptera present in treated crops and habitats adjacent to treated areas. As such, strict colony hygiene, frequent disinfection measures, regular quality control assays on OB pathogenicity and molecular screening for the presence of iflavirus contaminants are likely to be necessary in baculovirus mass-production facilities to maintain production of an effective product with registration-compliant levels of contaminant microorganisms.

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## CHAPTER V

# Co-infection by an iflavirus (SeIV-1) and alphabaculovirus (SeMNPV): effects on occlusion body structure and conformation

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### ABSTRACT

Occlusion bodies (OBs) of the multiple-nucleocapsid nucleopolyhedrovirus of *Spodoptera exigua* (SeMNPV) (*Baculoviridae*) were found to be physically associated with the iflavirus SeIV (*Iflaviridae*), based on PCR-based techniques and electron microscopy. These findings suggest that iflavirus particles were located in the polyhedrin-matrix of SeMNPV OBs generated after co-inoculation of both virus in a common host. Purification of OBs associated with SeIV through sucrose gradients revealed OB subpopulations with differential migration patterns. Two OB subpopulations were distinguished and separately purified to quantify viral loads (qPCR) and examine their conformation. While OBs from upper OB-band accumulated high loads of iflavirus genomes, the OBs from lower OB-band were the most abundant in SeMNPV genomes. An endpoint dilution assay showed that differences in viral genomes was not related to the number of ODV infection units per OB. Therefore, ODV conformation was examined. The quantification of ODV sucrose gradient fractions demonstrated that in OBs enriched in SeIV (upper OB-band), the proportion of single-nucleocapsids was higher than in those ODVs from OBs related to either low loads of SeIV or not associated to SeIV. In conclusion, the association of iflavirus particles with SeMNPV OBs altered ODV conformation, possibly during virion assembling in host cells harboring a co-infection of both viruses. The reduced infectivity in SeMNPV OBs associated with SeIV could be explained by the high proportions of single-nucleocapsid ODVs contained in those OBs. This conformation might affect viral spread during primary infection by reducing the possibility of nucleocapsids bypassing the nucleus during midgut cell infection, or other advantages conferred by multiple nucleocapsid packaging.

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## 1. INTRODUCTION

Lepidopteran populations frequently harbor cryptic infections involving one or more viruses (1, 2) that remain undetectable for long periods. Many aspects of asymptomatic cryptic infections remain unknown but their influence on host fitness can lead to a variety of responses ranging from an increase in host resistance to pathogens (3), to the modification of host behavior to favor pathogen dispersion (4). However, when more than one virus species is involved, the interaction scenario can become highly complex including host-pathogen together with virus-virus interactions (5).

Using next generation sequencing (NGS) two species of small RNA viruses, SeIV-1 (6) and SeIV-2 (7) were identified in the transcriptome of apparently healthy *S. exigua* larvae. These viruses were significantly more abundant than other RNA viruses (8). Another virus causing persistent infection in the same host is the nucleopolyhedrovirus of *Spodoptera exigua* (*Baculoviridae*, genus *Alphabaculovirus*), which can infect field populations of this insect (9). SeMNPV is widely used as a biological control agent in Europe (10), Asia (11) and America (12). SeMNPV has also been used as a model for persistent infections and vertical transmission studies conducted in laboratory and field conditions (13). The iflaviruses SeIV-1 and SeIV-2, together with SeMNPV are frequently found in *S. exigua* populations causing persistent mixed infection in laboratory cultures (14) or field collected insects (9). A physical association of SeIV and SeMNPV was revealed after productive infections of the baculovirus in hosts infected with SeIV. We demonstrated co-inoculation by both types of viruses resulted in the generation of OBs carrying both SeMNPV and SeIV genomes (15). This association benefited iflavirus persistence, in terms of resistance against UV light and high temperature (15), indicating a possible facultative phoresis of one virus by another. Bioassays involving SeMNPV-SeIV co-infection (16), revealed that iflavirus reduced the lethal medium concentration of SeMNPV OBs when co-infections were induced, but a reduction in OB production at larval death.

Interactions at the cell level might occur during the infection cycles of both viruses in a common host. The SeMNPV infective process starts when a larva ingests OBs. In the basic pH of the midgut, ODVs are released and cross the peritrophic membrane reaching epithelial cells where they enter by fusion with the

epithelial membrane (17). Once in the nucleus, new virions, named budded virus (BVs) are produced, by which the infection spreads to other tissues. After this, the newly assembled nucleocapsids acquire an envelope to form occlusion derived virions (ODVs). At the end of the cycle polyhedrin is produced and accumulates in the nucleus and condenses around the ODVs to form OBs (18, 19). At the end of the infectious process nuclear and plasma membranes breakdown releasing OBs into the hemocoel. In contrast, little is known about the iflavirus infectious process, but this virus replicates in the cytoplasm (20, 21) and has been found infecting all host tissues, especially the midgut (6) and reproductive tissues (22). Some studies indicate an association of iflavirus with the membranes or intracellular structures during the replicative process (23). Therefore, the association between both types of viruses can occur when NPVs cross the cytoplasm to the nucleus or when OBs are being formed and both viruses are co-occluded as a consequence of a co-infection.

OBs are responsible for the horizontal transmission of infection from an infected host to a healthy one (24). Morphologically OBs are complex structures. SeMNPV is a multiple NPV, thus single and multiple virions are wrapped alone or in groups to form ODVs. The co-enveloping of multiple nucleocapsids seems to be determinant for the establishment and spread of viral infection, since some nucleocapsids bypass the nucleus and bud out of the midgut cell basal membrane presumably to avoid the sloughing response of infected midgut cells and to propagate the infection as quickly as possible (25).

In this study, we examined the physical association between SeMNPV and SeIV-1 to determine whether iflavirus coinfection affects OB structure and conformation. For this we analyzed of the level of association and examined two OB subpopulations generated after co-infection with SeIV-1 that presented differences in size, number of nucleocapsids per virion and genome load.

## 2. MATERIALS AND METHODS

### 2.1 Insects, cell line and viruses

A virus free colony of *Spodoptera exigua* was reared on semi-artificial diet (26) in virus free conditions at  $25 \pm 1$  °C temperature and  $60 \pm 5\%$  relative humidity. OBs from two isolates of SeMNPV were used in this study: i) SeMNPV

OBs, isolate SP2, that consistently tested negative (qPCR) for SeIV-1 (15) and which has been maintained in the UPNA Baculovirus Collection (-20 °C) since 1996 (27), and ii) SeMNPV OBs associated with SeIV (qPCR) of the SeMNPV-AL1 isolate obtained by amplification in SeIV-1 positive insects during laboratory rearing (16, 28) (Figure 1C). Alternatively, SeMNPV/SeIV associated OBs were generated for this study by co-inoculation of iflavirus free SeMNPV-SP2 OBs and SeIV-1 purified suspension as described previously (16). Briefly,  $2.45 \times 10^5$  OB/ml were mixed with  $10^9$  genomes/ml of SeIV-1 and offered to fourth instar *S. exigua* larvae using the droplet feeding method (29). The resulting OBs were purified and tested for SeIV presence by qPCR as described below.

## 2.2 OB gradient centrifugation

To examine the conformation of OBs generated during SeIV-1 coinfection with SeMNPV in *S. exigua*-SeIV free insects, OB samples were purified through sucrose gradients (30). Briefly, 3 ml of  $10^8$  OB/ml suspension was loaded onto a 30 - 70% (w/w) continuous sucrose gradient and then centrifuged at  $40,000 \times g$  for 1 h at 4 °C. The OB bands were harvested by puncturing the tube with a needle and collecting the sucrose fraction in a syringe. OBs in sucrose were diluted in 2 vol. of  $1 \times$  TE buffer and centrifuged at  $40,000 \times g$  for 1 h at 4 °C. OBs in the resulting pellet were suspended in sterile double-distilled water.

## 2.3 ODVs gradient centrifugation

For ODV purification, 3 ml of  $10^{10}$  OBs/ml that had been produced in association with SeIV-1 were mixed with an equal volume of 0.5 M  $\text{Na}_2\text{CO}_3$  and incubated at 28 °C for 30 min. After low speed centrifugation ( $6,000 \times g$ , 5 min) debris was removed. The supernatant, containing ODVs, was placed on a continuous sucrose gradient (30 - 70%, w/w) and centrifuged at 4 °C for 1 h at  $40,000 \times g$  in a Beckman centrifuge using a SW 32 ti rotor. Two different methods were used to collect sucrose fractions: i) ultracentrifugation tubes were covered and sealed with parafilm and perforated at the bottom to harvest fractions. Subsequently, 16 fractions, 2 ml each, were collected (Figure 1) and, ii) four fractions were collected by needle perforation; the first, second and third ODV bands were collected and, the rest of the ODV bands were harvested together in a single sample (Figure 5). Each of all fractions was diluted in 5 vol. of TE buffer (10

mM Tris, 1 mM EDTA) and viruses were pelleted by centrifugation at 4 °C at  $40,000 \times g$  during 1 h and resuspended in 200  $\mu$ l of sterile water.

#### 2.4 Determination of infectious particles per ODV

Mean ODV content from SeMNPV OBs and SeMNPV OBs associated with SeIV-1 were determined by end-point dilution assays in Se301 insect cells. For this, ODVs were released from samples of  $2 \times 10^7$  OBs in a volume of 200  $\mu$ l by incubation with 1:1 of 0.1 M  $\text{Na}_2\text{CO}_3$  at 28 °C for 30 minutes. The resulting suspension was passed through a 0.45  $\mu$ m filter and serially diluted by a factor of 10 ( $10^{-1}$  to  $10^{-9}$ ) in HyClone™ CCM3 medium + 5% fetal bovine serum (FBS). Se301 cells were titrated in a 96-well microtiter plate where 20  $\mu$ l of each dilution was mixed with 180  $\mu$ l of  $5 \times 10^4$  cells and plates were sealed with masking tape and incubated at 28 °C for 7 days. Titers were analyzed using Spearman-Kärber method (31) to estimate 50% tissue culture infectious dose ( $\text{TCID}_{50}$ ). The  $\text{TCID}_{50}$  values were converted to infectious units per  $5 \times 10^6$  OBs. The results were analyzed by an ANOVA in SPSS v.21. This experiment was performed in triplicate.

#### 2.5 DNA and RNA purification from OBs and ODVs

For the viral load determination, both RNA and DNA were extracted from purified OB and ODVs samples and then viral genomes were quantified by qPCR. Briefly, for RNA extraction 60  $\mu$ l of  $10^9$  OBs/ml were incubated with 30  $\mu$ l DAS-3 $\times$  ( $\text{Na}_2\text{CO}_3$  0.3 M, NaCl 0.5 M, EDTA 0.03 M, pH 10.5) for 1 h, at 37 °C. Then, 700  $\mu$ l Trizol® Reagent (Invitrogen) was added and centrifuged at  $12,000 \times g$ , 15 min, 4 °C. The upper layer was transferred with 3  $\mu$ l of Glycogen® (Roche) and precipitated with isopropanol by centrifugation at  $12,000 \times g$ , 10 min, 4 °C. The pellet was washed with 70% RNase free ethanol, and resuspended in 20  $\mu$ l of DEPC (diethylpyrocarbonate)-treated water and stored at -20 °C. For DNA extraction, 100  $\mu$ l of  $10^9$  OBs/ml were incubated with 100  $\mu$ l  $\text{Na}_2\text{CO}_3$  0.5 M, 50  $\mu$ l SDS 10% and 250  $\mu$ l distilled water at 60 °C, for 10 min. Then, samples were centrifuged at 8,000 rpm, 5 min, and the supernatant incubated with 25  $\mu$ l of proteinase K (20 mg/ml) at 50 °C, for 1 h. DNA was extracted by two passes of phenol and one of chloroform followed by 13,000 rpm for 5 min, centrifugation, precipitated with 0.1 vol. 3 M sodium acetate (pH 5.2) and 2.5 vol. of 96% ethanol, washed with 500  $\mu$ l 70% ethanol and centrifuged for 5 min at 8,000 rpm. The DNA

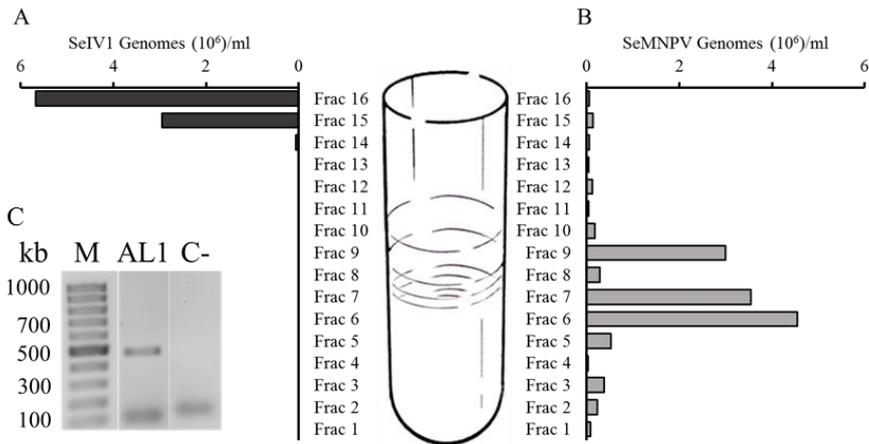
pellet was resuspended in 20  $\mu$ l of bi-distilled water. DNA/RNA extraction from ODV samples followed the same protocol except for the lysis of OB polyhedrin matrix by DAS-3 $\times$  or Na<sub>2</sub>CO<sub>3</sub> and SDS in RNA and DNA, respectively. cDNA performed by reverse transcription of 4  $\mu$ g of RNA to cDNA using the specific primer and SuperScript II Reverse Transcriptase (Promega). The reverse transcription mix consisted of 2  $\mu$ l 5 $\times$  buffer (Promega), 1.2  $\mu$ l MgCl<sub>2</sub> (25 mM), 0.5  $\mu$ l dNTP mix (10 mM), 0.8  $\mu$ l DEPC water and 1  $\mu$ l ImProm-II reverse transcriptase (Promega). The mixture was added to 4  $\mu$ l RNA samples and incubated at 25 °C for 5 min, followed by 42 °C for 60 min and 70 °C for 15 min.

Viral genomes were quantified by qPCR using specific primers for each virus targeted at DNA (SeMNPV, F, 5'-CCGCTCGCCAACTACATTAC-3', R, 5'-GAATCCGTGTCGCCGTATATC-3') and cDNA (SeIV-1 F, 5'-TGTGAAGTTAGACACGCATGGAA-3', R, 5'-CGACTTGTGCTACTCTCTTCATCAA-3') as described elsewhere (9). DNA/cDNA samples were subjected to quantitative PCR (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad) using the software Bio-Rad CFX Manager™ (v 3.1). Genome quantification between OBs treatments and between OBs subpopulations was compared by t-test in the SPSS Statistics program (v. 21).

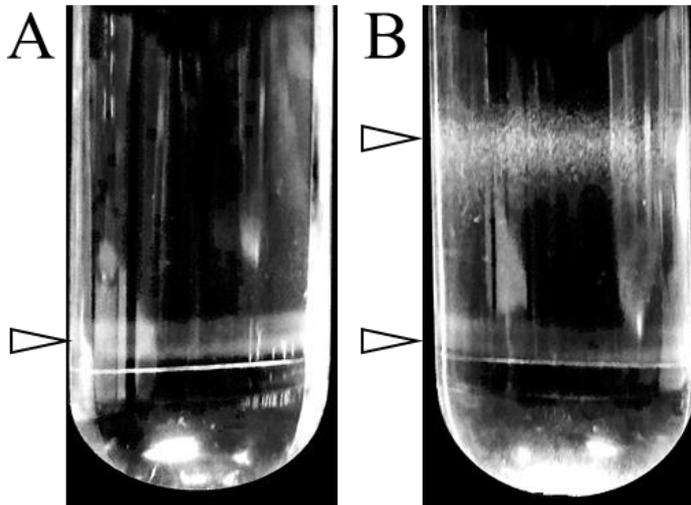
### 3. RESULTS

#### 3.1 ODVs-iflavivirus association

Continuous sucrose gradient for ODVs purification from SeMNPV-AL1 OBs, proved positive for SeIV-1 association, were performed. The gradient was divided into 16 fractions and DNA and RNA were extracted and quantified for the SeMNPV and SeIV-1 genomes in each fraction. SeIV-1 was most prevalent in fraction 16, followed by fraction 15 ( $5.68 \times 10^6$  and  $2.95 \times 10^6$  SeIV-1 genomes/ml, respectively) (Figure 1A), whereas fractions 9, 7, and 6 harbored the highest loads of SeMNPV genomes, that corresponded to the ODVs bands ( $2.99 \times 10^6$ ,  $3.54 \times 10^6$ , and  $4.54 \times 10^6$  SeMNPV genomes/ml, respectively) (Figure 1B). No correlation was detected between the abundance of SeMNPV and SeIV-1 genomes through the fractions (Pearson;  $r=-0.182$ ;  $N=16$ ;  $P = 0.50$ ), indicating there was no association between each type of viral genomes at the ODV level, although OBs were RT-PCR positive for the presence of SeIV (Figure 1C).



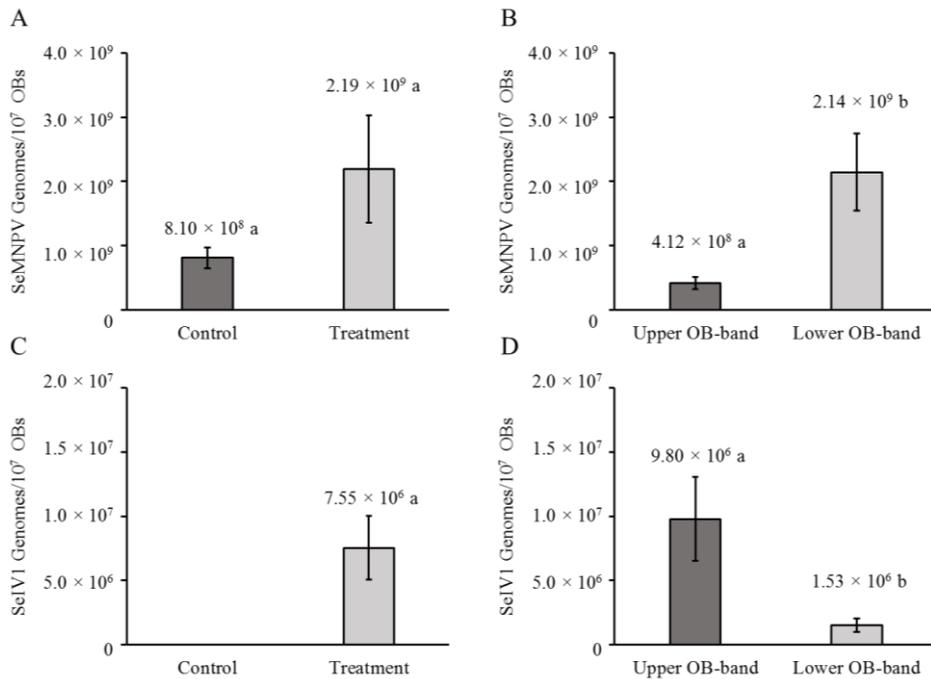
**Figure 1:** Quantification of viral genomes of SeIV-1 (A) and SeMNPV (B) in 16 fractions collected from a continuous sucrose gradient. Agarose gel for SeIV-1 electrophoresis for specific detection of the RdPd gene of SeIV-1 by RT-PCR (C), in RNA extracted from OBs of SeMNPV-AL1 (AL1) and water control (C-).



**Figure 2:** Sucrose gradient tubes of OBs generated in one larval passage of SeMNPV treatment (A) and SeIV-1 co-inoculated with SeMNPV (B). OBs from larvae treated with only SeMNPV migrate through the sucrose homogeneously and formed one band, while OBs of SeMNPV associated with SeIV-1 formed two bands (arrows indicate OB band positions) due to differential migration.

### 3.2 OBs subpopulation separated throughout sucrose gradient

OB conformation was examined in OBs generated following inoculation of SeMNPV-SP2 OBs compared with OBs produced by co-inoculation with SeMNPV-SP2 and SelV-1 in *S. exigua* larvae. Sucrose gradient separation revealed a single OB band in OBs from larvae treated with SeMNPV alone, whereas two OB bands were observed in OBs generated from co-inoculation with SelV-1 (upper and lower OB bands) (Figure 2), two OB subpopulations with different physical characteristics resulted after SeMNPV co-infection with SelV-1. Three OB-bands were collected separately for subsequent experiments.



**Figure 3:** Viral genome quantification for SeMNPV (A and B) and SelV-1 (C and D). Panels A and C show means for control OBs (generated by single inoculation with SeMNPV, dark) or OBs from treatment with SelV (generated by co-inoculated with SelV-1, light). Panels B and D show means for OB subpopulations, collected in upper and lower-OB bands of the sucrose gradient of OBs associated with SelV (B). Columns labelled with different letters indicate significant differences between treatments (*t*-test, *P* < 0.05).

### 3.3 SeMNPV and SeIV genomes quantification in OBs

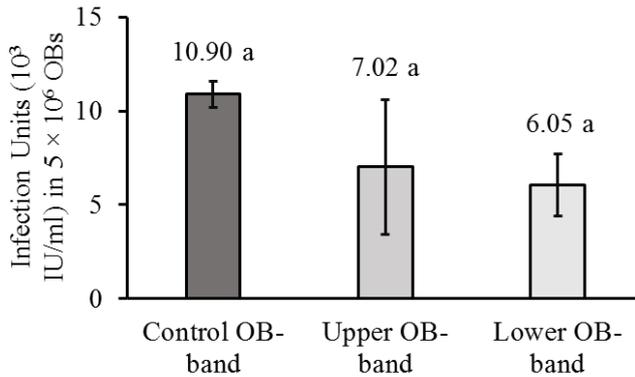
In order to further study OB subpopulations in the sucrose gradient separation, genomes were quantified from the three OB bands: i) control OBs (SeMNPV-treatment) and ii) OB-upper and iii) OB-lower band (co-inoculated iflavirus and SeMNPV). Overall, SeIV-1 co-inoculation did not affect the number of SeMNPV genomes measured in OB progeny ( $8.10 \times 10^8$  SeMNPV genomes/ $10^7$  OBs for control vs.  $2.19 \times 10^9$  SeMNPV genomes/ $10^7$  OBs for treatment;  $t$ -test=1.86;  $df=22$ ;  $P = 0.079$ ) (Figure 3A). As expected, SeIV-1 genomes were abundant for OBs generated in insects treated with iflavirus (no SeIV-1 genomes for control vs  $7.55 \times 10^6$  SeIV-1 genomes/ $10^7$  OBs for treatment) (Figure 3C). Viral loads for both iflavirus associated OB subpopulations differed significantly. SeMNPV genomes were significantly more abundant in the lower OB subpopulation ( $4.12 \times 10^8$  genomes/ $10^7$  OBs for upper OB-band vs  $2.14 \times 10^9$  genomes/ $10^7$  OBs for lower OB-band;  $t$ -test=2.46,  $df=19$ ;  $P < 0.05$ ) (Figure 3B). The average number of SeIV-1 genomes was 6-fold higher in the upper OB-band compared to the lower OB-band ( $9.80 \times 10^6$  genomes/ $10^7$  OBs for upper OB-band vs.  $1.53 \times 10^6$  genomes/ $10^7$  OBs for lower OB-band;  $t$ -test=2.49,  $df=6$ ;  $P < 0.05$ ) (Figure 3D).

### 3.4 Quantification of infectious units per OB

An endpoint dilution assay was performed to determine the number of ODVs per OB in OB subpopulations generated by co-inoculation of SeMNPV and SeIV, compared to the control treatment. The number of infectious units did not differ significantly across OB groups, including both OB subpopulations (upper OB and lower OB-bands) and control OBs (ANOVA;  $F_{2,6}=1.223$ ;  $P = 0.358$ ) (Figure 4). These findings suggest that the simultaneous infection of SeIV-1 did not alter the average number of ODVs occluded within each OB.

### 3.5 ODV banding profile for different OB populations

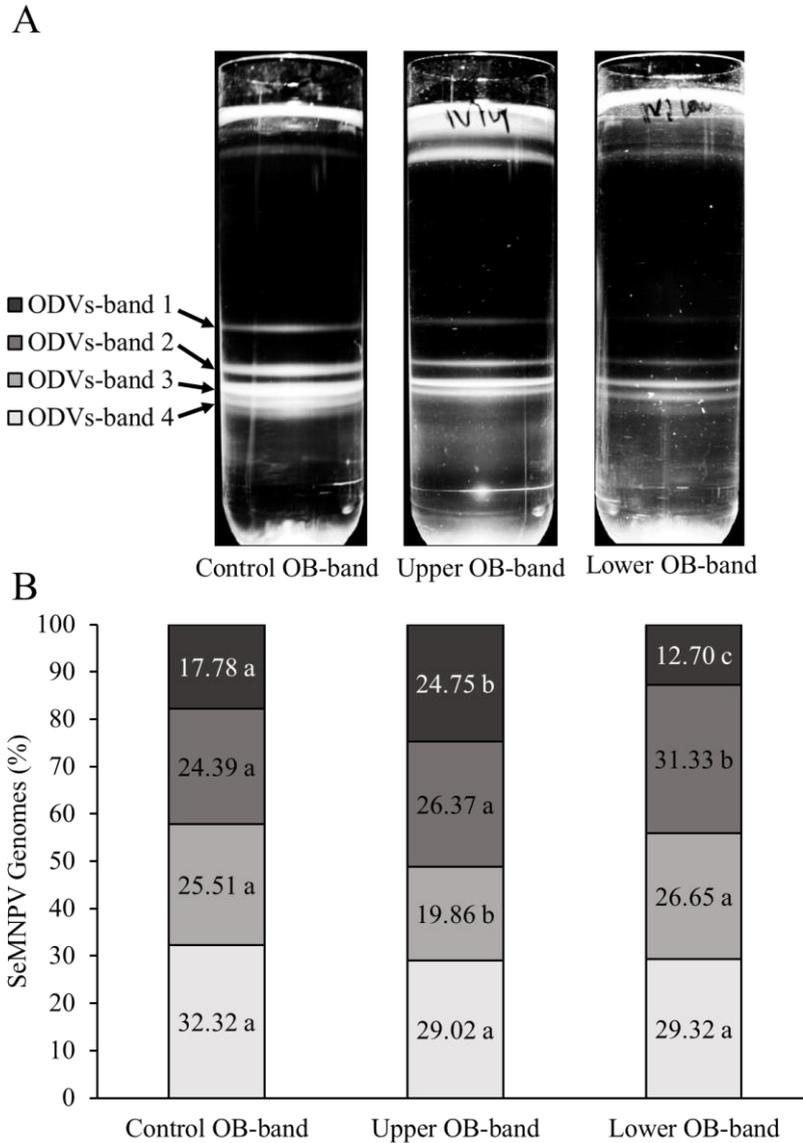
A total of  $10^{11}$  OBs/ml generated from SeMNPV-SP2 inoculation and SeMNPV-SP2 co-inoculation with SeIV-1 were purified by sucrose gradient ultra-



**Figure 4:** Endpoint dilution assay in Se301 cells inoculated with ODVs from  $5 \times 10^6$  OBs from the control treatment (larvae inoculated with SeMNPV OBs alone) and the lower and upper OB-band subpopulations obtained in the sucrose gradient fractions (larvae co-inoculated with SeMNPV and SelV). Columns labelled with identical letters did not differ significantly (ANOVA,  $P > 0.05$ ).

centrifugation. Three OB-bands including the control OB-band and both upper and lower OB-bands were collected separately. OB samples were dissolved and ODVs were subjected to gradient centrifugation to examine the ODV banding profile. The banding profile corresponding to the upper OB-band appeared to be more abundant in single nucleocapsid virions, while the lower OB-band showed more intensity of multiple nucleocapsid virions (Figure 5A). Four gradient fractions corresponding to ODV bands were collected and genome quantification performed. The percentage of SeMNPV genomes from each ODV band were subjected to a Chi-square test to compare differences between the relative abundance of fractions across treatments ( $\chi^2=62.503$ ;  $df=6$ ;  $P < 0.001$ ). ODV-band 1 in OBs from the upper OB-band accounted for 24.75% of the total genomes, which was significantly more abundant than in OBs from the lower OB-band (12.70%) or control OBs (17.78%). The ODV-band 2 was the most abundant for OBs from lower OB band with 33.3% of total genomes, which was higher than the control (24.39%) and upper OB-band (26.37%) OB-subpopulations. The ODV-band 3 was less abundant (19.86%) in OBs from the upper OB-band than any of the other groups (25.52% control OB-band; 26.66% upper OB-band). No

differences were detected in the relative abundance of genome estimates in band 4 across OB subpopulations (Figure 5B).



**Figure 5:** Quantification of ODV fractions in different OB subpopulations. A, ODV-band profiles of OB populations of control OB band, upper OB band and lower OB band. Arrows indicate differential patterns of banding. Arrows on left indicate the positions of gradient fraction collected for ODV-band 1, ODV-band 2, ODV-band 3 and ODV-band 4 . B, relative proportion of ODV-bands of the three OB subpopulations. Different letters indicate significantly differences between treatments ( $\chi^2$  test,  $P < 0.05$ ).

#### 4. DISCUSSION

In this study the physical association between SeIV particles and SeMNPV OBs and ODVs was examined. In a previous study, OBs containing both types of genomes were generated after co-inoculation of SeMNPV with purified particles of SeIV (15). Electron microscopy analysis revealed the presence of particles resembling iflavirus virions inside SeMNPV OBs, after PCR-based detection of iflavirus genomes in the OB. OBs contaminated by SeIV were found to be less pathogenic, but no effects on OB production and infectivity were attributable to the presence of iflavirus (15).

During purification of the OB population obtained from SeMNPV single inoculation and co-inoculation with SeIV-1 by continuous sucrose gradient, an unusual pattern of OB migration was detected. An extra OB band was observed in the OB sample from co-inoculated insects. This sample consisted of two OB subpopulations of different densities. The lower and upper OB bands were analyzed and the abundance of SeIV-1 genomes was significantly higher in the upper than the lower OB bands, while the number of SeMNPV genomes was significantly higher in the lower than the upper OB bands. Accordingly, the enrichment in iflavirus loads may be responsible for the differential density of the OBs, suggesting differences in OB structure including a different number of ODVs embedded in the polyhedrin matrix or a different distribution of nucleocapsids per ODV.

To assess whether ODV numbers differed in each subpopulation, an endpoint dilution assay was performed and no differences between subpopulations were found. These findings indicated that OBs from the different populations share similar numbers of infection units despite the fact that OBs associated with high loads of SeIV contained fewer SeMNPV genomes. Therefore, the differences might be related to the numbers of nucleocapsids per ODV.

Quantification of viral loads of different fractions of ODV demonstrated that the proportion of single-nucleocapsid ODVs was higher in OBs containing a higher concentration of SeIV-1 than in control OBs or in OBs with low loads of SeIV. This might explain previous findings showing that SeMNPV/SeIV associated OBs were less pathogenic than SeIV-1 free OBs (28).

Multiple packaging of nucleocapsids in ODVs is characteristic of the virion morphology of NPVs which ensures the horizontal transmission of genotypic diversity. Early studies indicated the importance of multiple nucleopolyhedrovirus morphotype for the infective traits of the multiple (OpMNPV) and single (OpSNPV) nucleocapsid morphotypes of *Orgyia pseudotsugata* NPV (32). Mixed infections of both morphotypes resulted in mainly multiple-nucleocapsid ODVs in OBs from virus-killed larvae (33). More recently, two main advantages have been described in relation to multiple morphotypes: i) the ability to overcome virus genome mutations and deletions after replication by introducing multiple copies of the virus into the same cell and, ii) the MNPVs ability to bypass the midgut cells to more rapidly spread the infection to the rest of the host body (34). This 'passage effect' accelerates the virus infection avoiding the necessity to complete virus replication in midgut cells and may reduce the host's ability to eliminate infected midgut cells (35). Although it is not known at what level the iflavirus infection produces altered SeMNPV infection, it seems that ODV assembly might be affected by reducing the prevalence of multiple nucleocapsid packaging. As a result, OBs may have a reduced infectious capacity due to a reduced ability to propagate the secondary infection. In conclusion, SeIV-1 association with SeMNPV altered OB conformation by reducing the proportion of ODV containing multiple nucleocapsids. These differences might explain the reduced pathogenicity observed in OBs associated with SeIV-1, following productive SeMNPV infections in hosts with persistent iflavirus infections.

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## CHAPTER VI

# Impact of iflavirus infection on host fitness-related variables and susceptibility to baculovirus infection in *Spodoptera exigua*

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### ABSTRACT

Naturally occurring covert infections in lepidopteran populations involve multiple virus species that result in virus-virus interactions. In this study, interactions between the two RNA viruses, *S. exigua* iflavirus 1 (SeIV-1) and *S. exigua* iflavirus 2 (SeIV-2) (family *Iflaviridae*) that are frequently found in natural populations of *Spodoptera exigua*, and the *S. exigua* multiple nucleopolyhedrovirus, SeMNPV (family *Baculoviridae*) were examined for their implications on the properties of SeMNPV as a biological control agent. Orally transmitted SeIV-1 infections detrimentally affected host developmental parameters, by reducing larval weight gain and decreasing pupal survival. In contrast, SeIV-2 infections did not result in measurable effects on host development. A study on the infection process over time showed increasing loads of SeIV-1 genomes after egg contamination, whereas SeIV-2 loads remained constant during all the developmental stages. Low levels of SeIV-2 were detected in all groups of insects tested, including those from superficially decontaminated egg masses, or eggs that had been superficially contaminated with a high concentration of iflavirus mixtures (SeIV-1 and SeIV-2). However, a similar treatment with SeIV-1, resulted in up to 1000-fold increase in SeIV-1 loads across developmental stages of treated insects. Inoculation of iflavirus in *S. exigua* second instars followed by super-infection with SeMNPV was performed to study whether the insecticidal properties of SeMNPV were altered. The 50% lethal concentration (LC<sub>50</sub>) of SeMNPV was reduced nearly 4-fold and the mean time to death was faster by 12 h, suggesting a synergic effect of SeIV-1 when present in the insect host. These results suggest that inapparent iflavirus infections may be able to modulate the host response to a new pathogen, a finding of particular interest for SeMNPV-based biological control products.

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## 1. INTRODUCTION

Viral infections of insects have mainly been studied in terms of the virulence of the diseases they cause and their potential to kill species that impact negatively on the economy (1) or human health (2). There is now a growing literature on virus infections that cause asymptomatic infections or without obvious signs of disease (3-5). Such hidden infections have been found to elicit certain host responses in a changeable environment (6). For example, in baculovirus, covert infection may play a significant role in the ecology of the virus in relation to seasonal outbreaks of host populations (7, 8). Also, field applications of these viruses may result in asymptomatic infections that can be successfully reactivated after exposure to chemical compounds (9) or other pathogens (4, 8, 10).

Covert infections are associated with opportunities for vertical transmission (11, 12) and as such, are expected to impose few biological costs on host fitness. Covert infection involves a low level of replication and a low level of transcriptional activity (13, 14). The pathogen also avoids clearance by the host immune response by different mechanisms (15) including microRNA interference (16, 17). Host benefits has been observed in some pathogen-host systems. Such a relationship was observed when a nucleopolyhedrovirus was used to challenge *H. armigera* larvae that were persistently infected by a densovirus (18). Complex relations might arise when more than one virus species is involved (19-21). A recent study classified virus-virus interactions into three categories: i) direct gene to gene (or the gene products) interactions; ii) indirect interactions through the alteration of the host environment; and iii) indirect immunological interaction (22). As an example, in a baculovirus system was observed that the first inoculated virus genotype conditioned the entry of a second variant during a temporal window up to ~16 h following the initial infection (23).

The *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV), currently represents the active ingredient of a number of NPV-based insecticides that are among the most effective biological agents for control of this pest. This virus has been shown to produce sublethal infections with measurable effects on host fitness, such as a reduction in pupal weight, adult emergence, fecundity and fertility (24). SeMNPV-covert infections can also be transmitted from sublethally infected parents to their offspring over several generations (25). Covertly infected

offspring were also found to be more susceptible to superinfection by the same virus (24).

New generation sequencing techniques (NGS) have led to the discovery of viruses in covertly infected insects that may be useful as bioinsecticidal agents (26-28), especially RNA viruses, that have remained unappreciated because they usually do not cause lethal disease. Iflaviruses are positive ssRNA viruses (29) from the order *Picornavirales*, family *Iflaviridae* (30), first reported causing lethal infections in economically important species such as silkworms and honeybees (31). Recently, the focus has shifted to studies of novel iflavirus species producing persistent infections, such as *Antheraea pernyi* iflavirus (ApIV) (32), *Lymantria dispar* iflavirus 1 (LdIV1) (33), *Opsiphanes invirae* iflavirus 1 (OilV-1) (34) or *Helicoverpa armigera* iflavirus (HaIV) (35), and the potential of these viruses in host population regulation.

Using NGS, the *Spodoptera exigua* iflavirus 1 (SeIV-1) (36) and *Spodoptera exigua* iflavirus 2 (SeIV-2) (37) were discovered in the *S. exigua* transcriptome. Also, both iflaviruses and SeMNPV were detected in natural populations of *S. exigua* (20) in single, double or triple mixed infections, which stimulated additional studies on the interaction between these viruses. Recent studies involving co-inoculation of SeMNPV, SeIV-1 and SeIV-2 revealed that the insecticidal properties of SeMNPV were significantly affected by the presence of the iflaviruses (38). Physical association also was observed in OBs producing in insects with mixed infections. OB-association provide physical stability to iflavirus, but reduced OB infectivity (39). To date, no studies have been conducted on the influence of SeIV covert infection on host fitness, and the consequences of covert infection by iflaviruses on the performance of the baculovirus in insects.

Here we examine differential baculovirus performance when complex interactions involving covert infections by iflaviruses are present in the host. Specifically, we first assessed the fitness costs of SeIV infection, including larval weight gain, developmental time to pupae, and adult fecundity and fertility. Second, we examined SeMNPV traits such as OB pathogenicity, virulence, and OB production upon death.

## 2. MATERIAL AND METHODS

### 2.1 Insects and virus stock

The *Spodoptera exigua* colony used in the experiments was originally obtained from Andermatt Biocontrol (Switzerland) (SUI colony) (40) as eggs masses. Insects were maintained in the facilities of the Universidad Pública de Navarra, in continuous rearing on semi-artificial diet (41) at  $25 \pm 2$  °C,  $50 \pm 10\%$  relative humidity and a 16:8 h, light:dark photoperiod. When a persistent infection of iflavirus was initiated in insects they were reared separately in disinfected bioclimatic chambers used exclusively for this propose.

OBs of the Spanish isolate SeMNPV-SP2 (42) used in this study were amplified in iflavirus-free *S. exigua* larvae and consistently proved negative for the presence of SeIV-1 and SeIV-2 by RT-qPCR (39).

SeIV-1 and SeIV-2 particles were isolated from larvae previously found to harbor a persistent infection. Briefly, virus particles were purified from guts dissected from ~200 fourth instar larvae (39). The tissues were lyophilized and homogenized in 0.01 M potassium phosphate buffer (pH 7.4) with 0.45% (w/v) diethyldithiocarbamic acid (DIECA) and 0.2% (v/v)  $\beta$ -mercaptoethanol (2.5 ml of buffer per gram of larva), sonicated for 20 sec and filtered through two layers of cheesecloth. The filtrate was loaded on to 10, 30 and 60% discontinuous sucrose gradient and centrifuged for 6 h at  $60,000 \times g$ , 10 °C. The fraction between 30 and 60% corresponding to the virus band, detected by qRT-PCR (see below), was collected and RNA was extracted using RNAzol® RT (Sigma-Aldrich) following the manufacturer's protocol. Quantitative PCR (qPCR) was undertaken as described below to determine the concentration of SeIV-1 and SeIV-2 genomes per sample. For this study three iflavirus populations were selected: SeIV-1 enriched in SeIV-1 (95% of SeIV-1 genomes), SeIV-2 enriched in SeIV-2 (98% of SeIV-2 genomes) and SeIV-1 + SeIV-2 in which both iflavirus species were present in equal numbers (50% SeIV-1 + 50% SeIV-2).

### 2.2 *S. exigua* fitness cost by SeIV infection

Fitness cost studies were performed at Universitat de València using the same insect colony and reared under similar conditions as described above. Different fitness-related parameters were measured in insects that had been orally

infected with the different viral preparations. For this, groups of 30-40 first instar larvae from a virus-free sample of the colony were allowed to drink from viral suspensions by the droplet feeding method (43). The final viral concentration in the suspension was of  $1 \times 10^9$  iflavivirus genomes/ $\mu\text{l}$  for the three viral treatments. Larvae were allowed to drink for 20 min and ingestion was tracked by the presence of phenol red dye in the larval digestive tube. For each treatment and replicate, twenty larvae that consumed the inoculum were selected and reared on artificial diet in plastic boxes (10 cm (W)  $\times$  10 cm (D)  $\times$  10 cm (H)) at  $25 \pm 2$  °C to monitor larval growth, survival to pupa and mean time to pupae. For each of the replicates, four treatments were evaluated in a side-by-side manner: i) Control larvae (C); ii) SeIV-1-infected larvae (SeIV-1); iii) SeIV-2-infected larvae (SeIV-2); and iv) SeIV-1 + SeIV-2-infected larvae (SeIV-1 + SeIV-2). Larval weight and instar was recorded daily and three independent replicates were performed using different batches of larvae. Insects from each group that reached the pupal stage were selected and used to evaluate adult fecundity and fertility. For that, four females and four males from each treatment and replicate were selected at the pupal stage, allowed to emerge and mate under standard conditions and the number of laid eggs and their hatching rate were counted for the following 10 days. In addition, pupae that were not used for mating experiments were used to estimate viral abundance by RT-qPCR, as previously reported (40). Larval weight, mean time to pupa, survival to pupa and fecundity were compared between control and iflavivirus treatments using ANOVA and Bonferroni post hoc test. Fertility was compared by Kruskal-Wallis test using the SPSS package (v. 21 IBM).

### 2.3 Establishment and course-time of iflavivirus infection

We assessed the establishment and course time of iflavivirus infections to determine the optimal timing to induce the second infection by SeMNPV, with the aim of ensuring high levels of iflavivirus in the insect samples. An inoculative method involving immersion of host egg masses in viral suspension was developed to ensure virus acquisition by the neonates, to emulate natural conditions. The insects challenged with iflavivirus were then reared through to the adult stage, and samples were taken and analyzed for the presence of SeIV-1 and SeIV-2 by qPCR.

We knew from previous studies that SeIV was vertically transmitted (20) and rapidly spread in conditions of artificial rearing (36), so surface decontamination of eggs was applied to minimize cross-infection. For that, egg masses were submerged in 0.5% sodium hypochlorite for 10 min, thoroughly rinsed in water, and allowed to dry. Three groups of hypochlorite-treated egg masses were inoculated by immersion in one of three concentrations of SeIV-1 + SeIV-2:  $10^7$ ,  $10^8$ , and  $10^9$  genomes/ $\mu\text{l}$  in total. As controls, one egg mass without any treatment (non-treated, NT) and another hypochlorite-treated (NaClO) followed by immersion in water instead of virus were included. All egg masses were placed in 100  $\text{cm}^3$  plastic containers until hatching. Four day-old larvae were individualized in 24-compartment plates (Corning, New York, USA) provided with semiartificial diet and reared through to the adult stage in the rearing conditions describe above. To test for the presence of viruses 6 individuals were randomly collected at second, third, fourth and fifth larval instars, pupae and adult stages. Insect samples were frozen at  $-20\text{ }^\circ\text{C}$  before use for total RNA extraction and iflavirus quantification as describe below. The entire bioassay was performed three times.

## 2.4 Pathogenicity and virulence bioassays

In order to study the effect of iflavirus infections on baculovirus traits, a superinfection by SeMNPV was performed in insects that had previously been inoculated with iflavirus preparations. *S. exigua* egg masses were treated either with iflavirus suspension or water (control) following the method described above. Bioassays were conducted in both insect lines in parallel to assess the effect of SeIV infection on SeMNPV OB pathogenicity (median lethal concentration,  $\text{LC}_{50}$ ) and virulence (mean time to death, MTD). For this, SeMNPV oral inoculation was performed in second instar larvae by the droplet-feeding method (43). Briefly, groups of 30 pre-molt *S. exigua* first instars from each insect colony were starved overnight, and once molted, larvae were fed with one of five OB concentrations:  $2.54 \times 10^5$ ,  $8.18 \times 10^4$ ,  $2.72 \times 10^4$ ,  $9.09 \times 10^3$  and  $3.03 \times 10^3$  OBs/ml, previously demonstrated to kill between 95% and 5% of the tested larvae. A group of 30 mock-infected larvae from each insect colony was included as control. Larvae were allowed to drink from the OB suspension for 10 min and only those visibly inoculated were individually placed in 24-compartment plates (Corning, New York,

USA) containing artificial diet. Inoculated larvae were maintained at  $25 \pm 1$  °C,  $50 \pm 5\%$  RH and NPV mortality was recorded for 7 days post-inoculation at either 8 h intervals for those insect challenged with the highest concentration or, 24 h for the rest of the treatments.

OB production upon larval death was also estimated for larvae inoculated with the highest OB concentration ( $2.54 \times 10^5$  OBs/ml). OB-treated larvae showing clear signs of NPV-disease were confined in 1.5 ml vials when they did not response to mechanical stimuli and stored at room temperature for 24-48 h. Cadavers were homogenized in 1 ml of double-distilled water and OB suspensions were counted in triplicate using a Neubauer hemacytometer (Hawksley, Lancing, UK) under contrast phase microscope at  $400\times$ .

Concentration-mortality data were subjected to Probit regression analysis using the POLO-PLUS program (44).  $LC_{50}$  values and relative potencies were estimated when a parallelism test confirmed that the regressions for each treatment could be fitted with a common slope. Time-mortality data recorded for larvae dosed with the highest concentration ( $2.54 \times 10^5$  OBs/ml) was subjected to Weibull survival analysis in GLIM 4 program (45). The validity of the Weibull model was determined by comparing fitted values with Kaplan–Meier survival function estimated values. The  $\log_e$  (mean OB production) from each insect colony were compared by ANOVA using SPSS 21.0.

## 2.5 Virus detection and quantification

For detection of covert infections total RNA was isolated from insect tissues using the Master Pure Complete RNA Purification kit (Epicentre Biotechnologies). One of the three following insect samples: i) entire larva; ii) pupa or; iii) dissected adult abdomen, was placed individually in a 2-ml microfuge tube with ceramic beads, 300  $\mu$ l tissue lysis solution and 1  $\mu$ l proteinase K (50 ng/ $\mu$ l). Samples were homogenized using MP FastPrep-24 tissue cell homogenizer at 4 m/s for 20 s and incubated at 65 °C for 15 min at 1,000 rpm orbital agitation. For RNA extraction protein precipitation reagent was added, centrifuged at  $10,000 \times g$  for 10 min and the DNA was precipitated with isopropanol. Nucleic acid pellets were treated with RNase-free DNase buffer and 5  $\mu$ l of DNase for 30 min at 37 °C. A volume of 200  $\mu$ l of 2  $\times$  T and C lysis solution was added and vortexed for 5 s followed by 200  $\mu$ l of protein precipitation reagent and vortexed for 10 s. The

debris was pelleted by centrifugation and the supernatant was precipitated once with isopropanol and twice with 70% ethanol. Lastly, RNA was resuspended in 20  $\mu$ l DEPC water and stored at  $-20$  °C. Blank extraction samples containing only water were processed in parallel to control for cross-contamination during the extraction process. All equipment and reagents were previously sterilized and treated with DEPC to remove RNases.

To quantify viral loads in *S. exigua*, reverse transcription quantitative PCR (RT-qPCR) was performed for SelV-1 and SelV-2 separately. Specific primers for detection of SelV-1 and SelV-2 (40) sequences were used (Table 1). The PCR program used was as follows: 3 min at 95 °C, 10 s at 95 °C and 30 s at 62 °C for 45 cycles followed for a melting curve of 5 s per 0.5 °C from 65 to 95 °C. Total cDNA was used as template for amplification to detect the iflaviruses. To obtain cDNA, one  $\mu$ g of RNA was reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Promega). The reverse transcription mix consisted of 2  $\mu$ l of 5 $\times$  buffer (Promega), 1.2  $\mu$ l MgCl<sub>2</sub> (25 mM), 0.5  $\mu$ l dNTP mix (10 mM), 0.8  $\mu$ l DEPC water and 1  $\mu$ l ImProm-II reverse transcriptase (Promega). The mixture was added to RNA samples and incubated at 25 °C for 5 min, followed by 42 °C for 60 min and 70 °C for 15 min. qPCR based on SYBR Green fluorescence was carried out in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) in 96-well reaction plates. A 9  $\mu$ l mastermix containing 5  $\mu$ l SYBR, 0.5  $\mu$ l of both primers (10  $\mu$ M) and 3  $\mu$ l water was added to 1  $\mu$ l of cDNA template. For the construction of standard curves, the PCR products generated with specific primers of SelV-1/2 were cloned into a pGEM®-T Easy cloning vector (Promega). Plasmid DNA was quantified using a spectrophotometer (Eppendorf BioPhotometer Plus) and eight-fold serial dilutions in sterile MilliQ water (from  $1 \times 10^{-1}$  to  $1 \times 10^{-7}$  ng/ $\mu$ l) were used in duplicate as standards for the construction of a standard curve. Data was acquired and analyzed using Bio-Rad CFX Manager 3.1 software (Bio-Rad). The regression parameters of the standard curves exceeded  $R^2 = 0.95$  in both cases with slopes of -3.50 and -3.61 for SelV-1 and SelV-2, respectively, with an efficiency between 90 and 110% (46). In all cases, the last standard concentration,  $10^{-7}$  ng/ $\mu$ l, represented the limit of detection and showed correct amplification curves and the expected melting temperatures of 77.0 and 79.5 °C for SelV-1 and SelV-2, respectively. The corresponding Ct values were assigned as cut-off points for each virus, so that higher values were considered virus-free samples. SelV Ct

among different insect-stage samples values were normalized using the corresponding values of the references gene *ubiquitin/ribosomal protein L40* (*RPL40*), as previously reported (40) (Table 1). Quantifications of treatments and host stages for SelV-1 and SelV-2 were compared by ANOVA in SPSS (v. 21 IBM).

**Table 1:** Primer sequences and description of the genomic region targeted.

Primer	Sequence	Description
SelV-1q	F: 5'- TGTGAAGTTAGACACGCATGGAA-3' R: 5'-CGACTTGTGCTACTCTTCATCAA-3'	Amplifies a 97-bp in the RNA-dependent RNA polymerase (RdRp) region from SelV-1 (36).
SelV-2q	F :5'-CCGCTCGCTTATTGAAACGT-3' R: 5'-CATGAGACAGCTGGAATTGGAA-3'	Amplifies a 78-bp in the RNA-dependent RNA polymerase (RdRp) region from SelV-2 (37).
<i>ubiquitin/ribosomal protein L40</i> ( <i>RPL40</i> )	F: 5'-GTTGCTGGTCTGGTGGGATT-3' R: 5'-AGGCCTCAGACACCATTGAAA-3'	Amplifies a 72-bp in ribosomal protein L40 from <i>S. exigua</i> (40).

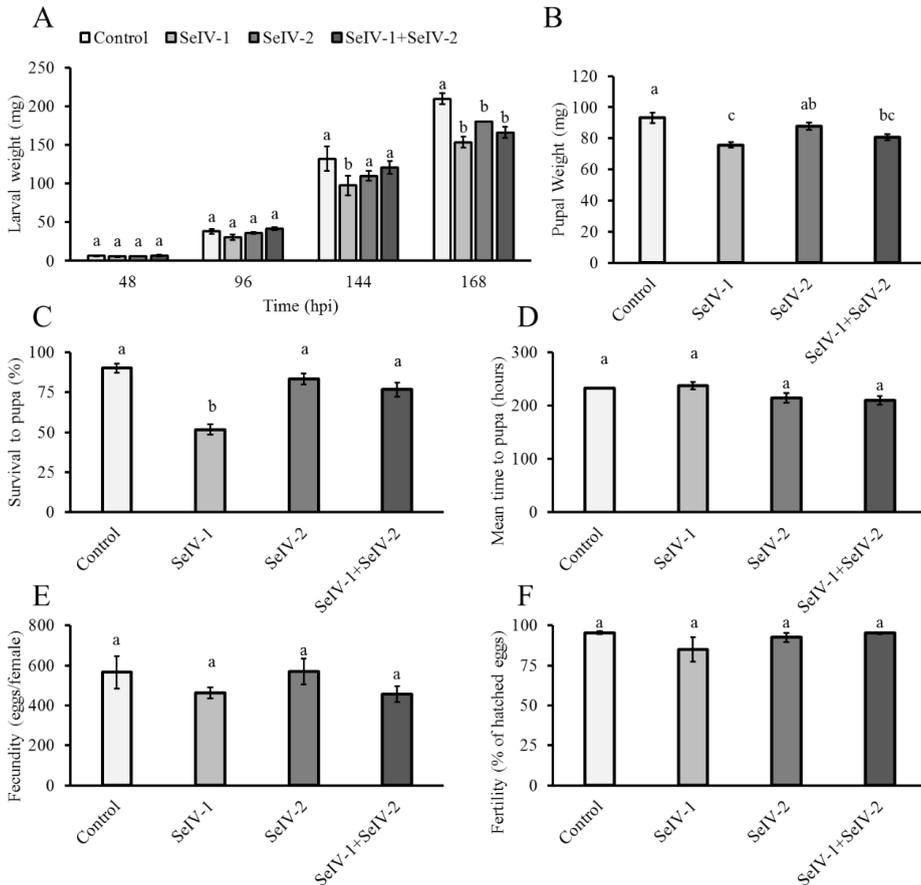
### 3. RESULTS

#### 3.1 Iflavirus effect on insect fitness

Developmental parameters for *S. exigua* inoculated with either SelV-1, SelV-2 or an equal mixture of SelV-1 + SelV-2 were compared with the corresponding values estimated for mock-infected insects. Larval weight records did not differ significantly at 48 and 96 hours post infection but differed significantly at 144 hpi and 168 hpi (2-way ANOVA,  $F_{9,14}=4.371$ ;  $P < 0.05$ ), as decreasing weight gain were registered in the presence of SelV-1 infection (Figure 1A). SelV-1 + SelV-2 infections, probably due to the presence of SelV-1, also showed a significant decrease in weight gain at 168 hpi. In line with these results, pupal weights for iflavirus infected insects were lower than observed in mock-infected insects ( $F_{3,8}=10.163$ ;  $P < 0.05$ ) (Figure 1B). The lowest pupal weight values were registered in insects treated with SelV-1 ( $75.78 \pm 1.61$  mg) and SelV-1 + SelV-2 ( $80.68 \pm 1.90$  mg), that were significantly lower than the weights of SelV-2-treated pupae ( $87.72 \pm 2.41$  mg) (Bonferroni,  $P < 0.05$ ).

The survival to the pupal stage was markedly reduced in the SelV-1-treated insects, whilst no significant differences were found between either SelV-2

or SeIV-1 + SeIV-2 and the control groups (Figure 1C) (ANOVA;  $F_{3,8}=22.436$ ;  $P < 0.001$ ). Larval developmental time was not affected by iflavirus infections (ANOVA;  $F_{3,8}=3.595$ ;  $P = 0.066$ ), ranging from averages of 209.9 to 237.3 h corresponding to the SeIV-1 + SeIV-2 and SeIV-1 treatments, respectively (Figure 1D).



**Figure 1:** Effects of iflavirus infection by SeIV-1, SeIV-2 and SeIV-1 + SeIV-2 on *Spodoptera exigua* in terms of larval weight gain (A) observed at 48, 96, 144 and 168 hours-post-inoculation, pupal weight (B), percentage of survival to pupa (C), mean time to pupa (D), fecundity (E) and median percentage of egg fertility (F) of insects treated with SeIV-1 and SeIV-2 and controls. Different letters indicate significant differences between virus-treatment groups by post-hoc Tuckey HDS test ( $P < 0.05$ ) or Kruskal-Wallis test ( $P < 0.05$ ), respectively. Bars indicate the standard error (A-D) or interquartile range (E).

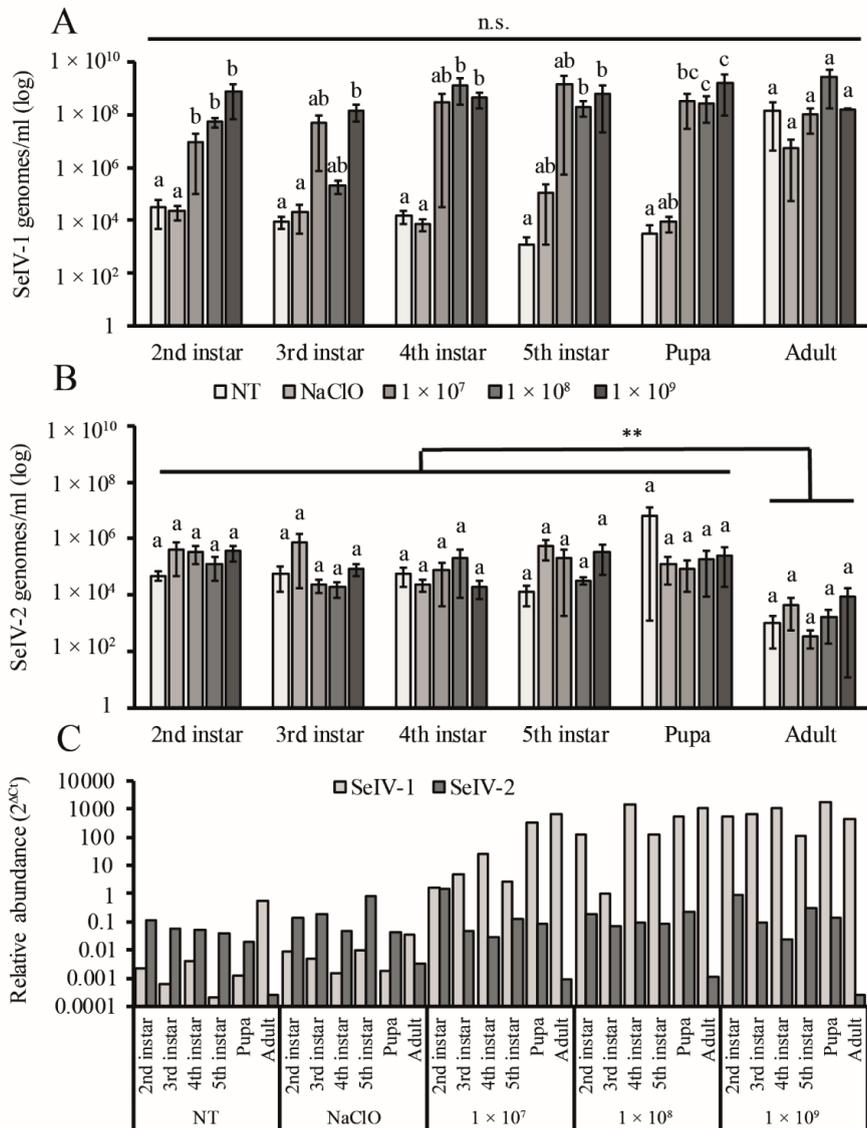
Reproductive traits were measured in terms of the numbers of eggs laid by adult females (fecundity) and the percentage of hatched eggs (fertility).

Fecundity did not differ significantly across virus treatments (ANOVA;  $F_{3,8}=1.231$ ;  $P = 0.360$ ), although egg masses from SelV-1-treated females tended to be smaller (462.58 eggs for SelV-1 and 456.00 eggs for SelV-1 + SelV-2) than those of mock-infected (565.66 eggs) or SelV-2-treated females (569.58 eggs) (Figure 1E). Similarly, no significant differences were found in the percentage of hatched eggs across iflavirus treatments and the control (Kruskal-Wallis;  $\chi^2=1.769$ ;  $df=3$ ;  $P = 0.622$ ) (Figure 1F).

### 3.2 Time-course of iflavirus infections

Insects from egg masses inoculated with iflaviruses ( $10^7$ ,  $10^8$  or  $10^9$  genomes/ $\mu\text{l}$  of SelV-1 + SelV-2) were reared throughout to adults and samples taken at each larval instar, pupa and adult stages for SelV-1 (Figure 2A) and SelV-2 (Figure 2B) genome load quantification. All insects were carefully treated reared and manipulated but this was not sufficient to prevent the control insects from cross-contamination as low viral titers were detected in all the analyzed insects. Nevertheless, the SelV-1 treatment resulted in a 100- to 1,000-fold increase in viral titer when compared to control groups (NT and NaClO-treated) (Figure 2A). In contrast, SelV-2 loads were very similar across the different treatments. A similar trend was found for all larval groups exposed to the iflavirus and analyzed at different stages. Viral titers in the adult reached similar loads across treatments groups, including controls for SelV-1 (ANOVA;  $F_{4,9}=2.578$ ;  $P = 0.110$ ), although no significant differences were found for SelV-2 (ANOVA;  $F_{4,9}=0.346$ ;  $P = 0.841$ ). No significant differences were found for SelV-1 loads over the course of insect development (ANOVA;  $F_{5,68}=1.184$ ;  $P = 0.326$ ). However, a significant decrease in adults was found for SelV-2 comparing to the larval stages (ANOVA;  $F_{5,79}=8.817$ ;  $P < 0.001$ ). No evidence of interference between the quantities of each virus was detected (data pooled for treatment and stages), as viral loads varied independently (Pearson;  $r=-0.197$ ;  $N=90$ ;  $P = 0.062$ ).

SelV-1 and SelV-2 titers were normalized using *RPL40* gene expression as a reference (Figure 2C), to control for host growth effects. SelV-1 expression exceeded the reference gene expression for all groups of insect iflavirus but not for control groups, indicating a positive relationship between viral expression and time post-infection. SelV-2 expression levels did not exceed that of the reference gene at any development stage, and independently of treatment concentration the

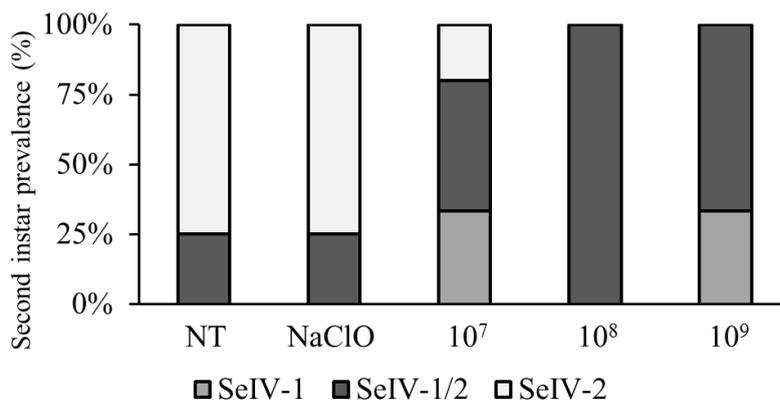


**Figure 2:** Absolute quantification of SeIV-1 (A) and SeIV-2 (B) and SeIV-1/SeIV-2 relative abundance (C) over *S. exigua* development following treatment of egg masses with iflaviruses. Viral loads were estimated by RT-qPCR for non-treated (NT), decontaminated (NaClO) or virus-treated egg masses with  $10^7$ ,  $10^8$  and  $10^9$  genomes/ $\mu$ l of a mixture of SeIV-1 and SeIV-2 (50:50). Bars show means quantified of second, third, fourth, fifth larval instars, and pupa, and adult stage. Different letters mean significant differences between viral treatments for each instar/stage group by Tukey test ( $P < 0.05$ ). SeIV-1 genomes quantification did not differ across instar-stages (n.s.) and SeIV-2 genomes quantification significantly differed between larval and adult stages (\*\*) by ANOVA test ( $P < 0.01$ ). Mean  $2^{\Delta Ct}$  values were calculated by subtracting Ct values for *RPL40* from Ct values of the target genes.

relative abundance of SeIV-2 was near constant across treatments and developmental stages.

### 3.3 Effects of iflavirus infection on SeMNPV insecticidal properties

Two insect groups previously treated with the iflavirus mixture SeIV-1 + SeIV-2 and the untreated control were used in bioassays to determine their susceptibility to SeMNPV infection. Egg masses were inoculated with SeIV-1 + SeIV-2 mixture  $10^8$  genomes/ $\mu$ l in total and used in bioassays when the larvae reached the second instar. A high prevalence of SeIV-2 infection was detected in control groups, and these differed in the prevalence of SeIV-1 infections (25% control vs 100% in iflavirus-treated insects) (Figure 3). Consequently, the possible influence of iflavirus treatment on susceptibility to SeMNPV was mainly attributable to the presence of SeIV-1 infection.



**Figure 3:** Prevalence of SeIV-1 and SeIV-2 in second instar larvae after iflavirus treatment. Percentage of individuals RT-qPCR positive in second instar larvae for SeIV-1 and SeIV-2 or both viruses simultaneously after egg mass treatments at different concentrations of the iflavirus mixture (SeIV-1 + SeIV-2). NT = not treated, NaClO = hypochlorite treatment.

Larval susceptibility to SeMNPV OBs significantly increased in the iflavirus treatment (SeIV-1 infection) (Table 2). SeMNPV OB pathogenicity ( $LC_{50}$  value) was reduced by ~4-fold for the iflavirus treated insects when compared to the control insects.

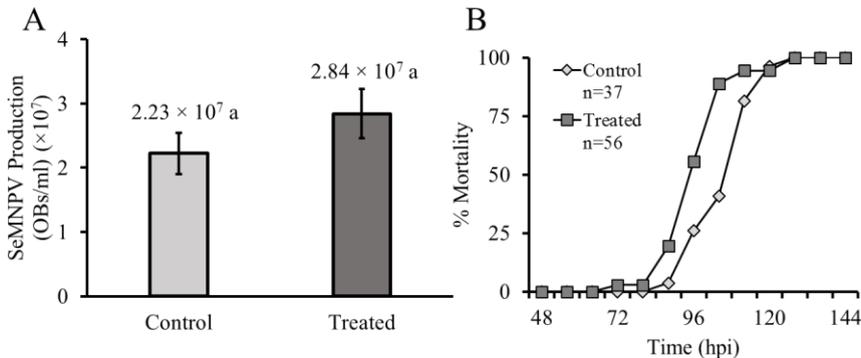
Between 37.5% and 61.1% of larvae treated with the highest concentration of SeMNPV ( $2.54 \times 10^5$  OB/ml) died from lethal polyhedrosis in the

control and iflavirus-infected groups, respectively. No mortality was registered in mock-infected larvae of either insect colony.

**Table 2:** LC<sub>50</sub> values, relative potencies and mean time to death (MTD) for *S. exigua* second instars inoculated with the SeMNPV on controls or colonies with persistent infections of SelV-1 + SelV-2.

Insect colony Persistent Infection	LC <sub>50</sub> ( $\times 10^4$ OBs/ml)	Relative Potency	95% Confidence limits		$\chi^2$	MTD (h)	95% Confidence limits	
			Low	High			Low	High
Control	5.87	1.00	-	-	5.27	90.77a	88.83	92.74
Treated with SelV-1/2	1.56	3.76	2.23	6.35	5.93	78.93b	77.62	80.26

Probit analysis was performed using the Polo-Plus program. The hypothesis of equality was rejected ( $\chi^2=31.93$ ;  $df=2$ ;  $P < 0.05$ ) and a test for non-parallelism was not significant ( $\chi^2=2.56$ ;  $df=1$ ;  $P = 0.11$ ), such that regressions were fitted with a common slope of  $0.989 \pm 0.116$  (mean  $\pm$  S.E.). Relative potency was calculated as the ratio of effective concentrations relative to the control. Mean time to death values (MTDs) were estimated by Weibull analysis. Mean values labeled with different letter differed significantly by *t*-test ( $P < 0.05$ ).



**Figure 4:** OB production (A) and SeMNPV-mortality in second instar *S. exigua* over post-infection time (B) for untreated (control) and iflavirus-treated insect colonies. Bars show means and standard error. Same letters indicate no significant difference by one-way ANOVA test ( $P > 0.05$ ). Percentage of mortality recorded every 8 h after SeMNPV inoculation.

OB production and time-mortality response was assessed in these groups. SeMNPV speed of kill was significantly affected by iflavirus treatment, resulting in a 12 h reduction in the mean time to kill compared to the control group

(Table 2, and Figure 4B) (Weibull GLM;  $t=5.16$ ,  $df=94$ ;  $P < 0.05$ ). However, OB production upon death (Figure 4A) did not differ significantly between larvae carrying either single ( $2.23 \times 10^7 \pm 3.21 \times 10^6$  OBs/ml) or double iflavirus infections ( $2.84 \times 10^7 \pm 3.88 \times 10^6$  OBs/ml) (ANOVA,  $F_{1,92}=1.38$ ;  $P = 0.24$ ).

#### 4. DISCUSSION

In this study, we aimed to evaluate the fitness-related costs associated with infection with different types of iflaviruses, including possible changes in susceptibility to another virus pathogen, SeMNPV. Iflaviruses have been mainly studied in economically important species, such as *Bombyx mori* (47) and *Apis mellifera* (48). Infectious Flacherie Virus (IFV) produces an acute infection on silkworms, in which the midgut epithelium is primarily infected (49). This infection spreads to neighboring cells finally leading to a lethal diarrhea. A silkworm IFV infection was estimated to result in 27 – 35% of losses (50). Deformed wing virus (DWV) does not cause honeybee death but produces deformed wings, paralysis, altered abdominal shape, reduced life span and learning deficits (48, 51). However, studies conducted on *S. exigua* iflavirus have not previously focused on the pathology of the virus. Indeed, no symptoms or sign of infections were previously observed in insect cultures harboring high iflavirus loads (40). However, by examining developmental parameters, here we detected differential biological effects exerted by each iflavirus species in *S. exigua*. Notably, SelV-1 infection resulted in a detrimental effect on larval development indicated by reduced weight gain that lead to lower survival in the pupal stage. However, reduced weight gain was not as important as reduced fertility and fecundity, as observed in studies on baculovirus sublethal effects (24). SelV-2 effects on developmental parameters was minimal and when mixed with SelV-1, the detrimental effect caused by SelV-1 seemed to be attenuated. Comparing both species genome sequences, SelV-1 and SelV-2 share 40% of nucleotide identity (40). Phylogenetic analysis carried out between a total of 24 fully-sequenced iflavirus genomes, revealed that SelV-1, and SelV-2 were closely related but occupied different clades, with SelV-1 closest to the Infectious Flacherie Virus, whilst SelV-2 was more similar to *Perina nuda* virus or *Ectropics oblicua* virus (52). However, viruses that share the same host species might be evolutionary diverse and involve complex relationships. Due to

its economic importance, one of the best investigated groups of iflaviruses are those species affecting *Apis mellifera* (52). Bees can act as hosts of a complex of up to four different iflavirus species, showing a diverse spectrum of symptoms and pathological effects on the host, sometimes driven by virus-virus interactions at different levels including recombination events (53). For instance, the picornavirus-like Deformed wing virus (DWV) and Kakugo virus (KV), despite sharing 98% of their genome sequence (aa identity), drastically differ in their virulence and infectious symptoms (54). High titer infections of DWV affect honeybee development, producing developmental deformities, premature ageing and even death (55), while effects of KV have been related to increases of aggressiveness of the working bees (56).

The pathological and symptomatic effects of iflavirus infections were likely to be related to the viral loads accumulated in the host. Low level infections by iflaviruses have been related to asymptomatic infections, whereas high levels may result in measurable disease effects or host death (57, 58). For instance, when low-level persistent DWV infections in bees, were vectored by *Varroa* mites 1000-fold amplification and extremely virulent viral outbreaks occurred in the host colony (57). Similarly, asymptomatic bees were shown to harbor SBV, though at lower levels than those observed in individuals showing signs of sacbrood disease (58). In the present study, low viral loads of iflavirus were detected in the untreated group, indicating a persistent low-level of infection. Notably, SeIV-1 loads dramatically increased up to 1,000-fold in iflavirus-treated groups, proving that the pathogen replicates during insect development. In contrast, SeIV-2 loads did not increase from low persistent virus levels when eggs were inoculated, even at an inoculum concentration of  $10^9$  genomes/ $\mu$ l. Interestingly the relative proportions SeIV-1/SeIV-2 increased as the insect reached the pupal stage, such that SeIV-2 loads nearly overtook that of SeIV-1, despite the proportion of this virus in the inoculum (data not shown). In line with this, adult loads of SeIV-2 decreased compared to those present in the larval stage, suggesting a limited amplification of this virus. Taken together, these results indicate that SeIV-1 successfully established an infection despite the presence of a persistent infection by SeIV-2.

Viral production has been related to the transmission of iflaviruses. Depending on the route of transmission, the DWV virus could cause either symptomatic infection, characterized by high levels of virus, or asymptomatic

infection, at a low level of virus (52). Usually, vertically transmitted iflaviruses do not reach high levels and do not produce symptomatic infections (59). This dual transmission system is frequently reported among iflavirus groups and it may be applicable to SeIV-1 and SeIV-2 and their opportunities for vertical and horizontal transmission. In a previous study, we observed a rapid spread of SeIV-1 infections in insect colonies (36), likely mediated by food contamination as reported in *Helicoverpa armigera* IV (35). Other study revealed SeIV-1/SeIV-2 transgenerational transmission detected in 20 - 40% of the offspring from field-caught adults (25). Here we did not observe any effect of surface decontamination treatment on SeIV titers, so that the low-level infection detected in control insects might be due to transovum rather than transovarial transmission. Similarly, HaIV infections were less efficient in vertical (28.3%) than horizontal transmission (75%) (60). Interestingly, iflavirus transmission can occur via vectors such as *Varroa* mites for DWV infections in bees (59), whereas for SeIV-1 the association with SeMNPV OBs resulted in marked improvements in environmental persistence of the iflavirus (39). Here we observed divergent trends between the iflavirus species during host development. SeIV-1 showed high levels of transcription, suggesting high levels of replication and opportunities for horizontal transmission route. In contrast the reduced loads and limited amplification of SeIV-2 suggest persistent and relatively benign infection and potential opportunities for vertical transmission. In this sense, the iflavirus of *Lymantria dispar* (LdIV1) was capable of producing persistence infections in cell culture as a result of viral suppression of host-mediated RNA silencing (33).

Iflaviruses appear to be widespread in *S. exigua* colonies from different origins (40), including field-caught and laboratory-reared individuals (25). Here we demonstrated the influence of iflavirus covert infection on host responses to a different pathogen, SeMNPV. Iflavirus infections altered the insecticidal properties of SeMNPV as a biological control agent. Inoculation of *S. exigua* with SeIV-1 increased SeMNPV-induced mortality in terms of lethal concentrations and speed of kill. Similar synergic effects were observed when SeMNPV and SeIV-1/2 were simultaneously inoculated in *S. exigua* larvae (38), suggesting a virus-virus interaction between both types of virus. The changes in host susceptibility may be a result of differences in the host environment due to sequential infection (22). The effects of the first virus on host defense mechanism, such as changes in the

peritrophic membrane (61), the activation of innate antiviral immunity responses (62), apoptosis (63) and RNA interference activation as a cellular defense mechanism (16), may make change host susceptibility to a subsequent baculovirus infection. For example, in vertebrates a second infection with a DNA virus blocked the immune system pathway and led an RNA virus to increase its replication (64). Iflavirus infections have been observed to activate some of this type of defense mechanism by up-regulating antimicrobial peptides and down-regulating the expression of phenoloxidase genes that form part of the insect humeral immune response (57). Further studies are required to elucidate the mechanisms behind iflavirus persistent infections and their influence on the host immune system.

Considering the ubiquitous nature of iflavirus populations and the potential effect on host development of SeIV-1 persistent infection, it seems likely that these viruses affect the efficacy of SeMNPV-based insecticides. We conclude that SeIV-1 persistent infections in *S. exigua* populations could improve the efficacy of SeMNPV-based insecticides. However, previous studies by us (38) revealed a negative impact of iflavirus infection on SeMNPV OB production. The biosecurity implications of the presence of iflaviruses in biological insecticides and the insect colonies used to produce these products remain unclear and this is an issue that merits further study.

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## CHAPTER VII

# General discussion

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*Spodoptera exigua* cause important economic losses in most of the template areas worldwide, including greenhouses of Almería, Spain (1). To avoid or reduce the economic losses caused by this pest, the applications of NPV-based bioinsecticides can play an important role in the implementation of integrated pest management (IPM). These viruses present clear advantages over chemical pesticides (2), since they do not produce most of the problems associated with chemical control and they can be considerate environmental friendly in terms of toxic residues in fields and crops. In addition to the mortality caused by virus infections, field application of NPVs can produce other effects due to virus persistence, producing horizontal and vertical transmissions occurring after realizing the NPV inocula (3). The *S. exigua* multiple Nucleopolyhedrovirus (SeMNPV) have been developed as the active ingredient of commercial bioinsecticides (2, 4, 5), which promising expectations to be implemented in the organic sweet pepper production system of the greenhouses of Almería (4-6).

Novel sequencing techniques such as NGS, have revealed increasing numbers of RNA viruses infecting insects (7) stating the importance of knowledge about how these viruses affect host populations ecology including the effect of virus-virus interactions (8, 9). The number of virus identified in the *Iflaviridae* family markedly increased in the last few years. These single stranded positive RNA viruses (ss(+)RNA) are characterized by a unique large open reading frame (ORF) that encodes both structural and non-structural proteins content in not enveloped, icosahedral particles approximately 30 nm in diameter (10). Novel RNA viruses belonging to the *Iflaviridae* (11) family was discovered in the transcriptome of *S. exigua* colonies (8), and were later characterized as *S. exigua* iflavirus 1 (SeIV-1) (12) and *S. exigua* iflavirus 2 (SeIV-2) (13).

Despite some of the best known iflavirus species affect insect beneficial species such as *Apis mellifera* (14) or *Bombyx mori* (15), many other species of RNA viruses are capable of infecting insects which cause pests, destroy crops and produce important economic losses. Some of them have been seen to be useful in

pest control as active ingredient of a bioinsecticide (16) or contrary, may be useful in other more technically developed approaches including genetically engineered crop plants (17) and baculovirus expression vectors (18).

Previous studies have addressed the wide range of factors affecting the SeMNPV field application efficiency (19). However, very little is known about the sanitary condition of the natural insect population. Our findings in *S. exigua* field-caught adult (20) showed that more than half part of individuals tested harbor covert infection of SeMNPV (54%) and a relative high prevalence of iflavirus (21%), involving dual, and triple species combinations in most of cases (9%). In addition, SeMNPV covertly infected *S. exigua* populations have found to result in increasing susceptibility to a superinfection of the same virus (21). This and other similar findings (22) suggest that hidden infections might alter host populations dynamics.

Since the *S. exigua* iflavirus persistence in field populations (20) and the simultaneous SeMNPV field application can occur we hypothesized two different scenarios: i) iflavirus covert infections would improve the effects of SeMNPV as a bioinsecticide, producing an increase in its insecticidal properties; or ii) the iflavirus covert infections negatively affect the baculovirus properties by alerting the host immune response system, which prevent the baculovirus infection to progress.

In this context, RNA viruses' discoveries lead us to study the abundance of both iflavirus in different laboratory populations (**Chapter II**). It is interesting to mention that different geographical origin *S. exigua* populations revealed different iflavirus relative abundances. While in Almerian populations SeIV-2 was more abundant than SeIV-1, other colonies such as Mexico or Switzerland mainly harbor SeIV-1 (23), indicating different rearing conditions might favor each one species amplification or transmission traits. These different quantities allow us to obtain experimental SeIV populations comprising different proportions of the virus to study the biological characteristics of each species.

Interestingly, after SeIV and SeMNPV co-inoculations, we consistently found that OBs generated upon larval death include SeIV-1 particles (23, 24). That association increase SeIV survival abilities out of the host (23). However the association was less beneficial for SeMNPV, as the OB pathogenicity significantly decrease (**Chapter III**) (25), as medium lethal concentration (LC<sub>50</sub>) was nearly 2-fold higher than SeIV-free OBs. Not differences in OB production or larval weight

gain were detected. These preliminary approximations to baculovirus-iftavirus association raised a concern about whether the insecticidal properties of SeMNPV might be negatively affected by the presence of iftavirus.

It is also noticeable the effect of iftavirus infections on the pathogenicity of SeMNPV, but it is not consistent across the different experimental approaches tested, probably due to a dose-dependent effect of iftavirus response. The  $LC_{50}$  were reduced between 2 - 4 times either when the iftavirus were co-inoculated (**Chapter IV**) or when the iftavirus were inoculated previously as neonates and then treated with OBs dosage as second instar (**Chapter VI**). Contrary the  $LC_{50}$  result in higher values for those OBs associated with the iftavirus SeIV-1 particles within the polyhedrin matrix of OBs. The average ( $\pm$ SE) number of SeIV-1 particles per OB was estimated at  $18.9 \pm 2.3$  particles/OB. As such, the ratio of iftavirus particles to OBs radically increase in the co-inoculation approach, being from  $4 \times 10^6$  to  $3.3 \times 10^7$  SeIV particles per OB in the inocula consumed by second instar larvae. As a result, the differences in pathogenicity are likely to be due to the higher concentrations of iftavirus particles present in inocula mixtures with OBs used. Similar dose-dependent effects on the host mortality response to a mixed infection has been previously observed. For instance, the mixture of *Bacillus thuringiensis* (Bt) and *Helicoverpa armigera* nucleopolyhedrovirus (HaNPV) resulted in additive effects on the mortality of *Plutella xylostella* that depended on the concentration of the bacteria in the mixture (26).

Also, other studies have been conducted on the effect of the gut microbiota (27), or vertically transmitted persistent bacteria on baculovirus infections showing divergent results. *Wolbachia* persistent infections in *S. exempta* has been related with an increasing susceptibility to baculovirus (28). Other RNA viruses belonging to densovirus family produce host resistance against both baculovirus (HaNPV) and *B. thuringiensis* infection in *Helicoverpa armigera* persistently infected population (29).

Another explanation for OBs differential pathogenicity is that the OBs may have undergone modifications during assembly in cells co-infected by iftavirus. In **Chapter V**, we studied the physical association between both viruses and whether structural modification can affect SeMNPV pathogenicity, as previously mentioned (25). By separation of gradient centrifugation, we determine that iftavirus genomes are not associated with ODVs by qPCR detection and

quantification. These is line with TEM previous studies indicating the presence of iflavirus in the polyhedrin-matrix (25). During OBs purifications in sucrose gradient of OBs associated with SeIV-1, two different subpopulations were separated into two groups of different physical properties. Viral load quantification indicated that upper OB-band harbor more iflavirus genomes whereas lower OB-band harbor more baculovirus genome respectively. These findings suggested that the association with SeIV-1 might pose a reduction in the number of ODVs per OBs that explain the lack of pathogenicity observed in SeIV-1 associated OBs. However, an endpoint assay with *in vitro* *S. exigua* cells (Se301) indicated that the differences in SeMNPV genome loads between both OB subpopulations did not depend on the number of ODV infective units (30). Alternatively, the difference might relay on the number of nucleocapsids per ODV, and the proportion of single or multiple virions per OB. Viral load quantifications of ODV gradient sucrose assays corroborated that the ODV-band corresponding to single-nucleocapsid ODVs, were proportionally more abundant in those OB samples enriched in SeIV-1 loads. As a result, a different ODV assembling seem to occur when iflavirus infections are simultaneously infecting a common host. The results of ongoing experiments will be reported aimed at determining the number of infections units (ODVs) and OB physical characteristics by electron microscopy (TEM).

This different ODV conformation might have an influence on the baculovirus infections establishment. During the primary infection affecting epithelial midgut cells, naked nucleocapsids enter to the cells reaching the nucleus for first replication (31). When several nucleocapsids are available some nucleocapsids by-pass the nucleus, cross the cytoplasm and rapidly reached the hemocele or tracheal cells, avoiding being cleared by cell sloughing (32). Single-nucleocapsids ODVs, promoted in SeIV-1 associated OBs might result in disadvantages during the infections first steps including virions spread comparing to multiple-nucleocapsid ODV (33). That difference explains the need of higher OB quantities to establish a successful infection detected in terms of increasing effective doses for the SeIV associated OBs. Other advantages conferring by multiple ODV conformation are related to the ability to overcome virus genome defectiveness and the inability of replication of deleted genomes by introducing multiple copies of the virus into the same cell.

Covert viral infections play an important role in viral persistence (34, 35). Namely, baculoviruses are known to shift from an horizontally to a vertically transmission pathways in low density populations (36). Benefits in persistence can be drawn for the NPV-based insecticides with respect to conventional insecticides, since the virus remains after spraying on survival adults. Covertly infected adults are able to propagate the infections to their offspring or spread it to other crops field areas (37), and may also contribute to transgenerational viral transmission and long-term pest suppression (38, 39). The effect of covert infections caused by other virus on the vertical transmission traits of SeMNPV has not been undertaken so far. Here we observed that the iflavirus might also modify transgenerational transmission abilities of SeMNPV. In the iflavirus-baculovirus co-inoculations of **Chapter IV**, SeMNPV prevalence in co-inoculated insects did not differ with control treatment, but SeMNPV viral loads experiment a significant increase in those individuals treated with the SeIV-2, either in single or double infections with the SeIV-1. High SeMNPV viral load might lead to a vertical transmission improvement, as we previously observed that high viral loads of SeMNPV are correlated with high levels of prevalence in the offspring of *S. exigua* females populations (40).

Despite initially iflavirus infections were considered asymptomatic, due to non-specific or obvious symptoms are detected during infections, they might have a biological cost for the host. In **Chapter VI**, the fitness cost of SeIV-1/SeIV-2 inoculation is examined looking at several biological parameters. We found that SeIV-1 infections lead to lower rates of larval weight gain and pupae final survival. This effect on larval development negatively affect the process of OB formation when the SeMNPV are ingested simultaneously by the host. Co-inoculations of SeMNPV and SeIV consistently result in weight gain reduction, and decreasing quantities of OB production per larva. The effect of larval weight on final OB production is well documented on baculovirus literature (41-43). We did not found the same response when we induce the baculovirus infection in iflavirus infected larvae (**Chapter VI**), probably because this effect has been hidden by the synergistic effect of iflavirus infection on mortality induced by NPV, which lead to prompt larval death.

Conversely, SeIV-2 did not produce measurable effects on *S. exigua* development when was inoculated at high concentrations ( $10^9$  genomes/ml).

These results coupled with the different time-course of infection in terms of viral loads, seems to point out different transmission and survival strategies between both species, as already described for other species of baculoviruses (34, 37, 38, 40) and iflaviruses (14, 44, 45). SeIV-1 inoculation result in viral amplification across host development as high viral loads accumulates in host tissues, leading to horizontal transmission. SeIV-2 maintained constant low viral loads of persistent infection, apparently without any effect on the host fitness, leading to increase their survival opportunities, and vertical transmission. In other *Iflavirus* species, low levels of infections have been related to asymptomatic infections, while high viral levels turn up to measurable symptoms or even host deaths (46, 47). For instance, when asymptomatic low-persistently DWV-infected honeybees, undergone mite-vectored 1,000-fold amplification, extremely virulent viral outbreak occurs in the host colony (46). Similarly, the quantification of SBV in individual honeybees allowed to distinguished asymptomatic bees that harbor lower levels SBV, and high levels of viral related to individuals showing sacbrood disease symptoms (47).

In conclusion, both *Iflavirus* species infecting *S. exigua* have an effect on both the host fitness and, consequently on SeMNPV insecticidal properties during coinfections. Remarkable differences between both iflavirus in the course of infection and trends of transmission has been appreciated. SeIV and SeMNPV interactions widely vary depending on the conditions of host inoculations, and virus concentrations. As such, iflavirus pre-inoculation or co-inoculation result in synergistic effect on the baculovirus mortality, still detrimental OB production at larval death. Altogether, these findings could be considered for baculovirus mass-production procedures to ensure product effectiveness and biosecurity. These findings also point out the risk of fortuitous contamination by RNA viruses infecting insect cultures, similar to RNA virus causing human disease (48, 49). To avoid undesirable outcomes, strict colony hygiene measures, including regular quality control assays, and molecular screening for the presence of iflavirus contaminants are recommended.

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## CONCLUSIONES

1. Las poblaciones de *Spodoptera exigua* analizadas en este trabajo están afectadas, con una sola excepción, por infecciones persistentes causadas por uno o más virus de las familias *Baculoviridae* o *Iflaviridae*. La abundancia relativa de los virus de la familia *Iflaviridae*: iflavirus 1 (SeIV-1) e iflavirus 2 (SeIV-2), es muy variable dependiendo del origen de la población huésped.
2. Las infecciones con iflavirus producen alteraciones en las características biológicas de *S. exigua*. El SeIV-1 afecta negativamente a la ganancia de peso de la larva, el peso de la pupa y la supervivencia del adulto. En cambio, el tiempo de desarrollo larvario, así como la fertilidad y la fecundidad, no se ven afectados. La inoculación del SeIV-2 no produce variaciones en ninguno de las características estudiadas del desarrollo del huésped.
3. La carga del SeIV-1 aumenta durante el desarrollo del huésped, indicando un alto nivel de replicación; mientras que la concentración del SeIV-2 se mantiene siempre a niveles bajos. El aumento de la carga viral en el huésped se ha relacionado con un aumento de la transmisión horizontal del iflavirus, mientras que bajas concentraciones parecen mantenerse debido a la transmisión vertical.
4. Las propiedades insecticidas del nucleopoliedrovirus múltiple de *S. exigua* (SeMNPV) se ven alteradas, en un sentido u otro, cuando interactúan con especies de iflavirus (SeIV) en alguna de las siguientes situaciones: i) cuando ambos virus están físicamente asociados en los OBs de SeMNPV (OBs mixtos; SeMNPV + SeIV-1); ii) cuando el huésped es coinfectado por una mezcla de OBs y SeIV; y iii) cuando el huésped sufre una infección persistente causada por SeIV y es inoculado con OBs del SeMNPV.
5. La patogenicidad de los OBs mixtos (SeMNPV + SeIV-1) (situación i) disminuye, es decir, el valor de la  $CL_{50}$  aumenta cerca de 40%, con respecto al valor correspondiente de los OBs libres de iflavirus, pero no

- hay diferencias en el tiempo medio de mortalidad (TMM). En cambio, el peso de la larva y la producción de OBs/larva no se ven afectados.
6. Los productos resultantes de la disolución alcalina de los OBs mixtos presentan distinta movilidad cuando son sometidos a ultracentrifugación en un gradiente continuo de sacarosa. Los ODVs generan bandas concretas de ODVs en función del número de nucleocápsidas por ODV; mientras que las partículas de iflavirus tienen una movilidad distinta. Esto sugiere que los SeIV están asociados a los OBs, pero no a los ODVs.
  7. Los OBs mixtos (situación i) muestran diferencias estructurales respecto a OBs libres de iflavirus. Los OBs mixtos con altas concentraciones de SeIV-1 contienen una mayor proporción relativa de ODVs con una sola nucleocápsida con respecto a los OBs libres de SeIV-1. Esto implica que los OBs mixtos contienen menos número de genomas de SeMNPV que los OBs libres de SeIV-1 o con bajas cantidades de SeIV-1. Esta estructura morfológica de los OBs enriquecidos en SeIV puede explicar la más baja patogenicidad de los mismos.
  8. En los huéspedes coinfectados con una mezcla de OBs y SeIV-1, SeIV-2 o una mezcla de ambos (situación ii) se produce un aumento significativo de la patogenicidad, es decir, una disminución del valor de  $CL_{50}$  de los OBs. El TMM no se ve afectado de forma significativa, en cambio, la producción de OBs se reduce estadísticamente. En los huéspedes coinfectados, la menor producción de OBs guarda relación con un menor incremento de peso de las larvas lo cual se atribuye a la infección por iflavirus.
  9. La coinfección de SeMNPV y SeIV (situación ii) no altera la prevalencia de SeMNPV en adultos de *S. exigua*. Sin embargo, aquellos tratamientos que contienen el SeIV-2 aumentan la carga viral del baculovirus, indicando que las infecciones por SeIV-2 pueden contribuir positivamente a la transmisión vertical del baculovirus.
  10. Las larvas de *S. exigua* que albergan infecciones persistentes causadas por SeIV-1 (situación iii) son más susceptibles a la infección por SeMNPV ya que los valores de la  $CL_{50}$  y del TMM son significativamente menores. En este sentido, se concluye que las condiciones sanitarias de las

poblaciones naturales de *S. exigua* pueden alterar la eficacia de las aplicaciones de bioinsecticidas basados en baculovirus.



## CONCLUSIONS

1. The *Spodoptera exigua* populations analyzed in this work are affected, with one exception, by persistent infections caused by one or more viruses from the *Baculoviridae* and *Iflaviridae* families. The relative abundance of the *Iflaviridae* family viruses: iflavirus 1 (SeIV-1) and iflavirus 2 (SeIV-2), is greatly variable depending on the origin of the host population.
2. The iflavirus infections produce alterations in the *S. exigua* biological characteristics. SeIV-1 negatively affect the larval weight gain, the pupal weight and the adult survival. However, the larval development time, as well as fertility and fecundity, are not affected. The SeIV-2 inoculation did not produce variations in the studied host development characteristics.
3. The SeIV-1 load increase during the host development, indicating a high level of replication; while the SeIV-2 concentrations always remains at low levels. Increased of host viral loads has been associated with increased of iflavirus horizontal transmission, while low concentrations appear to be maintained due to vertical transmission.
4. The insecticidal properties of the nucleopolyhedrovirus multiple of *S. exigua* (SeMNPV) are altered, in one way or another, when they interact with iflavirus species (SeIV) in one of the following situations: i) when both viruses are physically associated in the SeMNPV OBs (mixed OBs; SeMNPV + SeIV-1); ii) when the host is co-infected by a mixture of OBs and SeIV; and iii) when the host undergo a persistent infection caused by SeIV and is inoculated by SeMNPV OBs.
5. The pathogenicity of mixed OBs (SeMNPV + SeIV-1) (situation i) decreases, namely the LC<sub>50</sub> value increases about 40%, with respect to the corresponding value of iflavirus-free OBs, but there is no difference in mean time to death (MTD). In contrast, larval weight and OB production per larva are not affected.
6. Products resulted from the alkaline dissolution of mixed OBs present different mobility when they are subjected to a ultracentrifugation in a

continuous sucrose gradient. The ODVs generate specific bands of ODVs due to the number of nucleocapsids by ODV; while iflavirus particles have different mobility. This suggests that SeIV are associated with OBs, but not with ODVs.

7. Mixed OBs (situation i) show structural differences with respect iflavirus-free OBs. Mixed OBs with high concentrations of SeIV-1 contain a higher relative proportion of ODVs with a single nucleocapsid with respect to SeIV-1-free OBs. This suggests that mixed OBs contain fewer SeMNPV genomes than either SeIV-1-free OBs or SeIV-1 low amounts OBs. This OBs morphological structure enriched in SeIV may explain their lower pathogenicity.
8. In co-infected hosts with a mixture of OBs and SeIV-1, SeIV-2 or a mixture of both (situation ii) there is a significant increase of the pathogenicity, namely a decrease of the OB  $LC_{50}$  value. The MTD is not significantly affected, however, the OB production is statistically reduced. In coinfecting hosts, the lower OB production is related to a larvae lower weight gain which is attributed to iflavirus infection.
9. SeMNPV and SeIV co-infection (situation ii) does not alter the SeMNPV prevalence in adults of *S. exigua*. However, those treatments containing SeIV-2 increase baculovirus viral loads, indicating that SeIV-2 infections may positively contribute to baculovirus vertical transmission.
10. *S. exigua* larvae harboring persistent infections caused by SeIV-1 (situation iii) are more susceptible to SeMNPV infection since  $LC_{50}$  and MTD values are significantly lower. In this sense, it is concluded that the sanitary conditions of the natural populations of *S. exigua* can alter the efficiency of baculovirus-based bioinsecticide applications.

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The beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae) is an important pest in temperate areas worldwide causing important economic losses in field crops and greenhouses. Recently, bioinsecticides based on the *S. exigua* nucleopolyhedrovirus (SeMNPV) (*Baculoviridae*) have been developed to control this pest. The environment in which SeMNPV is applied could determine its effectiveness as a biological control agent. Besides, Next Generation Sequencing (NGS) techniques have revealed a large amount of RNA viruses that infect Lepidoptera with no apparent disease symptoms and novel *S. exigua* iflaviruses (SeIV) (*Flaviridae*) were later characterized as SeIV-1 and SeIV-2. The study of the co-infection and superinfection of both baculovirus and iflaviruses on the same host could allow to know the interference of the SeIV over the SeMNPV bioinsecticide properties



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