On the competitive fitness of baculoviruses in insects

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PREFACE

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ABSTRACT

Mixed infections of baculoviruses in insect hosts are quite common in nature. This leads to 'within-host' and 'between-host' competition between virus variants. Because both levels of selection will contribute to overall biological fitness, both must be included in assessments of the fitness of fast-acting recombinant baculoviruses. We investigated baculovirus fitness parameters in single and mixed infection of insect larvae, in single and serial passage experiments in lepidopteran hosts (*Helicoverpa armigera*, *Spodoptera exiqua* and *Trichoplusia ni*) in laboratory, greenhouse and field settings. Median time to death in third instar larvae of *H. armigera* (Hübner) was lower in insects challenged with a mixture of wild type (HaSNPV-wt) and mutant (Δ*egt*, HaSNPV-LM2) *Helicoverpa armigera* SNPV, than in larvae infected with only HaSNPV-wt. The results from a behavioral study on cotton (glasshouse, field) indicated that the transmission of HaSNPV-LM2 is not modified by the absence of the *egt* gene, whereas in the case of the HaSNPV-AaIT (Δ*egt*, + AaIT) lower virus yield as well as altered caterpillar behavior could compromise virus fitness. Virus transmission in greenhouse and field was not reduced, when HaSNPV-LM2 was used in mixed infections with HaSNPV-wt. However, a reduction of 'between host' transmission was recorded when *H. armigera* larvae were co-infected with HaSNPV-wt and HaSNPV-AaIT. Serial passage experiments with *S. exigua* and *T. ni* showed positive selection for wild type SeMNPV and AcMNPV over genetically modified variants (Δ*egt*, + AaIT in the case of SeMNPV, and Δ*egt* in the case of AcMNPV) over passages. These findings can help to understand long-term dynamics of virus genotypes in virus-insect-host plant systems. They can also help foresee potential consequences of the introduction of genetically-modified or exotic baculoviruses in agro-ecosystems.

Keywords: baculovirus, insects, mixed infections, competition, transmissio

CONTENTS

Chapter 1

GENERAL INTRODUCTION

Insects are the most diverse group of animals living on earth. Often they are considered beneficial (bumble bees), appreciated for their natural beauty (butterflies) or function as key players in the ecosystem (food chain, scavengers). However, from a human perspective insects are also quite often considered a nuisance (mosquitoes) or an indirect (disease vectors of plants and animals) or a direct (caterpillars) pest. Insects can indeed dramatically reduce food and fiber production in agriculture and forestry (Fig.1), with an estimated loss that reaches up to 30% of the total crop production in the field and in storage (Erlandson, 2008). Control of these pest insects is therefore pivotal to meet the current and future demands of food and fiber production and to improve and secure human and animal health.

Since the 1940's insect pests in agriculture and insects affecting human and animal health have been controlled using chemical insecticides. The overuse of these agents has not only induced resistance in target insects, but also contributed to the environmental pollution issue (residues) and caused major health problems in humans (intoxication, cancer).

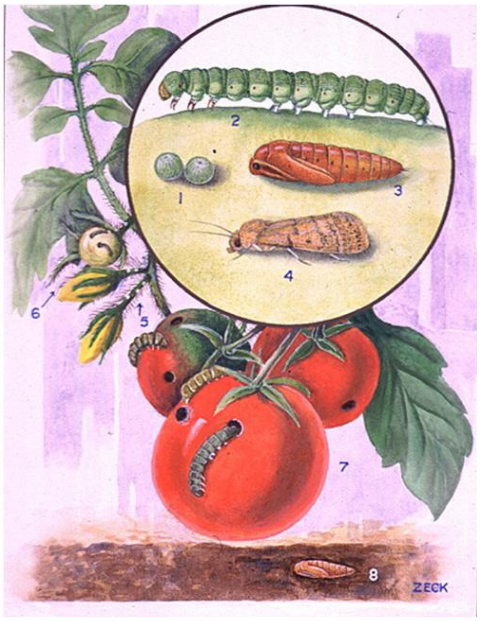

Figure 1. Holometabolous life cycle of insect belonging to family Lepidoptera: Mostly 2 to 4 generations occur in a year. (1) eggs, (2) larval stage which cause damage on crops, (3) pupa, (4) adult stage (5) females lay several hundred eggs on all parts of the plant (6) emergence of the larvae (7) mostly severe damage is caused by attack on reproductive parts such as flower buds and flower heads (8) overwintering in the soil (from: NSW Agriculture, 2006).

After Rachel Carson's 'Silent Spring' (1962) alternatives for chemical insect pest control were sought. These alternatives were found using integrated pest management (IPM) strategies to harness beneficial and biological control strategies including the use of natural enemies, such as parasites, predators, nematodes and microbials. Among the latter are insect viruses, notably baculoviruses, and these disease agents are in focus in this thesis.

Baculoviruses

Viruses of the family *Baculoviridae* infect invertebrate, mostly insect larvae and often cause mortality. An early example has been a virosis decimating silkworm cultures in Italy and France in the 16th-19th century. These baculoviruses have a large, double-stranded circular DNA molecule, which is wrapped inside a protective protein capsid. The association of nucleic acid and capsid is called a nucleocapsid.

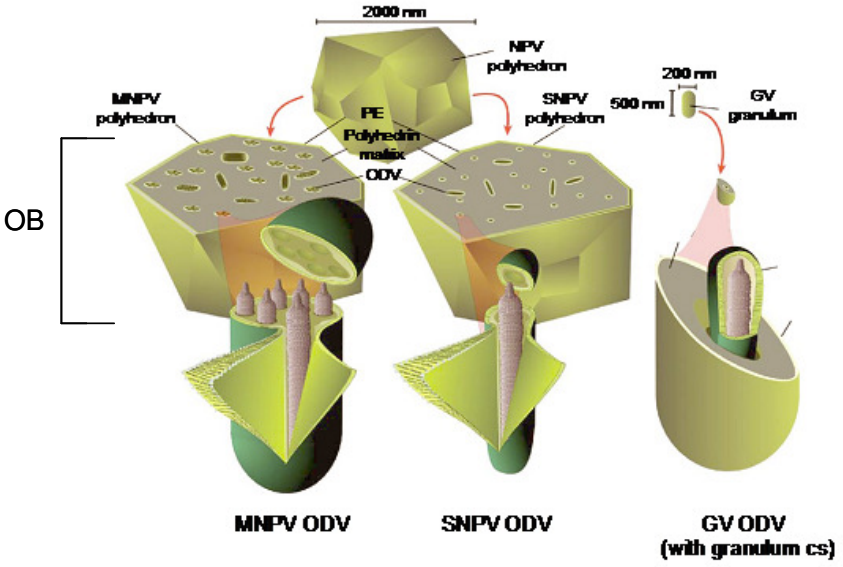

Figure 2. Occlusion derived virus (ODV; foreground) and occlusion bodies (OB; background) in three groups of baculoviruses. Granuloviruses (GV; shown at the right) have a capsule-shaped OB that contains a single virion. The single nucleopolyhedroviruses (SNPV; shown in the middle) have a polyhedron-shaped occlusion body that contains multiple virions, and each virion contains a single nucleocapsid. The multiple polyhedroviruses MNPV; shown at the left) also have multiple virions per polyhedron, and each virion contains multiple nucleocapsids. (from Slack & Arif, 2007).

Nucleopolyhedroviruses are subdivided into two groups: the multiple nucleopolyhedrovirus group (more than one nucleocapsid in each virion) (MNPV) and single nucleopolyhedrovirus group (one nucleocapsid in each virion) (SNPV). The virus particles (virions) are rod-shaped, hence the family name (baculum = rod in Latin) (Fig. 2). The virions are occluded into large proteinaceous capsules, often called polyhedra,

granula or occlusion bodies (OB). These OBs are another very characteristic feature of this group of viruses. The family *Baculoviridae* is composed of four genera: the Alphabaculoviruses, containing nucleopolyhedroviruses (NPVs) of Lepidoptera, the Betabaculoviruses, encompassing the granuloviruses (GV) of Lepidoptera, the Gammabaculoviruses, accommodating NPVs of Hymenoptera, and the Deltabaculoviruses, encompassing NPVs of Diptera (Jehle et al., 2006; ICTV, 2008). The NPV of *Autographa californica* (AcMNPV) and the GV of *Cydia pomonella* granulovirus (CpGV) are the type species of Alphabaculoviruses and Betabaculoviruses, respectively. About 700 baculoviruses have been reported, but most of these are poorly characterized biologically or genetically.

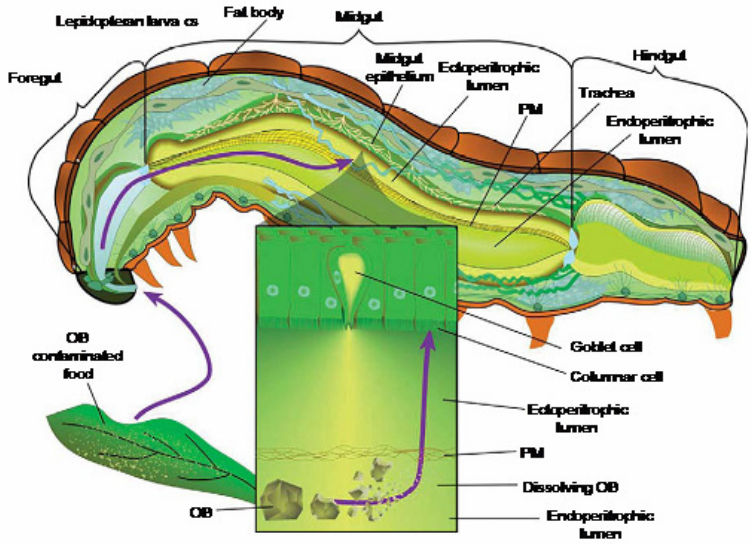

Figure 3. Schematic representation of *per os* infection of larvae with the contaminated food with baculoviruses. OBs pass through the foregut and enter the midgut where they dissolve in the alkaline midgut lumen and release occlusion derived virions (ODVs). Released ODVs past the peritrophic membrane (PM) to midgut columnar epithelial cells where they cause infection and replication of viruses takes place. (from Slack and Arif, 2007).

Baculovirus infection is initiated by uptake of OBs while feeding on plants or from soil and dissolution of these capsules in the midgut lumen to release the occlusion-derived virions (ODV), which are then able to infect midgut epithelial cells (Fig. 3). Upon replication of the virus in the nucleus of these cells, a second virion morphotype is produced, which acquires its membrane from the basal side of the cell upon egress into the hemocoel or trachea. This budded virus (BV) form, which is genetically identical but phenotypically different to ODVs, is responsible for cell-to-cell transmission within the

infected insect. Within a few days the infected larva dies and disintegrates to release millions of OBs onto plants and soil. The virus in the form of OBs can persist for many years in the environment (Krell, 2008). So, BVs are responsible for the spread of the virus within the insect, whereas the OBs are the vehicles to spread the virus horizontally in insect populations. Baculoviruses also transmit vertically, from parent to offspring, but to which extent and in what mode is not well characterized.

Baculoviruses as biological control agents

Baculoviruses are natural disease agents and can cause epidemics that reduce the size of insect populations of agricultural and forest pest insects. This has led to successful inoculative and inundative control strategies using baculoviruses produced in insect larvae. To be successful microbial insecticides in the field, they must be sufficiently infectious and virulent to the target insect host in order to be able to limit economic plant damage (Erlandson, 2008). Baculoviruses are specific insect pathogens, which showed considerable success in biological control of lepidopteran and hymenopteran insect pests, such as the cotton bollworm, velvetbean caterpillar, codling moth, etc. (Bonning & Nusawardani, 2007). Baculoviruses provide an effective and selective means for biological insect control in agricultural crops, providing a welcome, ecologically sound and sustainable alternative to chemical pesticides (Moscardi, 1999). Baculoviruses are host specific, easy to apply, can be produced in a variety of formulations and are safe for non-target invertebrates and vertebrates including humans (Hunter-Fujita et al., 1998). Baculoviruses occur ubiquitously in nature. As a consequence their use should leave less of an environmental imprint in comparison with synthetic chemical insecticides (Krell, 2008). Furthermore, insects quickly develop resistance against these chemical agents. Currently, a number of baculoviruses is registered as insect control product worldwide (Table 1). One of the first semi-commercial baculovirus pesticides was ‘Elcar’ (Sandoz) to control *Heliothis zea* in cotton (1975). In China HaSNPV is produced as a commercial biopesticide since 1991 and has been widely used for the control of *H. armigera* for many years on large acreages of cotton (Zhang, 1989; Sun & Peng, 2007) and vegetable crops (Table 2). In Thailand there is a record of successful small scale local production of HaSNPV and SeMNPV to control cotton, tomato and tangerine (Jones & Burges, 1998). SeMNPV of the beet armyworm, *Spodoptera exigua* (Hübner), forms the basis for a number of effective biopesticide products that are marketed in the USA, Southern Asia and some European countries (Hunter-Fujita et al. 1998; Kolodny-Hirsch et al., 1993, ibid 1997; Smits & Vlak, 1994) and some new preparations of this virus are in the phase of testing (Lasa et al., 2007). The most successful commercial application of baculoviruses in Europe is the control of codling moth with a GV in more than 100,000 ha of orchards (Rechcigl, 1998).

There is natural variability in baculovirus isolates or preparations, some strains kill faster than others. Fast killing viruses are more suitable for crop protection, providing better control than slow killing viruses. In Spain and Portugal HaSNPV field strains were reported, which had higher pathogenicity and virulence compared with the already known Iberian genotypic variants of HaSNPV (Figueiredo et al., 2009).

Many baculoviruses produce *egt*, an ecdysteroid UDP-glucosyl transferase, adding a sugar moiety to ecdysone, thereby inactivating this hormone. The *egt* gene is not required for the baculovirus infection or replication at the molecular and cellular level, but has a major effect on insect larvae (O'Reilly & Miller, 1991; Flipsen et al., 1995). The normal function of *egt* is delaying or blocking normal larval development, which prevents cessation of larval feeding (Harrison, 2009). Natural deletion of some or all of the *egt* coding sequences was observed in insect cell culture infected with AcMNPV (Kumar & Miller, 1987) and in natural isolates of AgipMNPV (Harrison, 2009). In the AgipMNPV case results indicated that *egt* deletion may have a growth advantage in cell culture, because the *egt* deletion genotype produced moderately higher BV quantities than the wild-type virus. It has been reported that deletion of the *egt* gene enhances the virus virulence, for example in the case of HaSNPV (Δ*egt*) the LT_{50} (median time to death of infected hosts) decreased 27% compared to wild-type HaSNPV (Sun et al., 2004). A similar situation exists for LdMNPV (Slavicek et al., 1999). Absence of the *egt* gene is a common feature in the *Spodoptera frugiperda* MNPV. Variant genotypes with the deletion of the part of this gene were found in the Nicaraguan field isolate of SfMNPV (Simon et al., 2004) and field isolates from SfMNPV isolated in Missouri, USA (Harrison et al., 2008). Naturally occurring, fast-killing virus isolates may lead to further advances in the development of this NPV as an insecticide and ecologically friendly means of controlling insect pests.

A limitation of wild-type baculoviruses as biocontrol agents is, among others, their slow speed of kill. To eliminate this drawback baculoviruses have been genetically engineered to improve their insecticidal properties and reduce crop damage (see Inceoglu et al., 2006; Szewczyk et al., 2006, Sun et al., 2009). Several approaches have been used to produce fast acting baculoviruses, such as (i) insertion of a foreign gene, e.g. specific toxin, hormone or enzyme, (ii) deletion of a baculovirus gene (exp. *egt*) or (iii) incorporation of a (Bt) toxin into the OB (Chang et al., 2003).

Table 1. Registered and experimental virus-based bioinsecticides developed as commercial microbial control agents for Lepidoptera (from Rechcigl, 1998; Huber, 1998; Moscardi, 1999; Erlandson, 2008)

Virus	Product names	Target pest	Production/crop system
Baculoviruses			
Lymantria dispar multiple nucleopolyhedrovirus	Gypcheck, Disparvirus, Virin-ENSH	Gypsy moth	Forestry
Orgyia pseudotsugata multiple nucleopolyhedrovirus	TM Biocontrol	Douglas fir tussock moth	Forestry
Neodiprion sertifer nucleopolyhedrovirus	Neocheck-S, Virox, Sentifervirus, Monisärmiövirus	European spruce sawfly	Forestry
Neodiprion lecontei nucleopolyhedrovirus	Leconteivirus	Redheaded pine sawfly	Forestry
Adoxphyes orana granulovirus	Capex 2	Summer fruit tortrix moth	Orchard
Cydia pomonella granulovirus	Madex 3, CYD-X, Granupom,Granusal, Carposin, Virosoft CP-4, Virin-Gyap	Codling moth	Orchard
Autographa californica multiple nucleopolyhedrovirus	VPN 80	Multiple pest targets	Horticulture, Glasshouse and field crop, cottons
Anagrapha falcifera multiple nucleopolyhedrovirus		Multiple pest targets	Horticulture, Glasshouse and field Crops
Anticarsia gemmatalis multiple nucleopolyhedrovirus	Polygen, Multigen	Velvet bean caterpillar	Soybean
Heliocoverpa armigera single nucleopolyhedrovirus	Virin-Hs	*Heliothis/Helicoverpa* complex	Cotton
Helicoverpa zea multiple nucleopolyhedrovirus	Gemstar	*Heliothis/Helicoverpa* complex	Cotton, Horticulture
Heliothis virescence multiple nucleopolyhedrovirus	Elcar	*Heliothis/Helicoverpa* complex	Cotton
Mamestra brassicae multiple nucleopolyhedrovirus	Mamestrin, Virin-EKS	Multiple pest target	
Mamestra configurata multiple nucleopolyhedrovirus		Bertha armyworm (*M. configurata*)	Canola
Spodoptera exigua multiple nucleopolyhedrovirus	Spod-X, Ness-A, Ness-E	Beet armyworm (*S. exigua*)	Horticulture, glasshouse, and field crops
Spodoptera littoralis NPV	Spodopterin	*Spodoptera littoralis*	Cotton
Cypoviruses			
Dendrolimus spectabilis cypovirus 1	Matsukemin	Pine caterpillar	Forestry
Anticarsia gemmatalis NPV	VPN, Baculoviron	*Anticarsia gemmatalis*	Soybean
*Agrotis segetum*GV	AGROVIR		

Table 2. Viruses authorized as commercial insecticide in China.
(from Sun & Peng 2007).

Virus name	Target insects	Crops	No. of producers
Helicoverpa armigera NPV	Cotton bollworm	Cotton, pepper, tobacco	12
Spodoptera litura NPV	Cotton leafworm	Vegetable	2
Autographa californica NPV	Alfalfa looper	Vegetable	3
Spodoptera exigua NPV	Beet armyworm	Vegetable	2
Gynaephora sp. NPV	Meadow caterpillar	Grass	1
Buzura suppressaria NPV	Tung tree geometrid	Tea	1
Ectropis oblique NPV	Apple geometrid	Tea	2
Leucania separate NPV	Oriental armyworm	Wheat, corn	1
Pieris rapae GV	Cabbage white butterfly	Vegetable	1
Plutella xylostella GV	Diamondback moth	Vegetable	2
Pseudaletis separate GV	Armyworm	Wheat, corn	1
Dendrolimus punctatus CPV	Masson pine moth	Pine	1

However, baculoviruses which lack the *egt* gene (Δ*egt*) produce fewer OBs because the absence of *egt* (the enzyme) reduces the insect lifespan and OB yield (O'Reilly & Miller, 1991). The Δ*egt* virus genotypes are, therefore, assumed to be ecologically impaired and most likely have lower fitness than wild-type viruses. For crop protection Δ*egt* viruses are desirable because they act faster (O'Reilly & Miller, 1991; Treacy et al., 1997).

Field trials were done to test generated baculoviruses which express neurotoxin from the scorpion *Androctonus australis* Hector (AaIT), such as AcMNPV-AaIT (Cory et al., 1994; Black et al.; 1997) and HaSNPV-AaIT (Sun et al. 2005, ibid 2009). Another toxin used to generate a fast-acting AcMNPV recombinant came from the scorpion *Leiurius quinquefasciatus* Hebraeus (*Lqh*IT2). Expression of this toxin in AcMNPV-*Lqh*IT2 recombinant protected cotton from damage better than the wild-type virus (Smith et al., 2000). AcMNPV recombinant with straw itch mite, *Pyemotes tritici,* Txp-1, has

higher pathogenicity and virulence for second and fourth instar *Trichoplusia ni* larvae (Burden et al., 2000). Also the insecticidal properties of baculoviruses were accelerated by incorporating basement membrane-degradating protease, cathepsin L (ScathL), from the flesh fly *Sarcophaga peregrina* (Harrison & Bonning, 2001). The HaSNPV-cathL protected cotton from *H. armigera* feeding damage better than wild-type virus (Sun et al., 2009).

The decision to release recombinant baculoviruses as biocontrol agents in the environment depends on the outcome of the risk assessment evaluation of the product. For that purpose the baculovirus fitness parameters (i) speed of kill and virus production (yield), (ii) behavior of infected larvae, (iii) transmission to the next generation and (iv) persistence in the soil compartments need to be assessed.

Baculovirus fitness parameters

Long-term persistence of virus genotypes in agricultural systems depends among others on their competitive interactions with other either closely related virus variants or different baculovirus species at the individual population and the eco-system level. It is quite common that wild-type baculovirus isolates consist of a mixture of genotypes (Knell and Summers, 1981; Gettig & McCarthy, 1982; Cherry & Summers, 1985; Muñoz et al., 1998; Cory et al., 2005). Important elements (outcomes) of the competition between virus genotypes are lower virus yield in insects and changes in transmission characteristics (Figs. 4 and 5). The behavior of viruses and hosts in mixed infections is a key component of this competitive process between virus strains. The co-infecting pathogens during mixed infection can act independently (in status of equilibrium), synergistically (beneficial for both genotypes) (Tanada, 1959; Wang & Granados, 1997; Malakar, 1999; Washburn et al., 2000, Thomas et al., 2003) or antagonistically (negative for one or both of them) (Hackett et al., 2000; Ishii et al., 2002) depending on the environmental condition, order of infection, host response to infection, etc. Based on available literature until now we can not draw a firm conclusion about virus population dynamics over time and space in a microcosm or ecosystem. The effect of mixed virus infection on the population dynamics of insects is not known, but attracted considerable attention in recent years in view of the potential use of genetically engineered baculoviruses in insect control or the displacement of resident baculovirus populations.

(i) Speed of kill and virus production (yield)

The ability of the virus for OBs production (yield) is an important parameter to predict long-term fate of the baculoviruses in the environment. Competition of two genotypes in mixed infection may modify the survival of the co-infecting genotypes, but may also affect the within-host parasite growth, host survival and reproduction (Vizoso & Ebert, 2005). The difference in virus yield between wild-type and the recombinant

AcMNPV was used to predict the reduction of the secondary cycling of recombinant baculoviruses in the environment in comparison to wild-type baculoviruses (Hammock, 1992). The yield of the wt AcMNPV was 1.25-2.42 times greater than a recombinant AcMNPV containing the AaIT gene (Ignoffo & Garcia, 1996). Sun et al., (2005) showed for three HaSNPV recombinants that the virus yields were significantly affected by the larval instar and related to time to death. Two recombinants HaSNPV-Δegt and HaSNPV-AaIT caused larvae to die earlier, compared with the wild-type HaSNPV and the yield in the former case was significantly reduced. This can help to predict the capacity of a recombinant baculovirus in the environment relative to a wild-type baculovirus. It is known that in the environment insect hosts are exposed and frequently infected with more than one genotype of the same pathogen (Reed & Taylor, 2000). On the basies of the results from single infections we can not draw a firm conclusion about the outcome of mixed virus infections in nature. Also the genetic diversity in baculovirus isolates within naturally acquired infections has not been well studied so far.

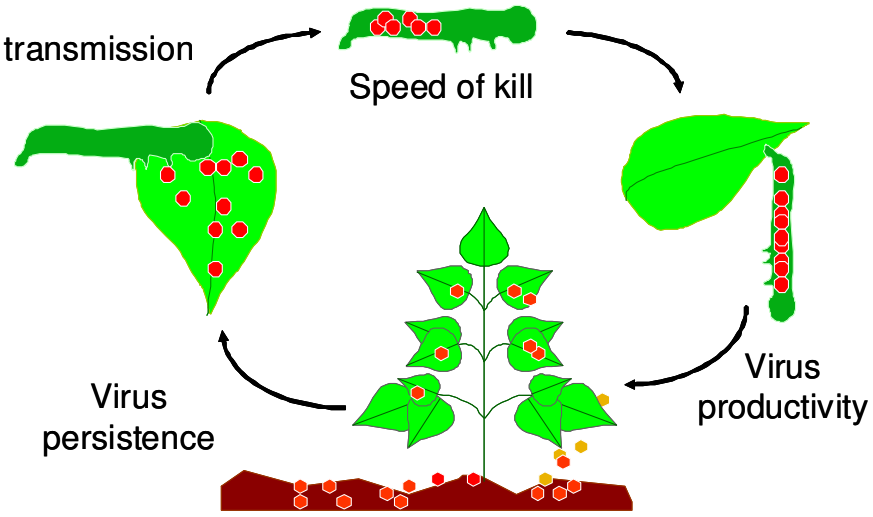

Figure 4. Baculovirus fitness parameters in the ecosystem: speed of kill, virus productivity (virus yield), virus persistence and transmission.

(ii) Influence of baculovirus on larval behavior

The behavior of baculovirus-infected larvae differs from the behavior of healthy larvae and larvae infected with a genetically modified baculovirus as the virus infection plays a significant role in the transmission of baculoviruses. These behavioral changes in infected larvae are thought to enhance rapid dissemination of the virus and to increase horizontal transmission, an important factor in initiating new virus infections (Evans & Allaway, 1983; Cory & Myers, 2003). Mostly, larvae infected with a wild-type baculovirus move up their host plant, leading to a phenomenon known as 'tree top

disease' (Vasconcelos et al., 1996; Hernandez-Crespo et al., 2001) or 'Wipfelkrankheit'. However, there is also evidence of an opposite behaviour, where larvae infected with an NPV moved downwards on their host plant and mostly die on the stem (Raymond et al., 2005). AaIT expressing baculoviruses cause paralysis causing larvae to fall from the plant (Hails, 2001). This resulted in a reduction of contact rate between host and pathogen, which consequently led to reduction in baculovirus transmission. The recombinant baculoviruses were selectively removed from the plant, which means that they were protected from sunlight and inactivation by the UV light. However, this does not mean that they are less capable of causing a new infection, since there is evidence that they can be directly translocated from the soil to the plant by rain splash or wind blown dust (Fuxa et al., 2007).

It is not known how the behavior of larvae with mixed infections of a fast and slow-acting baculovirus is affected, compared to larvae with infections with the respective single baculovirus genotypes. Detailed information on mixed infections influencing the effect on host insect behavior and dispersal characteristics are therefore of great interest. Larval behavior is an important parameter, which influences transmission of baculoviruses in the field, in terms of where infected insect die and the possibility of healthy insects to acquire the virus (Cory & Evans, 2007).

(iii) Transmission of baculoviruses

The epizootiology and use of baculoviruses as microbial control agents depends on their ability of host to host transmission (Fuxa, 2004). The transmission process is critical in the understanding of insect pathogen dynamics (Hails et al., 2002). Horizontal transmission of baculoviruses (from one insect to another) is affected by the encounter rate between infected and susceptible individuals, and the rate at which contacts result in new infections. This encounter rate depends on the density and behavior of larvae in the system, i.e. on the plant. Experimentally it was estimated that baculovirus transmission increased with inoculum density (Zhou et al., 2005). D'Amico et al., (1996) observed a decline in the transmission constant as the densities of both healthy larvae and pathogen increased. The number of cadavers remaining on the foliage has a greater influence on transmission than the yield of virus from those cadavers (Hails et al., 2002). Zhou et al., (2005.), study the influence of larval stage on transmission of baculovirus in *H. armigera*. They showed that horizontal transmission of HaSNPV variants was greatest when 3rd instar inoculated larvae were used as infectors, intermediate with 2nd instar infectors and lowest with 1st instar infectors. In contrast, Goulson et al., (1995) found no significant difference in transmission rate when different instars were used. Transmission of baculoviruses can be facilitated by birds and other vertebrates (aerial dispersal), which helps in their long distances dissemination (Entwistle et al., 1993). Alternative route of

horizontal transmission is when larvae transfer viable virus to the environment before death through either defecation or regurgitation (Vascancelos, 1996).

Another way of virus dispersal in the eco-system is via vertical transmission from the adults to their progeny. This can be achieved by surface contamination of the eggs (transovum transmission) or when virus is passaged within the egg (transovarial transmission) (Kukan, 1999; Fuxa et al., 2004; Myers & Rothman, 1995).

There are a number of different approaches available for the modeling insect-pathogen interactions (Dwyer & Elkington, 1993; Godfray et al., 1997; Dwyer et al., 2000; ibid, 2005 Dushoff & Dwyer, 2001; Bianchi et al., 2002; Bonsall et al., 2005; Sun et al., 2006).

Transmission is a very important fitness parameter of baculoviruses for further dissemination of the virus in the ecosystem, but this parameter has not been investigated in mixed infections. Interaction between baculovirus genotypes in mixed infections may strongly affect the likelihood of virus transmission.

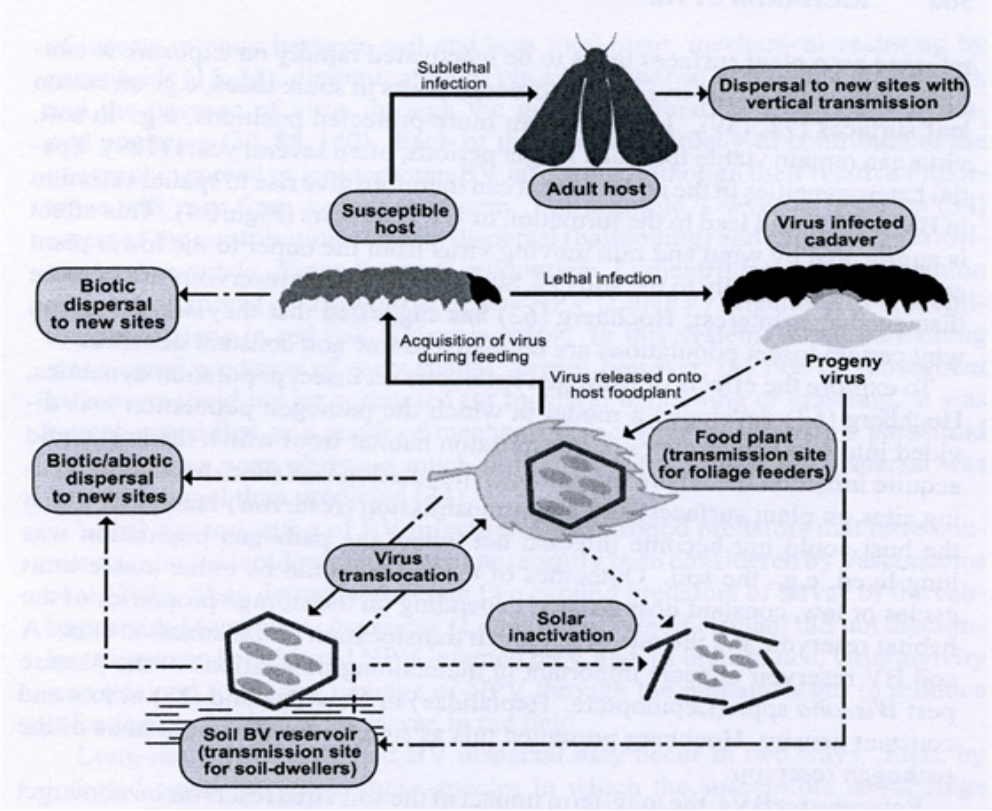

Figure 5. The fate of lepidopteran BVs in the environment. BV transmission routes, *solid arrow*; BV dispersal routes, *long-dash/short-dash arrow*; BV inactivation, *short-dash arrow* (from Richards et al., 1998).

(iv) Persistence of baculoviruses in the environment

Soil is the natural reservoir for the baculoviruses where they can persist for decades, from which they can be translocated abiotically to new insect host plants to initiate epizootics (Fuxa et al., 2001, 2007). Studies on insect population dynamics pay

considerable attention to the long-term conservation of viruses in the insect habitat so that epizootics appear during population peaks increasing the probability of the insect-virus contact. It has been reported that the baculoviruses can persist in the soil for many years serving as a potential source of infection for the next generations of insects and causing natural epizootics (Il'inykh, 2007). Murray & Elkington (1989) reported that natural epizootic of LdMNPV virus from Massachusetts (United States) can cause infection of up to 24,000 caterpillars per ha. The virus of pine sawfly *Diprion sertifer* remained active on the needles (Kaupp, 1983) and in soil (Olofsson, 1988) for at least 2 and 13 years, respectively. One of the longest studies of virus persistence in the soil was the example of virus of the Douglas-fir tussock moth *Orgyia pseudotsugata,* which persisted in soil up to 41 years (Thompson et al., 1981). Numerous factors influence the virus ability to accumulate and persist in the soil compartments (Young, 2001). Fuxa et al. (2001) showed that wild-type HzSNPV accumulated 2.3 times as many OBs as HzSNPV-LqhIT2 in soil in the cotton-cotton bollworm system. This can be due to the fact that wild-type HzSNPV has a higher replication in the host insect and accumulates a greater number of OBs in the soil. The exposure to direct solar radiation can decrease virus infectivity. Recombinant AcMNPV- vHSGFP had in virus infectivity by a factor of 100, which occurred when virus was incubated at a temperature above 45ºC (Michalsky et al., 2008). CpGV larvicidal activity declined after 40 weeks at 35 ºC, compared to 3 years at 2 ºC (Lacey et al., 2008). There is a report that LdMNPV can be completely inactivated within one week (Podgwaite & Mazone, 1982). So, the literature shows wide variety in persistence of baculoviruses as affected by environmental factors such as UV radiation, temperature and plant chemicals.

There is a limited knowledge on the persistence, i.e. the survival in the eco-system over time, of fast acting baculoviruses in pest populations, measured over multiple generations. Long-term persistence of genetically modified baculoviruses is seen as undesirable, because it would prolong the exposure time of non-target biota, even though the effects of such exposure are considered to be harmless because of the host specificity of baculoviruses. From a population genetic perspective it is expected that viruses with increased speed of kill, due to deletion of the *egt* gene or insertion of toxin genes (such as AaIT), would be ecologically less fit because the survival of infected insects is shortened, compared to insects infected with wild-type viruses. Hence, the virus yield of recombinants in terms of OBs is much lower than that of wild-type baculoviruses, generally by a factor of 10.

To increase the biosafety of genetically modified baculoviruses in the environment it has been suggested to use baculovirus vectors lacking genes which could not replicate and spread naturally. This would reduce not only their environmental fitness in the environment, but also the viral load. The approach of using a polyhedron-negative baculovirus results in severely disabled viruses that are of little value in agriculture

(Hammock, 1992). A recent study from Zwart et al. (2009) suggests that AcMNPV–Δ*egt* can be a safe biocontrol agent since such recombinant baculovirus will be outcompeted in mix-infected insect by the wild-type AcMNPV. These authors show that *egt* negative baculoviruses have a lower competitive fitness as a result of within host competition. It is important to quantify 'within-host' and 'between-host' competitive fitness of recombinant baculoviruses with altered insecticidal properties compared with wild-type baculovirus, as evidenced from serial passage experiments (Hamblin et al., 1990; Huang et al., 1991, Zwart et al., 2009). The 'within-host' selection in mixed infections is very relevant for the risk assessment and environmental safety of recombinant viruses as biological control agent (Cory, 2000).

The hypothesis tested in this thesis is that baculovirus recombinants, which have altered properties, such as higher speed of action and lower yield, are rapidly replaced in nature by the conspecific wild-type baculovirus because of the lower ecological fitness of the former. To make progress towards decisions on ecological safety of genetically modified baculoviruses, we also need to perform field studies directed towards baculovirus persistence, to approach the fundamental question on the ecological fitness of wild-type and recombinant baculoviruses.

THE MAIN OBJECTIVES OF THE PROJECT AND OUTLINE OF THE THESIS

The main objectives of the thesis are the following:

1. to obtain insight in the competition between wild-type and genetically modified baculovirus genotypes in mixed infections in host insects;
2. to determine the effect of the different baculoviruses in single and mixed infection on the larval behaviour.
3. to quantify the transmission rate of genetically modified and wild-type baculoviruses in the single and mixed infections, in the cotton-cotton bollworm system, particularly to test the influence of density and time post virus release on plant.
4. to compare persistence of biological activity of wild-type, genetically modified and mixtures of both baculoviruses in soil.
5. to obtain insight in the competition between wild-type and genetically modified baculovirus genotypes in mixed infections in host insects, in multiple insect host generations.

To reach these objectives three virus-host systems have been used: firstly, the cotton-cotton bollworm-HaSNPV system to relate laboratory with field evaluations, secondly, the beet armyworm-SeMNPV system and thirdly the beet armyworm-AcMNPV system. In all cases recombinant baculoviruses with different biological properties were available and allowed a comparative approach.

In **Chapter 2** the biological properties of wild-type and recombinant HaSNPV were studied in single as well in mixed infections of cotton bollworm larvae, using a bio-assay in which pathogenicity and virulence of different HaSNPV genotypes were assessed. In **Chapter 3** a combination of greenhouse and field experiments was performed to measure the effect of the different HaSNPV genotypes and their interaction via mixed infections on the behavior of cotton bollworm larvae. **Chapter 4** is an extension of the experiments in chapter 3 and studies in more detail the relationship between inoculum density and the time post release of recipient larvae on the plant and the impact on transmission. Furthermore the persistence of wild-type and recombinant HaSNPVs in the soil was measured to help assessing the ecological safety of genetically modified viruses. **Chapter 5** describes the competition of SeMNPV viruses with different biological properties, when passaged several cycles in *S. exigua* insect larvae in different ratios. **Chapter 6** describes the competition between an AcMNPV recombinant baculovirus lacking an *egt* gene, but with enhanced speed of action and wild-type AcMNPV in insect larvae level when different ratios of virus mixtures were used in single and multiple insect generations. Chapters 5 and 6 are meant to investigate whether, in a different baculovirus-insect system, the hypothesis that faster viruses are less fit, holds up. **Chapter 7** summarizes the most important results from this thesis and discusses these with reference to the current literature in the area of baculovirus ecology and to the use of baculoviruses and their genetically modified derivatives as biological control agents in crop protection.

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Chapter 2

DOSE DEPENDENCY OF SURVIVAL TIME IN SINGLE AND MIXED INFECTIONS WITH A WILDTYPE AND EGT DELETION STRAIN OF *HELICOVERPA ARMIGERA* NUCLEOPOLYHEDROVIRUS

SUMMARY

Recombinant insect nucleopolyhedroviruses lacking the *egt* gene generally kill their hosts faster than wild type strains, but the response of insects to mixtures of virus genotypes is less well known. Here, we compared the survival time, lethal dose and occlusion body yield in third instar larvae of *H. armigera* (Hübner) after challenge with wild type *Helicoverpa armigera* SNPV (HaSNPV-wt), a strain with a deletion of the *egt* gene, HaSNPV-LM2, and a 1:1 mixture of these two virus strains. A range of doses was used to determine whether the total number of OBs influenced the response to challenge with a mixture of virus strains versus single strains. At high virus doses HaSNPV-LM2 killed *H. armigera* larvae significantly faster (ca. 20 h) than HaSNPV-wt, but at low doses, there was no significant difference in survival time between the viruses. The survival time after challenge with mixed virus inoculum was significantly different from and intermediate between that of the single viruses at high doses, and not different from that of the single viruses at low doses. No differences in lethal dose were found between single and mixed infections or between virus genotypes. The number of occlusion bodies produced per larva increased with time to death and decreased with virus dose, and no significant differences among virus treatments were found. These experiments show that the outcome of mixed infections depends on dose and this should be taken into account when evaluating the ecological consequences of release of viral types with different biological properties.

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INTRODUCTION

Baculoviruses occur naturally in insect populations, and some have been developed as microbial insecticides because, due to their specificity, they are generally safe to non-target organisms and the environment. However, only few baculoviruses have become a commercial success because of their slow speed of action, UV sensitivity and limited host range (Fuxa, 1991; Moscardi, 1999; Szewczyk et al., 2006; Erlandson, 2008).

Molecular engineering tools have been used to generate genetic modifications in wild type baculoviruses, in particular to accelerate speed of kill. These modifications include the deletion of viral genes and the insertion of genes that express insect specific toxins or metabolic enzymes (Bonning et al., 1992; Inceoglu et al., 2006). An important example of the former is the deletion of the baculovirus *egt* gene. This gene encodes the enzyme ecdysteroid UDP-glucosyltransferase (*egt*), which modifies ecdysteroid hormones by adding a carbohydrate moiety. These modifications render these hormones inactive thereby delaying or inhibiting the molt in virus-infected insects (O'Reilly & Miller, 1990, 1991). Infection with an *egt*-deletion virus results in normal progression of the larval molt, and often produces a reduction in time to death of virus-infected insects and an attendant reduction in crop damage (Hoover et al., 1995; Cory et al., 2004). The (molecular) mechanism responsible for the shortened time to death in insects infected with *egt* deletion strains is not known, but the Malpighian tubules seem to be involved (Flipsen et al., 1995).

In the field, baculoviruses occur as mixtures of conspecific genotypes (Miller, 1995; Hodgson et al., 2001). This genotypic diversity in a baculovirus population can be the result of genetic drift from a single parent strain, due to mutations, deletions and insertions during virus replication; or it can result from mixing and recombination of baculovirus strains (Williams, & Otvos, 2005; Lauzon et al, 2005; Jakubowska et al., 2005). How exogenous conspecific genotypes, such as genetically engineered recombinants, will interact with existing genotypes over time is unknown. The pertinent question is, what is the ecological fitness of fast killing, genetically modified, baculovirus strains, compared to more slowly killing, wild type strains?

The persistence of genetically engineered baculoviruses in an ecosystem will depend on competitive processes between virus strains at several levels of organization. Both processes within host insects (e.g. virus production) and processes at the ecosystem level, e.g. virus transmission due to encounters between larvae and inoculum, may be involved. These competitive interactions between recombinant and wild type strains will determine whether recombinant baculoviruses will persist in an ecosystem, and for how long. Given the high levels of heterogeneity in natural populations of baculoviruses, competition between virus strains within hosts is highly likely. There are many reports of mixed virus infections in insects (Steinhaus, 1963; Tanada & Chang, 1964; Thomas et al.,

2003). Co-infection of insect hosts with multiple viral species can be "antagonistic" [(Hackett et al. 2000 (GV/NPV); Muñoz & Caballero, 2000 (conspecific strains); Ishii et al. 2002 (NPV/EPV)], "neutral" [Milks et al. 2001 (conspecific including a recombinant)], or "synergistic" [(Tanada, 1985 (NPV/GV); Hukuhara et al., 1987 (NPV/GV); Wang et al., 1994 (GV/NPV); López-Ferber et al., 2003; Hodgson et al., 2004 (conspecific strains)].

When an insect is challenged with a mixture of a fast and a slow killing genotype of a baculovirus, different situations are theoretically possible: the time to kill could be similar to that of the fast acting virus strain in the mixture, it could be similar to that of the slow acting virus, or it could be intermediate. For an *egt*-deletion virus genotype the first possibility is less plausible because the faster speed of kill is due to the absence of a gene product in single infection. If a wild type virus is also present, then this gene product, *egt,* is again available. The second possibility, in which time to death is similar to that of infection with wild type virus, is plausible because the gene product of *egt* will be present in a mixed infection, as it is in a single infection with wild type virus. An intermediate time to kill is also possible since the quantity of *egt* in insects infected by a mixture of a wild type and *egt*-deletion virus may be reduced in comparison to the concentration in an infection with pure wild type virus. Thus, we have three alternate hypotheses: (1) time to death in larvae challenged with a virus genotype mixture is similar to that of larvae infected with the pure *egt* deletion strain, (2) time to death is similar to that of larvae infected with the wild type strain, and (3) time-to-death is intermediate between that of larvae with single genotype infections.

Here, we study mixed infection with the baculovirus HaSNPV in the L3 stage of the cotton bollworm, *Helicoverpa armigera*. The cotton bollworm is an economically important pest insect that attacks at least 35 crop and 25 wild host plants (Greathead & Girling, 1982). In Asia, India, and South Africa, *H. armigera* is a key pest on cotton (van Hamburg & Guest, 1997; Cherry et al., 2003). Whilst transgenic resistance to the cotton bollworm is available in cotton, it is essential that alternative control options are also studied and optimized, because the transgenic resistance may be broken (Tabashnik et al., 2003), transgenic resistance may not be available in minor crops, and the cotton bollworm is highly resistant to pesticides (Armes et al, 1992; Brèvault et al., 2008; Ugurlu & Gurkan, 2008).

Experiments were conducted with a wild type HaSNPV (HaSNPV-wt) and an *egt*-deletion mutant, HaSNPV-LM2, with enhanced speed of kill. Sun et al., (2004) reported that the *egt*-deletion mutant, HaSNPV-CXW1 showed significantly quicker speed of action than wild type virus in third instars. Therefore, cohorts of third instar larvaes were challenged with an equal dose of a 1:1 mixture of HaSNPV-wt and HaSNPV-LM2 or an equivalent dose of one of the two individual biotypes, using a range of 10 different doses.

The results of the experiments were analyzed to determine lethal dose (LD_{50}), survival time (ST_{50}) and occlusion body (OB) yield.

MATERIALS AND METHODS

Viruses

The baculoviruses used in this study were: 1) purified wild type HaSNPV, isolated from *H. armigera* larvae from China (named HaSNPV-G4) (Sun et al, 1998), further referred to in the text as HaSNPV-wt; 2) a recombinant HaSNPV lacking *egt* and generated in cell culture, further referred to as HaSNPV-LM2, and 3) a 1:1 mixture of these two baculoviruses, further referred to as HaSNPV-mix.

HaSNPV-LM2 was generated by co-infection with HaSNPV-CXW2 DNA (-*egt*; +GFP) (Chen et al., 2000) and plasmid pHaLM2. Most of the *egt* open reading frame (ORF) in this transfer vector was deleted by insertion of the AaIT gene (Inceoglu et al, 2001) and a SV40 transcription termination sequence flanked by *Hin*dIII sites (Chen et al., 2000; Fig. 1). The AaIT gene is not expressed in this construct because the *egt* promoter is absent, which was confirmed by nucleotide sequencing. The co-transfection was carried out using *Helicoverpa zea* Hz-AM1 cells, grown in CCM3 medium supplemented with 10% fetal bovine serum. Recombinant HaSNPV-LM2 was re-isolated after three cycles of plaque purification in Hz-AM1 cells (McIntosh & Ignoffo, 1983). All viruses were amplified in fourth instar *H. armigera* reared in the laboratory on artificial diet (Green et al., 1976). Occlusion bodies (OBs) were purified from infected larvae by homogenization and sucrose gradient centrifugation (Allaway & Payne, 1984). Concentration of OBs of the viral stock solutions was determined in three independent counts using an Improved Neubauer chamber (Hawsksley, Lancing, UK) by phase-contrast microscopy (× 400). Virus stocks were stored at 4°C until use.

Insects

H. armigera used in the experiments were obtained from an insect colony maintained at the Department of Entomology, Public University of Navarra, Pamplona, Spain. The colony was reared continuously on artificial diet (Green et al., 1976) at 25ºC, 70% relative humidity (RH), and a 16L: 8D h photoperiod.

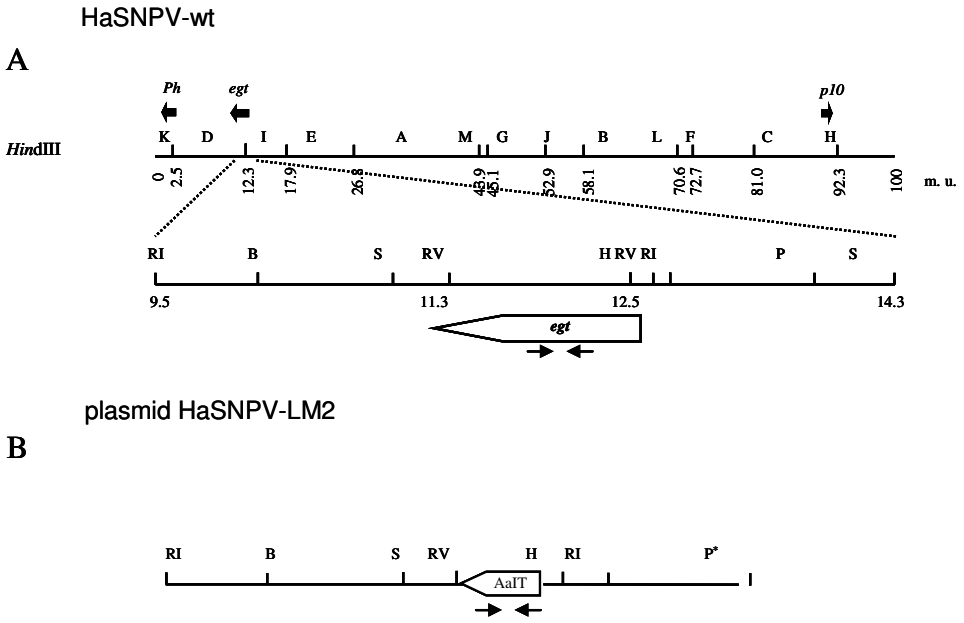

Figure 1. (a) Schematic representation of HaSNPV-wt (A) and HaSNPV-LM2 (B) at the *egt* gene locus of the HaSNPV genome (Chen et al., 2000). (B) The transfer vector pHaLM2 was used to construct the recombinant HaSNPV-LM2. The restriction sites indicated here are B, *Bam*HI, RI, *Eco*RI, RV, *Eco*RV, H, *Hin*dIII, P, *Pst*I, S, *Sst*I. Thick arrows indicate the orientation of the genes *ph* (polyhedrin), *egt* and *p10*. Thin arrows indicate the forward and reverse primers on the *egt* and AaIT genes, respectively.

Bioassays: Determination of lethal dose (LD_{50}) and survival time (ST_{50})

Third instar *H. armigera* were challenged with a range of doses of HaSNPV-wt, HaSNPV-LM2 or a 1:1 mixture of these two viruses to determine mortality and survival time. Second instar larvae exhibiting head capsule slippage were held without food for 16 h at 25ºC. Larvae that had molted to the third instar were orally inoculated by the droplet feeding method (Hughes et al. 1981), by exposing the larvae to an aqueous suspension containing 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue (food dye), and relevant concentrations of OBs. The following serial dilutions of each virus were used: 3×10^7, 1×10^7, 3×10^6, 1×10^6, 3×10^5, 1×10^5, 3×10^4, 1×10^4 , 3×10^3 and 1×10^3 OBs/ml. Sun et al. (2004) determined that third instar *H. armigera* under the same conditions ingested a volume of approximately 1 µl; thus the mean ingested dosage was estimated at 1, 3, 10, 30, 100, 300, 1000, 3000, 10000 and 30000 OBs/larva, respectively. Only larvae that imbibed the virus solution, as evidenced by the blue coloration of their midgut, within 10 min after exposure to the virus solution, were transferred onto fresh artificial diet.

Controls (N = 25 larvae) consisted of larvae handled in the same manner but fed virus-free solution instead.

Inoculated larvae were reared individually at 25°C, 79 % RH, and a 16L: 8D light-dark regimen; insects were monitored at approximately 8 h intervals until they died or pupated. Bioassays were repeated six times with minor variations in the range of doses used, i.e. each experiment included most of the above-mentioned doses but not necessarily all. The number of larvae was varied with dose to obtain a sufficient number of infected insects at each dose; from 25 larvae at high doses to 70 at low doses.

Median lethal dose values (LD_{50}) were determined by probit analysis using the computer program POLO-PLUS (Russell et al., 1977). Survival time (ST_{50}) was estimated using the Kaplan-Meier Product Limit Estimator in JMP SAS (2008) in non-parametric survival analysis. Non-parametric Cox's Proportional hazards model was fitted to the data by dose with virus treatment and experiment as the independent variables in JMP SAS (2008).

Bioassays: Determination of OB yield

OB yield was determined for two out of the six bioassays from approximately five cadavers, randomly selected for each combination of dose and virus treatment. Individual cadavers were homogenized in 0.5 ml of sterile water, filtered through a plastic filter with a fine metal net (mesh size 120 to 200 µm, pore diameter 70 nm) and then centrifuged at 6,000 *g* for 5 min. The supernatant was discarded and the OB pellet was resuspended in 500 µl of sterile water. Virus yield was determined by counting the number of OBs using an Improved Neubauer hemocytometer by phase contrast microscopy, in three independent counts. Concentrations are reported as OBs/ml.

OB yield was analyzed using regression analysis in SPSS (SPSS Inc., 2003 and Genstat (Procedure REML). The REML procedure in Genstat is equivalent to an ordinary least squares regression; in REML (Restricted Maximum Likelihood), the model is fitted using maximum likelihood instead of least squares. Yield was log-transformed before analysis to obtain identically distributed normal errors (IDNE). Residuals were checked visually for departures from the assumption of IDNE. Experiment (random) and virus treatment (fixed) were used as factors in the analysis, time to death as covariable, while dose was alternately included as a factor (10 levels) or as a covariable (1 df).

RESULTS

Mortality

Mortality increased with dose in all treatments (Fig. 2). Mortality responses were shallow indicating large variation in susceptibility among individual larvae (Ridout et al., 1993). The LD_{50} of the virus treatments were similar, and not significantly different in

pairwise comparisons any of the six experiments as shown by overlapping confidence intervals. For example, in Experiment 6, the LD_{50} for HaSNPV-wt was 13 (7-24) OBs/larva, df = 8, $\chi^2 = 8.46$), the LD_{50} for HaSNPV-LM2 was 10 (5-18) OBs/larva, df = 8, $\chi^2 = 8.25$) and the LD_{50} for HaSNPV-mix was 18 (7-49) OBs/larva, df = 7, $\chi^2 = 16.79$).

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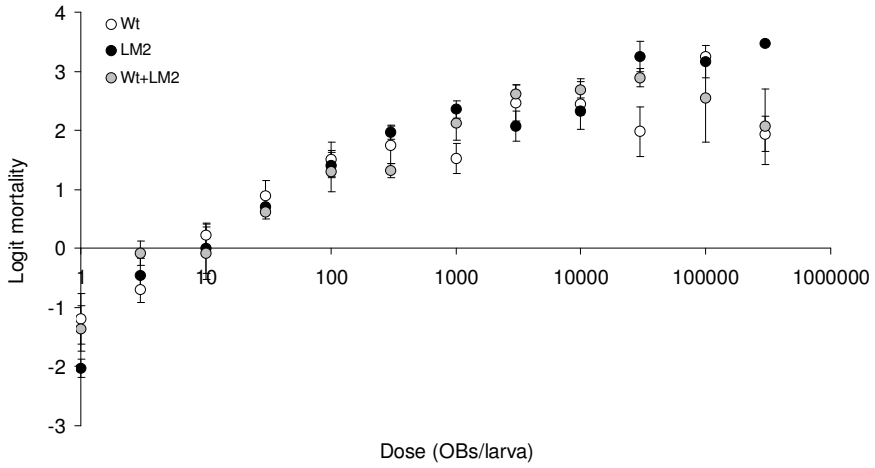

Figure 2. Dose–logit mortality responses of third instar *H. armigera* exposed to one of three HaSNPV preparations (OBs/larva): HaSNPV-wt, the *egt* deletion mutant HaSNP-LM2, or a 1 : 1 mixture of HaSNPV-wt and HaSNP-LM2.

Survival time (ST_{50})

ST_{50} decreased with increasing dose in all virus treatments, from approximately 150 h at the lowest doses to approximately 80 h at the highest (Fig. 3). At virus doses of 300 OBs/larva and higher, significant differences among virus treatments were observed. HaSNPV-mix killed larvae significantly faster than the wild type at most viral doses above 300 OBs (Table 1).

Table 1. Survival times (ST_{50}s) of third instar *Helicoverpa armigera* infected with wild-type and recombinant viruses.

Dose	Virus	ST_{50}s	95% fiducial limits		SE	df	χ^2	P
(OBs/larva)		(h.p.i.)[1]	CI low	CI high				
1	HaSNPV-wt	136	136	160	6.3	2	1.70	0.4269
	HaSNPV-mix	144	128	160	5.1			
	HaSNPV-LM2	160	136	160	7.0			
3	HaSNPV-wt	128	112	136	4.4	2	1.91	0.3839
	HaSNPV-mix	128	120	136	3.2			
	HaSNPV-LM2	136	120	144	4.1			
10	HaSNPV-wt	144	144	152	2.3	2	1.87	0.3924
	HaSNPV-mix	136	136	144	2.6			
	HaSNPV-LM2	136	128	136	3.3			
30	HaSNPV-wt	136	136	144	2.4	2	1.68	0.4315
	HaSNPV-mix	152	136	160	2.6			
	HaSNPV-LM2	136	136	144	2.3			
100	HaSNPV-wt	136	136	136	1.5	2	3.02	0.2201
	HaSNPV-mix	136	136	136	1.9			
	HaSNPV-LM2	136	136	144	1.6			
300	HaSNPV-wt	136 a	128	136	1.8	2	44.16	0.0001
	HaSNPV-mix	128 a	120	136	2.1			
	HaSNPV-LM2	120 b	112	120	1.5			
1000	HaSNPV-wt	120 a	120	128	1.7	2	24.60	0.0001
	HaSNPV-mix	120 a	120	128	2.1			
	HaSNPV-LM2	112 b	104	112	1.6			
3000	HaSNPV-wt	120 a	120	128	1.9	2	29.34	0.0001
	HaSNPV-mix	104 b	104	112	2.0			
	HaSNPV-LM2	96 c	96	104	1.8			
10 000	HaSNPV-wt	112 a	104	120	1.8	2	25.87	0.0001
	HaSNPV-mix	96 b	96	104	2.4			
	HaSNPV-LM2	96 c	96	96	1.4			
30 000	HaSNPV-wt	104 a	96	112	1.8	2	11.71	0.0029
	HaSNPV-mix	96 a	96	104	2.1			
	HaSNPV-LM2	88 b	88	96	1.7			
100 000	HaSNPV-wt	96 a	96	96	3.5	2	12.25	0.0022
	HaSNPV-mix	88 b	72	88	4.3			
	HaSNPV-LM2	80 b	72	88	3.7			
300 000	HaSNPV-wt	88 a	88	96	3.5	2	26.48	0.0001
	HaSNPV-mix	80 b	72	88	5.0			
	HaSNPV-LM2	72 c	63	72	2.6			

1 Median survival times were determined by survival analysis using the Kaplan Meier Product Limit estimator (Collett, 1994). h.p.i.= hours post inoculation. Different letters after ST_{50} values for each dose are significantly different at $p < 0.05$.

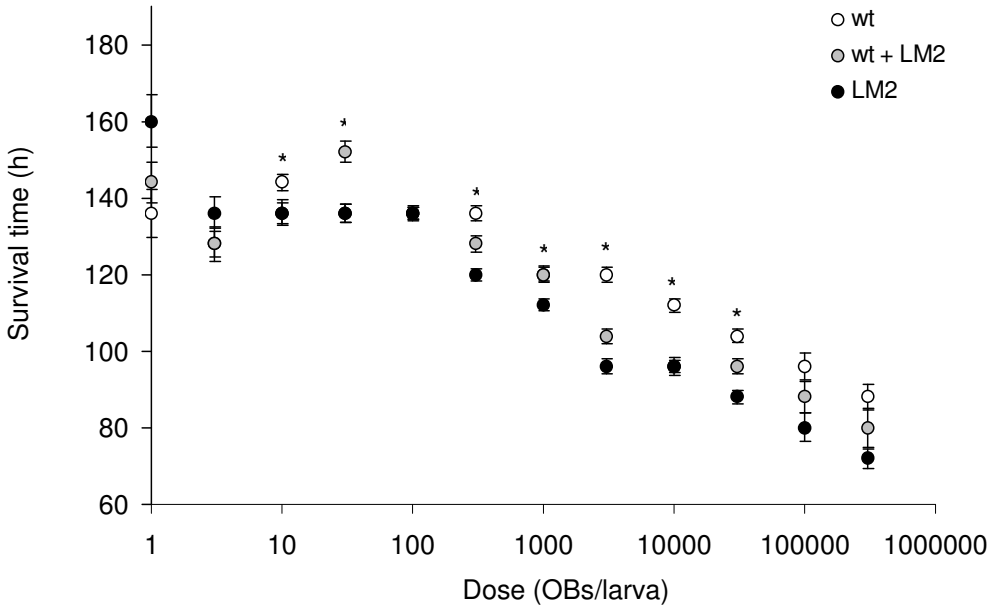

Figure 3. Survival time (ST_{50}) of third instar *H. armigera* exposed to one of three HaSNPV preparations, plotted against dose (OBs/larva). Data were analyzed as the average of the median survival times (ST_{50}) across the experiments, separately for each viral dose. Vertical bars represent standard errors of the means. Asterics were added on the treatments that are significantly different from each other, for each dose. Significant difference is at level $P < 0.05$.

At low doses (1, 3, 10, 30 and 100 OB/larva), there was no significant difference in ST_{50} between wild type and HaSNPV-LM2 (Fig. 3, Table 1). At doses ≥ 300 OBs/larva the survival time (ST_{50}) for HaSNPV-LM2 was approximately 8-24 h shorter than for HaSNPV-wt (Fig. 4). For the mixture of viruses, at the higher viral doses (> 300 OBs/larvae), ST50 was approximately 8-16 h shorter than with HaSNPV-wt. When HaSNPV-LM2 and HaSNPV-mix were compared, the pure recombinant killed faster than the mixture at the doses of 300 OBs/larva or more, except at a dose of 100,000 OBs/larva (Table 1).

Survival curves at doses of 10 and 100 OBs per larva in three experiments are shown in Fig. 5, to exemplify the variability in response among replicates, observed at low doses. At 10 OBs per larva, HaSNPV-LM2 killed faster than HaSNPV-wt in Experiment 1 (Fig.5A), but not in Experiments 2 and 3 (Fig 5 C and E). At a dose of 10 OBs per larvae, the mixture killed the larvae faster than the wild type virus in Experiment 1 (Fig. 5 A), but not in Experiments 2 and 3 (Fig.5 C and E). At 100 OBs per larva, these

trials show more consistency. In all replicates (Fig. 5 B, D, F) HaSNPV-LM2 killed faster than HaSNPV-wt and HaSNPV-mix. The large variability in treatment effects at low doses is reflected in large standard errors in Fig. 4, and wide confidence intervals for ST_{50} (Table 1).

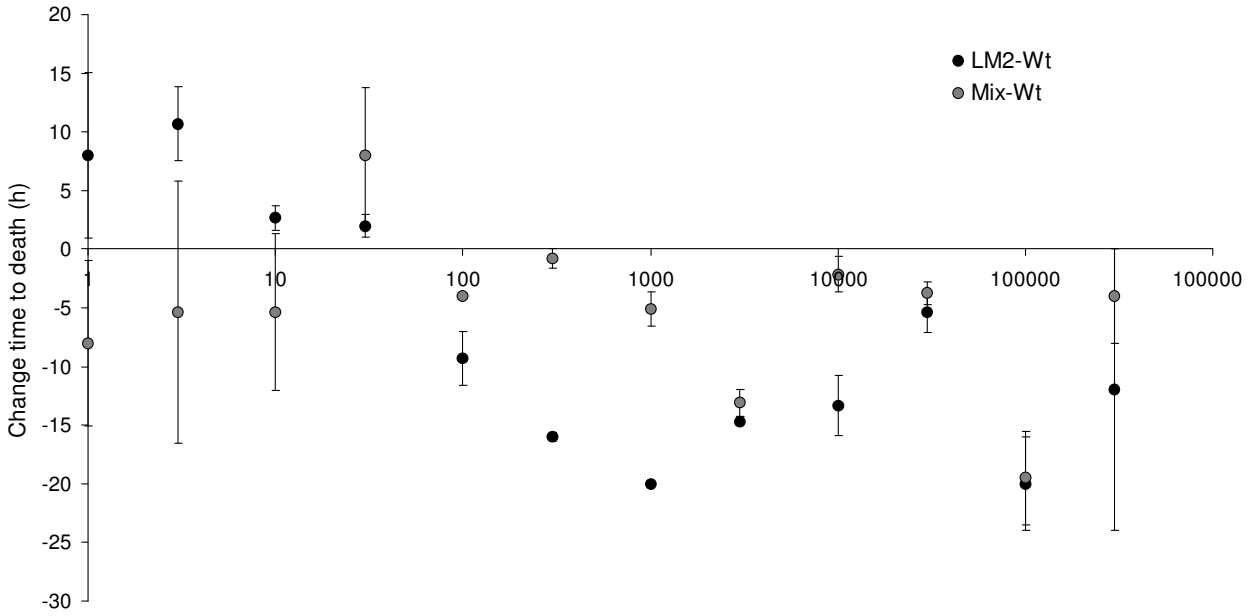

Figure 4. Differences in survival time (ST_{50}) of third instar *H. armigera* challenged with HaSNPV-LM2 or the mix in comparison with the ST_{50} of larvae challenged with HaSNPV-wt. Symbols represent the average survival time for the six experiments for each virus/dose combination. Standard error bars are shown.

When survival curves were compared by dose using virus treatment and experimental replicate as main effects in non-parametric survival analysis, times to death differed significantly as a function of both main effects. Significant differences among the three virus treatments were found at doses $\geq$ 300 OBs/larva. Variability among experiments in the relative effect of viruses showed up as significant interactions when survival analysis was conducted separately at each dose (Table 2).

Table 2. Non-parametric survival analysis of larvae inoculated with 1 of 10 viral doses. The survival function was modeled using the factors of virus treatment (wild type HaSNPV-wt; recombinant HaSNPV-LM2 and 1:1 mix HaSNPV-wt and HaSNPV-LM2), experiment as (repetitions), and the interaction between treatments and experiments (Exp. × trt).

Dose OBs/larva	Term	Log Likelihood	χ^2	df	P
1	Full model	281.87	16.5	8	0.0361
	Experiments		7.7	2	0.0208
	Treatments		2.1	2	0.3482
	Exp. × trt		4.8	4	0.3090
3	Full model	858.83	75.5	8	0.0001
	Experiments		69.7	2	0.0001
	Treatments		6.2	2	0.0445
	Exp. × trt		9.9	4	0.0407
10	Full model	1129.22	106.8	11	0.0001
	Experiments		81.9	3	0.0001
	Treatments		0.01	2	0.9915
	Exp. × trt		16.9	6	0.0096
30	Full model	1481.81	68.1	11	0.0001
	Experiments		41.7	3	0.0001
	Treatments		1.65	2	0.4390
	Exp. × trt		15.8	6	0.0146
100	Full model	3591.37	73.2	17	0.0001
	Experiments		31.8	5	0.0001
	Treatments		1.0	2	0.5899
	Exp. × trt		38.5	10	0.0001
300	Full model	2832.32	89.2	17	0.0001
	Experiments		43.2	5	0.0001
	Treatments		34.3	2	0.0001
	Exp. × trt		18.8	10	0.0429
1000	Full model	2555.51	63.6	17	0.0001
	Experiments		21.4	5	0.0007
	Treatments		12.9	2	0.0016
	Exp. × trt		19.4	10	0.0359

Table 2. Continued.

Dose OBs/larva	Term	Log Likelihood	χ^2	df	P
3000	Full model	2352.69	92.1	17	0.0001
	Experiments		45.9	5	0.0001
	Treatments		14.9	2	0.0006
	Exp. × trt		188	10	0.0042
10000	Full model	2177.8	87.3	17	0.0001
	Experiments		38.4	5	0.0001
	Treatments		19.3	2	0.0001
	Exp. × trt		32.0	10	0.0004
30000	Full model	2002.1	55.5	17	0.0001
	Experiments		23.1	5	0.0003
	Treatments		6.9	2	0.0313
	Exp. × trt		21.6	10	0.0175
100000	Full model	804.9	10.4	5	0.0654
	Experiments		0.9	1	0.3363
	Treatments		9.1	2	0.0104
	Exp. × trt		0.7	2	0.7086
300000	Full model	631.5	23.8	5	0.0002
	Experiments		5.9	1	0.0144
	Treatments		18.2	2	0.0001
	Exp. × trt		0.09	2	0.9535

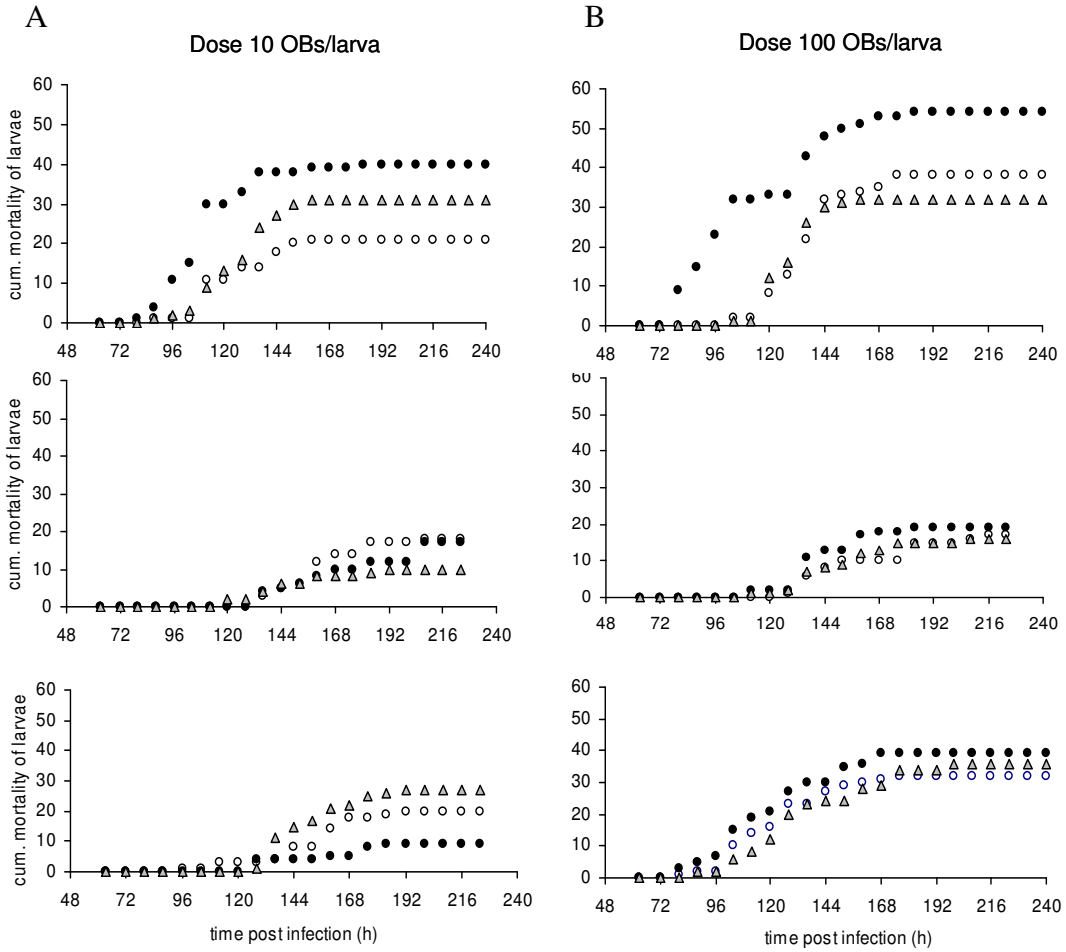

Figure 5. Cumulative mortality of third instar *H. armigera* larvae plotted over time (h) for three experiments at two viral doses: (A) Dose of 10 OBs/larva or (B) 100 OBs/larva. Data points are HaSNPV-wt: open circles; HaSNPV-LM2: black circles; HaSNPV-mix: grey triangles.

Virus yield

Yield varied with dose, and a tendency was observed towards higher yields at intermediate doses (Fig. 6) In an analysis of two replicate experiments with REML, using experiment (1 df), virus treatment (2 df), and dose (9 df) as factors, the effect of dose was significant ($P < 0.001$; Table 3), while there was no effect of virus treatment or interaction between dose and virus treatment. There was a significant difference between the experiments ($P < 0.001$). Survival time in this two replicates was shortest at the highest doses (Fig. 7), and survival time was significantly influenced by dose ($P < 0.001$), virus treatment ($P = 0.043$) and their interaction ($P = 0.013$) (Table 3). When survival time was included in the regression analysis of virus yield (Table 3), it explained most of the variability in the yield data (Wald/d.f. = 70.34; $P < 0.001$) while there was also a significant effect of dose (Wald/d.f. = 2.11; $P = 0.025$). The relationship between survival

time and virus yield is illustrated in Fig. 8. A test for differences between the regression slopes for different virus treatment, using Procedure "F special" in Genstat, gave no significant results.

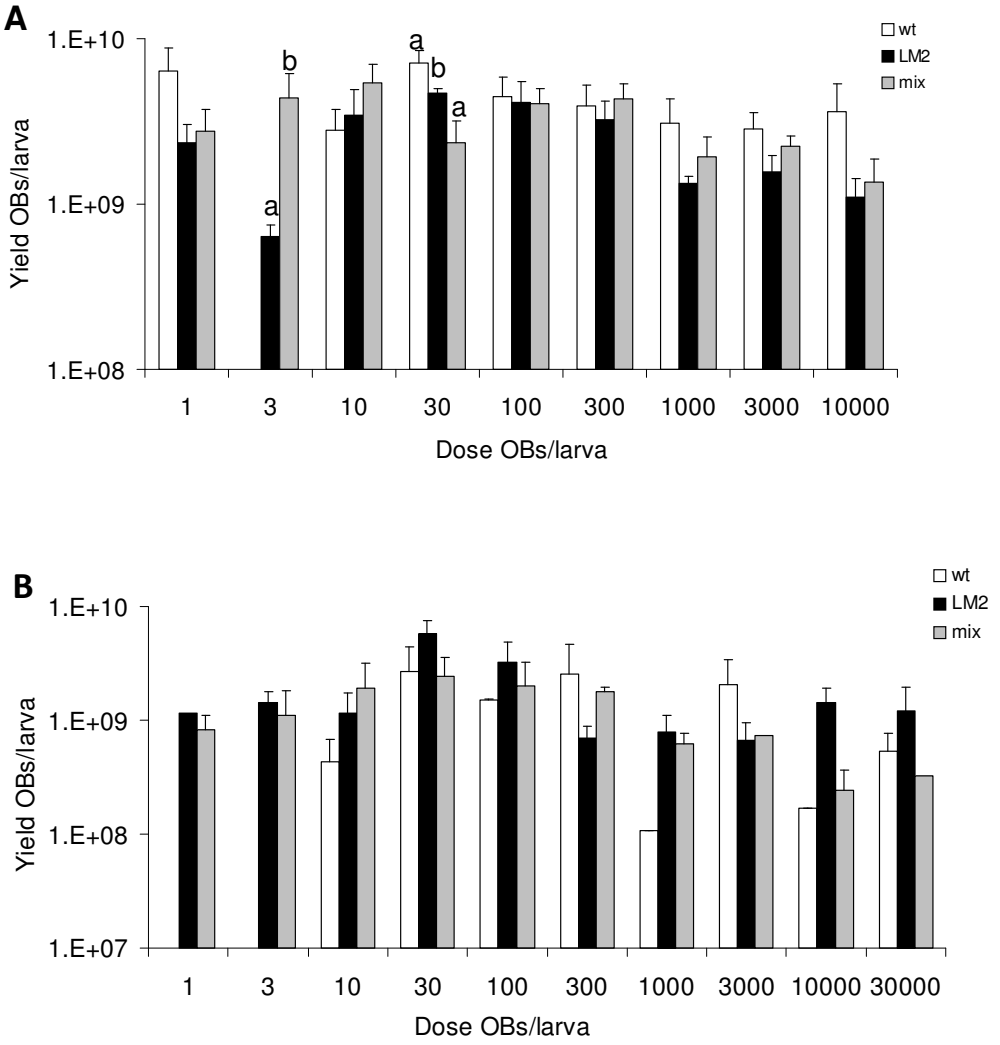

Figure 6. Relationship between dose and average yield (OBs/larva) of third instar *H. armigera* larvae infected with 10 different doses of HaSNPV-wt; recombinant HaSNPV-LM2 or a 1:1 mixture of wild type and recombinant HaSNPV. Standard errors show variation of yield among larvae per dose. (A) Experiment 1, and (B) Experiment 2. Different letters show significant difference at $P < 0.05$.

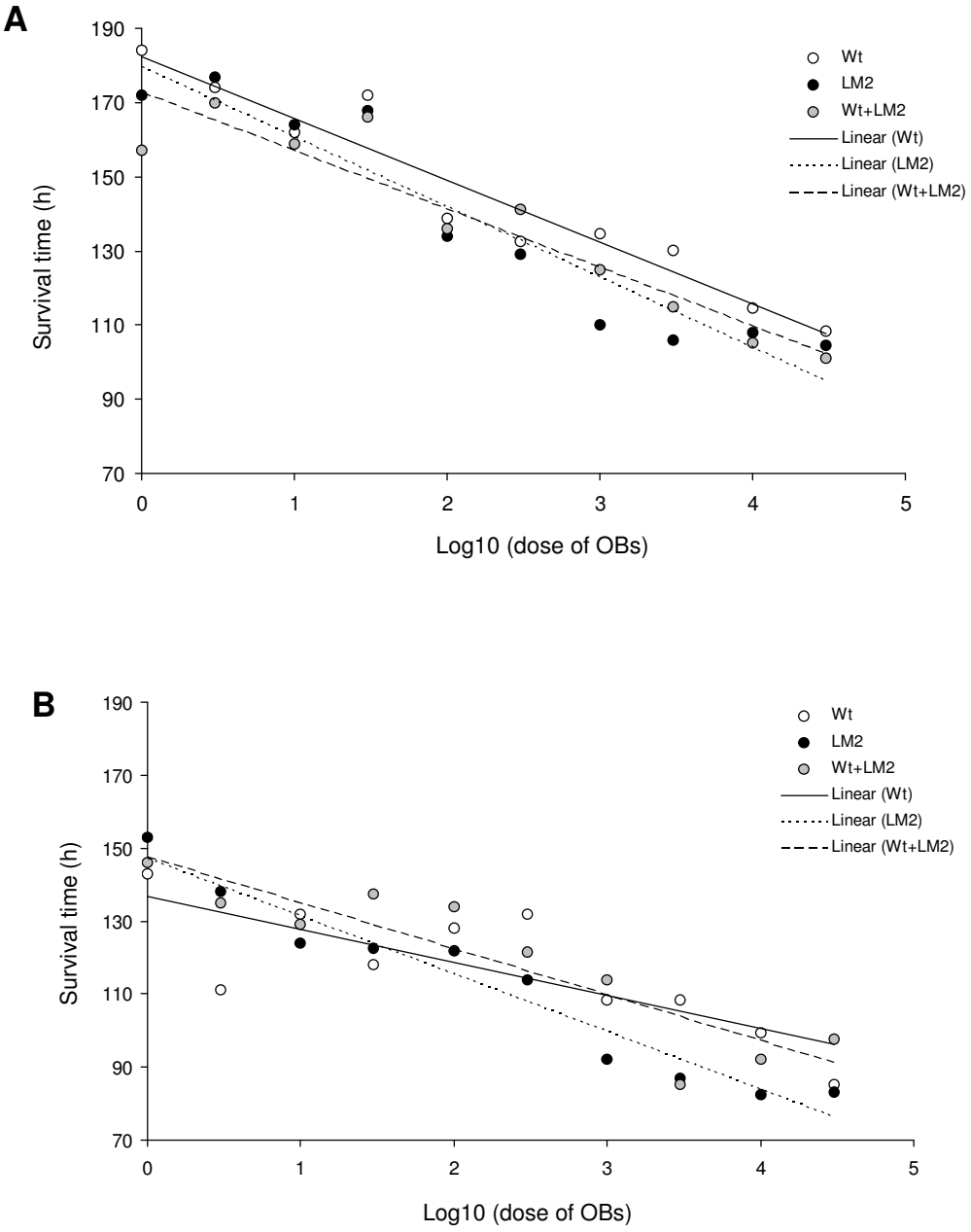

Figure 7. Dose-survival time relationship of third instar *H. armigera* larvae infected with HaSNPV-wt; recombinant HaSNPV-LM2 or a 1:1 mixture of wild type and recombinant HaSNPV. (A) Experiment 1, and (B) Experiment 2.

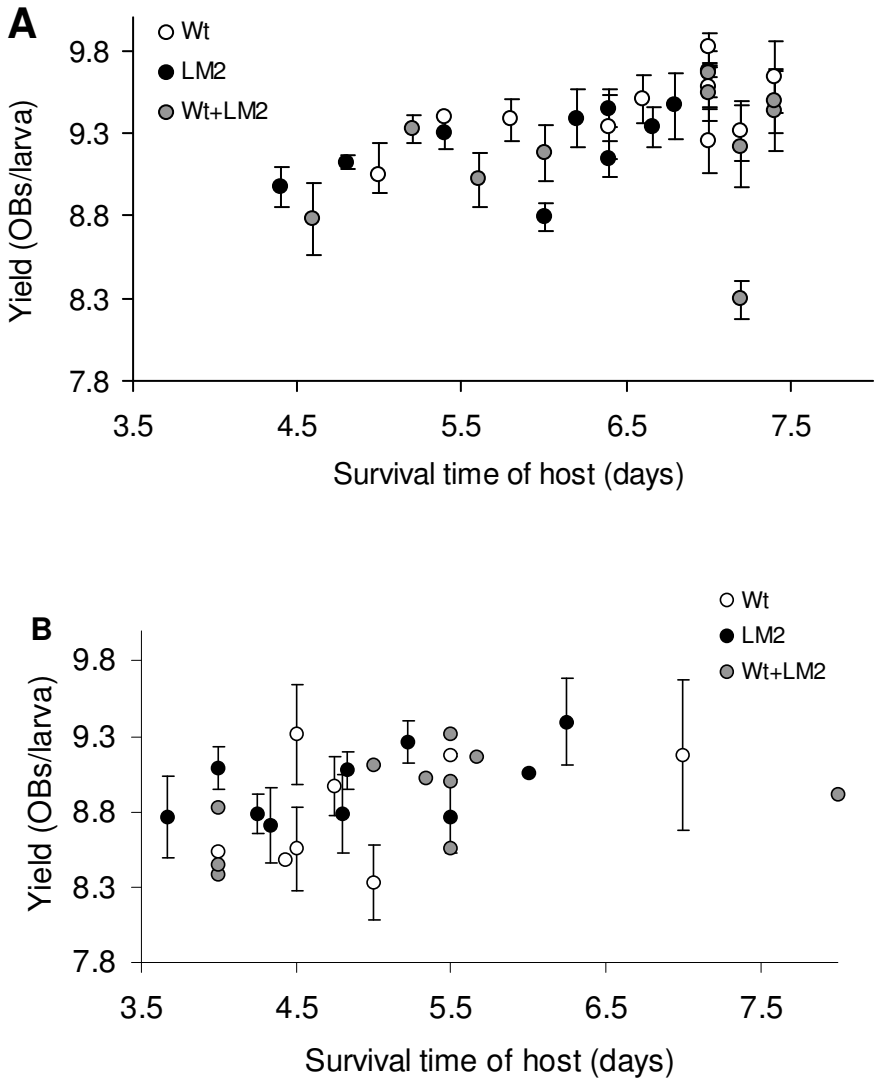

Figure 8. Relationship between time to death and yield of progeny virus (OBs/larva) of third instar *H. armigera* larvae infected with 10 different doses of HaSNPV-wt; recombinant HaSNPV-LM2 or a 1:1 mixture of HaSNPV-wt and HaSNPV-LM2. Standard errors are shown for each dose. (A) Experiment 1, and (B) Experiment 2.

Table 3. Two-way ANOVA of the log-transformed OBs yield/larva of 3rd instars *H. armigera* challenged with one of three HaSNPV preparations, HaSNPV-wt, HaSNPV-LM2 or HaSNPV-mix.

	Mean effects of factors				
	SS	**df**	**MS**	**F**	**P**
Exp. 1					
trt	0.72	2	0.36	2.85	0.0620
dose	4.71	9	0.52	4.15	0.0001
trt × dose	3.18	18	0.18	1.40	0.1440
error	14.6	116	0.13		
total	12743	146			
Exp. 2					
trt	0.22	2	0.11	0.45	0.640
dose	4.72	9	0.53	2.15	0.036
trt × dose	2.21	16	0.14	0.56	0.901
error	17.4	71	0.25		
total	7958	99			

DISCUSSION

Our experiments show that the survival time of larvae challenged with a mixture of a fast and slow killing genotype of HaSNPV is intermediate between the survival times of the two genotypes separately. Survival time decreased with increasing virus dose for all treatments. Differences in ST_{50} between virus treatments depended on dose, and were significant only at doses of 300 OBs per larva or higher. Survival time decreased substantially with dose, which is commonly observed in baculovirus infections (e.g. Milks et al., 2001), while variability in the estimated survival time increased at lower doses. This increase in variability may have multiple causes. One cause is the lower numbers of insects responding, even though the number of challenged insects had been raised at low doses to compensate for lower mortality. It is also conceivable that at lower challenge doses stochastic events in the infection process at the organismal level cause intrinsically greater variability in response. Finally, differences in dose due to the method of droplet feeding may have exacerbated variability especially at the low doses where the value of each OB in the infection processes is maximized (i.e. the flip side of the law of diminishing returns).

The findings support the second of hypotheses formulated and indicate that the effect of *egt* on survival time depends on the quantity of gene product, and is not a qualitative response to either presence or absence in the inoculum of a virus coding for *egt.* The quicker death following challenge with an *egt*-negative HaSNPV genotype

confirms findings of Sun et al. (2004) with another *egt*-negative *Ha*SNPV variant, HaSNPV-CXW1. Also the same was observed in egt negative baculoviruses in other insect host-virus systems AcMNPV in *T. ni* (Cory, 2004). The relation between dose and survival time may be related to the number of founders of the viral infection. At low dose, with an attendant low mortality, the number of virions that initiate infection, may be very low (Zwart et al., in press). Virus spread may be slowed down by anti-viral responses that may occur in the host larva, particularly at the level of the midgut by sloughing of infected midgut cells (Hoover, et al., 2000; Li,. et al., 2007) making it less likely that multiple foci of infection are established. With a low number of foci of infection in the insect it night take longer for the virus to colonize the host and kill it.

In a previous report, shorter ST_{50}s resulted in lower OB yields (O'Reilly & Miller, 1991; Cory et al., 1994; Ignoffo & Garcia, 1996 Burden et al., 2000; Hernandez-Crespo et al., 2001; Sun et al., 2005). Studies to date have shown overall lower OB yields and reduced virus transmission (Muñoz & Caballero, 2000; Sun et al., 2005). In contrast to these findings, Hodgson et al. (2001) reported increased yield in mixed genotype infections compared with single infections and the authors observed no difference in times to death. Here we found that the best predictor for the yield from virus-infected cadavers was the time to death, with no significant influence of virus treatment. To a large extent, the effect of virus dose on virus yield could be accounted for by survival time. Thus, our conclusion here is that in our system, the effect of mixed virus infection on virus yield can be entirely explained by the effect of survival time, i.e. the yield from mixed infections is expected to be intermediate between that of the pure virus variants used in the study.

In summary, detailed analysis of a set of six independent bioassays comparing two biotypes of HaSNPV that differ in a single trait (*egt*) showed high variability in survival times within and among experiments at low viral dosages, but not at intermediate and high dosages. The results obtained in this paper imply that only at intermediate and high dose there will be an effect of mixed infections on the response of the larval host. This should be taken into account when the ecological consequences of release of viral types with different biological properties are evaluated.

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Chapter 3

EFFECTS OF SINGLE AND MIXED INFECTIONS WITH WILD-TYPE AND GENETICALLY MODIFIED *HELICOVERPA ARMIGERA* NUCLEOPOLYHEDROVIRUS ON MOVEMENT BEHAVIOUR OF COTTON BOLLWORM LARVAE

SUMMARY

Naturally occurring insect viruses can modify the behaviour of infected insects and thereby modulate virus transmission. Modifications of the virus genome could alter those behavioral effects. We studied the distance moved and the position of virus-killed cadavers of fourth instar larvae of *H. armigera* infected with a wild-type genotype of *Helicoverpa armigera* nucleopolyhedrovirus (SNPV) or with one of two recombinant genotypes of this virus on cotton plants. The behavioral effects of virus infection were examined both in larvae infected with a single virus genotype, and in larvae challenged with mixtures of the wild-type and one of the recombinant viruses. An *egt*-negative virus variant caused more rapid death and lower virus yield in fourth instars, but *egt*-deletion did not produce consistent behavioral effects over three experiments, two under controlled glasshouse conditions and one in field cages. A transgenic virus containing the AaIT-(*Androctonus australis* Hector) gene, which expresses a neurotoxin derived from a scorpion, caused faster death and cadavers were found at lower regions of the cotton plants. Larvae that died from mixed infections of this recombinant and the wild-type virus died at intermediate positions, compared with infection to the pure viral strains. The results indicate that transmission of *egt*-negative variants of HaSNPV will likely be affected by lower virus yield, but not by behavioral effects of *egt* gene deletion. In contrast, the AaIT-recombinant will produce lower virus yields as well as modified behaviour, which together can contribute to reduced virus transmission under field conditions.

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INTRODUCTION

Baculoviruses are a large and diverse group of insect pathogens. They cause natural epizootics in insect pest populations, especially in forests (Cory & Myers, 2003), and they are used for insect pest management in forestry, horticulture and arable agriculture (Moscardi, 1999). Baculoviruses are slow killers compared to synthetic chemical insecticides. This is a disadvantage in curative crop protection strategies as it gives the insects more time to feed and cause damage. Their slow speed of kill has prompted attempts to develop faster acting recombinant baculoviruses, for example, through the incorporation of insect-specific toxin genes (Tomalski & Miller, 1991; McCutchen et al., 1991; Chejanovsky et al., 1995; Hughes et al., 1997; Chen et al., 2000). There is concern, however, about the ecological safety of such genetically modified baculoviruses, especially about possible effects of genetic modification on host range and long-term impacts after release in the environment (Cory & Hails, 1997; Hernandez-Crespo et al., 2001; Hails et al., 2002; Cory & Myers, 2003;). It is therefore pertinent to study factors that affect the long-term persistence of wild-type (wt) and genetically modified viruses in agro-ecosystems. It is particularly relevant to study factors affecting the transmission of wt and modified viruses, especially in situations where genetically-modified viruses could occur in host insects in mixed infections with wild-type viruses.

The persistence of virus genotypes in ecosystems depends on their competitive interactions at individual and eco-system level. These competitive interactions between genetically modified and wt genotypes will determine whether recombinant baculoviruses will persist in an ecosystem. Persistence of baculoviruses in the environment is directly related to their ability to be transmitted from one host to another, which in turn is related to host behaviour upon virus infection (Hoover et al., 1995). There is ample evidence that baculoviruses can induce modified behaviour in their hosts, including enhanced locomotor activity and a tendency to climb to elevated points on the host plant shortly before death (negative geotaxis); this climbing behaviour results in a phenomenon known as ‘tree top disease’ or ‘Wipfelkrankheit’ (Andreadis, 1987; Tanada & Kaya, 1993; Goulson, 1997). Elevation seeking behaviour is often accompanied with disintegration of infected larvae due to enzymes encoded by the viral cathepsin and chitinase genes (Hawtin et al., 1997). Thus, the occlusion bodies (OBs) containing the transmission stages of the virus can be spread over large parts of the plant when it rains, thereby potentially increasing the likelihood of transmission (Vasconcelos et al., 1996; Goulson, 1997; Zhou et al., 2005). In addition, cadavers at the ends of branches will be more apparent and accessible to scavenging birds that can carry the virus to distant locations (Entwistle & Evans, 1985; Fuxa, 2004). Behavioural changes in infected insects could also hamper virus transmission, however, and thereby be adaptive to the host. An upward movement of infected specimens, away from healthy conspecifics, could reduce virus transmission. A recent report (Raymond et al., 2005) shows negative phototaxis in baculovirus-infected

larvae of the winter moth, *Operophtera brumata*. In this system, most of the virus was deposited on the stems rather than on leaves. The more protected location of the virus could enhance virus persistence and hence foster transmission in the long term. All in all, the effects of baculoviruses on hosts appear diverse and different from one system to another. Likewise, the consequences of altered behaviour for transmission are system - specific.

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a polyphagous insect pest species with a worldwide distribution. It is an important pest in several crops, e.g. cotton, tomato, tobacco, maize, chickpea and sorghum (Zalucki et al., 1986; Fitt, 1989). Cotton bollworm has developed resistance to most of the available insecticides (Torres-Vila et al., 2002; Ramasubramanian & Regupathy, 2004). The protection of cotton against *H. armigera* is nowadays mainly based on the use of transgenic cotton varieties that express *Bacillus thuringiensis* (Bt) toxins (Liang et al., 1998; Liang et al., 2008). However, pesticides are also still used, e.g. on non-genetically modified varieties (Men et al., 2005). Resistance to Bt has already been demonstrated in field populations of *Plutella xylostella* (Huang, 2006) and glasshouse populations of *Trichoplusia ni* (Janmaat & Myers, 2005). There is, therefore, a continued need for sustainable control options. One such opportunity is offered by insect baculoviruses, even though they are also not immune to resistance development in pests (Asser-Kaiser et al., 2007). *H. armigera* nucleopolyhedrovirus (HaSNPV) has been developed as a commercial biopesticide to control *H. armigera* on cotton in China (Sun et al., 2004, 2009; Zhou et al., 2005) and on chickpea in India (Cherry et al., 2000).

Two HaSNPV isolates have been sequenced (Chen et al., 2001, HaSNPV-G4; Zhang et al., 2005, HaSNPV-C1). HaSNPV-G4 has been used as the basis for the generation of a recombinant virus into which the AaIT-(*Androctonus australis* Hector) insect-selective toxin gene (McCutchen et al., 1991) has been inserted to replace the viral *egt* gene (Chen et al., 2000). Sun et al. (2004) demonstrated superior performance of an AaIT-positive recombinant of HaSNPV, compared to the wild-type virus, in cotton crop protection in the field.

In this study we focus foremost on the behavioural effects of an *egt*-negative strain of HaSNPV (HaSNPV-LM2) in comparison with the wild-type virus, HaSNPV-G4, both in single infections with one virus strain and in mixed infections with both strains. Secondly, we included in one of the experiments an AaIT-positive HaSNPV, called HaSNPV-4A (Sun et al., 2004), with markedly shorter time to kill and expected behavioural consequences of the expressed toxin gene (Hernandez-Crespo et al., 2001). The inclusion of HaSNPV-4A is not only interesting in its own right, but it also provides a "positive" control, indicating whether the experimental approach and set up allows detection of behavioral effects, which – in the case of a gene deletion – might be subtle.

Studies were conducted with the L4 stage of *H. armigera*. Fourth instar larvae were chosen for this study because they cause more damage on the plant than younger larvae (Goulson et al., 1995). Earlier studies showed that *egt*-deletion variants of HaSNPV cause a faster death of infected L3 larvae of *H. armigera* (Georgievska et al., 2009) and showed that virus yield was decreased (Sun et al., 2005).

The preliminary questions that are addressed first are the following:

Does deletion of the *egt* gene modify survival time and virus yield in the fourth larval instar of *H. armigera* like it does for the third instar larvae (Sun et al., 2004)?

What is the survival time and virus yield upon challenge with a mixture of HaSNPV-G4 (wild-type) and HaSNPV-LM2 (*egt*-negative), compared to challenge with virus strains HaSNPV-G4 and HaSNPV-LM2 alone?

These preliminary questions were addressed in laboratory studies. We then conducted glasshouse and field studies to address the following questions:

Does infection with HaSNPV-G4 modify the behaviour of *H. armigera* and is a behavioral effect of virus infection altered when the virus is genetically modified by gene deletion (HaSNPV-LM2) or gene deletion and insertion (HaSNPV-4A)?

How do mixed wild-type recombinant virus infections influence the behaviour (movement and location) of the larvae, as compared to single viruses?

MATERIALS AND METHODS

Virus stocks

Wild-type HaSNPV was initially isolated from diseased *H. armigera* larvae collected in Hubei province in China in 1998. The genotype HaSNPV-G4 was isolated by in vivo cloning (Sun et al., 1998) and is called here HaSNPV-wt. The recombinant HaSNPV-LM2, lacking the *egt* gene (-*egt*), was generated by co-infection of Hz-AM1 cells in cell culture with HaSNPV-CXW2 DNA (-*egt*; +GFP) (Chen et al., 2000) and plasmid pHaLM2. This recombinant thus, lacks the ecdysteroid UDP-glucosyltransferase (*egt*) gene. A second recombinant HaSNPV-4A (-*egt*; + AaIT) has a deletion of the *egt* gene and at the same time contains a LacZ marker gene and a selective insect neurotoxin gene (AaIT) from the scorpion *Androctonus australis* Hector (McCutchen et al., 1991), which is expressed from a chimeric promoter derived from the p6.9 and polyhedrin genes of HaSNPV (Sun et al., 2004). HaSNPV-wt and HaSNPV-LM2 were amplified by injecting budded virus from the original infected cell culture supernatant into fourth instar *H. armigera* larvae and harvesting OBs upon death.

HaSNPV-4A was amplified by oral infection of third instar *H. armigera* larvae with a dose causing more than 99% mortality. OBs were isolated by grinding virus-killed cadavers in sterile water and filtering the homogenate through two layers of muslin. OBs were purified using centrifugation at 11,200 g for 60 min on a 30-60% continuous sucrose

gradient. OB concentration from the sediment was determined using the improved Neubauer counting chamber. Virus inoculum was prepared by serially diluting the OB stock solutions with sterile dH2O. The concentration was checked by three independent counts by phase contrast microscopy at × 400 magnification. OB stocks were maintained at 20°C until further use.

Insect culture

H. armigera used in Experiments 1-3 (see below) were from a colony maintained in the Laboratory of Virology, Wageningen, The Netherlands. Starting material for the culture was obtained from UPNA (Department of Entomology, Public University of Navarra, Pamplona, Spain). *H. armigera* larvae were reared individually in individual 1 ml plastic containers to prevent cannibalism. They were fed on bean diet (Zenda, BBA, Germany) and were kept at constant temperature of 27 ± 3°C, 70% R.H. and a L:D 14:10 h light regime. Pupae were sexed and transferred to 1.5 L plastic cylinders with vermiculite on the bottom and gauze on top. Thirty to forty male and female pupae were kept per cylinder. The inner walls were covered with paper for egg deposition by the adults after the pupae had eclosed and the adults mated. Eggs were surface-sterilized in 4% formaldehyde for 15 min, rinsed in tap water and air-dried. Larvae were reared on cotton leaves to adapt them to the plant food until used in the experiments, except for Experiment 3, for which the insects were reared on artificial bean diet. The insects used in Experiment 4 were obtained from China Cotton Research Institute (CCRI), Anyang, China. To prevent cannibalism from first instar larvae until pupation, larvae were reared individually in glass tubes. Insects were reared on cotton leaves in a controlled environment (27°C, 70% relative humidity (RH) and a 14:10 light-dark (LD) regime).

Experiment 1: Survival time and virus yield upon infection with HaSNPV-wt, HaSNPV-LM2 or a mixture of HaSNPV-wt and HaSNPV-LM2

The first experiment addressed the preliminary questions on time to death and virus yield in L4 larvae infected with the virus recombinant HaSNPV-LM2, or a mixture of this variant and the wild-type virus. Newly molted fourth instar larvae were starved for 16 h at 25ºC, and afterwards were inoculated with HaSNPV-wt, HaSNPV-LM2 or a 50:50 mixture of HaSNPV-wt and HaSNPV-LM2, using the droplet feeding method (Hughes & Wood, 1981). Larvae were challenged with a previously determined LD_{90} dose for this instar, 42,000 OBs/larva (Sun et al., 2004). Further, larvae were reared individually in plastic cups with artificial diet and checked for mortality at 8 h intervals.

Virus yield was measured by homogenizing individual cadavers in 1 ml of sterile water. The macerate was filtered through a plastic filter with a fine metal net (mesh size 120 to 200 μm, pore diameter 70 nm) and centrifuged at 6000 *g* for 5 min. Supernatant was removed and 500 μl of sterile water was added to disperse the OBs. The

concentration of the OBs/ml was determined with an improved Neubauer counting chamber using phase contrast microscopy.

Behavioural observations

Three experiments were conducted to determine the effects on larval behaviour of infection with wild-type HaSNPV, a recombinant virus, or mixtures between a recombinant and the wild-type virus. Two experiments (Experiments 2 and 3) were conducted in a glasshouse in Wageningen and one experiment was conducted in the field in China (Experiment 4). In all three experiments early L4 larvae were inoculated and released on experimental plants, after which their position was recorded three times per day until the 5th molt or virus-induced death.

Distance travelled by each larva was measured by marking its position with a small sticker placed either on the plant (leaf or steam) or on the soil at each observation time point. Larvae were observed and positions marked daily at 8:00, 14:00 and 20:00 h, until infected insects died. The distance travelled between two time points was measured using a ruler (in cm) and total distance travelled calculated as the sum of all observations for each larva.

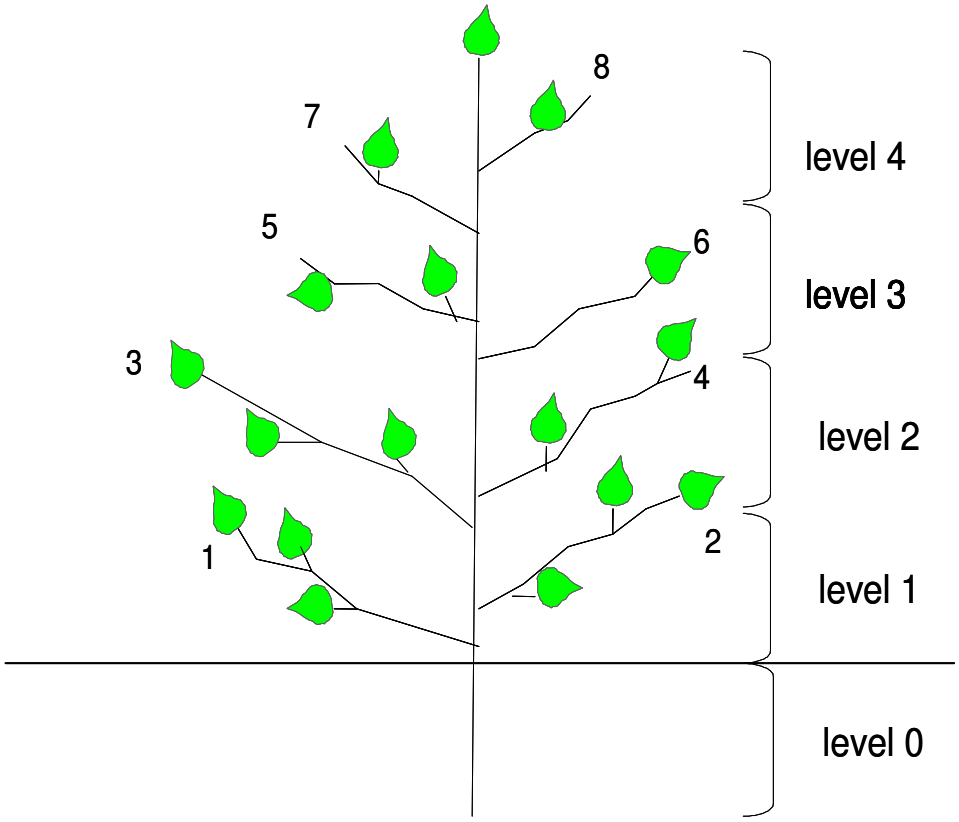

Figure 1. Diagram of a cotton plant, showing the coding system for recording the position of the larvae level 0 (ground); level 1 (branch 1 and 2); level 2 (branch 3 and 4); level 3 (branch 5 and 6) level 4 (branch 7 and 8) level 5 (branch 9 and 10) etc.

The position of each larva on the plant was also recorded. Data recording was based on the separation of plant branches at different levels (Fig. 1). All leaves belonging to the first and second branch of the cotton plant were classified as level 1 and so on, moving up the plant. Insects found on the ground were recorded as level 0 (see Fig. 1 for cotton plant architecture). Larvae were initially placed on cotton plants at level 3 or 4 (middle section of the plant).

Experiment 2: Behaviour of *H. armigera* upon challenge with wild-type HaSNPV, HaSNPV-LM2, or a virus genotype mixture on cotton plants in a glasshouse

Cotton plants (var. Zhongmiansuo #35) were sown on 3 January 2004 and grown in a glasshouse (UNIFARM, Wageningen University, The Netherlands) at a constant temperature of 28 ± 3°C, L:D 14: 10 h photoperiod and a relative humidity of 60-70%.

Fourth instar *H. armigera* larvae were infected individually by feeding them a 5 mm diameter cotton–leaf disc, coated with 200,000 OBs ($5 \times LD_{99}$ for this instar of *H. armigera*) (Sun et al., 2004). Larvae were assigned to one of four treatments: (1) uninfected (control), (2) HaSNPV-wt, (3) HaSNPV-LM2 and (4) a 1:1 mixture (HaSNPV-mix) of HaSNPV-wt and HaSNPV-LM2 (each 100,000 OBs/disk). Larvae were kept individually for 24 h in 24-well plates at 27°C until release. Larvae that had completely consumed the leaf discs were released on the middle section of the plant on 23 April 2004, when the plants were 16 weeks old and in the boll formation stage. One larva was released on each plant and the plants were enclosed individually in nylon mesh nets to prevent larvae from escaping and avoid cross contamination of viruses. Each treatment was replicated 10 times.

Experiment 3: Behaviour of *H. armigera* on cotton plants in a glasshouse upon challenge with wild-type HaSNPV, HaSNPV-LM2, HaSNPV-4A or with mixtures of wild-type and recombinant

Cotton plants (var. Zhongmiansuo #35) were sown on 13 August 2007 and grown in a glasshouse (UNIFARM, Wageningen University, The Netherlands) at a constant temperature of 28 ± 3°C, L:D 14:10 photoperiod and a relative humidity of 60-70%. At the time of the experiment the plants were 8 weeks old (Fig. 2 A and B).

In this experiment, larvae were assigned to one of six treatments: (1) uninfected (control), (2) HaSNPV-wt; (3) HaSNPV-LM2, (4) HaSNPV-4A, (5) a 1:1 mixture of HaSNPV-wt and HaSNPV-LM2, and (6) a 1:1 mixture of HaSNPV-wt and HaSNPV-4A.

The fourth instars used in the second glasshouse experiment were infected using the droplet feeding method. Newly molted fourth instar *H. armigera* were individually separated to prevent cannibalism and were starved for about 16 h at 24 ºC. The virus solution consisted of an aqueous suspension of 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue (food dye) and a dose of approximately 42 000 OBs/larvae, based on

ingested volume (Sun et al. 2004). Larvae showing blue coloration in the foregut after 10 min were transferred to 24-well plates containing cubes of artificial diet and kept at a constant temperature of 27°C. After one day the larvae were released on the cotton plants, on 13 October 2007. The plants were approximately 50-60 cm high, in the vegetative stage, and they had 10-15 full grown leaves (Figure 2).

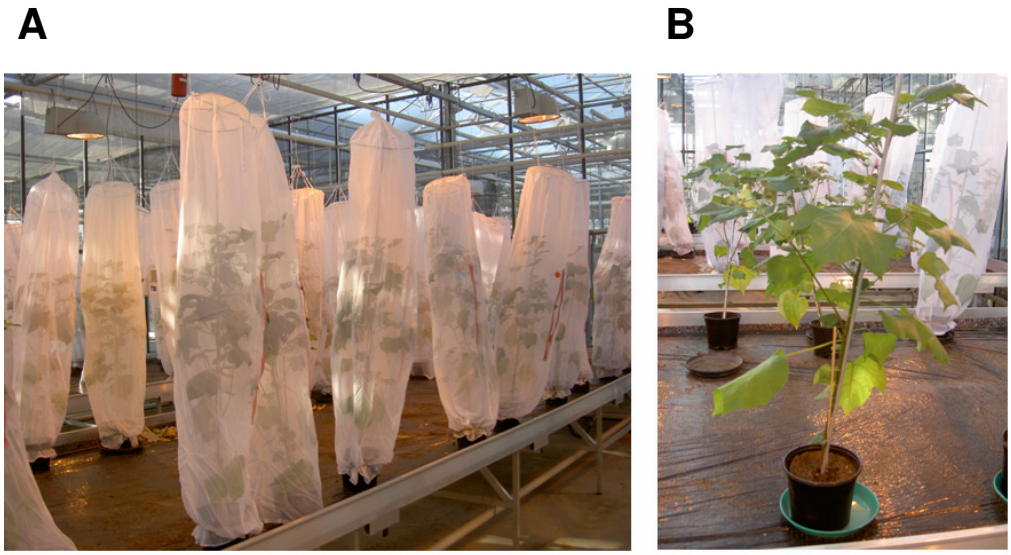

Figure 2. For Experiment 3 cotton plants in a glasshouse were netted individually to prevent larvae from escaping. (A) Each plant represents one replicate. (B) Cotton plant in the flowering stage.

Experiment 4: Behaviour of *H. armigera* on cotton plants in field cages upon challenge with wild-type HaSNPV, HaSNPV-LM2, or a virus genotype mixture

A field experiment was carried out at the China Cotton Research Institute (CCRI) Anyang, Henan Province. Cotton plants (var. Zhongmiansuo #35) were sown on 15 April 2005. Groups of six plants were grown in 2 × 2 × 2 m field cages with sides of fine mesh nylon netting and a plastic sheet roof top (Fig. 3 A and B). The sides of the cages were buried 10 cm into the soil to prevent larvae from escaping.

H. armigera used in the field experiment were obtained from the insect culture reared at CCRI (China Cotton Research Institute, Anyang, China). When plants were in the fruiting and flowering stage on 5 September 2005, one fourth instar larva was released on each plant in each cage. Larvae were assigned to one of four treatments: (1) uninfected (control); (2) HaSNPV-wt; (3) HaSNPV-LM2; and (4) a 1:1 mixture of HaSNPV-wt and HaSNPV-LM2.

The method of infection and the viral dose were the same as in Experiment 2. There were 4 cages, each with six plants, and each plant per cage represented a separate replicate. The position of the larvae on each plant in each cage was recorded three times daily, at 7:00, 13:00 and 19:00 h, for seven days post release.

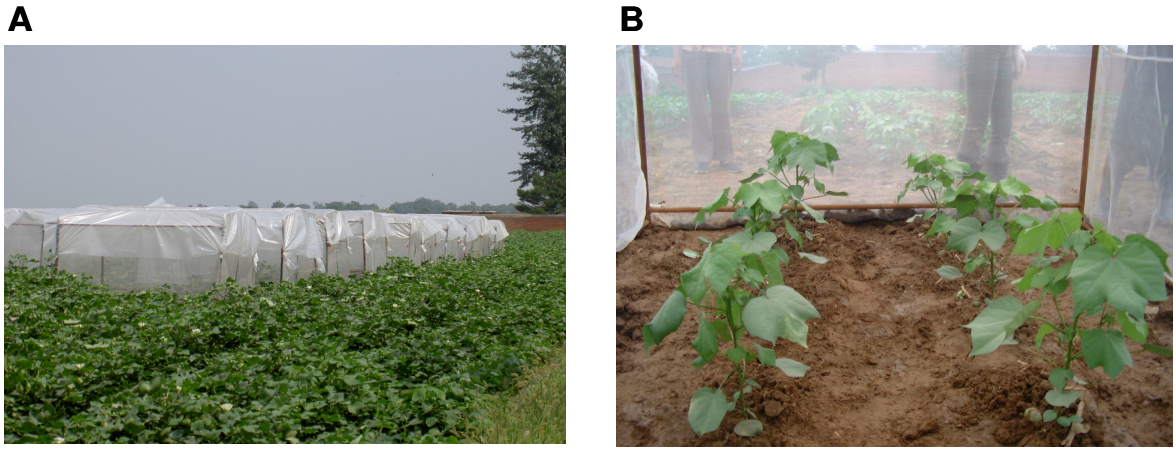

Figure 3. (A) Field cages used in Experiment 4 in Anyang, China. The experiment consisted of four cages and each cage contained one of the virus treatments. (B) Per cage there were six cotton plants, each infested with a single larva. Each plant served as a replicate.

Statistical analysis

The survival time (ST_{50}) values of fourth instar larvae were determined by survival analysis, using the Kaplan-Meier Product Limit Estimator in JMP SAS (2008). Virus yield was analysed, using virus treatment as a fixed factor, by ANOVA in the General Linear Model Procedure in SPSS (SPSS Inc., 2003). Daily distances (cm) travelled by larvae were square root transformed to normalize residuals. Differences in distance moved among treatments were analysed by univariate repeated–measures analysis of variance (ANOVA) using "virus type" as a "between-subject factor" and "time" (day) as a "within-subject (repeated) factor". Tests of sphericity and normality were performed to verify the assumptions of ANOVA (SPSS Inc., 2003). The daily position of larvae was compared among treatments using Kruskal-Wallis parameter free ANOVA and Mann-Whitney U-test for pairwise comparisons (SPSS Inc., 2003). In experiments each cotton plant was treated as a replicate.

RESULTS

Experiment 1: Survival time and virus yield upon infection with HaSNPV-wt, HaSNPV-LM2 or a mixture of HaSNPV-wt and HaSNPV-LM2

Survival time (ST_{50}) of fourth instar *H. armigera* differed significantly among virus treatments, according to the log rank test in the survival analysis (χ 2 = 74.95, df = 3, P < 0.0001) and all three pairwise comparisons between treatments were significantly different (Kaplan-Meier Product Limit Estimator, Table 1). The ST_{50} was 157 h in L4s infected with HaSNPV-wt; 133 h in larvae infected with the HaSNPV-LM2 and 109 h in larvae challenged with the 1:1 mixture of wild-type HaSNPV and HaSNPV-LM2, which was significantly shorter than wild-type-infected ones (Fig. 4A).

There were significant differences in virus yield among treatments ANOVA (F = 6.43, df = 2, 37, P = 0.004). The larvae infected with the recombinant virus produced significantly lower yields than the wild-type infected insects (t-test: t = 3.35, df = 28, P = 0.002). However, there were no significant differences in virus yield between the wild-type and the mixed infected larvae (t = 1.87, df = 23, P = 0.073) or between recombinant and mixed infected larvae (t = 1.61, df = 23, P = 0.12) (Fig. 4B).

Table 1. Survival time of fourth instar *Helicoverpa armigera* following *per os* inoculation with different preparations of HaSNPV, including HaSNPV-wt, recombinant HaSNPV-LM2 (-*egt*) and genotype mixtures of wild type and the recombinant, in comparison with a wild type isolate.

	Survival time (ST_{50})	**95% Fiducial limits**	
		CI low	**CI high**
HaSNPV-wt	157 a	157	171
HaSNPV-mix	109 b	109	123
HaSNPV-LM2	133 c	133	140

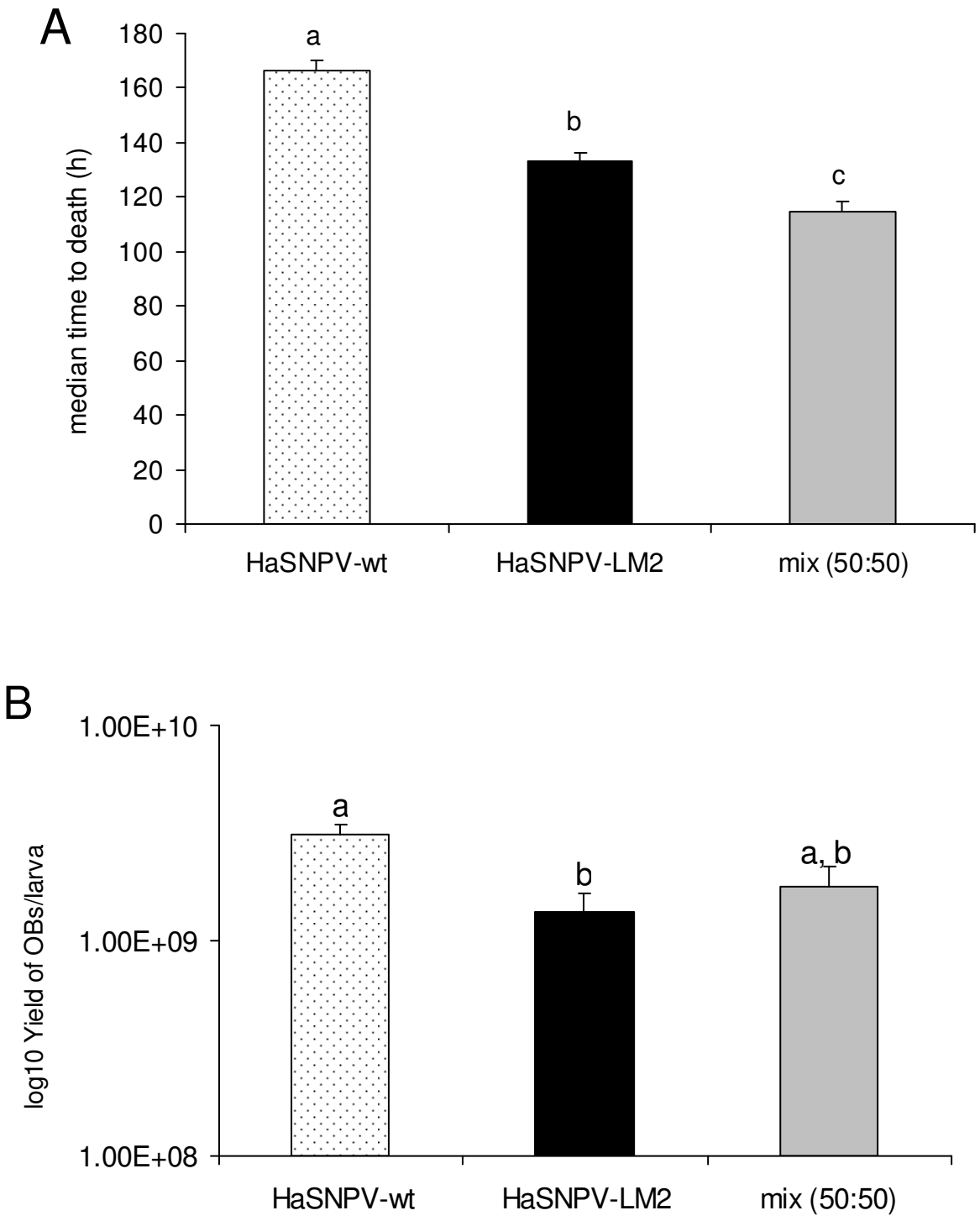

Figure 4. (A) Median survival time of fourth instar *H. armigera* infected with wild type HaSNPV, recombinant HaSNPV-LM2 (*-egt*) and a mixture of the two viruses (mix 50:50). All three viruses are shown with standard error bars. (B) Log yield per larva for the different virus treatments with a different letter over the bar indicates a significant difference at $P < 0.05$ level.

3.2 Experiment 2: Behaviour of *H. armigera* on cotton plants in a glasshouse upon challenge with wild-type HaSNPV, HaSNPV-LM2 or a virus genotype mixture

Total distance travelled by the control insects (mean ± SE) was 416 ± 43 cm, in contrast to the movement of the wild-type (308 ± 47 cm), HaSNPV-LM2 (185 ± 55 cm) and mixed infected (304 ± 65 cm) larvae (Fig. 5A). There was a significant difference in distance travelled among virus treatments ($F = 5.0$, $df = 3, 27$, $P = 0.007$; Figs. 7, 8). Larvae infected with the recombinant virus HaSNPV-LM2 moved significantly less than control larvae ($t = 2.15$, $df = 15$, $P = 0.048$), or wild-type-infected larvae ($t = 2.91$, $df = 15$, $P = 0.011$). There was also a significant time effect on daily distance travelled (RM ANOVA, time, $F = 5.0$; $df = 3, 81$, $P = 0.003$), but the interaction between time and treatment was not significant ($F = 0.9$, $df = 9, 81$, $P = 0.476$) (Table 2). Fig. 7 provides a comparison of movement within treatment for each day following inoculation, where Fig. 8 represents comparison of movement among treatments for each day. On day 2 after infection, HaSNPV-wt infected larvae moved more than larvae infected with HaSNPV-LM2 ($t = 3.71$, $df = 15$, $P < 0.05$) (Fig. 8).

Table 2. Repeated-measures ANOVA of the effects of three HaSNPV variants: HaSNPV-wt, HaSNPV-LM2 and HaSNPV-mix on distance traveled of 4th instar *H. armigera* in three experiments.

Source of variation	MS	F	df	P
Experiment 2 (glasshouse)				
Within subject effects				
time	74.1	5.0	3	0.003
time × treatment	14.2	0.9	9	0.476
Between subject effects				
treatment	63.6	5.0	3	0.007
Experiment 3 (glasshouse)				
Within subject effects				
time	20.0	4.05	3	0.017
time × treatment	4.8	0.98	15	0.499
Between subject effects				
treatment	12	3.52	5	0.048
Experiment 4 (field)				
Within subject effects				
time	82.6	7.57	4	0.001
time × treatment	14.9	1.3	12	0.176
Between subject effects				
treatment	44.3	1.3	3	0.27

There were no significant differences in movement among virus treatments on any of the other days. There was also no significant difference between the positions of the larvae on the plant among virus treatments (Fig. 6A, Table 3).

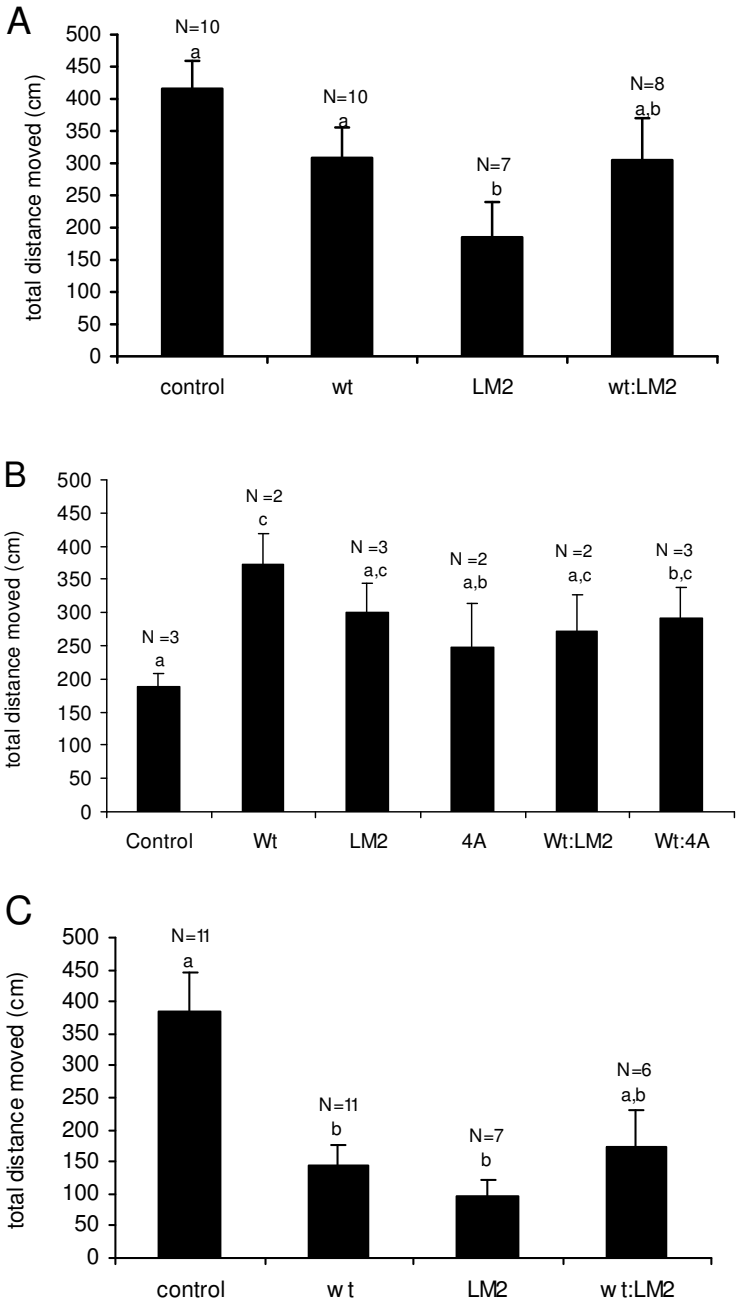

Figure 5. Total distance travelled (cm) by larvae by virus treatments in two glasshouse experiments (A: Exp. 2; B: Exp. 3) and one field experiment (C: Exp. 4). The number of larvae (N) in each treatment is indicated over the bars. Bars represent the total movement distance in four days ± standard error. Movement distances per day were square root transformed before statistical analysis with repeated measures ANOVA, the results of which are indicated with lettering above the bars. Significantly different means are indicated by a different letter over the bar ($P < 0.05$).

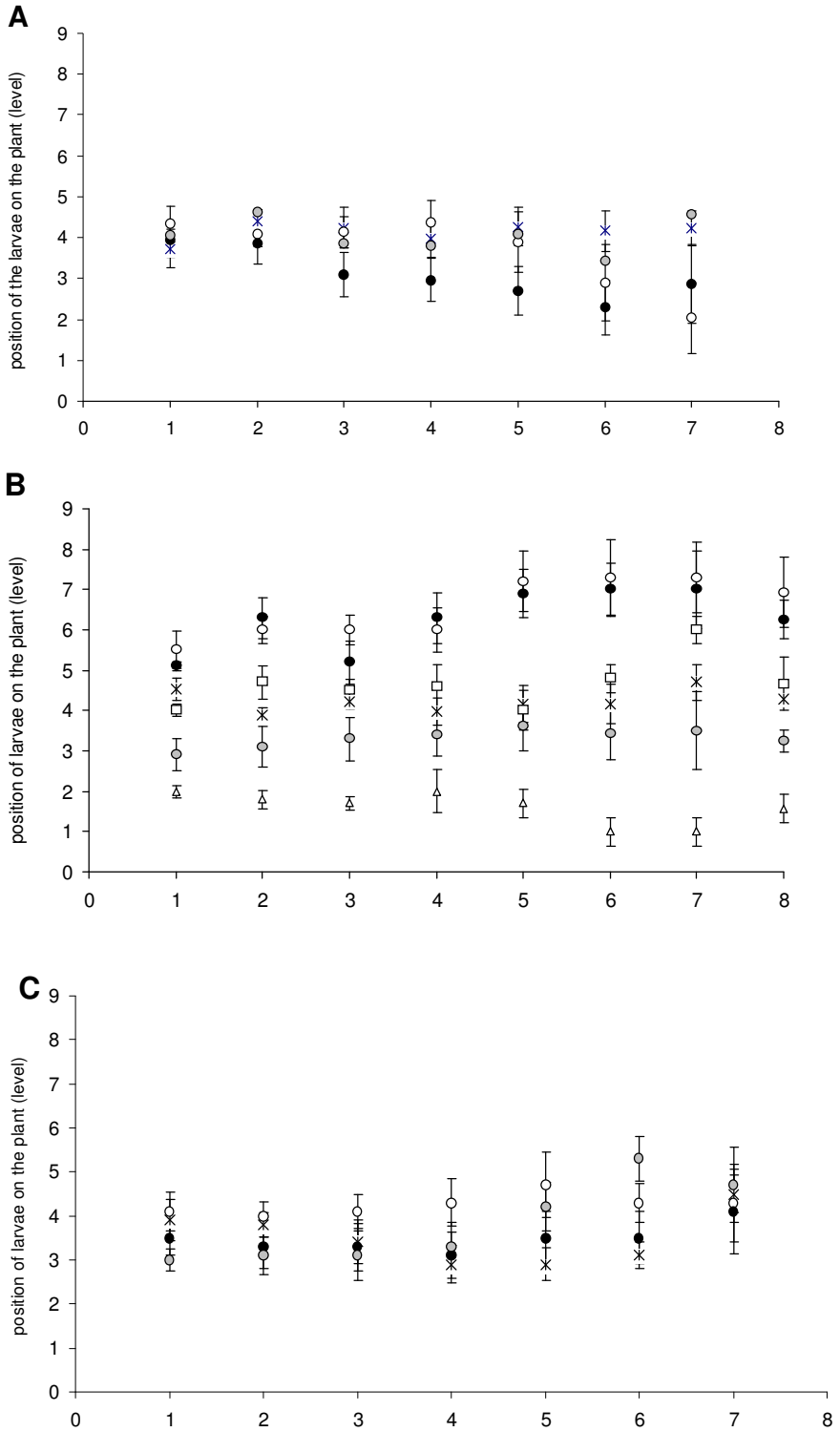

Figure 6. Daily position of *Helicoverpa armigera* larvae on cotton plants in different virus treatments in 3 experiments: (A) Experiment 2 and (B) Experiment 3 (two glasshouse experiments) and (C) Experiment 4 (the field experiment). Error bars indicate standard error of the mean. Asterisk =control; open circle = HaSNPV-wt; black circle = HaSNPV-LM2; grey circle = HaSNPV-wt: HaSNPV-LM2; triangles = HaSNPV-4A and squares = HaSNPV-wt: HaSNPV-4A

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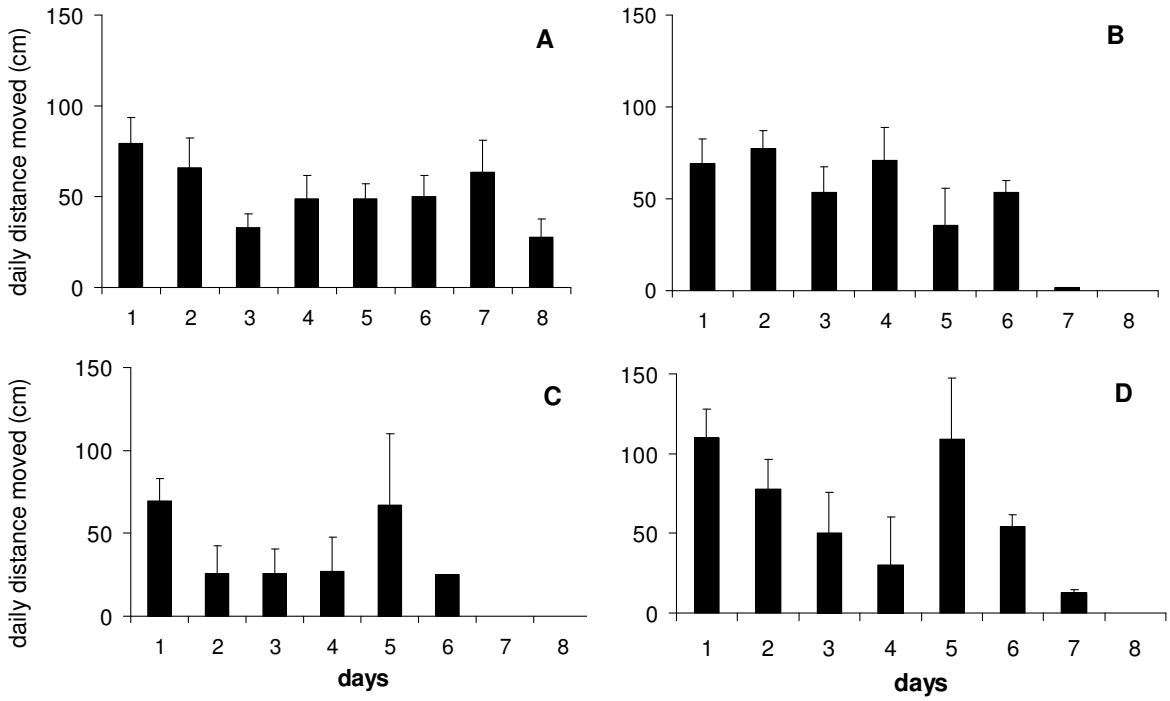

Figure 7. Mean distance travelled (cm) per day by fourth instar *Helicoverpa armigera* on cotton plants in a glasshouse (Exp. 2). There were four virus treatments: (A) control; (B) HaSNPV-wt; (C) HaSNPV-LM2 (-*egt*) and (D) 1:1 mixture of HaSNPV-wt and HaSNPV-LM2. Error bars indicate standard error of the mean.

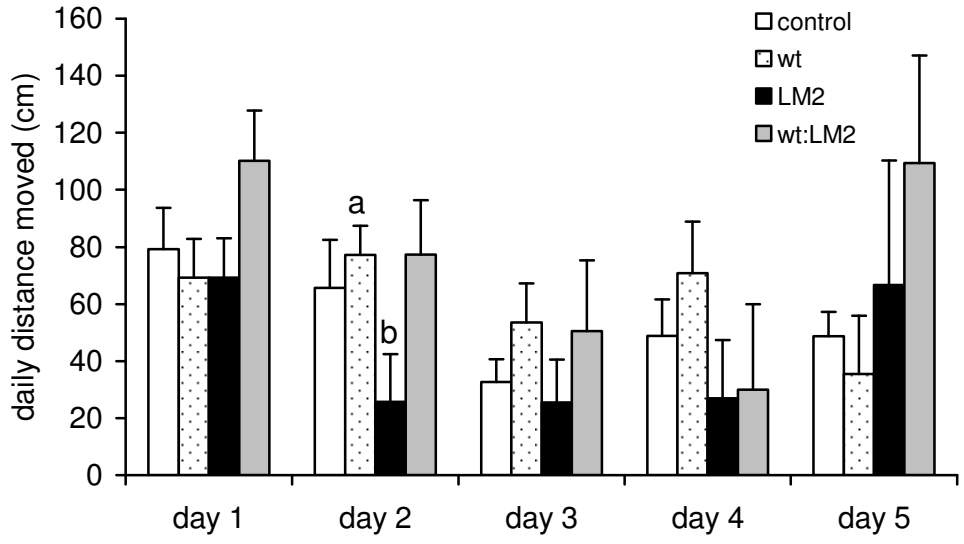

Figure 8. Mean distance travelled (cm) per day by fourth instar *Helicoverpa armigera* on cotton plants in a glasshouse (Exp. 2). There were four virus treatments: control (white); HaSNPV-wt (dotted); HaSNPV-LM2 (-*egt*) (black) and 1:1 mixture of HaSNPV-wt and HaSNPV-LM2 (grey). Error bars indicate standard errors of the mean. Different letters over the bar on the same day indicate a significant difference between treatments at $P < 0.05$.

Experiment 3: Behaviour of *H. armigera* on cotton plants in a glasshouse upon challenge with wild-type HaSNPV, HaSNPV-LM2, HaSNPV-4A or with mixtures of wild-type and recombinant virus

Total distance moved by wild-type HaSNPV-infected larvae on cotton plants was significantly greater than that of control larvae (189 ± 20.3 cm) and that of larvae challenged with HaSNPV-4A (248.5 ± 65.5 cm) (Fig. 5B). Larvae infected with the mixture of HaSNPV-wt and HaSNPV-4A (292 ± 44.7 cm) travelled more compared with the control larvae (189 ± 20.3 cm) (Fig. 5B). There were no other pairwise significant differences.

There was a significant effect of time on daily distance travelled, (RM ANOVA, time, $F = 4.05$, $df = 3, 27$, $P = 0.017$) while the interaction between the time and treatment was not significant ($F = 0.98$, $df = 15, 27$, $P = 0.499$) (Table 2; Fig. 9). There was a significant effect of virus treatment on distance moved ($F = 3.52$, $df = 5, 9$, $P = 0.048$).

Differences between treatments were first seen on the second day post infection when larvae infected with HaSNPV-wt moved more than those infected with HaSNPV-LM2 ($t = 2.47$; $df = 13$; $P = 0.028$). At the third day post infection, larvae infected with HaSNPV-LM2 moved more than insects with the mixture of HaSNPV-wt and HaSNPV-4A ($t = 2.81$; $df = 8$; $P = 0.023$) (Fig. 10). No further pairwise comparisons within days were significant.

As expected many larvae infected with the recombinant virus HaSNPV-4A (*egt*-deletion, AaIT-positive) dropped from the plants around 5 days post infection. A few cadavers were found on the lower parts of the plant (level 1.5), whereas cadavers infected with HaSNPV-wt were found much higher up the plant (level 6.3).

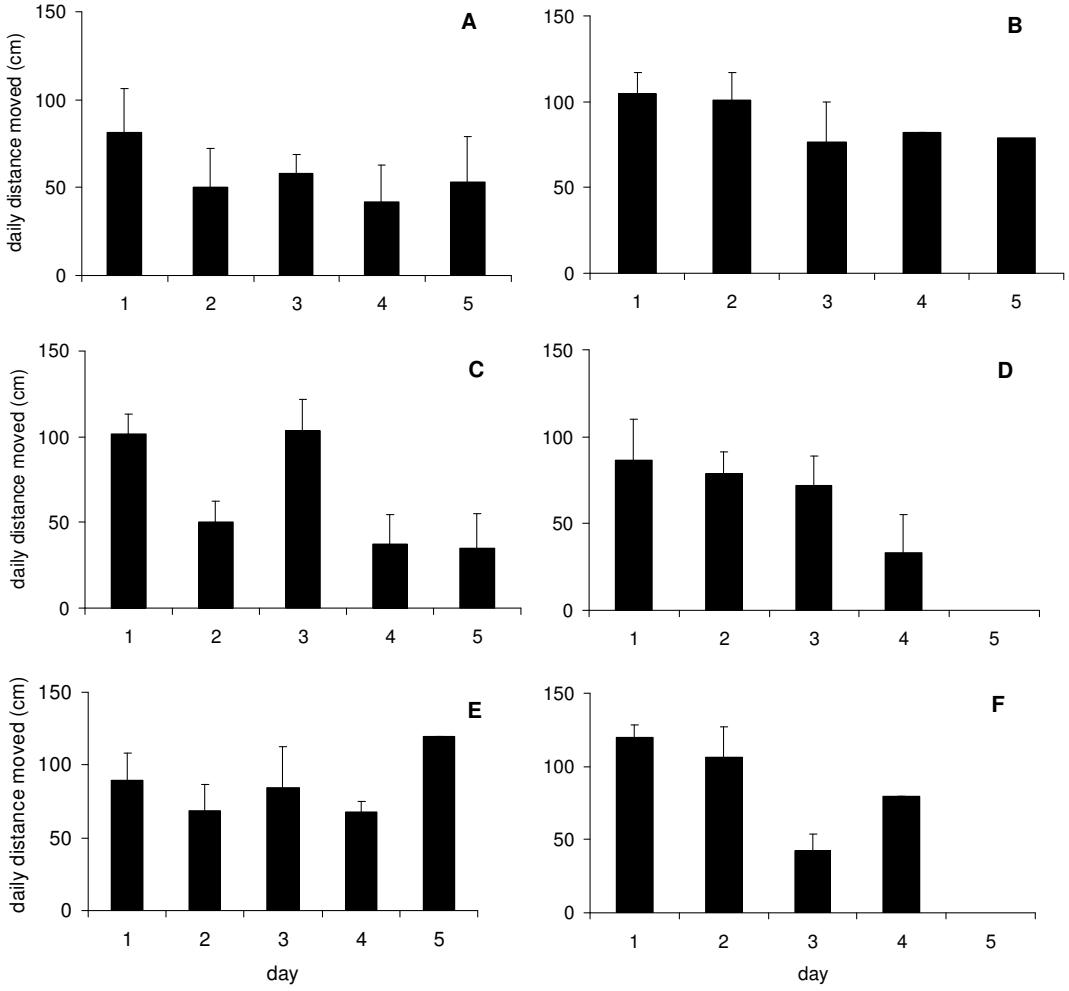

Figure 9. Mean distance travelled (cm) per day by fourth instar *Helicoverpa armigera* on cotton plants in a glasshouse (Exp.3). There were six virus treatments: (A) control; (B) HaSNPV-wt; (C) HaSNPV-LM2 (-*egt*); (D) HaSNPV-4A (-*egt*; +AaIT); (E) 1:1 mixture of HaSNPV-wt and HaSNPV- LM2 (F) and a 1:1 mixture of HaSNPV-wt and HaSNPV-4A. Error bars indicate standard errors of the mean.

Table 3. Final position of dead larvae on cotton plants

	Glasshouse		Field exp.
	Exp. 2	**Exp. 3**	**Exp. 4**
HaSNPV-wt	4.1 ± 0.87 a	6.3 ± 0.3 a	4.9 ± 0.3 a
HaSNPV-LM2	3.4 ± 0.84 a	6.2 ± 0.7 a	3.3 ± 0.8 a
Mix Wt:LM2	4.7 ± 0.88 a	4.6 ± 1.0 a, b	3.6 ± 0.6 a
HaSNPV-4A		2.1 ± 0.8 b	
Mix Wt+4A		3.3 ± 0.0 b	

There was a significant difference in the position of cadavers infected with HaSNPV-wt compared with those infected with HaSNPV- 4A ($U = 2.5$, $z = -1.94$, $P = 0.052$) and cadavers infected with a mixture of HaSNPV-wt and HaSNPV-LM2 ($U = 1.0$, $z = -1.95$, $P = 0.052$). Dead larvae infected with recombinant HaSNPV-4A containing the AaIT insect toxin gene were found at lower positions on the plant compared with HaSNPV-LM2 ($U = 2.5$, $z = -2.2$, $P < 0.05$). Dead larvae inoculated with a mixture of HaSNPV-wt and HaSNPV-LM2 were found on positions significantly lower than the singly infected HaSNPV-LM2 ($U = 1.0$, $z = -2.1$, $P < 0.05$) (Table 3).

During the course of the experiment HaSNPV-4A infected larvae were found mostly on the lower part of the plant or on the soil (Fig. 6B).

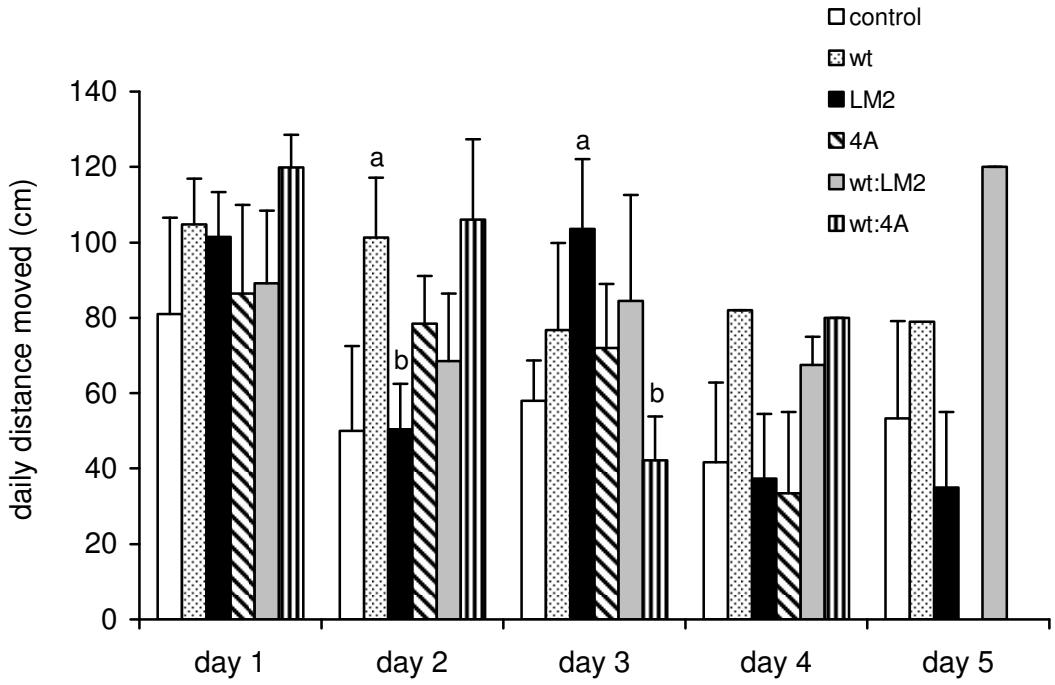

Figure 10. Mean distance travelled (cm) per day by fourth instar *Helicoverpa armigera* on cotton plants in a glasshouse (Exp. 3). There were six virus treatments: control (white); HaSNPV-wt (dotted); HaSNPV-LM2 (*-egt*) (black); HaSNPV-4A (*-egt*; +AaIT) (diagonally cross-hatched); 1:1 mixture from HaSNPV-wt and HaSNPV-LM2 (grey) and a 1:1 mixture from HaSNPV-wt and HaSNPV-4A (vertically striped). Error bars indicate standard errors of the mean. Different letters over the bars within the same day indicate significant differences between treatments at $P < 0.05$.

Experiment 4: Behaviour of *H. armigera* on cotton plants in field cages upon challenge with wild-type HaSNPV, HaSNPV-LM2 or a virus genotype mixture

The total distance moved by the control larvae (384 ± 62 cm) was greater than the distance covered by larvae infected with HaSNPV-wt (146 ± 30 cm) or HaSNPV-LM2 (95 ± 28 cm). The insects with mixed infections of wild-type and HaSNPV-LM2 covered a total distance of 173 ± 58 cm (Fig. 5C). Fig. 11 shows comparisons of mean distance travelled of larvae within treatments, and Fig. 12 of larval movement among all four treatments in the experiment.

There was a significant difference between times ($F = 7.57$, df = 4, 104, $P = 0.001$). There was no significant interaction between time and the treatment ($F = 1.3$, df = 12, 104, $P = 0.176$) and there were not significant differences among virus treatments in distance traveled by larvae ($F = 1.3$, df = 3, 26, $P = 0.27$; Table 2). There were significant differences in the position of the larvae on the plant (Fig. 6C, Table 3).

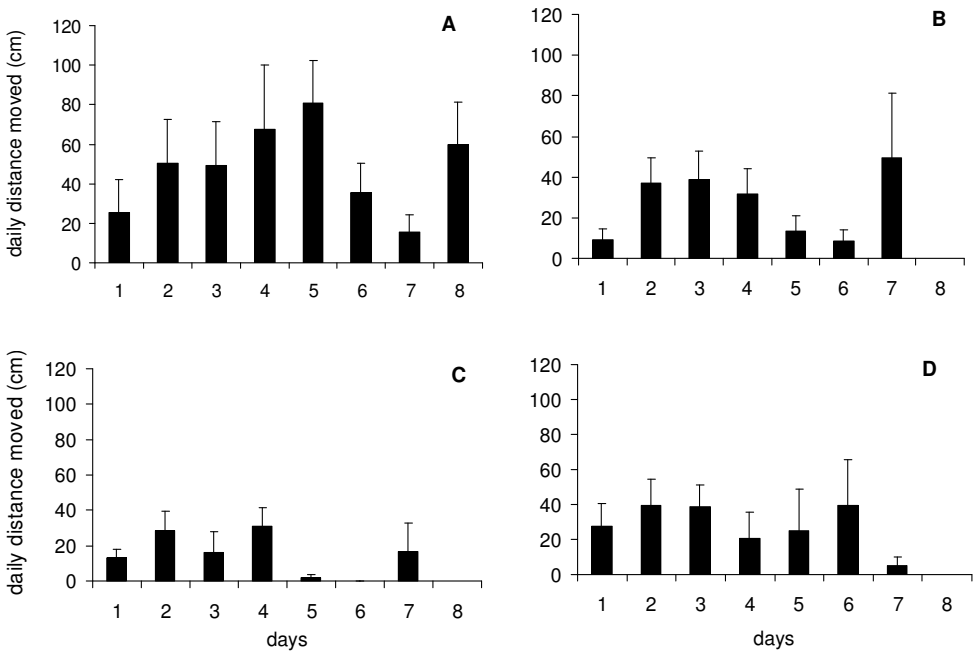

Figure 11. Mean distance travelled (cm) per day by fourth instar *Helicoverpa armigera* on cotton plants in the field (Exp. 4). There were six virus treatments: (A) control; (B) HaSNPV-wt; (C) HaSNPV-LM2 (-*egt*); (D) 1:1 mixture of HaSNPV-wt and HaSNPV-LM2 (-*egt*; +AaIT). Error bars indicate standard error of the mean.

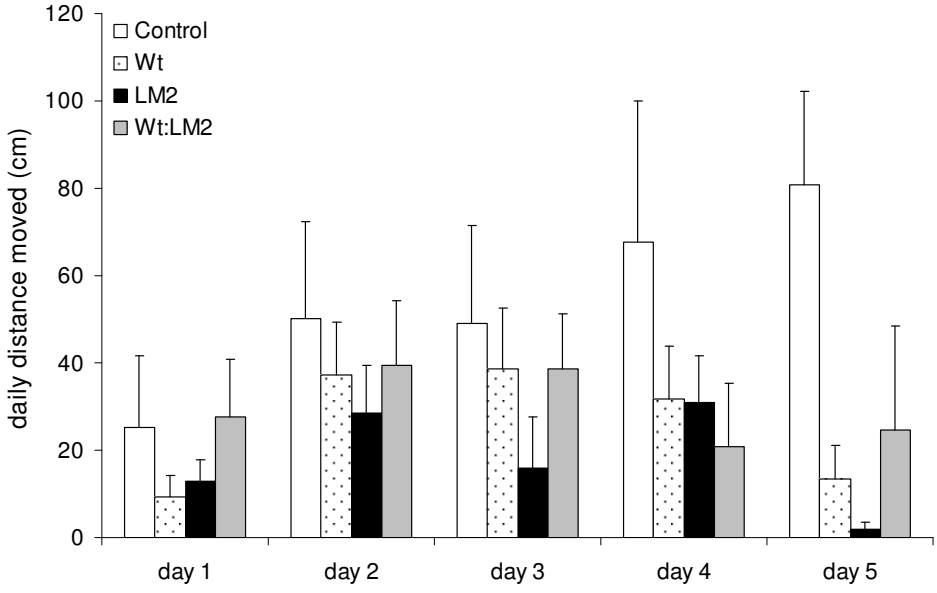

Figure 12. Mean distance in (cm) traveled by fourth instar Helicoverpa armigera on cotton plants in the field. Observations were done each 8 h in a period of 5 days. (Exp. 4). There were four virus treatments, as in Experiment 2: control (white); HaSNPV-wt (dotted); HaSNPV-LM2 (-*egt*) (black), and a 50:50 mixture of HaSNPV-wt and HaSNPV-LM2 (grey). Error bars indicate standard error of the mean.

DISCUSSION

We found ambiguous effects of virus infection on the behaviour of 4th instars of *H. armigera.* In one glasshouse study virus infected larvae moved more than healthy larvae, indicating a restlessness induced by virosis. However, in the other glasshouse experiment and the field study, the movement of infected larvae was less than that of healthy larvae, which was not surprising since once infected larvae are symptomatic they are often subsequently less mobile (Vasconcelos et al., 2006). Behaviour of larvae infected with the *egt*-negative variant HaSNPV-LM2 was mostly indistinguishable from that of larvae infected with the wild-type HaSNPV, whereas the toxin-positive recombinant HaSNPV-4A caused significantly reduced movement and a change in location of the cadavers, compared with larvae infected with the wild-type virus. The effect of a virus mixture of wild-type and HaSNPV-4A was intermediate between that of the pure infections, while larvae infected with the mixture of the HaSNPV-wt and HaSNPV-LM2 assumed a lower position on plants at death compared with those from single infections of these two viruses.

Differences in results from glasshouse and field experiments may be due to many factors, including differences in climatic conditions, phenological stage of the plant or influences of cages and nets. Temperatures in the field were extremely high (40ºC) during the day and cold (15ºC) during the night, while in the glasshouse temperature fluctuations between night and day were much more moderate.

The influence of mixed pathogen infections on host behaviour has received little attention. Consistent with our findings for HaSNPV-4A, Cory et al. (1994), Hoover et al. (1995) and McCutchen & Hammock (1995), reported that *Trichoplusia ni* and *H. virescens* larvae, respectively, infected with AcMNPV-AaIT, fell off the plants. In agreement with the data for *M. brassicae*, infected larvae with HaSNPV-G4 moved upwards (Vasconcelos et al., 1996) (Fig.6A, B, C).

Most insects infected singly with HaSNPV-4A (-*egt*; +AaIT) fell off the plants. This was expected as a result of expression of the AaIT gene, which causes tetanic paralysis and subsequently death. This knockdown induced behaviour from the recombinant virus reduces horizontal transmission (Hails et al., 2002; Zhou et al., 2005), but on the other hand it also might enhance deposition of virus in the soil and thus enhance virus persistence in agro-ecosystems. This might be beneficial in terms of longer term control by a biological control agent, but may be considered a negative attribute with respect to biosafety of using baculovirus recombinants.

The mobility of virus-infected larvae in the field and the position on host plants where they die, can affect the spread of the virus on the plant. Such behavioural effects could be altered when recombinant baculoviruses are introduced. Some preliminary conclusions can be drawn based on the movement of virus-infected larvae and where

larvae died on the plant. The HaSNPV-4A (-*egt,* AaIT) larvae showed the characteristic symptoms of paralysis before death and exhibited a different behaviour from HaSNPV-wt infected larvae, which may influence survival of the recombinant virus for the next generation of larvae (Table 3). By inducing larvae to fall off the plant, the 4A recombinant might result in reduced transmission of the virus to the next generation since survival time of these larvae will also be shortened, compared with larvae infected with wild-type virus. Also reduced transmission is expected as a result of reduced yields of OBs from larvae infected with 4A (Sun et al., 2005). This may be a useful property in the case of a multivoltine pest such as *H. armigera*. However, final position of insects infected with a mixture of HaSNPV-wt + HaSNPV-4A tends to be not different from the single virus infections.

In the introduction, we note that the effects of baculoviruses on the behaviour of insect hosts are system specific. Our experiments indicate that even within one system, cotton – *H. armigera* – HaSNPV, effects on behaviour vary from one experiment to another. Under field conditions (Experiment 4), virus-infected larvae moved less than healthy insects. In one of the glasshouse experiments (Experiment 3), virus infected larvae moved more than healthy larvae, but in another glasshouse experiment (Experiment 2) the virus infected larvae moved less. Clearest effects on behaviour were obtained with a toxin expressing recombinant, HaSNPV-4A, which caused larvae to take a low position on the plant. All in all, the behavioral effects of virus infection in these trials were modest, and varying from one experiment to another, indicating that virus transmission is influenced by the interplay between virus genotype and environmental conditions.

Transmission of HaSNPV might be impaired as the larvae are spatially separated from healthy larvae on the plant. Therefore, based on these findings, we predict that transmission of HaSNPV variants with an *egt* deletion is similar to that of the wild type, while that of variants with a toxin gene might be reduced. Based on the position of the diseased larvae on the plant, we predict that transmission of toxin variants might be enhanced when larvae are co-infected with the wild type virus, as these larvae were not so strongly separated from healthy larvae on the host plant. However, the transmission of such a toxin variant depends also on the competitive interaction between wild type and genetically modified virus within the host insect, and result in Chapter 5 and 6, and of Zwart et al. (in press) indicate that the competitive fitness of egt deletion variants within insect hosts is reduced.

The overall ecological fitness of genetically modified baculoviruses depends on the interaction between within host and between host competitive processes, and remains difficult to predict. Models can help to assess these interactions (Bianchi et al., 2002; Bonsall et al., 2005; Sun et al., 2006). A critical parameter that may be needed in models for competitive fitness of baculoviruses genotypes at agro-ecosystem level is the

transmission of virus genotype from mixed infected hosts. Almost no information on this is available, and hence, modelling studies remain explorative rather than predictive tools for the moment. In models at agro-ecosystem level, the possibility of spatial separation between infected hosts and healthy hosts is a factor to take into account.

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Chapter 4

TRANSMISSION AND PERSISTENCE OF WILD-TYPE AND RECOMBINANT HaSNPV BACULOVIRUS BY LARVAE OF THE COTTON BOLLWORM, *HELICOVERPA ARMIGERA*, ON COTTON

SUMMARY

Horizontal transmission of insect viruses is a key factor in their cycling in agro-ecosystems. Here we study the transmission of the baculovirus HaSNPV among larvae of the cotton bollworm in the cotton agro-ecosystem. Transmission of three HaSNPV genotypes was studied, from larvae infected with a single virus genotype as well as from larvae infected with two different genotypes. Genotypes included a wild-type virus, an *egt* deletion mutant with slightly enhanced speed of kill, and a neurotoxin-expressing genotype with a substantially increased speed of kill.

In three field experiments, no significant differences in virus transmission between treatments were demonstrated; i.e. the wild type and *egt* deletion virus variants and a mixture of the two had similar rates of transmission. Transmission increased with density of infector insects and decreased with time lapsed since the inoculation of the infector larvae. Side observations suggest that virus persistence of these HaSNPVs in soil is affected by solar radiation. Transmission of the neurotoxin expressing virus was lower than that of the other two genotypes in the glasshouse.

The studied genotypes of HaSNPV have significant differences in time to kill and virus yield. We found no significant consequences of these differences for rates of virus transmission at the crop level when HaSNPV-LM2 (-*egt*) was used. However, HaSNPV-4A (-*egt*; *AaIT*) had significantly reduced transmission. Based on these findings, we hypothesize that differences between virus genotypes (wild type and HaSNPV-LM2) expressed at the "within host" level may be more important determinants of the outcome of competition between viruses in the cotton agro-ecosystem than differences that are expressed at crop level.

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INTRODUCTION

Pathogens play an important role in the population dynamics of herbivorous insects and they sometimes cause a dramatic reduction in pest populations (Doane, 1970; Begon et al. 1996). Baculoviruses have been used worldwide against many insect pests, mainly Lepidoptera (Moscardi, 1999). Upon death of an insect host infected with a baculovirus, the integument disintegrates, the occlusion bodies (OBs) are released and when they are consumed by a susceptible host they can initiate a new virus infection (Miller et al., 1983). This horizontal, i.e. "within generation" transmission of baculoviruses is complemented by several modes of vertical transmission, i.e. transmission between generations. Here, the persistence of baculoviruses in the soil and on the plant is quite important, in addition to the transmission of virus infection from parent to offspring (Fuxa, 2004).

Horizontal transmission of baculoviruses depends primarily on the contact rate between susceptible hosts and infectious pathogens and on the susceptibility of the host (Reeson et al., 2000; Hails et al., 2002). Infection with a baculovirus can alter the behaviour of the host, resulting in a modified contact rate (Vasconcelos et al., 1996). Host plant architecture further affects the contact rate between 'infector' (= infected larvae producing OBs) and 'recipient' (= uninfected) larvae.

According to the literature, the relationship between baculovirus transmission and host density is not a matter of simple "mass action", indicating that rate of disease transmission is not directly proportional to the product of healthy larvae and the virus density (D'Amico et al., 1996). More is known about transmission of single viruses than about transmission of mixtures of viruses with different biological traits. Transmission from mixtures may be affected by the behavioral effects of both viruses that are expressed in the same insect host and do not necessarily have to be additive or average of the effect of single viruses. Multiple infections of a host by different genotypes of the same microparasite are common in nature (Ben-Ami et al., 2008).

Soil is reported to be a major reservoir for baculoviruses, as they are occluded in a protein matrix (OBs) to protect them against decay. The reservoir function of the soil facilitates the transmission of baculoviruses between hosts of the same or subsequent generations over space and time (Hostetter & Bell, 1985; Entwistle & Evans 1985; Fuxa, 2004, Olofsson, 1988 a, b; Young & Yearian, 1979, ibid.1986). Survival of baculovirus in the soil has been demonstrated for different amounts of time (Mohamed et al., 1982; Kaupp, 1983), e.g. for the Douglas-fir tussock moth, *Orgyia pseudotsugata* (Lepidoptera: Lymantriidae), in forest soil for 41 years in undisturbed soil (Thompson et al., 1981). OBs on plant foliage and on the soil are, however, rapidly inactivated by exposure to sunlight (Jaques, 1964, ibid. 1969; David et al., 1966, Roome & Daoust, 1976). Sun et al. (2004b) reported that a recombinant HaSNPV (*-egt*; *AaIT*) variant has a similar inactivation rate

on cotton tissue as the wild type virus. As inactivation of baculoviruses on foliage is very fast, within days, synchronization between 'infectors' and 'recipients' is potentially an important determinant of the transmission. Efficient virus transmission can be expected if 'recipients' encounter baculovirus deposited by 'infectors' on the host plant before the virus is inactivated by solar radiation. However, Zhou et al., (2005) showed that baculovirus transmission was not affected significantly by the different time of the release of the recipient larvae on the cotton plant.

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is an economically important pest in numerous agricultural and industrial crops including fiber crops and vegetables (Fitt, 1989; Zalucki et al., 1986). The *H. armigera* nucleopolyhedrovirus (HaSNPV) is one of the most common pathogens attacking field populations of *H. armigera* and is a potentially important bioinsecticide (Zhang, 1989).

The questions that are addressed in this study are: (i) How is transmission affected by genetic traits of the virus that affect time to death and virus yield, and how is transmission affected when the 'infector' larvae undergo a mixed infection? (ii) How does the density of 'infectors' influence transmission? (iii) How is transmission affected by the time that has elapsed between inoculation of infector larvae and release of the test larvae?

MATERIAL AND METHODS

Insects

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) insects used in the first field transmission experiments in 2005 were maintained at the China Cotton Research Institute (CCRI), Anyang, China. To prevent cannibalism, larvae were reared individually in glass tubes. Insects were reared on cotton leaves in a controlled environment (27°C, 70% relative humidity (RH) and a 14:10 light-dark (LD) regime). The insects used in experiments 2 and 3 were obtained from Wuhan Kernel Bio-pesticide Co., Ltd., Hubei, China. *H. armigera* used in Experiment 4 (2007) were from a colony maintained at the Laboratory of Virology, Wageningen University, Wageningen, The Netherlands, and originating from the Department of Entomology, Public University of Navarra, Pamplona, Spain. The colony was reared continuously on artificial diet (Green et al., 1976) at 25ºC, 70% RH, and a 16:8 h LD photoperiod.

Viral inoculum

Wild type HaSNPV (HaSNPV-wt) was initially isolated from diseased *H. armigera* collected in Hubei province in China. Genotype HaSNPV-G4 was isolated by in vivo cloning (Sun *et al.,* 1998). HaSNPV-LM2 was generated after co-infection of HaSNPV-CXW2 DNA (-*egt*; +GFP) and plasmid pHaLM2 in HaAM1 cells (Chen et al., 2000). HaSNPV-4A is a genetically modified variant (-*egt*, +AaIT) of HaSNPV-LM2

showing enhanced speed of action through the deletion of the *egt* gene and expression of an insect-selective scorpion toxin (AaIT) (Sun et al., 2004a). Viruses were propagated in fourth instar *H. armigera* larvae. OBs were isolated by grinding cadavers in a mortar. The resulting homogenate was filtrated through two layers of muslin, and the OBs in the eluate were separated from insect material using centrifugation at 11,000 *g* or 60 min on a 30 - 60% continuous sucrose gradient. Concentration of OBs of the viral stock solutions was determined in three independent counts using an Improved Neubauer chamber (Hawksley, Lancing, UK) and phase-contrast microscopy (× 400). Virus stocks were stored at 4°C until use.

Overall experimental approach

The laboratory-reared *H. armigera* larvae were inoculated individually with the various HaSNPV strains or with HaSNPV genotype mixtures, and were released as 'infectors' on cotton plants in field cages (experiments 1-3) or single plant enclosures in the greenhouse (experiment 4) (Fig. 1).

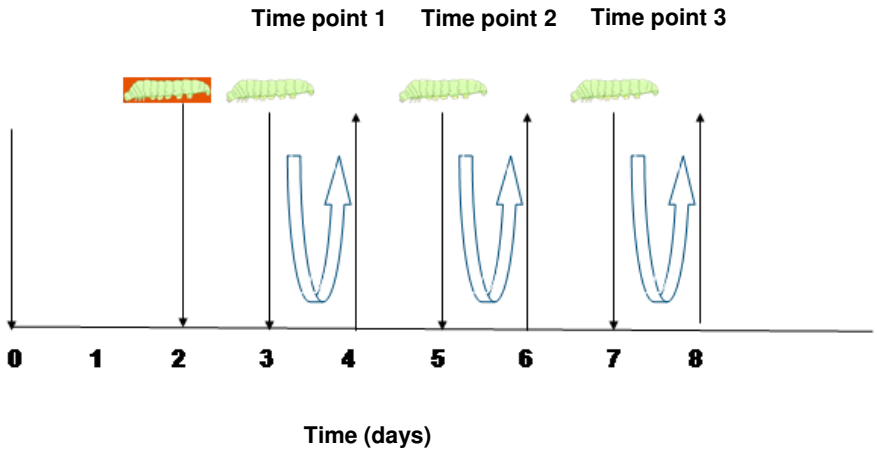

Figure 1. Schematic representation of Experiment 1. Second instar *H. armigera* were inoculated at time 0, and released on the plants, second instar *H. armigera* infector larvae (red) were released two days post infection. Six 3rd instar healthy larvae were released per plant at time point 1 (3 d.p.i.); time point 2 (5 d.p.i.) and time point 3 (7 d.p.i.) and recovered after one day.

Subsequently, test larvae ('recipients') were exposed to the same plants, and the proportion of these recipients contracting virus infection was scored. Factors investigated were: (1) virus treatment (all experiments); (2) infector density (experiments 2 and 4); (3) temporal coincidence between infectors and test larvae (experiment 1). As a side observation, availability of virus inoculum in soil was quantified in experiment 3.

Quantitative PCR was used to measure HaSNPV genotype ratios in a sample of virus-killed test larvae in Experiment 4. Details are given below.

Experiment 1: Anyang, China 2005

Experiment 1 was conducted at CCRI, Anyang, in 2005. Treatments were: (1) control (uninfected 'infectors'); (2) infectors inoculated with HaSNPV-wt; (3) infectors inoculated with HaSNPV-LM2 (-*egt*), (4) infectors inoculated with a 1:1 mixture of HaSNPV-wt and HaSNPV-LM2 (mix-in) and (5) mixture of infectors inoculated with either HaSNPV-wt or HaSNPV-LM2 (mix-pl). In treatment 5, equal numbers of larvae with either genotype were released on the same plant. The two different mixed infected treatments (4 and 5) were designed to measure at which level the competition takes place, at insect level (mix in) or at the crop level (mix pl). Cotton (var. Zhongmiansuo #35) was sown on 23 April 2005. To prevent larvae from escaping and exclude predators and rain, treatments were applied in 2 × 2 × 2 m metal framed cages with fine mesh nylon netting on the sides and plastic sheeting on top. The cages were buried up to 10 cm into the soil. Forty-five cages were used in the experiment: 5 treatments x 3 release times of test larvae x 3 repetitions. At the time of the experiment, the cotton plants had flowers and bolls. Second instar *H. armigera* larvae were used as infectors. They were inoculated in the laboratory with a dose of 10,000 OBs/larva, enough to reach 100% mortality. After two days, six HaSNPV-treated larvae were released onto each cotton plant and allowed to disperse and die. They were not recovered. At 3, 5 and 7 days post release of the infectors (i.e. 5, 7 and 9 days after the infector larvae had been inoculated) six third instar healthy *H. armigera* larvae were released onto each cotton plant. They are further referred to as 'recipient' larvae. The recipient larvae were recovered after one day, placed individually in a well of a 24-well tissue culture plate with artificial diet and reared at 27° C. The larvae were monitored daily until death or pupation.

Experiment 2: Ezhou, China 2006

Experiment 2 was conducted in a cotton field near Ezhou, Hubei Province, China from June until August 2006. Ezhou lies close to the Yangtze River, 750 km south of Anyang, and has a much hotter climate than Anyang. The physical setup was the same as in Experiment 1. (2005). There were five treatments: (1) control (uninfected infectors); (2) infectors inoculated with HaSNPV-wt; (3) infectors inoculated with HaSNPV-LM2; (4) infectors inoculated with a 1:1 mixture of HaSNPV-wt and HaSNPV-LM2 (mix in) and (5) mixture of infectors inoculated with either HaSNPV-wt or HaSNPV-LM2 (mix-pl). Cotton was sown on 15 April in beds and transplanted in the field on 25 May 2006, when the plants were in the 4-leaf stage. At the time of the experiment, the cotton plants were flowering. Second instar *H. armigera* larvae were inoculated on 8 July, with a dose 10,000 OBs per larva, which is 5 times the LD_{99} for this instar (Sun et al., 2004a). The virus solution was

applied onto 15 mm cotton leaf discs placed on a 1% agar support. Insects were kept on the leaf disc until they had finished it and taken up all the virus suspension. On 10 July the infected larvae (infectors) were released on the plants at one of two different densities: two or six infected larvae per plant. On 15 July four third instar healthy larvae were released per plant as recipient larvae, resulting in 24 larvae per cage. Recipient larvae were recovered after 2 days on 17 July, and reared individually until pupation or death in 24-well tissue culture plates containing artificial diet.

Experiment 3: Ezhou China, 2006

Experiment 3 was conducted in the same cotton field as Experiment 2, but a different section of the field was used. Experiment 3 started on 20 July and lasted until 2 August. 2006. The same virus treatments were applied as in Experiment 2, but only the higher level of infectors was used (six per plant), and there were now 10 repetitions for each treatment. At the time of the experiment, the cotton plants had bolls. Second instar larvae were inoculated on 20 July with a dose of 10,000 OBs per larva, using the leaf disk method as in Experiment 2 and released on the plant on 24 July, 4 days after inoculation. On 29 July, twenty third instar healthy larvae were released per plant as recipients. They were recovered after 4 days, on 2 August and reared individually on artificial diet until death or pupation.

Experiment 4: Wageningen (glasshouse), 2007

Experiment 4 was conducted on cotton plants in the glasshouse at Wageningen University, The Netherlands in October 2007. At the time of the experiment the cotton plants, sown on 14 August 2007, were two months old and in the vegetative stage. There were two factors: virus treatment and density of infectors. The experiment was laid out as a randomized block experiment, with location in the glasshouse as blocking factor. There were five blocks, and each plant constitutes one experimental unit. Virus treatments were: (1) control (uninfected infectors); (2) infectors inoculated with HaSNPV-wt; (3) infectors inoculated with HaSNPV-LM2 (-*egt*), (4) infectors inoculated with HaSNPV-4A (-*egt*, +AaIT), (5) infectors inoculated with a 1:1 mixture of HaSNPV-wt and HaSNPV-LM2 and (6) infectors inoculated with a 1:1 mixture of HaSNPV-wt and HaSNPV-4A. Two densities of infectors were used: one or ten per plant. On 10 October 2007, fourth instar larvae were inoculated with 200,000 OBs/larva, using droplet feeding. This dose is approximately a 5 × LD_{99} for four-instar larvae of *H. armigera* (Sun et al., 2004a). Four days later, on 14 October 2007, these larvae were released onto cotton plants in the glasshouse. The cotton plants were enclosed individually in nylon mesh nets to prevent larvae from escaping and to avoid cross contamination of viruses. Seven days later, on 21 October 2007, ten third instar healthy larvae were released onto each of the 60 plants. These recipient larvae were recovered at 48 h after their release and placed individually

into 24-well tissue culture plates containing artificial diet. They were reared at 26°C and 70 % R.H. until death or pupation. Cadavers were kept individually in Eppendorf tubes at – 20°C until analysis.

Measurements on virus persistence in soil

Following the release of infector larvae in Experiment 2, it is expected that the soil may become contaminated. To measure the flow of inoculum from the plant onto the soil compartment, samples were taken from soil underneath inoculated cotton plants at 3, 5, 8, 11, 15 and 22 days after release of the infectors. A bulk sample of soil (~ 200g) was collected from each cage, by scooping 5-6 table spoons of soil from the soil surface below the plants, to a depth of approximately 5 cm. Soil samples were returned to the laboratory and virus activity was measured by releasing 20 second instar *H. armigera* larvae onto 100 g of soil in a Petri dish and left to acquire the virus directly from the soil, for a period of 24 h (Roome & Daoust 1976). Larvae were subsequently reared individually on diet in 24-well tissue culture plates until death or pupation. Different spoons were used for different treatments to prevent cross contamination. After sampling the spoons were decontaminated with 0.5 % NaOH. During the first 5 sampling dates, the cotton plants were covered with cages, but 15 days after release of the infectors, the cages were moved for conducting experiment 3, and the sites were uncovered

Isolation of DNA and qPCR (Quantitative Polymerase Chain Reaction)

A sensitive qPCR analysis was used to quantify the ratio of each baculovirus present in the DNA isolated from the cadaver of each single larva of recipients for the mix infection treatment for glasshouse experiment. Quantitative PCR was performed using a RotorGene 2000 thermal cycler (Corbett Research, Germany). For each cycle, target nucleic acids were amplified and monitored by a fluorescence assay. Primers used for the amplification of viral genomic DNA for the qPCR assays were designed to target unique genes, i.e. the *egt* gene for HaSNPV-wt and the *AaIT* gene sequence that is specific for HaSNPV-LM2. The sequence of the specific *egt* primers were as follows: forward primer 5'GAAGAACTCGGAATCGGACGCAAC-3' and reverse primer 5' CTGTGTAGCGACTCTTGTTGTTGACGG-3'. A 100 bp product was produced. This 100 bp fragment derived from the *egt* gene was cloned into the pGMT-easy plasmid (Promega, Wisconsin) according to the manufacturers' instructions (Sambrook et al., 1989). Plasmid DNA was purified from the transformed bacteria using a midi prep kit (Geomed, Germany) and the DNA concentration was measured by UV spectrophotometry. The DNA was subjected to PCR and automated sequence analysis to confirm the expected *egt* sequence. This plasmid was used to set up standard curves in the qPCR, which were used to calculate the amount of viral DNA in OBs from cadavers using known copy numbers from the plasmid to make standard curves. A 100 bp coding

sequence for AaIT was targeted by using the forward primer 5'-ATGTCGTCAACAGTAATGTGGGTGTCAAC-3' and reverse primer 5'-TTGTTGCAGTAGTTAGACAGCAGG CATTC-3'. The same procedure was used for the *AaIT* sequence, where a fragment was cloned into pGMT-easy, and checked by PCR and DNA sequencing. The DNA was quantified with a Nanodrop (Nanodrop Technologies) and the number of plasmid copies were calculated based on DNA concentration.

OB DNA samples from individual larvae were analyzed in triplicate with both sets of primers, with sterile water as a negative control. For calibration, 10–fold dilutions of plasmids containing the cloned gene of interest were made to produce concentrations of 10^8, 10^7, 10^6, 10^5, 10^4 plasmid copies per µl. A 'master mix' for PCR was prepared with Qiagen Quantitect SYBR Green Master Mix (Qiagen, UK), to which the primers (10 µM each) and sterile water were added. Each PCR tube was filled with 20 µl of reaction mixture and a 5 µl aliquot of the isolated DNA sample at a 1:100 dilution. qPCR was performed with the following cycling program: 95°C for 15 min followed by 40 cycles of 15 sec at 94°C, 30 sec at 58°C, and 30 sec at 72°C. The products were examined by melting-curve analysis, followed by an increase in temperature from 58 to 99°C and continuous fluorescence recording. The CP (crossing points) and slope values for each treatment were used for the calculation of genotype ratios. The CP is defined as point at which fluorescence rises appreciably above background. Results are expressed as the ratio of the two amplified genes. Quantification analysis of the PCR amplification and melting curves were performed using LightCycler software (Rotor-Gene 6.0, Corbett Research). After plotting and analyzing of the melting curve from the PCR product generated from *egt* and *AaIT* primers, the results confirmed that the signal was due to the amplification of the desired product and not a random one. The total amount of the plasmid copies number per µl was calculated by direct comparison with a standard curve. In each run along with the sample DNA was used plasmid with desired insert in 10-fold serial dilutions, to serve as standard curves. The calculation of ratios between copy numbers of wild-type and recombinant viruses either (HaSNPV-LM2 or HaSNPV-4A) was done following the procedure by Zwart et al. (2008).

Statistical analysis

Virus transmission in the four experiments (Exp. 1-4) was analyzed with Genstat procedure REML. The REML procedure in Genstat is similar to an ordinary regression; however, in REML (Restricted Maximum Likelihood), the model is fitted using maximum likelihood instead of least squares. Significance was evaluated by comparing calculated deviance ratios to *F*-distributions (P = 0.05) (GenStat(r) Release 11, 2008). In the case of a significant effect of virus species, density or time post infection, a *t*-test was used to compare means in SPSS (SPSS 13, Inc.). The bioassay data (mortality) from the

persistence measurements were square root transformed, and analyzed with repeated measures ANOVA. Least significant differences (LSD) were used for comparisons among means (SPSS 13, Inc.). The non-parametric Mann-Whitney test was used to compare genotype ratios obtained by q-PCR on OBs of mixed infected cadavers (recipient). This test was performed separately for the two different mixtures wild-type: HaSNPV-LM2 and wild type: HaSNPV-4A in SPSS (SPSS 13, Inc.).

RESULTS

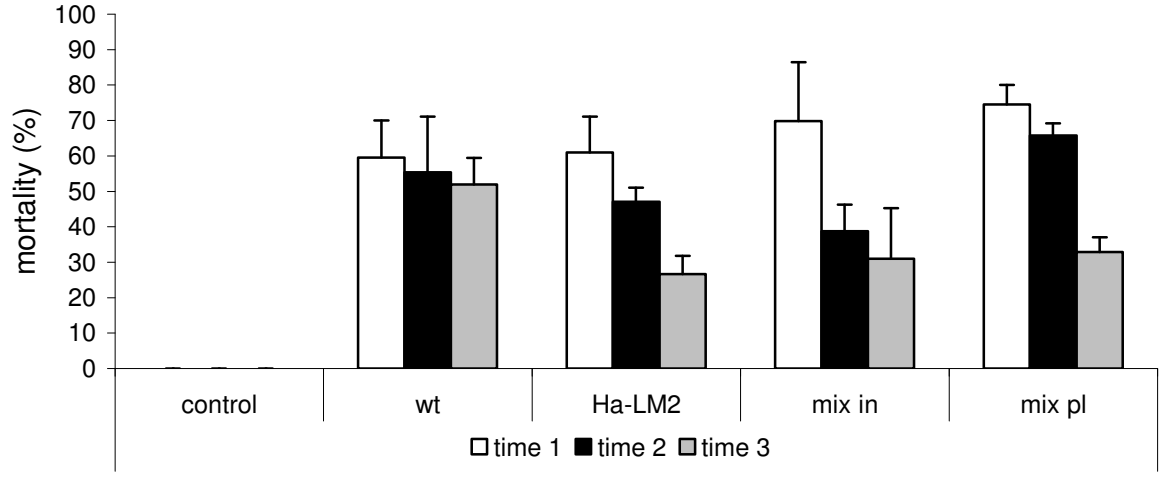

Figure 2. Percentage of third instar *H. armigera* larvae contracting virus when exposed for one day on cotton plants infested with virus-challenged *H. armigera* L2 larvae (Experiment 1). Virus treatments were (1) control; (2) HaSNPV-wt; (3) HaSNPV-LM2; (4) infectors were challenged with a 1:1 mixture of the two virus genotypes; (5) 50% of infectors with HaSNPV-wt and 50% of infectors with HaSNPV-LM2. Time between release of the infectors and the exposure of the recipients was varied: 3 days (white bars), 5 days (black bars), and 7 days (grey bars).

Experiment 1: Anyang, China, 2005

This experiment was meant to investigate whether there is a difference in the transmission rate between wild-type (HaSNPV-wt) and recombinant (HaSNPV-LM2) and a mixture of these viruses. Transmission from L2 infectors to L3 recipients diminished as the infectors were released onto the plants for a longer period of time (Fig. 2). Average mortality (across virus treatments) of recipients was 52.9 % with release at 3 d.p.i. (days post infection) of infectors on the plant), 41.4 % with release at 5 d.p.i., and 28.5 % with release at 7 d.p.i.. The differences between times were significant (Wald/df = 10.01; P <

0.001; Table 1), but differences between virus treatments were not significant (Fig. 2). (Wald/df = 1.2; P > 0.05; Table 1).

Table 1. REML (Restricted Maximum Likelihood) analysis, of transmission of viruses in terms of larval mortality for three field experiments (different virus treatments, virus density or time post infection) and one glasshouse experiment (different virus treatment and density).

Fixed term	Wald statistic	d.f.	χ^2 probability
(a) Field experiment 1 –Anyang 2005			
Virus Treatment	3.65	3	0.327
Time	20.03	2	< 0.001
Treatment x time	5.81	6	0.470
(b) Field experiment 2– Ezhou 2006			
Virus Treatment	0.63	3	0.733
Density	5.59	1	0.028
Treatment x density	3.16	3	0.230
(c) Field experiment 3– Ezhou 2006			
Virus Treatment	2.04	3	0.573
(d) Glasshouse experiment– 2007			
Virus Treatment	4.15	4	0.413
Density	9.70	1	0.005
Treatment x density	3.60	4	0.482

Experiment 2: Ezhou, China, 2006

A second transmission experiments was carried out, this time in Ezhou. The setup was similar to experiment 1, but in a different environmental setting. Mortality of the L3 recipients was 15.6 % at a density of 2 L2 infectors per plant and 29.8 % at a density of 6 infectors per plant (Table 1) and this difference is significant (Wald/df = 5.59; P = 0.028. (Fig. 3). There were no significant differences between virus treatments (Wald/df = 0.31 P > 0.05; Table 1).

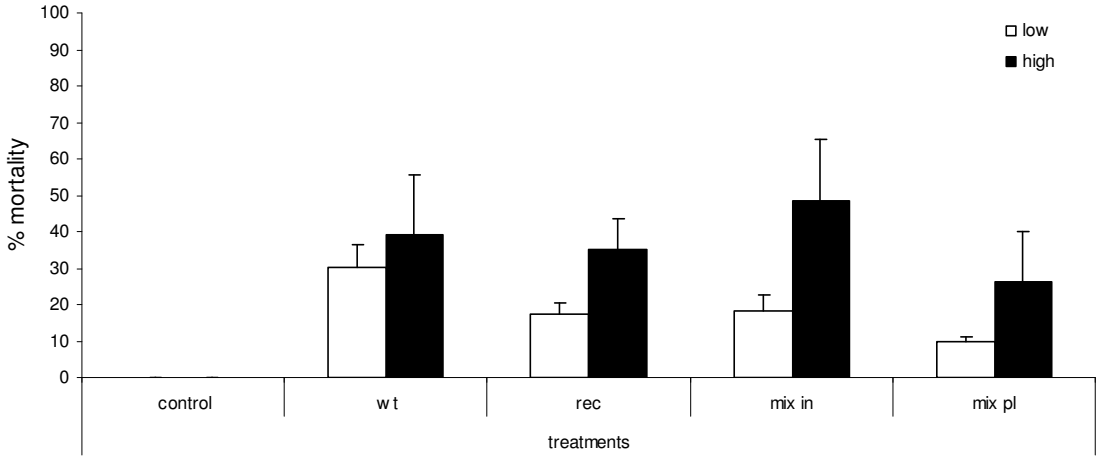

Figure 3. Percentages of third instar *H. armigera* larvae contracting virus when exposed for one day on cotton plants infested with virus-challenged *H. armigera* L2 larvae (Experiment 2). Virus treatments were (1) control; (2) HaSNPV-wt; (3) HaSNPV-LM2; (4) infectors were challenged with a 1:1 mixture of the two virus genotypes: (5). 50% of infectors with HaSNPV-wt and 50% of infectors with HaSNPV-LM2. White bars pertain to low density of infectors (2 / plant) and black bars to a high density of infectors (6 / plant).

Experiment 3: Ezhou China, 2006

In this experiment, also carried out at the Ezhou field site, the virus treatments of experiment 2 were repeated, now using only a high infector density of six larvae/plant, and a double number of replicates (10), in order to increase the power of the experiment to identify virus treatment effects. Mortality was very similar among treatments (mean ± SE): 20.7 ± 2.8% for HaSNPV-wt, 18.7 ± 4.6% for HaSNPV-LM2, 18.4 ± 3.3% for the infectors carrying HaSNPV-wt or HaSNPV-LM2 as a virus mixture (mix-in), and 25.4 ± 8.4% for the mixture of infectors carrying either HaSNPV-wt or HaSNPV-LM2 (mix-pl). Mortality was of the same order of magnitude as in Experiment 2, and no significant differences between virus treatments were identified (Wald/df = 0.68; $P > 0.05$ (Table 1).

Experiment 4: Wageningen (glasshouse), The Netherlands, 2007

In this experiment, L4 infectors were used to enhance transmission, compared to Experiments 1-3 in which L2 larvae were used as infectors. A toxin expressing recombinant, HaSNPV-4A, was included to obtain potential for greater differences in response between virus treatments. As in Experiment 2, density of infectors significantly affected transmission (Wald/df = 9.7; $P = 0.005$; Table 1), with an average (over virus treatments) of 73% mortality with 10 infectors per plant, and 33% mortality with a single infector per plant) (Fig. 4). There was no overall significant virus treatment effect in

analysis (Wald/df = 1.04; P = 0.41; Table 1). The pairwise contrast analysis testing of the means in IRREML showed that HaSNPV-4A treatment gave a significantly lower transmission rate than the HaSNPV-wt and mixture of HaSNPV-wt + HaSNPV-4A virus treatments at the higher density of infectors, but there was no significant difference at the lover density.

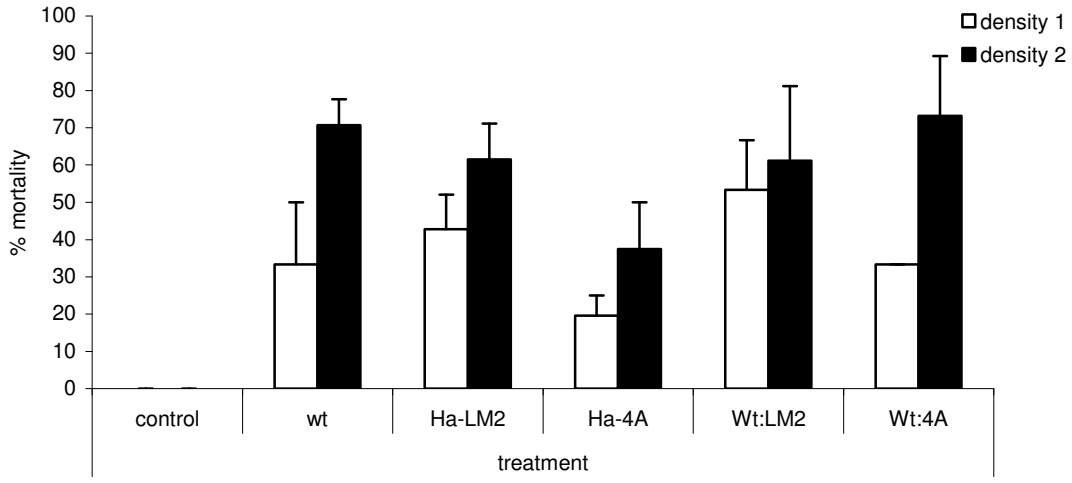

Figure 4. Percentage of fourth instar *H. armigera* larvae contracting virus when exposed for one day on cotton plants infested with virus-challenged *H. armigera* L4 larvae (Experiment 4). Virus treatments were (1) control; (2) HaSNPV-wt; (3) HaSNPV-LM2; (4) HaSNPV-4A; (5) infectors were challenged with a 1:1 mixture of the HaSNPV-wt and HaSNPV-LM2; (6) infectors were challenged with a 1:1 mixture of the HaSNPV-wt and HaSNPV-4A. White bars pertain to low density of infectors (1/plant) and black bars to a high density of infectors (10/plant). Standard error of the mean is indicated.

Persistence

This experiment was set up in order to determine the persistence of wild-type and recombinant HaSNPV in the soil. This was done alongside Experiment 2. Virus was recovered from the soil samples at multiple times after release of the infectors, including the first time, which was only 3 days after release of the infectors, equivalent to 5 days after the infectors had been inoculated with HaSNPV. The trend of virus availability in the soil at different times is erratic and does not show any clear pattern (Fig. 5). The strong decrease of virus availability on the last day of sampling, day 22 may be related to the removal of the cages on day 15, and exposure of the soil to intensive solar radiation. In repeated measures ANOVA no significant differences between the different HaSNPV variants were identified, while several time effects were significant (Table 2).The last sampling day (day 22) had a reduced mortality in all treatments in comparison with the rest of the days (Fig. 5).

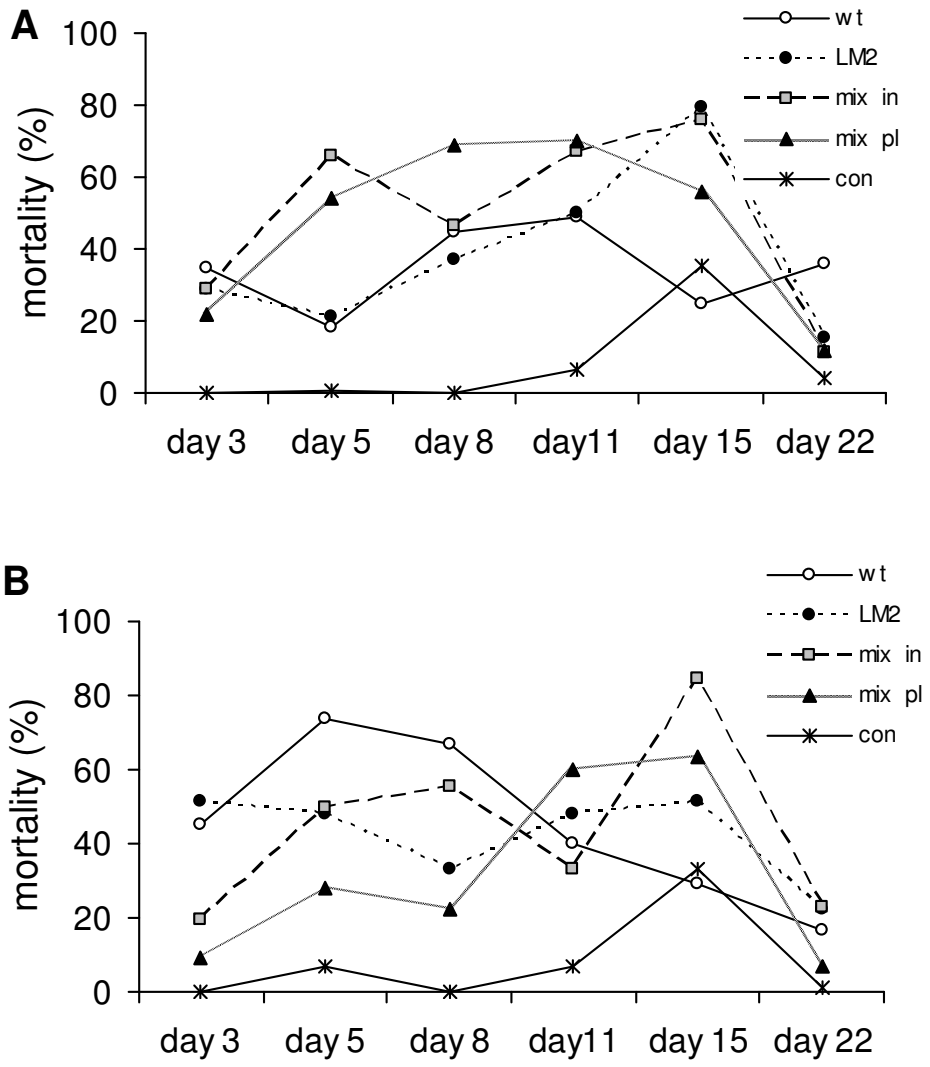

Figure 5. Virus persistence in soil. Y-axis is mortality (%) of second instar *H. armigera* larvae when exposed to soil from plots from different virus treatments in Experiment 2. Virus treatments were (1) control (asterix); (2) HaSNPV-wt (open circles); (3) HaSNPV-LM2 (black circles); (4) infectors were challenged with a 1:1 mixture of the two virus genotypes (grey squares); (5) 50% of infectors with HaSNPV-wt and 50% of infectors with HaSNPV-LM2 (black triangles). (A) At a low density of infectors of 2 L2s / plant and (B) – At a high density of infectors of 6 L2s / plant.

The results from the persistence experiment showed that persistence of the virus in the soil, when low density of infectors were used on the plants, was significantly affected by the day of sampling [$F(5, 50) = 3.56$, $P = 0.008$] (Table 2). In the case of the high density of infectors on the plants the Mauchy's test indicated that the assumption of sphericity had been violated [$\chi^2(14) = 33.31$, $P < 0.05$]. Therefore degrees of freedom

were corrected using Greenhouse-Geisser estimates of sphericity. The persistence of the virus in the soil, when high density of infectors were used on the plants, was significantly affected by the sampling day [F (3.4, 67.9) = 13.1, P = 0.0001]. Although there was a noticeable decrease of mortality over time, the soil-bioassay percentages of mortality did not differ significantly within virus treatment between low and high larval densities at all virus treatments.

Table 2. Repeated measures ANOVA of persistence in soil of three HaSNPV variants: HaSNPV-wt, HaSNPV-LM2 and HaSNPV-mix in a field experiment.

Source	*df*	MS	F	P > F
Measuring persistence when low density of infectors were used				
Within -subject effects				
Sampling date	5	29.25	3.56	0.008
Treatment * date	20	8.57	1.04	0.432
Error (date)	50	8.21		
Between- subject effects				
Treatment	4	10.94	19.88	0.0001
Error	10	0.55		
Measuring persistence when high density of infectors were used				
Within - subject effects				
Sampling date	3.39	158.31	13.10	0.0001
Treatment * date	13.58	14.88	1.23	0.275
Error (date)	67.92	12.08		
Between- subject effects				
Treatment	4	9.81	7.30	0.001
Error	20	1.34		

qPCR (Quantitative Polymerase Chain Reaction)

QPCR was used to measure the relative success of the competing genotypes within the individual host in mixed infected recipients insects with wild-type: HaSNPV-LM2 and wild-type : HaSNPV-4A at a high density of infectors from the glasshouse transmission experiment (Experiment 4).

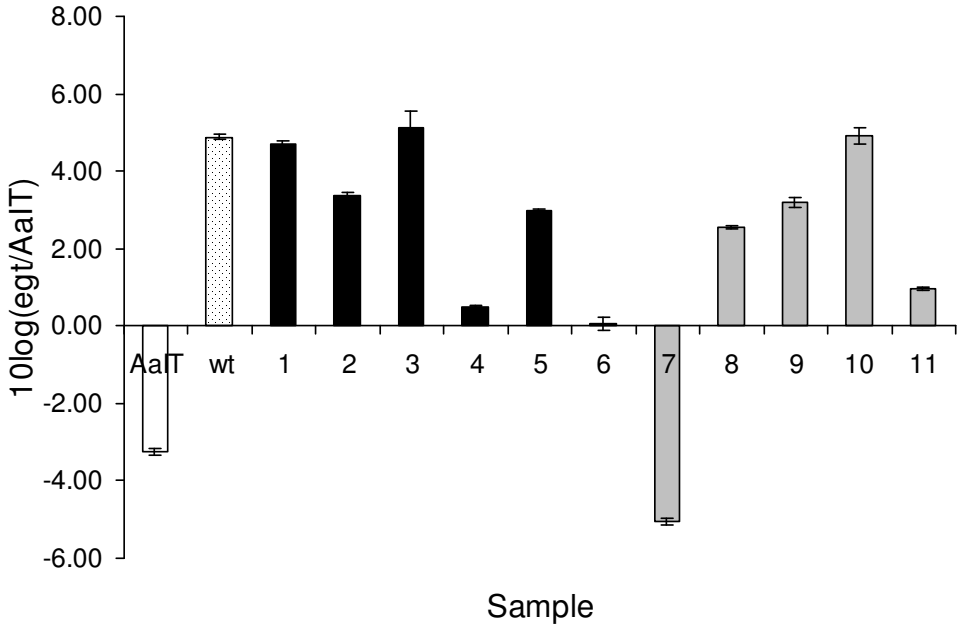

Figure 6. Co-infection of larvae with wild-type and HaSNPV-LM2 (*-egt*) (samples 1-6-black bars) and wild-type and HaSNPV-4A (samples 7-11-grey bars). Genotype ratios in mixtures obtained by qPCR for fourth instar *H. armigera* larvae obtained by infecting with a 1:1 ratio of polyhedra mixture of both viruses. Log-ratios greater than 4 in absolute value indicate that the larvae were singly infected. Error bars are the standard error of the log ratios. Values greater than 1 indicate that the copy number of *egt* (wild-type) exceeds that of AaIT (signaling recombinant virus), HaSNPV-LM2 or HaSNPV-4A, respectively.

The number of copies of recognized sequence for HaSNPV-wt (+ *egt*) was also compared to that of the *AaIT* sequence for the recombinant viruses HaSNPV-LM2 (where the *AaIT* gene is present, but not producing an active toxin) and HaSNPV-4A (where the *AaIT* gene is not only present but also active) (Fig. 6, samples 1-6). and HaSNPV-4A (where the *AaIT* gene is not only present but also active) (Fig. 6, samples 7-11). Six individual cadavers were tested from each treatment. Multiple infections were detected in the majority of insects, which had received an inoculum containing a mix of genotypes. The mean of the log transformed data shifted towards HaSNPV-wt although the starting ratio for infection was an equal mixture of genotypes (not shown), where first mixture set consists of (HaSNPV-wt : HaSNPV-LM2) (Fig. 6, samples 1-6) (Mann- Whitney test $Z = -2.739$, significance (2-tailed) = 0.006) and second sample set of HaSNPV-wt : HaSNPV-4A (Fig.6 samples 7-11) (Mann- Whitney test $Z = -2.205$; significance (2-tailed) = 0.032). The results from the analysis on the DNA isolated from single larvae initially infected with both viruses in equal ratio showed that the HaSNPV-wt was more prevalent after one passage compared with the recombinant viruses HaSNPV-LM2 and HaSNPV-4A. These findings indicate that the recombinant viruses have a lower fitness within the insect than the wild type virus.

DISCUSSION

The horizontal transmission of wild-type and *egt*-negative genotypes of HaSNPV was not identifiably different at the crop level, but there was a significant change in transmission when HaSNPV-AaIT expressing the AaIT toxin gene was used. The results lead us to conclude that significant differences in virus transmission among treatments, depends from various factors, different biological properties of virus genotypes, synchronization of infectors and recipients, and density of host insects. This is an important conclusion because from a biological safety point of view, a reduced transmission of genetically modified, fast killing, viruses, would count as a bonus.

Recombinant HaSNPV-LM2 is characterized by the absence of a functional *egt* gene (Chen et al., 2000). *H. armigera* larvae which are infected with this virus have a shorter survival time than larvae, which are infected with the wild type virus HaSNPV-wt (Sun et al., 2004a; O'Reilly & Miller., 1991). As a result, such insects cover a shorter distance on the host plant, and presumably they leave less of a virus trace on the plant as a result of lower virus yield. Furthermore, virus yield is decreased (Sun et al., 2005 and Chapter 3). An effect of these modifications on virus transmission was plausible, but did not materialize in our field experiments. Recombinant HaSNPV-4A is characterized by the absence of a functional *egt* gene, and the presence of a functional gene coding for an insect specific toxin (*AaIT*). Such a modification of baculoviruses in general results in a shortened survival of the larvae, in a reduced OB yield (Cory et al., 1994; Burden et al., 2000; Harrison, & Bonning 2000; Hernandez-Crespo et al., 2001; Sun et al., 2005, 2009) and often results in them falling off the plant, thereby diminishing the scope for virus transmission in the short term. These modifications are expected to result in reduced transmission, compared to the wild-type baculovirus (Hoover et al., 1995; Hails et al., 2002). Indeed, Zhou et al. (2005) observed reduced transmission of HaSNPV-4A compared to HaSNPV-wt on cotton in field-cages. Here, we confirm this effect from a glasshouse trial, but the difference in transmission between HaSNPV-wt and HaSNPV-4A was moderate, and the significance marginal. There was a minor difference in transmission among the individual viruses under the experimental conditions, as well as between HaSNPV-wt and the mixture of HaSNPV-wt and HaSNPV-4A.

Hails et al. (2002) and Dwyer, (1991) pointed out that the number of cadavers that remains on the plant has a greater influence on the transmission than the yield of the virus itself. Statistical analysis from our data showed that there was a significant reduction of transmission of HaSNPV-4A and mixture of HaSNPV and HaSNPV-4 A, when low density of the infectors was released in the glasshouse experiment.

In this study we were in particular interested in horizontal transmission of baculoviruses from mixed infected larvae. To be able to determine the 'winning' genotype in our study, we developed genotype specific primers. Results obtained from the qPCR analysis of the cadavers mixed infected with the wild-type and recombinant virus (HaSNPV-LM2 or HaSNPV-4A) in the glasshouse experiment demonstrated that both viruses were present. Although most of the larvae were positive for both types of HaSNPV-wt and recombinant (HaSNPV-LM2 or HaSNPV-4A), there was a significant shift towards the wild-type HaSNPV genotype from the 1 : 1 ratio. It might be that wild type HaSNPV has a higher fitness compared with the genetically modified HaSNPVs. Outcompetition of one genotype by another in a mixed baculovirus inoculation has been demonstrated earlier (Clarke et al. 1994; Zwart et al., 2009). This competitive displacement will depend on the ability of the genotypes to be more efficient in their resource use, which will help them to outcompete the less efficient genotypes (Vizoso et al, 2005).

Larvae dying from a baculovirus infection on the cotton plants are exposed and they may be washed off by rain or blown away by wind (Room & Daoust, 1976). The cotton plant has been recognized as a barrier for effective establishment and spread of baculoviruses in insect populations, because persistence of virus on the leaf surface is compromised (Streett et al., 1999). Our result showed that HaSNPV-wt and HaSNPV-LM2 (lacking the *egt* gene) and the mixture of these two baculoviruses are equally persistent in the soil. However, decrease of the amount of virus present in the soil over time was indicated. The sharp decrease in the persistence of the viruses at the last sampling date at 22 days post release of the infectors on the plant might be as a result of intensive exposure of the cotton plants to sunlight, after removal of the cages (Fig. 6). Although this study focuses on the short term persistence of the HaSNPV viruses in the soil, it is important to expand the study over a longer period of time, particularly to test how much virus is able to overwinter, to test what are the chances of starting new epizootics during the next growing season.

In terms of releasing of HaSNPV-LM2 (*-egt*) and HaSNPV-4A (*-egt*, *+AaIT*) as a microbial insecticide in the ecosystem, our data suggest that when these recombinants have HaSNPV-wt as a co-inoculant the former may be outcompeted within hosts.

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Chapter 5

COMPETITION IN INSECT LARVAE BETWEEN WILD-TYPE BACULOVIRUS (*SPODOPTERA EXIGUA* NUCLEOPOLYHEDROVIRUS) AND A MARKED RECOMBINANT WITH ENHANCED SPEED OF ACTION

SUMMARY

Competition between virus genotypes in insect hosts is a key element of virus fitness, affecting their long-term persistence in agro-ecosystems. Little information is available on virus competition in insect hosts or during serial passages from one cohort of hosts to the next. Here we report on the competition between two genotypes of *Spodoptera exigua* nucleopolyhedrovirus (SeMNPV), when serially passaged as mixtures in cohorts of 2nd instar *S. exigua* larvae. One of the genotypes was a SeMNPV wild-type isolate, SeUS1, while the other was a SeMNPV recombinant (SeMNPV-XD1) having a greater speed of kill than SeUS1. SeXD1 lacks a suite of genes, including the ecdysteroid UDP-glucosyl transferase (*egt*) gene. SeXD1 expresses the green fluorescent protein (GFP) gene, enabling the identification of SeXD1 in cell culture and in insects. The relative proportion of SeUS1 and SeXD1 in successive passages of mixed infections in various ratios was determined by plaque assays of budded virus from infected larvae and by polymerase chain reactions and restriction enzyme analyses. The SeUS1 genotype outcompeted recombinant SeXD1 over successive passages. Depending on the initial virus genotype ratio, the recombinant SeXD1 was no longer detected after six to twelve passages. A mathematical model was developed to characterize the competition dynamics. Overall, the ratio SeUS1/XD1 increased by a factor 1.9 per passage. The findings suggest that under the experimental conditions recombinant SeXD1 is displaced by the wild-type strain SeUS1, but further studies are needed to ascertain that this is also the case when the same baculoviruses would be used in agro-ecosystems.

Modified after:
Liljana Georgievska, Renate Velders, Xiaojiang Dai, Felix J.J.A. Bianchi, Wopke van der Werf & Just M. Vlak (2005). Competition in insect larvae between wild-type baculovirus (*Spodoptera exigua* nucleopolyhedrovirus) and a marked recombinant with enhanced speed of action. IOBC/WPRS Bulletin 28: 141-145.

INTRODUCTION

The beet armyworm, *Spodoptera exigua*, is a moth (Lepidoptera: Noctuidae) whose larvae cause economic losses in a wide range of cultivated crops throughout the temperate and subtropical regions of the Northern hemisphere. Control of *S. exigua* has for a long time relied almost exclusively on insecticides, resulting in widespread insecticide resistance in the insect (Brewer et al., 1995) and a quest for biological alternatives.

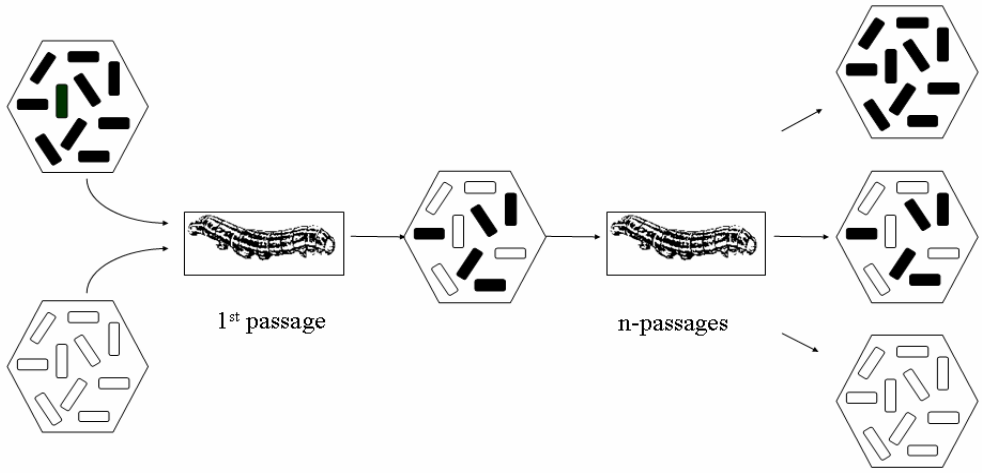

Figure 1. Mixed infection of *S. exigua* larvae with both wild-type SeUS1 (black) and recombinant SeXD1 (white) SeMNPV genotype. Upon successive passaging in insects three types of progeny genotypes are possible: the recombinant will dominate, co-exist with the wild-type virus or disappear.

Baculoviruses are naturally occurring insect pathogens that are suitable biological agents to control pest insects (Moscardi, 1999). They are often specific, are natural pathogens, safe for humans (vertebrates) and have little or no environmental impact. *S. exigua* multicapsid nucleopolyhedrovirus (SeMNPV) is an attractive bio-insecticide, since the virus is specific to the beet armyworm and highly virulent (Smits & Vlak, 1994). A successful product (Spod-X^R), produced by the Certis USA, has been on the market for well over a decade. A major drawback of baculoviruses is the long incubation time to show effect or cause disease, i.e. their action is relatively slow (a matter of days/weeks) when compared to most chemical insecticides (< day). Improvement of the speed of kill of SeMNPV has been achieved either by strain selection (Muñoz et al., 2000a) or by

genetic engineering (Dai et al., 2000). Genetic engineering approaches have been used predominately to increase the speed of action (McCutchen et al, 1991; Black et al., 1997; Inceoglu et al., 2001), such as deletion of the *egt* gene, encoding an ecdysteroid UDP-glucosyl transferase, or insertion of various insect-specific toxin genes. The question is whether recombinant baculoviruses can cause any harm to the environment or ecosystem, e.g. as a result of effects on non-target hosts. From the point of view of ecological safety and precaution it would be advantageous if genetically modified baculoviruses would not persist in agro-ecosystems after their use as pest control agents, but be outcompeted by wild-type baculoviruses over time. It is therefore pertinent and timely to study the fitness of recombinants as related to virus reproduction and survival (Bull et al., 2003).

Here we describe one aspect of the issue of virus fitness by setting up a model system to study competition of baculovirus genotype mixtures. Mixed infections with different ratios of wild-type and recombinant SeMNPV were carried out in serial transfers (passaging) in *S. exigua* larvae (Fig. 1); the progeny was used to infect a subsequent cohort of larvae. The relative proportion of each virus was measured over successive passages in the insects and a preliminary model to predict the competitive fitness was developed.

MATERIALS AND METHODS

Virus genotypes

The wild-type SeMNPV (SeUS1) was provided by Dr. B.A. Federici (UC Riverside, USA) and was propagated in laboratory-reared 4th instar *S. exigua* larvae. SeMNPV recombinant XD1 (SeXD1) was generated and described by Dai et al. (2000). This recombinant contains a gene for the green fluorescent protein (GFP) as a screening marker in lieu of the dispensable SeMNPV p10 gene. It further has a deletion of 10.6 kb sequence information, located between nucleotide 18513 to 29106, a region encompassing fourteen open reading frames (ORFs 15-28) (Fig. 2). Deleted genes include *egt*, gp37, chitinase, cathepsin and several other genes unique to SeMNPV and with unknown function. SeXD1 was isolated after recombination of SeUS1 and a plasmid construct carrying the GFP gene flanked by sequences of the p26 and p74 gene of SeUS1. The recombination was followed by alternate infection of Se301 insect cells, plaque purification of GFP-positive plaques and oral infection of *S. exigua* larvae. Budded virus (BV) was isolated from hemolymph and used to infect Se301 cells for a second round of plaque purification. This alternation between insect cells and insects was required to secure oral infection properties of SeXD1. SeXD1 has a significantly lower median survival time (ST_{50}) (-25%) than wild-type SeUS1, but a similar median lethal dose (LD_{50}) (Dai et al., 2000).

The *S. exigua* cell line Se301 was a gift from Dr. T. Kawarabata (Institute of Biological Control, Kyushu University, Kyushu, Japan). The cells were propagated at 27ºC in Grace's supplemented medium containing 10% fetal calf serum (FCS; Gibco).

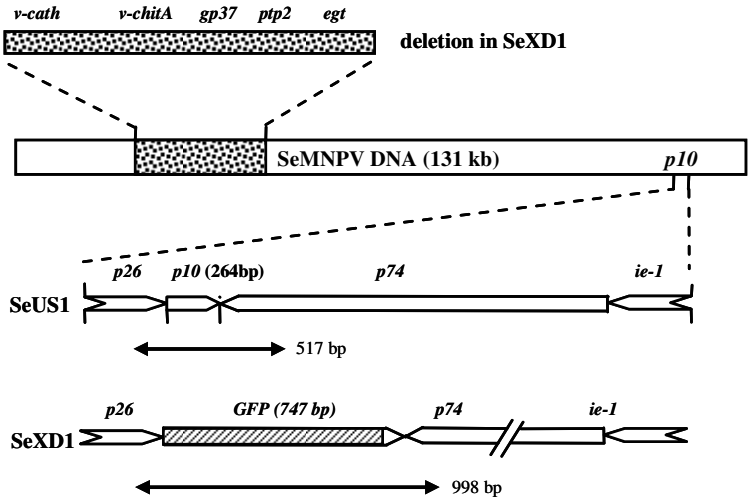

Figure 2. Schematic representation of wild-type SeMNPV (SeUS1) and recombinant SeMNPV (SeXD1). Deletion of *p10* gene and insertion of *gfp* are indicated in SeXD1 virus.

Serial transfers

Serial transfers were started by infecting 4th instar *S. exigua* larvae with mixtures of SeUS1 and SeXD1 in one of three different ratios: 5:1, 1:1 and 1:5, at a total challenge dose of 10^6 OBs per larva. This dose is 25 times the LD_{50} for this instar (Bianchi, 2001) and was administered to the larvae on diet plugs (Smits & Vlak, 1988). At least 30 larvae were used for each treatment. After the inoculated larvae had died OBs were extracted from 10 larval cadavers and purified by sucrose gradient centrifugation. The OB concentration of the viral stock solutions was determined in three independent counts using an Improved Neubauer chamber (Hawksley, Lancing, UK) by phase-contrast microscopy (× 400). Virus stocks were stored at 4°C until use. The resulting virus suspensions were used for the next inoculation through contaminated diet (passage) in *S. exigua* larvae with a dose of 10^6 OBs/larva. Only larvae that consumed the entire dose were considered. Twelve successive challenges were conducted for each SeMNPV mixture.

Plaque assay

A plaque assay in cell culture was carried out to count foci (plaques) with OBs (SeUS1 + SeXD1) and plaques showing GFP as well (SeXD1 only). BV was isolated from the haemolymph of twelve infected larvae 4 days post infection (d.p.i.). Insect haemolymph was collected in 200 µl Grace's medium and filtered using a 0.45 µm filter. The plaque-assay was performed as described by King & Possee (1992) using about 106

Se301 cells per dish. A haemolymph dilution of 10^4 or 10^5 was used to infect the cells using a 500 µl virus suspension. The plaque assay dishes were incubated in a moisted box at 27ºC for about 1 week. The plaques were analyzed and counted using light and a fluorescence microscopy. As each plaque originates from a single BV, absence of GFP means that a plaque with OBs started from a SeUS1 infection. The relative proportion of SeUS1 and SeXD1 plaques was recorded.

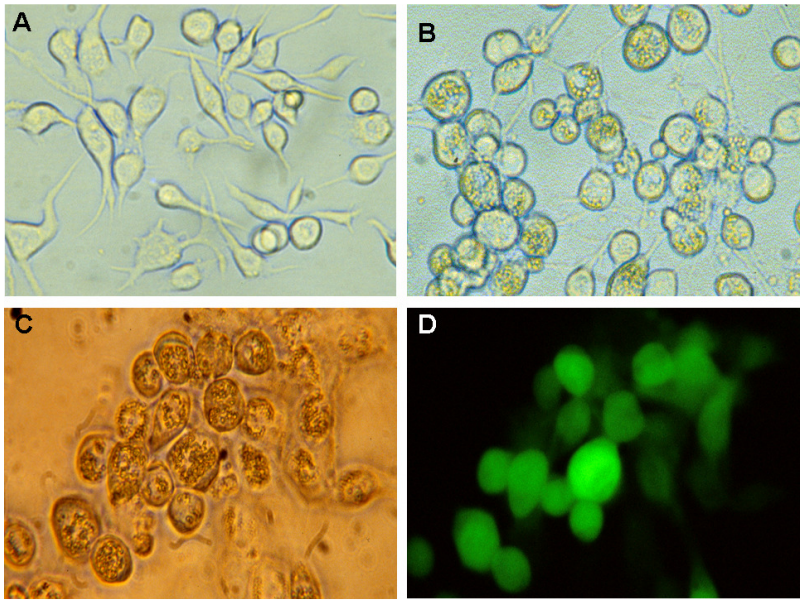

Figure 3. Phase-contrast and UV micrograph of the *S. exigua* Se301 cells. Se301 cells (A) were infected with wild-type SeMNPV (SeUS1) (B) and with SeXD1 (C and D) at 48 h p.i. OBs were observed in phase-contrast images of SeUS1- (B) and SeXD1-infected cells (C). The expression of GFP in SeXD1 infected cells is shown under UV light (D). From Dai et al., 2000.

DNA isolation and PCR

OBs were purified from 10 larvae. The DNA was isolated from OBs by incubation in a dissolution buffer (0.1 Na_2CO3, 0.01 M EDTA and 0.17M NaCl), followed by proteinase-K, phenol/chloroform extraction and dialysis (Sambrook et al., 1989). The purity and amount of the DNA was determined spectrophotometrically.

To monitor the presence of the two baculoviruses (SeUS1 and SeXD1) in the various samples after each passage, a semi-quantitative PCR was set up and the results were analyzed by agarose gel electrophoresis (Fig. 4). A PCR was performed with the Expand Long Template PCR system (Boehringer Mannheim) using forward primer p10-F (5«CGATTGGACGGACCTCTG3«) and reverse primer p10-RW (5«ATTTACGACGACAAACCAAC3«). These primers correspond to nt 123,624 to 123,642 and 124,121 to 124,141 of the SeMNPV genome (IJkel et al., 1999), respectively. This primer set has recognition sites outside the p10 open reading frame on the SeMNPV genome and gives PCR products with different sizes. This helps to distinguish the two baculovirus genotypes, with or without a p10 gene and with and without the GFP gene. For the wild-type SeMNPV genome the target gene was p10,

giving a product of 517 bp, and for the recombinant SeXD1 the gene of interest was the GFP gene, giving a product of 998 bp (Fig. 2). The GFP gene in SeXD1 has replaced the p10 gene. The following PCR program was used: denaturation at 94ºC (5min), 30 cycles of denaturation at 94ºC (40 sec), annealing at 45ºC (1 min), elongation at 72ºC (1 min) and final elongation at 72ºC (7 min) in GeneAmp PCR System 2400 (Perkin Elmer).

Restriction enzyme analysis

Fifteen µl of extracted DNA (about 1.5 µg) was digested in a total volume of 100 µl, using either *Hin*dIII or *Pst*I restriction enzymes according to the protocol as recommended by the manufacturer (Boehringer Mannheim). Digestions were performed for about 12 h in a 37ºC water bath. After digestion DNA was subjected to agarose gel electrophoresis for about 3 h in 0.5% agarose in 1 × TAE (TRIS, acetic acid, EDTA) buffer to separate the restriction fragments. The DNA was visualized using ethidium bromide in a 0.5 µg/ ml concentration. The size of the fragments, as bands on the gel, was estimated using a *Hin*dIII-restricted lambda (λ) DNA marker. After subjection to gel electrophoresis, the gel DNA fragments were analyzed for intensity using the 'Gel Pro' densitometer computer program (Muñoz et al., 2000b).

Model development

A model was developed to describe the dynamics of the competition between two baculoviruses with distinctive fitness (De Wit, 1960; Godfray et al., 1997). Following Godfray et al. (1997) we assumed that the ratio between baculovirus A (SeUS1) and B (SeXD1) evolves geometrically:

$$\frac{z(k)}{1-z(k)} = \frac{z(0)}{1-z(0)} w^k$$

where z (k) is the proportion of baculovirus A (SeUS1) after the k^{th} passage and z (0) the initial value of z. The proportion of baculovirus B (SeXD1) is 1-z(k). The parameter w is the (fixed) factor by which the genotype ratio changes upon each baculovirus passage in insect larvae. This model embodies a geometric time course of the genotype ratio, towards fixation of either the baculovirus A or B, depending upon whether w is greater than or smaller than 1, respectively. If w is not significantly different from 1, the competition between the two baculoviruses is neutral, i.e. both genotypes are equally fit. The relative proportions of SeUS1 and SeXD1 in samples, based on the plaque assays, were used as input for fitting the model.

To fit the model to data, the above equation was linearized by taking logarithms:

$$\ln\left(\frac{z(k)}{1-z(k)}\right)=\ln\left(\frac{z(0)}{1-z(0)}\right)+k\ln(w)$$

Linear regressions were conducted with SPSS v.13. A genotype ratio of 199:1 was used if one of the virus genotypes was not detected in the plaque assay..

RESULTS

Most of the larvae were successfully infected at the challenge dose of 106 OBs/larva: larvae usually died after about 6 days. Larvae infected with SeXD1 alone or with the virus mixture containing SeUS1 and SeXD1 in a 1:5 ratio usually turned black after death and showed limited liquefaction. This may be due to lack of chitinase and cathepsin genes in SeXD1 (Dai et al., 2000; Hauxwell, 1999). For each passage the OBs were used for PCR and restriction enzyme analysis, as well as for inoculum to initiate the next passage of infection. BVs were isolated from infected larvae at 3 d.p.i and subjected to a plaque assay

Plaque assay of BVs upon passage

The relative proportion of SeUS1 versus SeXD1 was determined by a plaque assay using BVs of infected larvae. SeXD1 expresses GFP giving a strong green signal (Fig. 3), which enables plaques of SeXD1 to be distinguished from those of the wild-type SeUS1. The counting of the plaques was done in two steps. First the total number of plaques that showed OBs was counted (wild-type SeUS1 and recombinant SeXD1). The second step was counting the OB-containing plaques that also showed GFP fluorescence (only SeUS1). From the total number of plaques counted the recombinant ones were deducted to generate the number of wild-type plaques (Table 1).

The proportion of SeXD1 decreases over passage (Table 1). After 6-9 passages in *S. exigua* larvae the proportion of SeXD1 approached zero. In mix 1 (SeUS1:SeXD1 = 1 : 1) the proportion of SeXD1 dropped quickly and recombinant was not observed after 6 passages. SeXD1 was still present after 9 passages, but was absent at passage 12 in mix 2 (SeUS1:SeXD1 = 1 : 5), whereas in mix 3 (SeUS1:SeXD1 = 5 : 1) SeXD1 was absent already in passage 9. Although the kinetics are somewhat irregular, the results clearly show that the proportion of SeXD1 decreases and finally disappears, independently of the proportions tested.

Table 1. The proportion of plaques formed from wild-type SeUS1 and recombinant SeXD1 virus for the three different starting ratios of mixtures of these two viruses, mix 1 (1 : 1) Se-US1 : Se-XD1, mix 2 (1 : 5) and mix 3 (5 : 1).

		Number of plaques			Proportion (%)	
mix	Passage	SeUS1	SeXD1	total	SeUS1	SeX
1	1	90	45	135	7	33
1	3	110	39	149	74	26
1	6	142	0	142	100	0
2	1	58	49	107	54	46
2	3	50	203	253	20	80
2	6	74	135	209	35	64
2	9	340	14	354	96	4
2	12	150	0	150	100	0
3	1	44	36	80	55	45
3	3	81	50	131	62	38
3	6	349	147	496	70	30
3	9	175	0	175	100	0
3	12	324	0	324	100	0

PCR analysis of viral DNA upon passage

Viral DNA was isolated from OBs produced by infected larvae upon each passage and subjected to PCR using SeMNPV primers from the flanking regions of the *p10* gene. Expected products for SeUS1 and SeXD1 are 517 bp and 998 bp, respectively.

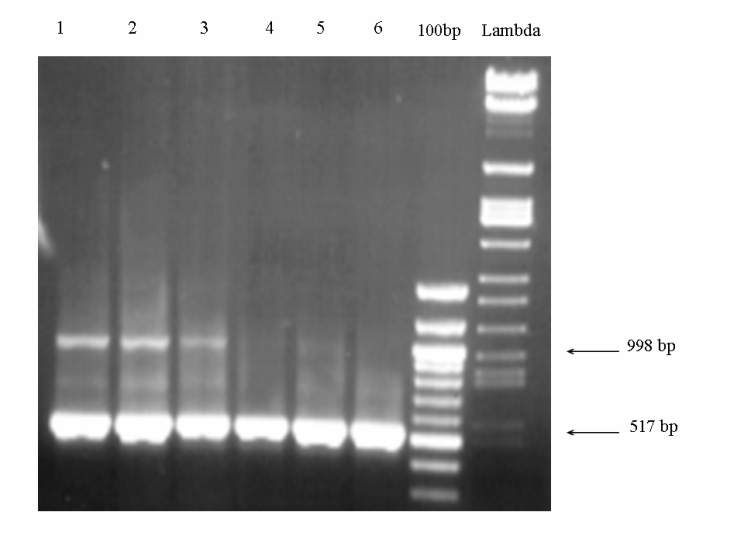

Figure 4. Agarose gel electrophoresis showing PCR results of mix 1 (SeUS1:SeXD1 = 1:1) as an example. Arrows indicate the visible bands with the given sizes. The band sizes are estimated using the 100 bp DNA ladder and λ DNA. Lane 1-6: passage 2-7, Lane 7: 100bp DNA Ladder, Lane 8: λ DNA marker.

For the mix with a virus ratio of 1 : 1 (SeUS1 : SeXD1) a p10-specific PCR product (517 bp) was present in roughly equal amounts in all passages. However, a SeXD1-specific PCR product (998 bp) gradually disappeared after 4 passages (Fig. 4). For mix 2 and mix 3 the GFP containing PCR fragments disappeared after passage 10 and 7, respectively (data not shown). These results are roughly in line with the results obtained from the plaque assay analysis presented in Table 1, indicating that the SeUS1 finally overtakes SeXD1 in a competitive situation.

Restriction enzyme analysis of viral DNA upon passage

Restriction enzyme analysis was performed to measure the relative proportion of wild-type (SeUS1) and recombinant (SeXD1) genotypes at the DNA level. The restriction digestion was performed on DNA from mix 5 : 1 (SeUS1 : SeXD1) (not shown). On the gel it was possible to visualize the expected bands (*Pst*I-C and D) and to distinguish between SeUS1 and SeXD1 by fragment intensity.

The gel was analyzed for intensity using densitometry and the 'Gel Pro' computer program. The relative proportions were calculated based on the relative presence of fragments *Pst*I-C and D (Table 2). Fragment *Pst*I-C is present both in SeUS1 and SeXD1 and fragment *Pst*I-D is only specific for the SeUS1. In SeXD1 this fragment is spliced because of the insertion of the GFP gene. On basis of the presence of both fragments the relative proportion was calculated and the results showed that the proportion of SeUS1 increases as the passage number goes up. The opposite was observed for recombinant SeXD1, where the relative proportion decreases in comparison to the SeUS1 over passage (Table 2).

Table 2. Average quantity of fragments *Pst*I-C and D from DNA isolated from the mix 5:1 (Se-US1:Se-XD1) upon digestion with the *Pst*I restriction enzyme. Gel fragment intensity (average box size of 0.8) was calculated using the 'Gel Pro' densitometer computer program.

	Relative quantity		Proportion (%)	
Passage	*Pst*I-C	*Pst*I-D	wt	Se-XD1
8	140.22	117.93	84	16
9	203.17	106.87	53	47
10	112.42	101.14	90	10
11	167.55	162.56	96	4
12	185.53	181.82	98	2

Modeling the competitive fitness

The logarithm of the ratio US1/XD1, as quantified in plaque assay, increased approximately linearly in all three mixes (Fig. 4). The slope of the regression was 0.96 ± 0.34 in mix 1, 0.54 ± 0.14 in mix 2, and 0.55 ± 0.18 in mix 5. The mean regression slope was 0.68, and the selection coefficient was quantified as exp (0.68) = 1.9.

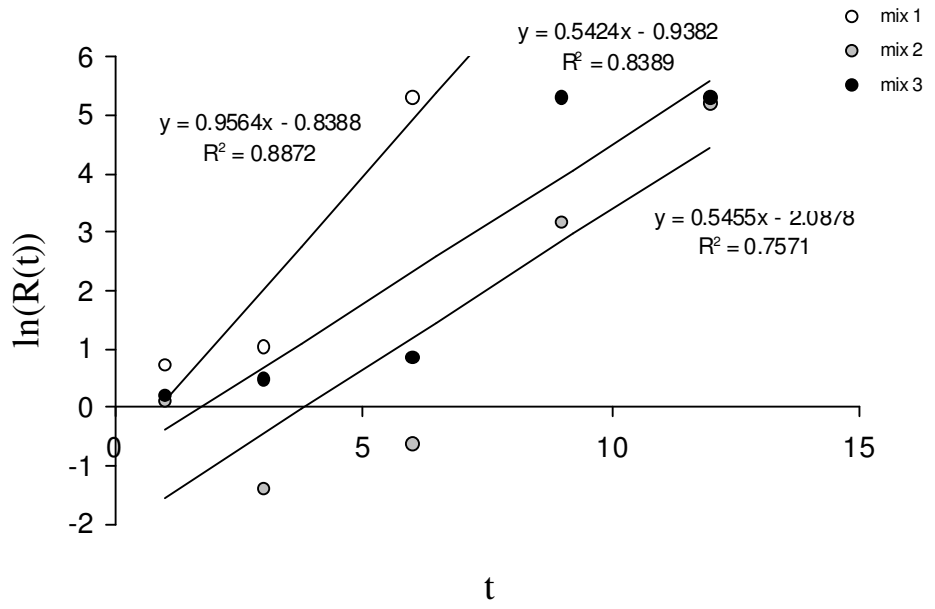

Figure 5. Relative proportion of wild-type SeMNPV (SeUS1) in serial passages of a mixture of wild-type and recombinant SeMNPV (SeXD1) in 4th instar *S. exigua*. Genotype ratios were measured by plaque-assay experiments. R(t) is the ratio US1/XD1 after passage t.

The value of w best fitting our data is 1.9, indicating that after each passage the ratio of SeUS1 over SeXD1 has increased by a factor of 1.9, signalling SeUS1 dominance upon passaging in insects.

DISCUSSION

The introduction of recombinant baculoviruses in the environment may result in their uncontrolled spread and in replacement of wild-type baculovirus populations. Information on the parameters that influence baculovirus behavior in the field may allow predictions on the behavior of recombinant baculoviruses in the environment (Vlak et al., 1995). Results from this preliminary study on the competition between wild-type SeMNPV (SeUS1) and recombinant SeMNPV (SeXD1) in mixed infections of S. exigua larvae clearly showed dominance of SeUS1 after several successive passages. This trend was noted using three independent techniques: plaque assay analysis of BVs from

infected larvae (Fig. 3, Table 1), analysis of OB DNA by PCR (Fig. 4, Table 2) and restriction enzyme analysis (densitometry). The cause of SeUS1 gaining dominance is not known. The virulence of both viruses in terms of LD_{50} is very similar (Dai et al., 2000). The genes that were deleted in SeXD1 apparently are not essential for virus infectivity and are not known to be related to baculovirus virulence in vivo (Heldens et al., 1998). It may be that SeXD1 has a replication disadvantage in the insect, although the genome itself is of smaller size (- 10.6 kb) and would therefore be expected to replicate faster.

SeXD1 also lacks the *egt* gene as part of the suite of genes that is missing from the wild-type SeMNPV genome. Our results of reduced fitness of SeXD1 as compared to SeUS1 are in agreement with those of Zwart et al. (2009), who found that an *egt* deletion mutant of AcMNPV was less fit in competition experiments with wild-type AcMNPV in *T. ni*. These findings collectively suggest that *egt* has a role in virus multiplication, spread or packaging in the insect. Lack of the *egt* gene may result in reduced budded virus production or spread in the larva as compared to SeUS1, or in incomplete packaging of occluded virions in OBs.

The outcome of this competitive fitness experiment clearly shows wild-type dominance over time and thus 'within host' competition. It is not clear whether this is at the organismal or at the cellular level. Experiments in cell culture with SeMNPV are difficult to value due to the severe 'passage effect' in *S. exigua* cells infected with SeMNPV (Pijlman et al., 2003). This effect results in the generation of defective SeMNPV that can only replicate in the presence of wild-type SeMNPV and reduce the production of BVs and OBs. This passage effect is also cell line dependent (Pijlman et al., 2003). Other cell lines may be found or developed that do not show this effect.

The results from these preliminary experiments may imply that also in the field wild-type SeMNPV will get dominance over time over a recombinant virus. This would imply that recombinant baculoviruses should be applied as mixtures with wild-type baculoviruses rather than as recombinants alone. The outcome of our experiments may also have implications for the introduction of new fast-acting 'wild-type strains' from elsewhere for biocontrol. When these new strains are selected for improved insecticidal activity, they also may displace the existing baculovirus population. However, our experiments suggest that this is not very likely to occur. These results are a good starting point for the field evaluation of recombinant baculoviruses in general and of SeMNPV in particular.

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Chapter 6

MIXED GENOTYPE INFECTIONS OF *AUTOGRAPHA CALIFORNICA* MULTICAPSID NUCLEOPOLYHEDROVIRUS IN *TRICHOPLUSIA NI* LARVAE; SPEED OF ACTION AND PERSISTENCE OF A RECOMBINANT IN SERIAL PASSAGE

Fast-acting recombinant baculoviruses have potential for improved insect pest suppression. However, the ecological impact of using such viruses must be given careful consideration. One strategy for mitigating risks of recombinant baculoviruses might be simultaneous release of a wild-type baculovirus, so as to facilitate a rapid displacement of the recombinant baculovirus by a wild-type. However, at what ratio must the two baculoviruses then be released? An optimum release ratio would ensure both fast action and the eventual fixation of the wild type baculovirus in the insect population. Here we challenged *Trichoplusia ni* larvae with different ratios of wild-type *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and a derived recombinant vEGTDEL, which has the endogenous *egt* gene (encoding for ecdysteroid UDP glucosyltransferase) deleted, and measured time to death and virus occlusion body (OB) yield. Five serial passages of three different mixtures of OBs of the two viruses were also performed. For the serial passage experiment (SPE), OBs from 10 larval cadavers were pooled and used to start the following passage. A 1:10 ratio (wild-type to recombinant) resulted in quick insecticidal action, while the wild-type baculovirus was maintained over five passages. However, the wild-type virus did not go to fixation in most replicates of the SPE, and there was no evidence for selection against the recombinant. Long term maintenance of a recombinant in serial passage suggests an ecosystem safety risk. We conclude that for assessing ecological impact of recombinant viruses, SPEs in both single and multiple larvae are relevant because of potential modulating effects at the between-host level.

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INTRODUCTION

Baculoviruses have shown potential as agents for biological control of pest insect species (Moscardi, 1999). These viruses are highly virulent (Bianchi et al., 2000b; Cory & Myers, 2003), and have a restricted host range (Federici, 1997). A major drawback of the use of baculoviruses for pest control is their slow speed of action (Moscardi, 1999). Therefore, crop damage after application of a baculovirus spray can be substantial, even if mortality in target insects is eventually high (Bianchi et al., 2000a). With the advent of recombinant DNA techniques, it has become possible to engineer baculoviruses with faster speeds of action (Stewart et al., 1991; Inceoglu et al., 2006). These fast-acting baculoviruses can provide improved protection of crops in comparison with wild-type baculoviruses (Cory et al., 1994; Sun et al., 2004b). However, the ecological impact of the release of fast-acting recombinant baculoviruses is not fully understood and deserves further consideration to avoid unintended non-target impacts on non-target insects.

Many studies have addressed different aspects of recombinant baculovirus fitness. The general tenor of these studies is that fitness of recombinant viruses is not distinguishable from that of the parental wild-type baculovirus (Bianchi et al., 2000b; Sun et al., 2004a) or reduced (Cory et al., 1994; Cory et al., 2004; Sun et al., 2005; Zhou et al., 2005). At the between-host level, genetically modified viruses are generally thought to be less fit than wild-type baculoviruses. This is due to the lower virus yield associated with shorter survival time of infected insects (Cory et al., 2004). These patterns do not necessarily extend to all susceptible species, however (Hernández-Crespo et al., 2001). In some instances insect behavior is also altered, which removes the virus-infected cadavers from plant surfaces (Hoover et al., 1995) and hereby reduces secondary transmission (Hails et al. 2002).

Two studies have addressed the within-host fitness of fast-acting recombinant baculoviruses derived from Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV). Milks et al. (2001) found that a recombinant AcMNPV expressing the scorpion-derived, invertebrate-specific toxin AaIT had unaltered within-host fitness, when in direct competition with its parental wild-type virus. Zwart et al. (2009) studied the behavior of vEGTDEL, an AcMNPV derived recombinant lacking the endogenous egt gene, and found decreased within-host fitness compared to its parental wild-type virus. Egt encodes the ecdysteroid UDP glucosyltransferase enzyme, which inactivates ecdysteroids and thereby modulates host development in a manner that results in a greater OB yield (O'Reilly & Miller, 1989; Cory et al., 2004; Zwart et al., 2009). Deletion of egt leads not only to a reduction in OB yield, but also to reduced cadaver weight and shorter time to host death (O'Reilly & Miller, 1989; Cory et al., 2004).

The available evidence thus suggests that fast-acting baculoviruses lacking the *egt* gene may be ecologically impaired at both the within-host and between-host levels, and

could be displaced by wild-type baculoviruses after release. Therefore, to hasten competitive displacement of the fast-acting recombinant, a wild-type baculovirus strain could be added to the inoculum of recombinant virus. However, can an application of both recombinant and wild-type baculovirus retain the improved speed of action of the recombinant virus, and at the same time result in the displacement of the recombinant virus in the longer term in agro-ecosystems?

Other strategies for mitigating the persistence of recombinant baculoviruses have been previously suggested. For example, Hamblin et al. (1990) suggested releasing 'co-occluded' OBs containing both a wild-type virus and a recombinant incapable of generating OBs autonomously (an AcMNPV-derived virus missing the polyhedrin gene, Ac-E10). Wood et al. (1993) and Hughes & Wood (1996) suggest releasing Ac-E10 virions (i.e. preoccluded virions) for biological control because (i) they are highly infectious, ensuring effective biological control, and (ii) they remain infectious only for short periods of time, ensuring that the virus will be quickly lost from the environment. The approach we study here releasing a mixture of OBs would be an attractive alternative because it is relatively simple, requiring only quantification of the different OBs. Moreover, because recombination between wild-type and recombinant viruses will occur in the field, understanding the competitive fitness of recombinant baculoviruses with polyhedrin is also relevant to evaluate the approaches suggested by Hamblin et al. (1990) and Hughes & Wood (1996).

Zwart et al. (2009) found that *Trichoplusia ni* (Hübner) larvae challenged with an LD_{80} dose of AcMNPV at a wild-type to recombinant ratio of 1:100 most larvae were solely infected by the recombinant virus, putatively because the founder number at an LD_{80} dose is too low to enable the presence of the wild type at such a low ratio (see Zwart et al., in press). On the other hand, at a wild-type to recombinant ratio of 1:10, all larvae contained both the wild-type and recombinant viruses at death, indicating a founder number well-above 10 virions per larva (Zwart et al., 2009). Thus, at an LD_{80} dose and a wild-type to recombinant ratio of 1:10, the wild-type virus will be present in a majority of infected insects. This inoculum fulfills one of the requirements for a strategy of use of a recombinant virus based on co-packaging of a wild-type virus in a biological control product, but we also need to know: (1) What is the speed of action at this dose and ratio? (2) Will the wild type virus go to fixation, and displace the fast-acting recombinant virus?

To answer these questions, we studied the co-infection behavior of the fast-acting vEGTDEL and its parental virus, AcMNPV Wt L1, in *T. ni* larvae. We first determined time to death for a range of different OB mixtures in a bioassay with an LD_{80}. An LD_{80} is suitable for initial evaluation of recombinant baculoviruses because medium to high OB doses will be required for effective biological control (Cory et al., 1994; Bianchi et al., 2002). A serial passage experiment (SPE) with different initial inoculum mixtures of the wild-type and recombinant viruses was then performed to test whether the wild-type virus

would outcompete the recombinant completely, and go to fixation. Alternatively, the recombinant might compete out the wild-type virus, or both viruses might be maintained in the population. Sustained maintenance of two genotypes over many generations would indicate that competition is neutral (no stronger competitor), or it could also indicate that both virus genotypes occupy somewhat different niches within the host, such that their co-occurrence is stable. Note that by 'maintenance' we mean that a genotype remains present over time in the virus population, irrespective of its frequency. By 'fixation' we mean that only a single genotype is represented in the population, and other genotypes have been competitively displaced.

In previously reported SPEs with recombinant baculoviruses, each replicate was performed in a single larva (Milks et al., 2001; Zwart et al., 2009). This experimental design focuses on the competitive process within a single host. In the field, however, a larva may ingest OBs originating from multiple larval cadavers. This is especially likely to happen when, during an epizootic, infectious larval cadavers occur at high densities. The two situations passaging in a single larva or passaging in ten larvae probably represent extremes compared to what may happen in the field. We chose to perform an SPE with inoculum preparation from multiple larvae at each passage. This approach complements previously reported data, as the ensemble of previous results and those reported here bracket the relevant range of the number of OB sources for insect larvae.

MATERIALS AND METHODS

Insects and viruses

Trichoplusia ni larvae were reared as previously reported (Zwart et al., 2009; see also Smits et al., 1986). Larvae were reared communally on artificial diet based on wheat germ, in plastic boxes with a paper towel as a lid. Diet composition was identical to that described by Smits et al. (1986), except that cornmeal was replaced with wheat germ. Larvae were allowed to spin cocoons and pupate on the paper towel lids. Moths were kept in cages, with paper towels placed along the sides for egg-laying. Paper towels with eggs were collected daily and surface sterilized as described by Smits et al. (1986). Eggs were occasionally kept at 4°C or 16°C for one or two days, in order to keep the insects in a regular rearing scheme. Larvae, pupae and moths were kept with a 16-hour photoperiod and at 27°C.

AcMNPV Wt L1 and vEGTDEL (O'Reilly & Miller, 1991) were amplified in 30 *T. ni* L4 larvae, inoculated at a high dose (approx. 100 × LD_{99}). Larval cadavers were collected upon death, stored at –20° C, and OBs were subsequently purified as described by Zwart et al. (2008). Briefly, larval cadavers were macerated, filtered through cheesecloth, centrifuged (2500 *g* for 15 min) washed thrice and then stored in 50% (v/v)

glycerol. Note that all virus ratios whether quantified OBs or quantitative real-time PCR ratios are given as the ratio Wt L1 to vEGTDEL.

Time to death bioassays and OB yield

We obtained developmentally synchronous final instar *T. ni* larvae (L5) by selecting insects which had slipped their head capsules within a 12-h interval. The chosen larvae were subsequently reared on diet at 27° C for a further 24 h, and then challenged with OBs. The artificial diet used for rearing purposes was also used for bioassays.

We quantified OBs of Wt L1 and vEGTDEL by counting with a haemocytometer (20 counts per virus). The OBs were subsequently serially diluted in water to give suspensions of 10^6 OBs ml-1. Two µl of the OB suspension was pipetted onto small diet plugs placed in 12-well plates. This should give a dose of 2000 OBs per larvae, which is equivalent to an LD_{80} for a 1:1 mixture according to dose-response data reported by Cory et al. (2004). The following OB suspensions were used: vEGTDEL alone, 1:1000 (Wt L1 to vEGTDEL), 1:100, 1:10, 1:1, 10:1 100:1, 1:1000, and Wt L1 alone. For a non-virus control 24 larvae were taken and 2 µl water added to the diet plug. For each of the 9 virus treatments 36 larvae were used. After 12 hours at 27°C those larvae which had eaten all the diet were individually transferred to new 12-well plates containing diet, and again kept at 27°C. The amount of diet added was usually sufficient to rear the larva to pupation, but more diet was added if necessary. Mortality was recorded every 12 hours until all larvae had died or pupated. The cadavers of dead larvae were collected and individually stored at -20°C in 1.5 ml Eppendorf tubes. The bioassay was performed thrice. For each replicate of each treatment mean time until death was calculated. A Jonckheere-Terpstra test was used to determine if more Wt L1 OBs in the inoculum mixture resulted in a longer mean time to death. This test is applicable because it is a non-parametric procedure and the independent variable (treatment) is ordinal (i.e. increasing amounts of Wt L1 OBs). Tukey's HSD, a post hoc multiple comparison test, was used to test for significant differences in mean time to death. All statistical analyses were performed in SPSS 15.0 (SPSS Inc., Chicago, IL).

A second bioassay was performed as described above, but with only two treatments: vEGTDEL alone and a 1:100 mixture of OBs. Five replicates of the bioassay were performed. Mean time until death was calculated and significant differences between treatments tested for with a pair-wise t-test.

All larvae which died in the first bioassay replicate were used for analyzing larval OB yield. Larval cadavers were macerated in 500 µl milliQ water. Counting of OBs with a haemocytometer was performed twice for a 1:100 dilution of each sample. Analysis of variance (ANOVA) was performed on square-root transformed OB yield, and between-treatment variation was partitioned into linear and quadratic components. Tukey's HSD

post hoc multiple comparison test was used to test for significant differences between treatments in OB yield.

Serial passage experiment with OB mixtures

A serial passage experiment (SPE) was performed with three different starting mixtures of OBs: 1:10, 1:1 and 10:1. Three replicates with a 1:1 starting mixture were performed; two replicates were taken for the 1:10 and 10:1 initial mixtures. Five serial passages were performed. Twenty-four larvae were challenged per replicate, with the same procedure and dose (2000 OBs) as described above. All larval cadavers were individually collected at death and stored. A random selection of 10 larvae for use in the next passage was then made. The 10 selected cadavers were pooled in 50 ml plastic tubes, and used for OB purification as described above. The concentration of OBs was then determined by counting in a haemocytometer (at least 2 counts per replicate). The next round of passage was then initiated with a dose of 2000 OBs. As a control the Wt L1 and vEGTDEL were passaged in a single larva.

DNA isolation and qPCR

For each replicate and every passage the Wt L1 to vEGTDEL ratio in OBs of the pooled larvae was determined by quantitative real-time PCR (qPCR). DNA isolation from OBs was performed as previously described (Zwart et al., 2008). All qPCR reactions and analyses were performed as described by Zwart et al. (2009), with all qPCR reactions being performed twice. In summary, specific primers for Wt L1 and vEGTDEL were used in separate PCR reactions, with SYBR Green I used as a fluorophore. Template concentration was then determined by comparative analysis, using RotorGene 6.0 software (Corbett Research, Sydney) and the virus ratio (Wt L1 to vEGTDEL) could then be calculated.

Calculation of selection rate constants

A model was developed to describe the dynamics of the competition between two baculoviruses with distinctive fitness (De Wit, 1960; Godfray et al., 1997). We assumed that the ratio between genotypes evolves geometrically over passages:

$$\frac{z(k)}{1-z(k)} = \frac{z(0)}{1-z(0)} w^k$$

where z(k) is the proportion of wild-type after the kth passage and z(0) the initial value of z. The proportion of recombinant is 1-z (k). The parameter w is the fixed factor by which the genotype ratio changes upon each baculovirus passage in insect larvae. This model embodies a geometric time course of the genotype ratio, towards fixation of either

genotype, depending upon whether w is greater than or smaller than 1. If w is not significantly different from 1, the competition between the two baculoviruses is neutral (i.e. both genotypes are equally fit). The relative proportions of wild-type and recombinant, determined from the qPCR-derived virus ratio, were used as input for fitting the model. The qPCR assay used cannot quantify virus ratios of less than 1:1000 and greater than 1000:1 (Zwart et al., 2009) and the model does not allow for fixation of either virus. We therefore considered all data less than 1:1000 or greater than 1000:1 including only vEGTDEL or Wt L1 signal to be a ratio of 1:1000 or 1000:1, respectively.

To fit the model to data the above equation was linearized by taking logarithms:

$$\ln\left(\frac{z(k)}{1-z(k)}\right)=\ln\left(\frac{z(0)}{1-z(0)}\right)+k\ln(w)$$

Linear regressions were conducted with SPSS 15.0, combining replicates of each initial inoculum mixture (1:10, 1:1, 10:1). Following analysis the selection rate constant w was estimated by taking the antilogarithm of the slope of the regression. Based on the chain rule of mathematical calculus:

$$\mathrm{SE}\left(f\left(x\right)\right)=\frac{\mathrm{d}f}{\mathrm{d}x}\mathrm{SE}\left(x\right),$$

the standard error of w is calculated by multiplying the standard error of the slope of the regression with the estimate of w:

$$\mathrm{SE}\left(w\right)=\mathrm{SE}\left(\exp\left(b\right)\right)=\frac{\mathrm{d}}{\mathrm{d}b}\exp\left(b\right)\cdot\mathrm{SE}\left(b\right)=\exp\left(b\right)\cdot\mathrm{SE}\left(b\right)=w\cdot\mathrm{SE}\left(b\right)$$

where b is the slope of the regression, SE(b) is the standard error of this slope, and w the selection rate constant.

RESULTS

Mean time to death for OB mixtures

In order to understand how virus mixtures affected time until death, bioassays were performed and mean time until death determined (Figure 1). A Jonckheere-Terpstra test indicated that mean time until death increased significantly as the amount of Wt L1 virus was increased (Standardized JT = 4.536, N = 27, $P < 0.001$). Tukey's post hoc multiple

comparison test showed that treatments with more than a 1:1 (Wt L1 to vEGTDEL) ratio of the viruses killed the host significantly slower than the vEGTDEL alone (Figure 1). Different OB mixtures caused similar levels of mortality (Kruskal-Wallis test; $\chi^2 = 2.134$, degrees of freedom = 8, P = 0.977) and there was no significant effect of the proportion of Wt L1 virus on mortality (Jonckheere-Terpstra test; Stand. JT = -0.42, N = 27, P = 0.966).

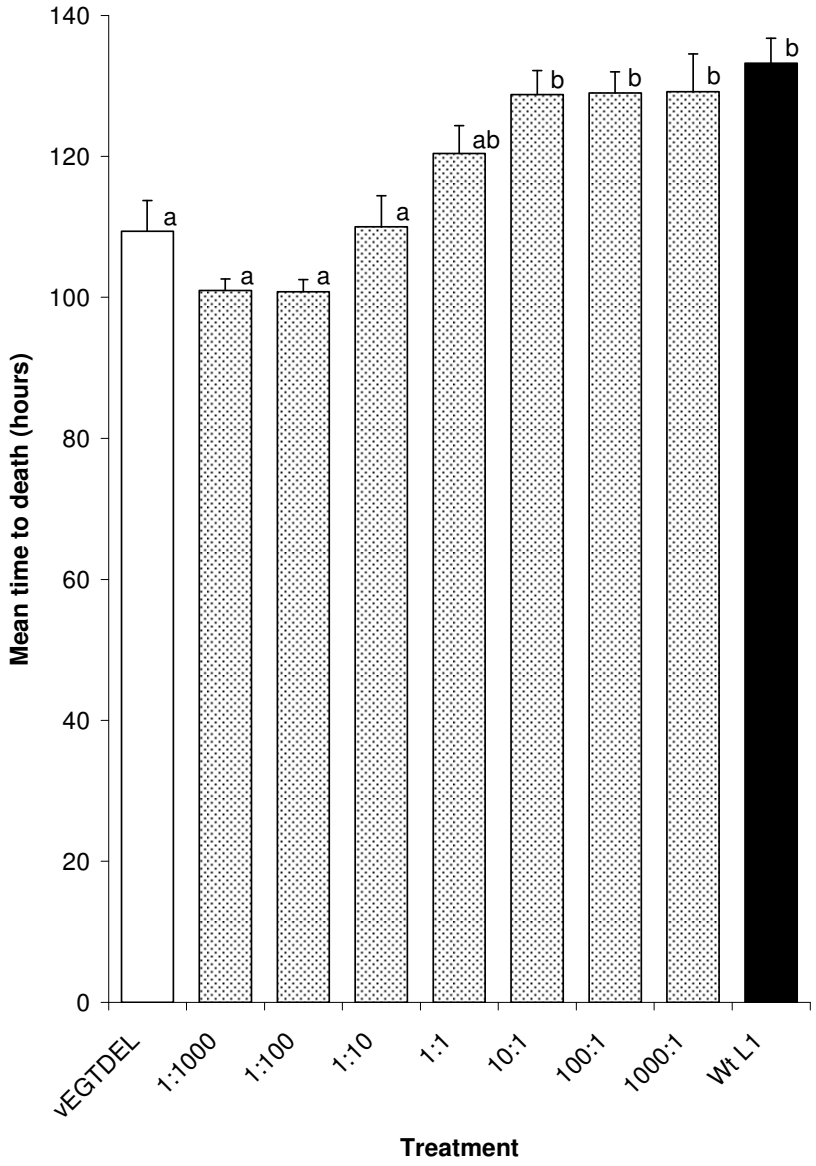

Figure 1. Mean time until death in hours is on the y-axis (with standard errors), for different bioassay occlusion body (OB) mixtures (dotted bars), vEGTDEL alone (white bar), and Wt L1 alone (black bar), on the x-axis. All ratios for OB mixtures are given as Wt L1 to vEGTDEL. Tukey's post hoc multiple comparison test was used to test for differences between treatments. Treatments that are not significantly different from the vEGTDEL treatment are marked with an 'a'. Treatments that are not significantly different from the Wt L1 treatment are marked with a 'b'. Note that for all significant differences P-values were smaller than 0.01.

Trends in the data (Fig. 1) suggest that the 1:1000 and 1:100 ratios may be killing larvae faster than the vEGTDEL virus alone, but differences were not significant with a post hoc test. A second bioassay comparing time to death between vEGTDEL alone and with a 1:100 mixture of wild-type and vEGTDEL was therefore performed. No significant difference in mean time until death was found (pair-wise t-test, t4 = -0.911, P = 0.414), confirming that the speed of kill of pure recombinant virus and mixtures with a small amount of wild-type virus is not appreciably different.

OB yield was determined for all larval cadavers from the first replicate of the mean lethal time bioassay (Figure 2). A one-way ANOVA indicated that there are differences in mean between inoculum OB mixtures ($F_{8, 239} = 2.541$, $P = 0.011$), although none of the OB mixtures differed significantly from the Wt L1 or vEGTDEL only conditions according to a post hoc test (Figure 2). There were statistically significant linear ($F_{1, 239} = 7.491$, $P = 0.007$) and quadratic ($F_{1, 239} = 6.095$, $P = 0.014$) components in the between-treatment variation for OB yield.

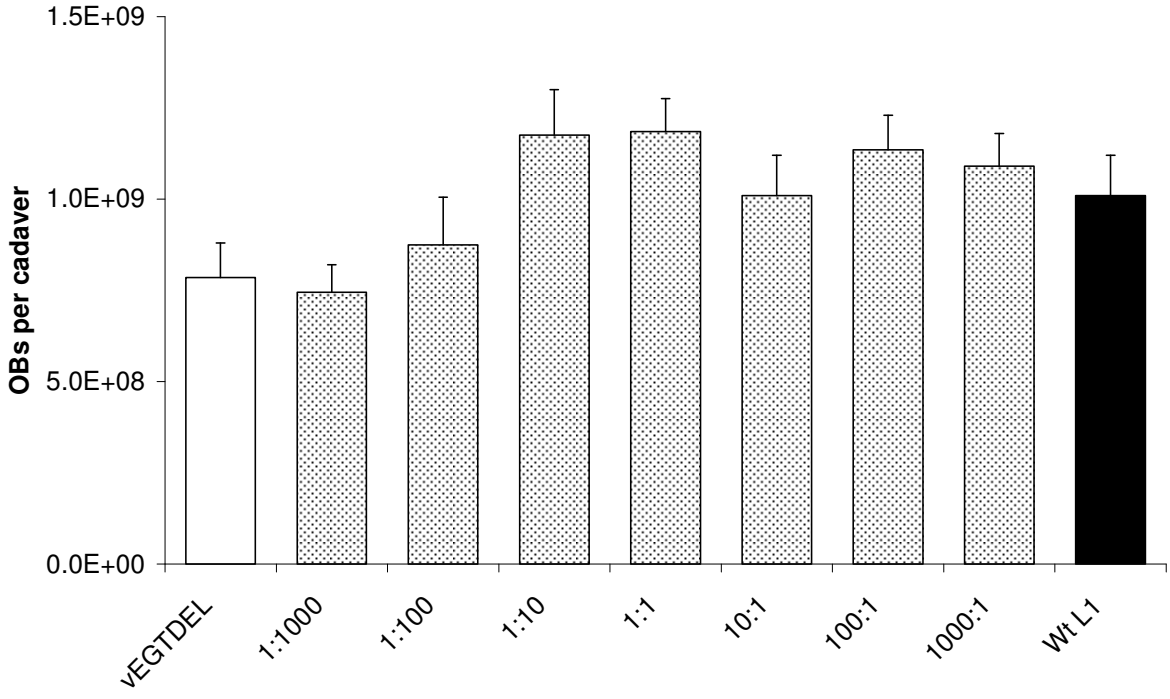

Figure 2. Mean occlusion body (OB) yield per cadaver is on the y-axis (with standard errors), for different bioassay occlusion body (OB) mixtures (dotted bars), vEGTDEL alone (white bar) and Wt L1 alone (black bar), on the x-axis. All ratios for OB mixtures are given as Wt L1 to vEGTDEL. None of the mixtures differ significantly from either single virus condition (Wt L1 or vEGTDEL alone), according to Tukey's post hoc multiple comparison test, but ANOVA showed significant linear and quadratic components in the response of yield to genotype ratio.

Serial passage experiment results

Mixtures of Wt L1 and vEGTDEL were serially passaged in *T. ni* L5, with an OB inoculum from 10 pooled larvae used for the next round of passage. The qPCR derived ratio of Wt L1 to vEGTDEL was determined for each replicate for every passage (Figure 3). Note that Zwart et al. (2009) previously reported that the Wt L1 produces larger polyhedra with 83% more virions than vEGTDEL. Hence, although a 1:1 mixture of OBs of the two viruses was used to start the experiment, the starting ratios of virions are biased towards the Wt L1 virus. When the initial OB ratio was 10:1, Wt L1 went to fixation in one of the two replicates, while in the other replicate both viruses were maintained. In all replicates starting with a 1:1 or 1:10 ratio, both viruses were maintained over five passages. Calculated selection rate constants (w) were not significantly different from 1 for any inoculum ratio (for 10:1 ratio: w = 1.65 ± 0.65; for 1:1 ratio: w = 1.00 ± 0.08; for 1:10 ratio: w = 1.05 ± 0.11; mean ± SE given).

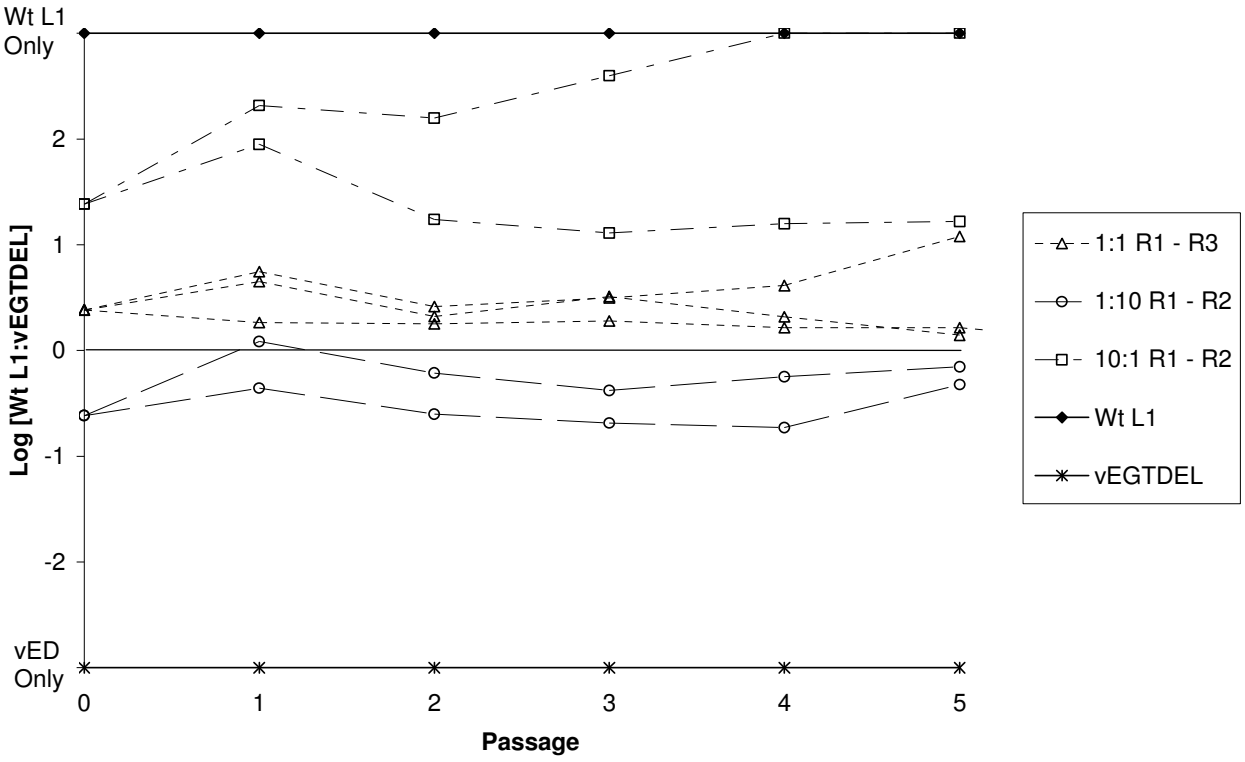

Figure 3. qPCR data of serial passage experiments. Within each treatment different replicates are denoted as R1-R2 or R1-R3. On the x-axis is the passage number, and on the y-axis the base 10 logarithm of qPCR ratio (Wt L1 to vEGTDEL) measured in ten pooled larval cadavers used for passaging.

DISCUSSION

We investigated the behavior of vEGTDEL, an AcMNPV-derived recombinant baculovirus, in mixed infections with its parental wild-type virus, Wt L1. We specifically considered what ratios of Wt L1 to vEGTDEL would result in (1) faster speed of action as compared to the wild-type, and (2), whether introduction of the wild-type virus ensured maintenance and eventual fixation of the wild-type virus in infected larvae, over multiple passages. Speed of kill is an indicator for biological control efficacy (Cory et al., 1994; Sun et al., 2004b) while fixation of the wild-type in serial passage is an indicator for ecological safety. A 1:10 mixture gave a time until death similar to vEGTDEL alone, differing in mean by only 2 hours, which was not a statistically significant difference (Figure 1). Zwart et al. (2009) previously demonstrated that with this ratio and dose, the Wt L1 virus will be present in all infected larvae. Therefore, a 1:10 mixture appears to be an optimum ratio for field applications of the two viruses used here; it pairs rapid speed of kill with the maintenance of the wild-type virus in infected larvae. However, will the wild-type virus then also go to fixation and displace the recombinant virus? A serial passage experiment was performed with different initial inoculum ratios (1:10, 1:1 and 10:1). Although the wild-type virus was maintained in all seven replicates, the wild-type virus went to fixation in only one replicate (Figure 3). Selection rate constants were not significantly greater than 1 at any of the initial inoculum ratios. We therefore conclude that while the wild-type virus is maintained when an inoculum mixture of 1:10 is used, there is no evidence that the wild-type virus is selected for or that it will go to fixation.

We previously found that there is within-host selection against vEGTDEL in an SPE in passage experiments in single larvae (Zwart et al., 2009). On the contrary, in the SPE with inoculum from pooled larvae, reported here, there is no sign of selection for the wild-type virus. The main difference in the experimental setups is the number of larvae used for passaging. How could the use of multiple larvae for producing inoculum nullify the selection for wild-ype virus observed in single larva passages? The SPEs in single and multiple larvae represent highly different experimental designs: when passaging in single larva virus genotypes compete only within the host; however when passaging in multiple larvae there is also between-host competition between virus genotypes.

One important factor in between host-competition is OB yield and previous experimental data (Zwart et al., 2009) suggest that intermediate mixtures may produce higher OB yields in *T. ni* L5 larvae. OB yield data obtained here (Figure 2) had a significant negative (i.e. downward curving) quadratic component in the between-treatment variation, indicating that intermediate mixtures may produce a higher OB yield. These observations may explain why there is little or no selection for the wild-type virus when passaging in multiple larvae: the within-host selection for the wild-type virus is

counterbalanced by higher OB yields for intermediate mixtures. Maintenance of both genotypes in the majority of SPE replicates could indicate that the virus genotypes occupy somewhat different niches within host larvae, congruent with a subtle but significant increase in virus yield at intermediate genotype ratios. Although this explanation requires further testing, the results indicate that selection for the wild-type virus in the field may not be as strong as suggested by our SPE in single larvae (Zwart et al., 2009), because larvae may typically ingest OBs originating from multiple cadavers. The exact patterns of baculovirus horizontal transmission are therefore relevant for evaluating the ecological safety of fast-acting recombinant baculoviruses, but there have been few theoretical or experimental studies which have addressed this topic.

We have conducted our experiments with a fixed dose (LD_{80}) in L5 larvae, which are more resistant than earlier instars (e.g. Bianchi et al., 2000b; Cory et al., 2004). This means that the infection probability per OB is the lowest in L5 larvae and that when exposing larvae of all instars to the same OB dose, the average number of viral founders of infection will be the smallest in L5 larvae, and higher in all the other instars. Co-infection with the wild-type virus should be expected in all treated stages, when the dose is high enough to give high frequency of co-infection in the least susceptible instar.

We have shown that mixtures of a wild-type and a fast-acting recombinant baculovirus may be advantageous for biological control. Our results show that in laboratory settings the OB ratio can be optimized for achieving fast speed of action and the maintenance of the wild-type virus. However, our SPE results do not provide evidence that the wild-type virus will go to fixation in due course. On the contrary, results obtained here point to long term maintenance of a deletion mutant of AcMNPV in serial passages, even though the same recombinant was outcompeted by wild-type AcMNPV in previous passage experiments with single larvae. Thus, selection for the wild-type virus may depend on the number of larvae used for passaging. Patterns of baculovirus transmission in the field are not clear, and are likely to vary depending on host density. Both SPE results from single or multiple larvae must therefore be given consideration. Although optimization of OB ratio could result in both efficient pest control and maintenance of the wild-type virus, there remain concerns for ecosystem safety, as the recombinant is also maintained if larvae typically ingest OBs originating from multiple larval cadavers. This means that the approach of mixing OBs from a wild-type and fast-acting recombinant is not in itself a satisfactory alternative to previously suggested strategies for mitigating the persistence of recombinant baculoviruses (Hamblin et al., 1990; Hughes & Wood, 1996). However, our approach could be combined with that of Hamblin et al. (1990): one could generate co-occluded OBs containing a 1:10 ratio of wild type virus and a fast-acting recombinant virus missing the polyhedrin gene. High in vivo multiplicity of cellular infection (Bull et al., 2001, 2003) ensures that such co-occluded OBs could even be generated in insect larvae. The resulting OBs would presumably be fast acting, while the

deletion of polyhedrin would further reduce fitness of the recombinant virus. Overall, the results presented here indicate that processes at the within-host and between-host levels co-determine the outcome of competition between insect virus genotypes in agro-ecosystems. Both levels of competition need to be considered in an ecological risk assessment for the use of genetically engineered virus in the biological control of insects.

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Chapter 7

GENERAL DISCUSSION

Lepidopteran insect pests are limiting factors in the successful cultivation of agricultural crops. One of the main principles of applied ecology over the last decades has been the natural control of populations of economically damaging insect species, particularly agricultural and forest insect pests, using natural control agents such as parasites, predators and pathogens. This in addition to the more conventional methods of chemical and integrated control using synthetic pesticides sometimes in combination with biological control agents. More recently transgenic crops are used that express one or more insect toxins, derived from *Bacillus thuringiensis*, which protect the crop effectively (Cannon, 2000). Extensive use of chemicals resulted and still results in the problem of resistance development in insects, in chemical waste and environmental decay, and cause human health risks. The evolution of resistance by insect and weed pests to commonly used chemical pesticides and to transgenic crops is a problem of increasing importance worldwide (Brattsten et al., 1986).

The future perspective in biological control of crops is to develop a more effective approach to control phytophagous insects. Baculoviruses are associated with insects living in different habitats, on different plants, in and on soil, and in fresh water. Baculovirus diseases are widespread in insects and often reduce insect populations by causing epizootics. A number of baculoviruses are commercially available on the market (Chapter 1) for inundative and inoculative control of pest insects. Control using baculoviruses is compatible with other insect control strategies such as biological and integrated control. However, they have a few drawbacks, such as slow speed of action, limited biological activity, UV sensitivity and a limited host range. Baculovirus recombinants have been engineered for improved insecticidal properties and tested in the field for their efficacy, but there is concern about their biosafety. In-depth analyses have been carried out on environmental impact of these genetically-modified baculoviruses in nature and to what extent they comply with safety regulations.

A major question is to what extent genetically-engineered baculovirus are able to transfer the transgene into another host. This possibility is related to the chance of encounter and related to the survival of the recombinant baculovirus in the ecosystem. Long term presence of recombinant insect viruses depends on their ecological fitness in comparison with wild-type viruses. The hypotheses presented in this PhD thesis require,

in order to be tested, basic information to predict what will be the consequence of mixed baculovirus infections in insect populations, when genetically modified baculoviruses are used as biocontrol agents. Different experimental settings and approaches in laboratory, glasshouse and in the field are used to obtain quantitative insight in components of competitive fitness (Figure 1). The work especially concentrated on the fate of the released recombinant baculovirus when it comes into contact with naturally existing wild-type genotypes.

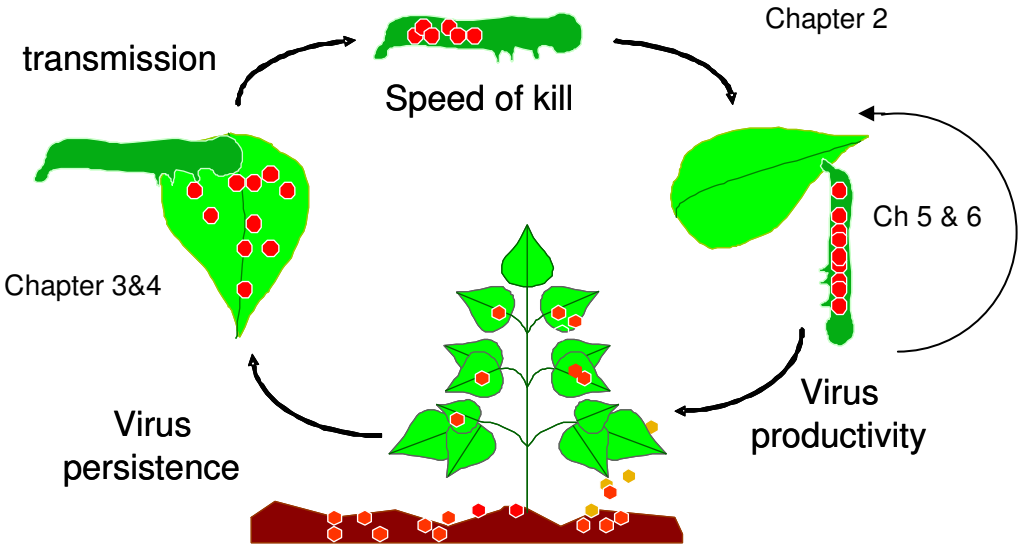

Figure 1. Schematic representation of the Chapters in the thesis.

Mixed infections

Multiple infections of the same host by different microparasite strains are commonly observed in natural populations (Taylor et al., 1997, Lipsitch & Moxon, 1997). Infection of one host by multiple pathogen genotypes is an important force in disease ecology and evolution. Recently, experimental and theoretical studies have provided more information on the mechanisms and conditions that could influence coexistence or exclusion of pathogens (Reed & Taylor, 2001). The interaction between different virus genotypes can only be fully appreciated if it is studied at multiple levels, within insects, as well as in insect populations in the laboratory, or in the field. There is relatively little information on the behaviour of mixed infections and how these affect the fitness of baculoviruses and their ecological impact upon release into the environment.

The work described in this thesis focuses on mixed infections of different baculovirus genotypes in a range of virus-host systems. Studies were made at the individual insect level (intra-host competition) and at population level (crop level or laboratory trials with insect cohorts). The overarching aim was to obtain insight in the ecology of mixed genotype baculovirus infections, and particularly in competitive

interactions between coinfecting baculovirus genotypes within mixed infected hosts, and the consequences of these interactions for virus cycling and persistence of genotypes in agro-ecosystems.

Theoretical studies of the pathogen interactions have emphasized the role of pathogenicity, transmission and interference in determining the outcome of the competition between the pathogens (Bremermann & Pickering, 1983; Hochberg, 1998; Ebert, 1998; May & Nowak, 1994; 1995). Invertebrate pathologists have multiple definitions for the term pathogenicity and virulence. According to Thomas & Elkinton (2004), virulence is defined as a severity of disease manifestation in infected individuals. Median time to death of an infected host (LT_{50}) is a measure of virulence. Pathogenicity is the ability of a pathogen to produce infectious disease in an organism. Pathogenicity includes transmission. It is characterized by median lethal dose, LD_{50}, and is measured in dose response bioassays.

Models of multiple infections fall into two classes. One class allows coexistence within the host under exploitative competition (coinfection models, May & Nowak, 1995). The second class is interference competition, where resident pathogens may be rapidly replaced by a superior genotype (superinfection models May & Nowak, 1994). There are a number of models of multiple co-infections (Bremermann, & Pickering, 1983; Ebert, 1998; Briggs, & Godfray 1995, Bell et al., 2001, Bonsall, et al., 2005), but there is lack of empirical evidence for the outcome of the competing pathogen genotypes at the population level.

In this study was addressed the issue of multiple infections among others with two biologically different, but near isogenic baculovirus genotypes (*Helicoverpa armigera* SNPV = HaSNPV) at the population level on cotton (Chapter 3 and 4). Two approaches were used to understand the dynamics of multiple infections by HaSNPV in this system. First, the behaviour of the larvae infected with a mixture of the both viruses was investigated. Secondly the virus transmission was studied under glasshouse and field experimental situations. The results indicate that larvae infected with HaSNPV–LM2 do not have major difference in behaviour in comparison with the wild type infected larvae. In contrast, the AaIT-recombinant induces modified behaviour in the infected insects: they drop off the plants. This together with lower yield can contribute to reduced virus transmission and lower virus fitness under field conditions. Larvae that died from mixed infections of this recombinant and the wild-type virus died at intermediate positions compared to those that died from either of the pure viral strains.

Insect virus fitness in an ecosystem depends on virus transmission and behaviour and these responses are influenced by survival time and virus yield. Therefore also data on a number of biological parameters were collected which might be important in determining the impact of within-host competition and competition at the population level. Chapter 2 and 3 measure properties of host –virus interactions (at insect level),

which may be relevant for competition at the population level. We also assessed the effect of dose (inoculum size) on mixed infections. It was found that the HaSNPV genotypes differ in survival time and OB yield. Later killing of the host is correlated with a higher production of the transmission stages (OBs). A wild-type genotype, with a lower virulence (e.g. a longer ST_{50}), is superior in terms of yield production in single infections, so this genotype will have an advantage in case when there will be a low incidence of multiple infections.

Since the fitness of most pathogens depends on horizontal transmission from host to host, this issue was addressed in Chapter 4. Our interest was to determine the influence of density of infectors and time post virus release on the plant, particularly in horizontal transmission of mixed baculoviruses. Here we demonstrated a few important findings related to the transmission of the virus depended on the virus genotype. Transmission increased with density of infector insects and decreased with time lapsed since the inoculation of the infector larvae. The genetic modifications in HaSNPV-4A influencing survival time and yield were positively correlated with reduced transmission. Effects on transmission were not observed in HaSNPV-LM2, a recombinant with a deletion of the *egt* gene but without an additional transgene. Results of quantitative PCR indicate that the wild-type virus dominates in the recipient insect hosts, suggesting that the wild-type virus was transmitted more successfully than recombinants from mixed infected infector larvae to the next generation of insects. This suggests that a recombinant will be competitively displaced over time. Furthermore, comparison in persistence of biological activity of wild-type, genetically modified and mixtures of both baculoviruses in soil was performed in the field. Our result showed that HaSNPV-wt and HaSNPV-LM2 (lacking the *egt* gene) and the mixture of these two baculoviruses are equally persistent in the soil. However, decrease of the amount of virus present in the soil over time was indicated.

Generally, host-pathogen interactions have been identified experimentally, in terms of pathogenicity, sublethal effects, disease transmission and so on, typically determined from short-term studies (a single generation). In order to evaluate the true impact of pathogens on host population dynamics, however, insect-pathogen relationships need to be studied continuously over multiple / many generations. Within-host dynamics is an important level of interaction between genotypes. Virus strains competing for a shared limited resource are in a “tragedy of the commons” situation. The expected situation is that the outcome of this competition will be that the strain with the highest growth rate will outcompete slower growing strains at the within-host level. These experiments mimic a scenario where viruses with different genetic properties co-infect the same host. In both cases prior to serial passaging of the viruses several larvae were pooled together.

The most successful competitor is the one that most effectively exploits shared resources. Chapters 5 and 6 have a different outcome from serial passage experiments. In Chapter 5 there is a competitive replacement of the recombinant with the wild-type virus,

which was not observed in Chapter 6. In Chapter 5 it was found that a fast acting recombinant virus (*Spodoptera exigua* MNPV recombinant Se-XD1) exhibits reduced 'within host' competitive fitness, i.e. at within host insect level. Since most of the experimental models predict that the genotype which has higher fitness (higher reproduction, speed of kill etc) will be more competitively fit and will overcome the wild-type baculovirus. The latter is bigger in DNA size and therefore requires more time for replication and thus has slower speed of action. However, our findings have a different outcome and the wild-type SeMNPV was found to be a superior competitor, despite a larger genome than the recombinant SeXD1. It has been reported that the expression of a heterologous marker gene (*β-gal*) leads to the reduced fitness of AcMNPV in mixed infections with wild-type AcMNPV in cell culture (Huang et al., 1991). The presence of the GFP marker gene in Se-XD1 might also influence the 'within-host' fitness of this virus since was proven that the marker genes are not neutral. Thus the findings suggest that under the experimental conditions recombinant Se-XD1 is displaced by the wild-type strain SeUS1, over successive passages.

To further explore and understand the competition between wild-type and genetically modified baculovirus genotypes in mixed infections, experiments were performed with wild-type and recombinant AcMNPV in multiple insect host generations in 5th instar *Trichoplusia ni* larvae, (Chapter 6). Continuous presence of two genotypes, wild-type and the recombinant, after several passages in the insect host indicates that the recombinant virus may be maintained over time in an infect population over many generations. In one serial passage line, started with a recombinant : wt ratio of 1 : 10, the wild-type virus eventually displaced the recombinant genotype completely. The findings in Chapter 6 contrast with those in Chapter 5, where the wild type virus was a superior competitor that displaced the recombinant virus. This might be related to a difference in the cell culture 'in vitro' in Chapter 5 versus a more complex environment in the insect body 'in vivo' in Chapter 6. In contrast, Zwart et al. (2009) previously found that an *egt*-deletion mutant of AcMNPV was outcompeted in serial passage experiments using inoculum from single infected larvae to infect the next generation of larvae. The explanation for such a difference in outcome, even though the same virus genotypes and insects were used in performing the two sets of experiments, might be a difference in methodology.

Conclusion and further perspectives

The results presented in this work are a further step in understanding the behaviour of insects mixed-infected with baculoviruses. However, in our case we always release the insects on a certain position of the plants, but in the future it might be interesting they be placed on the plastic container and are free to wander and search on the plant, because it was reported that there is learning process involved in the *H. armigera* (Cunningham,

2004). Further epizootical studies on the influence of the mix infection on the behaviour of the larvae movement should include also the different instars of the *H. armigera* larvae, because from recent studies there were indications that the distribution of the larvae in various instars is different (Vascancelos,1996).

Using the fast-acting HaSNPV-4A genotype in glasshouse transmission experiments confirmed the importance of the integration of several parameters involved (speed of kill, production of OBs), which are responsible for a difference in the performance of the various baculovirus genotypes. Also a question, which was not experimentally approached in this thesis, is the possibilities in future studies to include wild-type HaSNPV in mixed infections along side with the faster killing virus HaSNPV-4A in a field setting. This latter virus has better biocontrol properties than wild-type HaSNPV in terms of speed of kill, reduced yield, and reduced transmission in the co-infection studies in a field setting. In our field studies we have been able to investigate the host-pathogen interaction in a contained environment (cages), which is an approximation of the real situation. However, open field experiments are needed since many other factors play a role in insect-pathogen interaction and dynamics.

Our results from the QT-PCR analysis on the DNA isolated from single larva initially infected with both viruses in equal ratio, showed that the wild type is taking over the recombinant virus due to this results we assume that this virus has a lower fitness.

There is potential to further broaden our understanding of mixed infections by further empirical and theoretical studies. In the current study, the larvae were always challenged simultaneously, and we did not try sequential (asynchronous) infection of the larvae. Milks et al. (2001) found that the wild-type AcMNPV and genetically modified AcMNPV-AaIT are equally fit during intra host competition, but the outcome of the competition was strongly influenced by the dose and the synchrony of the infection of the two viruses. The virus that was administrated first to a larvae gained competitive dominance in asynchronous infections, which was not the case in synchronous infection where both viruses have a level playing field. This situation is analogous to competition between crop plants and weeds, where the outcome of competition depends on the timing of seedling emergence (e.g. Kropff & Spitters, 1991; Kropff & van Laar, 1993)

The fact that the Δ*egt* baculoviruses result in lower yield of OBs/larva can be of concern, as secondary transmission of baculoviruses is important for the success of the baculoviruses as a biological insecticide. The amount of virus produced per gram of dead larva, although lower in larvae killed by the *egt*, may be sufficient to allow efficient secondary transmission in the field. On the other hand the reduced yield from the Δ*egt* baculoviruses is positive since it may contribute to rapid disappearance from ecosystems after use, and thus enhance ecological safety.

The knowledge of baculovirus biology and ecology needs to be broadened further to not leave any doubt that biopesticides based on baculovirus formulations will cause

lower risk to the environment than classical chemical pesticides. This can be achieved by the combination of theoretical and empirical studies utilizing new advanced molecular techniques. For the time being, the evidence for ecological safety and non-persistence of genetically-modified baculoviruses in agro-ecosystems is insufficient to justify their release for large-scale practical use. Further studies will be needed to enhance familiarity and insight in the long term behaviour of virus genotypes in agro-ecosystems and build confidence that they can be used safely, or – alternatively – show that they pose intrinsic risks that cannot be ruled out beyond reasonable doubt.

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SAMENVATTING

Insectenplagen vormen een groot probleem in de gewasproductie en zijn steeds moeilijker verantwoord onder controle te houden met conventionele (chemische) middelen. Baculovirussen zijn ontwikkeld als een alternatief bestrijdingsmiddel en zijn reeds vele jaren ook commercieel in gebruik als biopesticide. Genetische modificatie is toegepast om de biologische eigenschappen van baculovirussen verder te verbeteren o.a. door het verwijderen van sommige baculovirusgenen (*egt*) of juist virusvreemde genen in te brengen (toxines, hormonen, etc.) zodat geïnfecteerde insecten sneller worden gedood en minder schade veroorzaken. Baculovirussen, die niet beschikken over een *egt*-gen (codeert voor een ecdysteroïd UDP-glycosyltransferase) of een insertie hebben van het insectspecifieke toxine-gen AaIT (afkomstig van *Androctonus australis* Hector), doden hun gastheer sneller dan wildtype baculovirussen. Er is weinig bekend over de respons van insectenlarven op een mengsel van wildtype en genetische veranderde virussen en ook is er nog betrekkelijk weinig kennis over de relatieve fitness van verschillende baculovirus varianten als deze gelijktijdig een insectenlarve infecteren. De relatieve fitness bepaalt de langetermijndynamiek en persistentie van deze recombinanten in agro-ecosystemen. Specifieke aandacht in dit proefschrift gaat uit naar het gedrag van menginfecties van wildtype en recombinante baculovirusvirusvarianten op insecten in het plant-insect-virus (katoen – katoendaguil – HaSNPV) systeem (hoofdstuk 1)

In hoofdstuk 2 worden metingen beschreven van de mediane overlevingstijd (ST_{50}) van derde stadium larven van de katoendaguil *Helicoverpa armigera*, wanneer deze besmet worden met het wildtype HaSNPV (HaSNPV-wt), een HaSNPV variant met een verwijderd *egt*-gen (HaSNPV-LM2) of met een 1:1 mengsel van deze twee virusvarianten. Bovendien zijn de mediane letale dosis (LD_{50}) en opbrengst aan ‘occlusion bodies‘ (OB = eiwitlichaampjes die virions bevatten) vastgesteld. Verschillende doseringen zijn ondermeer gebruikt om te bepalen of de virusdosis invloed heeft op de totale opbrengst aan OB’s of op de interactie tussen virusvarianten. Bij hoge virusdoseringen doodde HaSNPV-LM2 *H. armigera*-larven significant sneller (ongeveer 20 uur) dan HaSNPV-wt, maar bij lage doseringen was er geen significant verschil in overlevingstijd tussen de virusvarianten. De overlevingsduur na blootstelling aan een hoge dosis van een virusmengsel was significant verschillend van de overlevingsduur bij blootstelling aan één van de afzonderlijke virussen. Deze duur lag tussen die van de twee afzonderlijke virussen in, maar verschilde niet van die van een enkele virusvariant bij lage dosering. Er werden geen verschillen in (LD_{50}) gevonden tussen de virusvarianten of tussen enkele en menginfecties. Het aantal geproduceerde OB's per larve nam toe met

langere overleving en nam af met hogere dosis. Deze experimenten tonen aan dat de gevolgen van gemengde infecties voor insectenlarven afhangen van de ontvangen virusdosis. Dit zou in de beschouwing moeten worden betrokken wanneer de mogelijke ecologische gevolgen worden ingeschat van het toepassen van baculovirussen met verschillende biologische eigenschappen.

Een verandering in het gedrag van larven tijdens de virusinfectie kan de overdracht van het virus beïnvloeden, en als gevolg daarvan de fitness. In hoofdstuk 3 is onderzocht hoe actief de larven zich verplaatsen over de plant, en op welke plek ze doodgaan. Beide factoren zijn potentieel van invloed op de ontmoetingskans van virus met gezonde larven, met consequenties voor de virusoverdracht. De gedragseffecten zijn onderzocht met vierde stadium larven op katoenplanten, zowel met larven die geïnfecteerd waren met een afzonderlijke virusvariant als met larven die waren blootgesteld aan een mengsel van een wildtype HaSNPV en een deletiemutant. Het *egt*-ve virus, HaSNPV-LM2, veroorzaakte een snellere dood en een lagere virusopbrengst in het vierde larvestadium van de katoenrups, maar er werd over drie experimenten geen consistent effect op het gedrag van de geïnfecteerde larven gevonden. Twee gecontroleerde experimenten vonden plaats in een beschermde omgeving (kas), en een derde in een plastic kooi in een katoenveld. Een recombinant HaSNPV, dat het AaIT-gen bevatte (HaSNPV-4A), veroorzaakte een snellere dood en de kadavers werden gevonden op de lagere delen van de plant of op de grond. Larven die stierven door de 'gemengde' infecties van HaSNPV-4A en het wildtype virus deden dat op vergelijkbare plaatsen als larven geïnfecteerd met een van de afzonderlijke virusvarianten. De resultaten impliceren dat de overdracht van *egt*-negatieve varianten van HaSNPV te lijden kan hebben van de lagere virusopbrengst per larve, maar niet van gedragseffecten als gevolg van de afwezigheid van het *egt*-gen. In tegenstelling daarmee heeft het AaIT-gen een lagere OB productie tot gevolg en veroorzaakt het aangepast gedrag. Tezamen kan dit leiden tot een geringere overdracht van het virus en een lagere virusfitness onder veldomstandigheden.

In hoofdstuk 4 is de overdracht van het baculovirus HaSNPV bij larven van de katoendaguil bestudeerd in een katoen-agrosysteem. De overdracht van drie genetisch verschillende HaSNPV varianten is onderzocht, variërend van larven die zijn geïnfecteerd met een enkel virusgenotype tot larven die zijn gecoïnfecteerd met twee verschillende genotypen. Onder deze genotypen bevonden zich een wildtype virus, HaSNPV, een variant zonder *egt* gen (HaSNPV-LM2) met een licht verhoogde dodingssnelheid, en een HaSNPV-variant (HaSNPV-4A), die een neurotoxine tot expressie brengt en een versnelde dood van het insect veroorzaakt. In drie veldexperimenten zijn geen duidelijke verschillen gevonden in de virusoverdracht bij verschillende behandelingen, bijv.: zowel twee virusvarianten afzonderlijk als virusmengsels hadden een gelijke snelheid van virusoverdracht. De overdracht nam toe naarmate de dichtheid van het aantal gastheren toenam en nam af met het verstrijken van de tijd sinds de inoculatie van de geïnfecteerde

larve met virus. Andere waarnemingen gaven de indruk dat de persistentie van deze HaSNPV's in de bodem beïnvloed wordt door zonlicht. Significante verschillen in virusoverdracht tussen de virusgenotypen en hun mengsels zijn waargenomen in het kasexperiment. De onderzochte HaSNPV genotypen hebben duidelijke verschillen in dodingstijd en virusopbrengst. Er werd echter geen duidelijk gevolg van deze verschillen waargenomen op gewasniveau voor de snelheid van virusoverdracht, wanneer het *egt*-negatieve HaSNPV-LM2 gebruikt werd. Echter, HaSNPV-4A (*egt*-negatief, AaIT-positief) had een significant lagere overdracht. Gebaseerd op deze bevindingen werd de hypothese opgesteld dat de competitie tussen virusgenotypen (het wildtype HaSNPV en HaSNPV-LM2) op het gastheerinterne niveau een belangrijkere factor is in de competitie tussen baculovirussen in het katoen-agro-ecosysteem is dan de verschillen die optreden op gewasniveau.

De laatste twee hoofdstukken van dit proefschrift gaan nader in op de competitie tussen virusgenotypen binnen het insect, aan de hand van twee andere baculovirussen, SeMNPV en AcMNPV in twee andere gastheren, de floridamot *Spodoptera exigua* en de spanrups *Trichoplusia ni*. In hoofdstuk 5 wordt de competitie tussen twee genotypen van het *S. exigua* kernpolyedervirus (SeMNPV) bestudeerd in zogenaamde seriële passages. Daarbij werd een virusmengsel gedurende een aantal generaties overgezet in opeenvolgende cohorten van tweede-stadium larven van *S. exigua*. Een van deze genotypen was een SeMNPV wildtype (SeUS1); de ander was een SeMNPV-recombinant (SeMNPV-XD1). De laatste veroorzaakt een snellere doding dan SeUS1. SeXD1 mist een aantal genen, waaronder het *egt* gen; SeXD1 produceert een groenfluorescerend eiwit (GFP). Dit eiwit maakt de identificatie mogelijk van SeXD1 infecties in celkweken en in insecten. Het relatieve aandeel SeUS1 en SeXD1 in OB's van seriële gemengde infecties is bepaald door middel van plaque testen van virus uit geïnfecteerde larven op groeiende cellen (wit/groen screening), door polymerase kettingreacties (PCR) en restrictie-enzymanalyse (REN). Het SeUS1-genotype concurreerde het SeXD1-genotype volledig weg in achtereenvolgende passages. Afhankelijk van de oorspronkelijke verhouding tussen virusgenotypen werd de SeXD1-recombinant niet meer aangetroffen na zes tot twaalf passages in larvencohorten. De dynamica van de competitie werd beschreven met een eenvoudig wiskundig model. Gemiddeld nam de verhouding SeSU1/SeXD1 toe met een factor 1,9 per passage. Onder deze specifieke omstandigheden werd de SeXD1-recombinant vervangen door het wildtype virus SeUS1. Verder onderzoek is echter nodig om er zeker van te zijn dat dit ook het geval is wanneer deze baculovirussen gebruikt worden onder de complexere omstandigheden van een agro-ecosysteem.

In hoofdstuk 6 zijn *Trichoplusia ni*-larven blootgesteld aan diverse verhoudingen van het wildtype *A. californica* MNPV (AcMNPV) en de daarvan afgeleide recombinant vEGTDEL dat het endogene *egt*-gen mist. De mediane overlevingstijd (ST_{50}) werd bepaald voor beide genotypes, en uitgaande van drie verschillende mengverhoudingen

(1:10, 1:1 en 10:1) werd de genotype verhouding in vijf seriële passages bepaald. Bij elke passage werden tien geïnfecteerde larven de OB's geïsoleerd als inoculum voor de volgende passage. Een 1:10-verhouding (wildtype AcMNPV tegenover vEGTDEL) veroorzaakte een snelle actie van het virus, terwijl het wildtype virus na vijf passages behouden was gebleven. Het wildtype virus (AcMNPV) concurreerde in dit geval de *egt*-negatieve virusvariant vEGTDEL niet weg, en na vijf passages waren in vijf van zes passagelijnen beide virusgenotypen nog aanwezig. De persistentie van vEGTDEL plaatst vraagtekens bij de veronderstelde superieure competitiviteit van het wildtype virus. Dit suggereert wellicht een risico voor de veiligheid van het ecosysteem, omdat het recombinante virustype niet geheel weggeconcurreerd wordt, althans in deze laboratoriumopzet.

De resultaten beschreven in dit proefschrift laten zien dat de ecologische fitness van de baculovirussen bepaald wordt door zowel gastheerinterne als -externe factoren. Menginfecties kunnen resulteren in een tussenliggende uitkomst vergeleken met infecties door een afzonderlijk genotype (bijv. overlevingstijd; hoofdstuk 2) of in reacties die niet voorspeld hadden kunnen worden op basis van experimenten met enkele virussen (bijvoorbeeld de overmatig opbrengst bij een infectie met gemengde virussen; hoofdstuk 6). Precieze en gedetailleerde experimenten zijn noodzakelijk om genotypen van baculovirussen te selecteren met een lagere fitness vergeleken met een wildtype virus, opdat deze eerste, wellicht recombinante baculovirussen het in de competitie afleggen tegen het wildtype baculovirus en verdwijnen in de tijd. Deze experimentele resultaten en inzichten kunnen van waarde zijn voor het ontwikkelen van ecologisch veilige strategieën voor het gebruik van genetisch gemodificeerde baculovirussen in de biologische bestrijding van insectenplagen.

SUMMARY

Insect pests are a major impediment to the production of food crops and are increasingly difficult to control with conventional chemical insecticides. Baculoviruses have been developed as an alternative control method and they have been used as commercial biopesticides for many years. Genetic modification has been exploited to improve the biological properties of baculoviruses, by deletion of baculovirus genes or by insertions of foreign genes such as toxins, hormones, etc. Recombinant baculoviruses, which lack the *egt* gene (encoding for ecdysteroid UDP glucosyltransferase), or have an insertion of the AaIT (*Androctonus australis* Hector) insect specific toxin gene, generally kill their hosts faster than wild type strains. The response of insects to mixtures of virus genotypes – not unusual in nature - and its influence on several important baculovirus fitness parameters is less well known. Also the long-term fate of these recombinants in agro-eco systems is not well known, which depends on their ecological fitness. This thesis presents work on elements of ecological fitness. Particular attention is given to the behavior of mixed infections of two virus variants in insects and the plant-insect-virus cotton – cotton bollworm – HaSNPV (*Helicoverpa armigera* nucleopolyhedrovirus).

In Chapter 2 the median survival time (ST_{50}), median lethal dose (LD_{50}) and occlusion body (OB) yield were compared for third instar larvae of the cotton bollworm, *H. armigera* (Hübner) after challenge with wild-type HaSNPV (HaSNPV-wt), a strain with a deletion of the *egt* gene, HaSNPV-LM2, and a 1:1 mixture of these two virus strains. A range of doses was used to determine whether the total number of OBs influenced the response to challenge with a mixture of virus strains versus single strains. At high virus doses HaSNPV-LM2 killed *H. armigera* larvae significantly faster (ca. 20 h) than HaSNPV-wt, but at low doses, there was no significant difference in survival time between the two viruses. The survival time after challenge with mixed virus inoculum was significantly different from and intermediate between that of the single viruses at high doses, and not different from that of the single viruses at low doses. No differences in lethal dose were found between single and mixed infections or between virus genotypes. The number of OBs produced per larva increased with time to death and decrease with virus dose, and no significant differences among virus treatments were found. These experiments show that the outcome of mixed infections depends on dose and this should be taken into account when evaluating the ecological consequences of release of viral types with different biological properties.

Modifications of larval behaviour upon virus infection can influence virus fitness as a result of affecting the rate of virus transmission. In Chapter 3 the distance moved and position of virus-killed cadavers of fourth instar *H. armigera* larvae infected with

HaSNPV-wt or one of two recombinant genotypes of this virus (HaSNPV-LM2 and HaSNPV-AaIT) on cotton plants were studied. The behavioral effects of virus infection were examined both in larvae infected with a single virus genotype and in larvae challenged with mixtures of HaSNPV-wt and one of the recombinant viruses. HaSNPV-LM2 caused more rapid death and lower virus yield in fourth instars, but an *egt*-deletion did not produce consistent behavioral effects over three experiments, two under controlled glasshouse conditions, and one in field cages. A transgenic virus containing the AaIT gene (HaSNPV-AaIT), caused faster death and larvae died in lower strata of the cotton plants. Larvae that died from mixed infections of this recombinant and the wild-type virus died at intermediate positions compare to those that died from either of the pure viral strains. The results indicate that transmission of *egt*-negative variants of HaSNPV may be affected by lower virus yield, but not by behavioral effects of the *egt* gene deletion. In contrast, the AaIT-recombinant will produce lower virus yields and show modified behaviour, which together can contribute to reduced virus transmission and lower virus fitness under field conditions.

In Chapter 4 the transmission of the baculovirus HaSNPV among larvae of the cotton bollworm in the cotton agro-ecosystem was studied. Transmission of the three HaSNPV genotypes was studied, from larvae infected with a single virus genotype as well as from larvae infected with two different genotypes. Genotypes included (HaSNPV-wt HaSNPV-LM2 and HaSNPV-AaIT). In three field experiments no significant differences in virus transmission between treatments were demonstrated; i.e. two virus genotypes, as well as genotype mixtures, had similar rates of transmission. Transmission increased with density of host insects and decreased with time lapsed since the inoculation of the infector larvae. Side observations suggest that virus persistence of these HaSNPVs in soil is affected by solar radiation. Significant differences in transmission rate between virus genotypes and their mixtures were observed in the glasshouse experiment. The studied genotypes of HaSNPV have significant differences in time to kill and virus yield. No significant consequences of these differences were found for rates of virus transmission at the crop level when HaSNPV-LM2 (-*egt*) was used. However, HaSNPV-4A (-*egt*; AaIT) had significantly reduced transmission. Based on these findings, it was hypothesized that the competition between virus genotypes (HaSNPV and HaSNPV-LM2) at the "within host" level may be more important determinants of the outcome of competition between viruses in the cotton agro-ecosystem than differences that are expressed at crop level.

The remaining two chapters in this thesis describe the "within host" competition between virus genotypes using two different insect host species and two virus species different from HaSNPV. In Chapter 5 an account is given on the competition between two near-isogenic genotypes of *Spodoptera exigua* NPV (SeMNPV) when serially passaged as mixtures in cohorts of 2nd instar *S. exigua* larvae. One of the genotypes was a wild-type isolate SeMNPV, SeUS1, while the other was a SeMNPV recombinant (SeMNPV-XD1)

having a greater speed of kill than SeUS1. SeXD1 lacks a suite of genes, including the *egt* gene. SeXD1 expresses the green fluorescent protein (*gfp*) gene enabling the identification of SeXD1 in cell culture and in insects. The relative proportion of SeUS1 and SeXD1 in successive passages of mixed infections was determined by plaque assays of budded virus from infected larvae and by polymerase chain reactions and restriction enzyme analyses. The SeUS1 genotype outcompeted recombinant SeXD1 over successive passages. Depending on the initial virus genotype ratio, the recombinant SeXD1 was not anymore detected after six to twelve passages. A mathematical model was developed to characterize the competition dynamics. Overall, the ratio SeUS1/XD1 increased by a factor 1.9 per passage. The findings suggest that under the experimental conditions recombinant SeXD1 is displaced by the wild-type SeMNPV strain SeUS1, but further studies are needed to ascertain that this is also the case when the same baculoviruses would be used in agro-ecosystems such as greenhouses and field.

In Chapter 6 *Trichoplusia ni* larvae were challenged with different ratios of wild-type *Autographa californica* NPV (AcMNPV) and recombinant vEGTDEL, which has the endogenous *egt* gene deleted specifically. Five serial passages of three different mixtures of OBs of the two viruses were also performed. For the serial passage, OBs from 10 infected larvae were pooled and used to start the following passage. Then time to death was measured. A 1:10 ratio (AcMNPV versus recombinant vEGTDEL) ensures quick virus action, while the wild-type virus is also maintained over five passages. However, the wild type virus did not go to fixation in most replicates of the serial passage experiment, and there was no evidence for selection of the wild-type AcMNPV. This result suggests an ecosystem safety risk, as the recombinant virus may not be competitively displaced.

Results in this thesis illustrate that the ecological fitness of baculoviruses is determined both at 'within-host' and 'between-host' level, and that mixed infections may result in intermediate response compared to pure virus infections (e.g. survival time; Ch. 2) or in responses that cannot be predicted on the basis of experiments with pure viruses (e.g. over yielding in mixed virus infections; Chapter 6). Precise and detailed experiments and analyses are needed to select baculovirus genotypes with higher insecticidal activity but with lower fitness in comparison to a wild-type virus. This would result in competitive displacement of the recombinant baculovirus over time. These experimental results and insights can be of value for developing ecologically safe strategies for use of genetically modified baculoviruses in biological pest control.

KRATKA SODRZINA

Stetnite insekti se glaven limitiracki factor vo proizvodstvoto na prehranbeni kulturi i osobeno tesko se kontroliraat so konvencionalnite hemiski insekticidi. Bakulovirusite se razvieni kakao alternativnen kontrolen metod i se koristat mogu godini kako komercijalni biopesticidi. Genetskata modifikacija bese iskoristena za da se podobrat biloskite karakteristiki na bakulovirusite, preku otstranuvanje na bakulovirusnite geni ili preku vnesuvanje (vmetnuvanje) na nepoznati (tugji) geni kako sto se toksini, hormoni, itn.

Rekombinantnite bakulovirusi, na koi im nedostasuva *egt* genot (koj e odgovoren za proizvodstvo na ecdysteroid UDP glucosyltransferase), ili imaat vgradeno AaIT (*Androctonus australis* Hector) specificen gen na toxin za insekti, voobicaeno pobrgu go ubivaat domakinot od diviot vid na virusi. Reakcijata na insektite na mesavinata od virusni genotipovi- sto ne e nevoobicaeno vo prirodata-i nivnoto vlijanie na nekoi od vaznite bakulovirusni zivotni parametri ne e dovolno prouceno. Isto taka, ishodot od nivnoto dolgorocno prisustvo vo agro-eco sistemot ne e dovolno proucen, sto zavisi od nivnion ekoloska sposobnost. Ovaa teza gi iznesuva istrazuvanjata na elementite na ekoloska sposobnost. Posebno vnimanie se dava na odnesuvanjeto na insektite inficirani so mesavina od dvata virusi i odnosot pomedju rastenieto-insektot-virusot , pamukot-pamukovata gasenica-HaSNPV (*Helicoverpa armigera* nucleopolyhedrovirus).

Vo vtoroto poglavje, srednoto vreme na prezivuvanje (ST_{50}), srednata letalna doza (LD_{50}) i proizvodstvoto na virus (OB) bea sporedeni za *H. armigera* (Hübner) larva od tret stepen na razvoj, posle infekcija so div vid na virus (HaSNPV-wt), virus so otstranet *egt* gen, HaSNPV-LM2, i 1:1 mesavina od ovie dva virusi. Nekolku razlicni dozi bea koristeni so cel da se odredi kako vkupniot broj na virusni cesticki (OBs) vlijae na reakcijata na insektite na mesavina od virusi nasprema eden virus. Pri koristenje na visoki dozi, virusot HaSNPV-LM2 gi ubiva insektite znacitelno pobrzo (ca. 20 h) otkolku diviot vid na virus HaSNPV-wt, no pri niski dozi, ne bese zabelezana poglema razlika vo periodot na prezivuvanje pomedju dvata virusa. Vremeto na prezivuvanje na insektite inficirani so mesavina od dvata virusa bese znacitelno razlicno od vremeto na prezivuvanje na insektite inficiraani so visoki dozi od samo eden virus, a bez vidna razlika koga bea koristeni virusi vo niski dozi. Ne bese pronajdena znacitelna razlika vo letalnite dozi pomegju edinecnite i mesovitite infekcii ili pomegju virusnite genotipa. Brojot na virusi proizvedeni po edinica larva se zgolemi proporcionalno so vremeto do nastapuvanje na smrt na insektite i se namali so koristenata doza na virusite, no ne bese zabelezana nekoja pogolema razlika pomedju razlicnite metodi na tretiranje na virusite. Ovie eksperimenti potvrdija deka ishodot od infekcijata na insekti so mesavina na virusi zavisi

od dozata koja se koristi, i ova treba da zeme vo predvid koga se vrsi procenka na ekoloskite posledicite od upotreba na virusi so razlicni biloloski karakteristiki.

Modifikacijata vo odnesuvanjeto na larvite posle virusna infekcija moze da vlijanie na ekoloskata sposobnost na bakulovirusite kako resultat na vlijanieto vrz transmisijata na virusite. Vo tretoto poglavje bese ispituvano totalnoto rastojanie pominato od cetvrt stadium na larvi na pamukovo rastenie i pozicijata na mrtvite insekti koi bea inficirani so div vid na HaSNPV-wt ili genetski modificiranite virusi (HaSNPV-LM2 i HaSNPV-AaIT). Efektot na odnesuvanjeto na larvite bese ispituvano kora larvite bea inficirani so eden virusen genotip ili so mesavina od div vid (HaSNPV-wt) i eden od rekombinantnite virusi. HaSNPV-LM2 predizvikuva pobrza smrt i pomala produkcina na virus vo cetvrt stadium na razboj na insektite, dodeka kaj insektite inficirani so virus koj ima nedostatok od *egt* gene ne se dobi konstanten efekt vo odnesuvanjeto na insektite pomedju trite eksperimenti, dva eksperimenti bea izvedeni vo kontrolirani oranzeriski uslovi i eden bese izveden vo polski uslovi. Genetski modificiraniot virus koj sodrzi AaIT gen (HaSNPV-AaIT), predizvikuva pobrza smrt kaj larvite i tie uginuvaat na dolnite granki od pamukovite rastenija. Laravite koi umrele od mesana infekcija od div i rekombiniran virus najcesto umiraat na sredna pozicija sporedeno so larvite koi umiraa inficirani so eden od virusite. Rezultataite indiciraat deka prenesuvanjeto na HaSNPV virusot koj ima nedostatok na *egt* gen, moze da bide pod vlijanie na namalenata produkcija na virus, no ne e pod vlijanie na efektot koj *egt*-negativniot virus go ima na odnesuvanjeto na ovie inficirani insekti. Od druga strana, recombinantniot virus koj sodrzi AaIT gen kje ima namaleno proizvodstvo na virus i kje pokaze modificirano odnesuvanje, taka sto kombinacijata od ovie dva faktora moze do doprinese da se namali prenesuvanjeto na virusot i da se namali ekoloskata sposobnost na virusot vo polski uslovi.

Vo cetvrtoto podglavje bese ispituvana transmisijata (prenesuvanjeto) na HaSNPV virusot pomedju *H. armigera* larvite na pamukot vo pamukoviot agro-ekosistem. Transmisijata na tri HaSNPV genotipa bese ispituvana, i toa na larvi inficirani samo so eden virus ili na larvi inficirani so dva razlicni genotipa. HaSNPV genotipovite koi bea ispituvani se (HaSNPV-wt, HaSNPV-LM2 and HaSNPV-AaIT). Vo trite polski opiti, ne bese zabelezana pogolema razlika pomedju razlicnite virusni tretmani, kako na primer, dvata genotipa na virus kako i nivnata mesavina so diviot vid, imaa slicen stepen na transmisija. Transmisijata se zgolemuvase so gustinata na larvite koi sluzea kako izvor na infekcija i se namaluvase so vremeto pominato posle infekcija na larvite koi sluzat kako izvor na infekcija. Dopolnitelnite ispituvanja dadoa indikacija deka postojanosta na virusot vo pocvata zavisi od soncevata radijacija. Znacitelnata razlika vo stapkata na transmisija pomedju virusnite genotipovi bese zabelezana vo oranzeriskite opiti. Ispituvanite virusni genotipovi na HaSNPV imaat znacitelna razlika vo pogled na vremeto na ubivanje i virusna produkcija. Pri upotreba na HaSNPV-LM2, ne bese zabelezana pogolema razlika vo pogled na transmisijata. Medjutoa, HaSNPV-4A (-*egt*; AaIT) pokaza

sznacitelno namalena transmisija. Vrs osnova na ovie soznanija, bese postavena hipotezata deka konkurencijata pomedju virusnite genotipa (HaSNPV and HaSNPV-LM2) na nivo na "konkurencija vo samiot domakjin" moze da bide mnogu povazen factor za ishodot od konkurencijata pomedju dvata virusi vo pamukoviot agro-eko sistem, otkolku razlikite koi se pokazuvaat na nivo na rastenie.

Preostanatite dve poglavja vo ovaa teza ja opisuvaat "konkurencijata vo samiot domacin" pomedju virusnite genotipovi koristejkji dva razlicni vidovi na insekti kako domakjin i dva virusi razlicni od HaSNPV. Vo pettoto poglavjeto se opisuva konkurencijata pomedju dva genotipa od *Spodoptera exigua* NPV (SeMNPV) koga seriski se ispituvaat vo opiti od seriski pasaz vtor instar *S. exigua* larva inficirani so mesavina od virusi.

Eden od virusnite genotipovi bese div tip na izolat od SeMNPV, SeUS1, dodeka drugiot bese rekombinat od SeMNPV (SeMNPV-XD1), koj ima pogolema brzina na deluvanje od SeUS1. SeXD1 ima nedostatok na nekolku gena vklucuvajki goi *egt* genot. SeXD1 go proizveduva zeleniot fluroscenten (*gfp*) genkoj ovozmozuva negova identifikacija vo kultura na tkivo i insekti. Relativniot soodnos na SeUS1 and SeXD1 vo posledovatelni opiti na infekcii od mesavina na ovie virusi bese odredena so "plaque assays" (plaque analizi), od virus od inficirani larvi i koristenje na PCR i analiza so restrikcioni enzimi. SeUS1 genotipot go nadmina rekombinantniot SeXD1vo nekolku posledovatelni pasazi. Vo zavisnost od pocetniot soodnos na virusniot genotip, rekombinantniot SeXD1 ne bese povekje prisuten posle 6 do 12 pasazi (posledovatelni ispituvanja). So cel da se opise dinamikata na konkurencijata, bese napraven matematicki model. Opsto zemeno, soodnosot pomedju SeUS1/XD1 se zgolemuvase za koeficient od 1.9 po pasaz. Naodite posocuvaat deka pod ekperimentalni uslovi rekombinantniot SeXD1 se zamenuva so diviot vid od SeMNPV- SeUS1, no neophodni se ponatamosni ispituvanja za da se potvrdi deka ova kje bide slucaj koga istite bakulovirusi kje se koristat vo agro-eco sistemi kako sto se oranzeriite i polskite uslovi.

Vo sestoto poglavje, larvite od *Trichoplusia ni* bea inficirani so razlicen soodnos od div vid virus *Autographa californica* NPV (AcMNPV) and recombinanaten vid vEGTDEL, koj ima otstranet *egt* gen. Pet seriski pasazi od tri razlicni mesavini na OB od ovie dva virusi bea izvedeni. Za seriskiot passage, OB od 10 inficirani larvi bea grupirani i iskoristeni da se se zapocne sledniot pasaz. Potoavremeto od infekcija do nastapuvanje na smrt na insektite bese izmereno. Soodnos od 1:10 (AcMNPV nasproti recombinanten vEGTDEL) ovozmozuva brza virusna aktivnost, dodeka diviot vid na virus e istotaka prisuten i posle 5 pasazi. Vo sekoj slucaj, diviot vid na virus ne bese fiksiran vo povekjeto replikacii od seriskiot pasazen opit, i ne postoese dokaz za selekcija na diviot vid na AcMNPV. Ovoj resultat implicira bezbedonosen rizik za eco-sistemot, ako se zeme vo predvid deka rekombiniraniot virus mozebi nema celosno da bide zamenet.

Rezultatite vo ovaa teza ilustriraat deka ekoloskata sposobnost na bakulovirusite e odreden na nivo na dva vida na konkurencija: “ vo domakjinot” i “pomegju domakjinot”, i deka infekciite so mesavina od dvata virusa moze da rezultiraat so neposreden odgovor sporedeno so cisto virusna infekcija (na pr. vreme na prezivuvanje, vtoro poglavje) ili da dadat rezultati koi mozat da bidat tesko predvidlivi vrz baza na opiti so cisti virusi (na pr. zgolemeno proizvodstvo pri infekcii so mesavina od virusi,sesto podglavje). Precizni i detalni opiti i analizi se neophodni za da se izbere bakulovirusen genotip so povisoka insekticidna aktivniost, no so pomal fitness vo sporedba so diviot vid na bakulovirus. Po izvesen period, ova kje rezultira so konkurentska zamena na rekombiniraniot virus. Ovie eksperimentalni rezultati i sogleduvanja mozat da bidat od znacenje za razvivanje na ekoloski bezbedni strategii za upotreba na genetski modificirani bakulovirusi vo bioloskata zastita od stetnici.

ABBREVIATIONS

General

AaIT	*Androctonus australis* (Hector) insect toxin
bacmid	Full-length, biologically active baculovirus genome as a bacterial plasmid
bp	Base pair
BV	Budded virus
EGTDEL	Recombinant AcMNPV missing the *egt* gene
GV	Granulovirus
h	hours
IPM	Integrated pest management
kbp	kilo bp
LD50	Dose at which 50% of the larvae die
MNPV	Multiple Nucleopolyhedrovirus
MOI	Multiplicity of infection: the mean number of infectious virions offered per cell
NC	Nucleocapsid
MNPV	Multi-capsid nucleopolyhedrovirus
NPV	Nucleopolyhedrovirus
nt	Nucleotide
OB	Occlusion body
ODV	Occlusion derived virion
ORF	Open reading frame
NTC	Non template control
p.i.	Postinfection
PCR	Polymerase chain reaction
qtPCR	Quantitative real time PCR
SNPV	Single-capsid nucleopolyhedrovirus
SPE	Serial passage experiment
ST50	Median survival time

Viruses

AcMNPV	*Autographa californica* MNPV
AgipMNPV	*Agrotis ipsilon* MNPV
CpGV	*Cydia pomonella* GV
HaSNPV	*Helicoverpa amigera* SNPV
HaSNPV-LM2	HaSNPV missing the *egt* gene
HzSNPV	*Helicoverpa zea* SNPV
LdMNPV	*Lymantria dispar* MNPV
SeMNPV	*Spodoptera exigua* MNPV
SeMNPV-US1	Wild type SeMNPV
SeMNPV-XD1	Recombinant SeMNPV missing the *egt* gene
SfMNPV	*Spodoptera frugiperda* MNPV
TnMNPV	*Trichoplusia ni* MNPV

CURRICULUM VITAE

Liljana Georgievska was born on 11th of February 1973 in Skopje, Republic of Macedonia. From 1992-1998, she studied at the Agricultural Faculty at the "Sts Cyril and Methodius University" in Skopje, where she obtained her B.Sc. degree with specialization in fruit and crop production. From 1998 to 1999, she worked as a research assistant in Entomology at the Plant Protection Department, at the Agricultural Research Institute in Skopje. From 1999 to 2001, she studied at the National Pingtung University (NPUST) in Taiwan, where she got Master Degree in Entomology with the thesis research entitled "Isolation and Identification of Chemical Stimulants from *Solanum viarum* (Dunal) and Their Effects on the Oviposition of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuide)". From 19 April until 28 Jun 2002 she followed the training course on "Integrated Pest Management" at the International Agricultural Center, (IAC) Wageningen, The Netherlands. Since 2003, she has been involved in a Ph.D. and worked in the Laboratory of Virology and the Crop and Weed Ecology Group, at Wageningen University, The Netherlands. Currently she is a PostDoc researcher at the Iowa State University, Ames in the laboratories of Prof. Dr. Bryony Bonning and Dr. Allen Miller.

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of Literature (4.5ECTS)

- Competitive fitness and persistence of wild type and genetically modified baculoviruses in the biological control of the bollworm, *Helicoverpa armigera*, in cotton in China (2003)

Laboratory Training and Working Visits (4.3 ECTS)

- Practical experience on performing bioassays with lepidopteran insects; BBA, Darmstadt (2003)
- Competitive study bioasssays with *H. armigera*; UPNA, Pamplona, Spain (2007)

Post-Graduate Courses (2.8 ECTS)

- Advanced statistics; PE&RC (2004)
- Principles of ecological and evolutionary genomics; SENSE (2006)

Deficiency, Refresh, Brush-up Courses (1.4 ECTS)

- Basic statistics; PE&RC (2003)

Competence Strengthening / Skills Courses (2.4 ECTS)

- Time planning and project management; WGS (2006)
- PhD Competence assessment; WGS (2006)
- Techniques for writing and presenting scientific papers; WGS (2006)

Discussion Groups / Local Seminars and Other Meetings (10.3 ECTS)

- Dutch annual virology symposium (2003-2007)
- Work discussion meetings; Virology (2003-2008)
- WOTRO DAY Utrecht (2004)
- Seminar conventional & real-time PCR; Westburg, Leusden (2005)
- Seminar conventional & real-time PCR-Bio-rad; Nol in 't Bosch, Wageningen (2005)
- Netherlands entomological society meetings; Ede, the Netherlands (2005-2007)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (1.2 ECTS)

- PE&RC PhD Weekend (2003)
- PE&RC Annual meeting "Global climate change & biodiversity" (2003)
- PE&RC Annual meeting "Biological disasters" (2004)
- 10 Years anniversary Graduate School for Production Ecology & Resource Conservation (2005)

International Symposia, Workshops and Conferences (5.8 ECTS)

- IOBC Meeting; Salzau-Kiel, Germany (2003)
- Current themes in ecology (2004-2007)
- 37th Annual Meeting of the Society for Invertebrate Pathology (SIP); Helsinki (2004)
- 39th Annual Meeting of the Society for Invertebrate Pathology (SIP); Wuhan, China (2006)
- 42th Annual Meeting of the Society for Invertebrate Pathology (SIP); Park City, Utah, USA (2009)

Courses in Which the PhD Candidate Has Worked as a Teacher

- ENT 30306 Ecological aspects of bio-interactions (2005-2006); Laboratory of Virology; 21 days

Supervision of MSc Students

- Transmission and persistence of wild type and genetically modified HaSNPV baculoviruses in cotton in China; 4 months; 1 student

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