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# Effects of single and mixed infections with wild type and genetically modified *Helicoverpa armigera* nucleopolyhedrovirus on movement behaviour of cotton bollworm larvae

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**Abstract**

Naturally occurring insect viruses can modify the behaviour of infected insects and thereby modulate virus transmission. Modifications of the virus genome could alter these behavioural effects. We studied the distance moved and the position of virus-killed cadavers of fourth instars of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) infected with a wild-type genotype of *H. armigera* nucleopolyhedrovirus (HaSNPV) or with one of two recombinant genotypes of this virus on cotton plants. The behavioural 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 behavioural effects over three experiments, two under controlled glasshouse conditions and one in field cages. A recombinant virus containing the AaIT-(*Androctonus australis* Hector) insect-selective toxin gene, which expresses a neurotoxin derived from a scorpion, caused faster death and cadavers were found lower down the plant than insects infected with unmodified virus. Larvae that died from mixed infections of the AaIT-expressing recombinant and the wild-type virus died at positions significantly lower, compared to infection with the pure wild-type viral strain. The results indicate that transmission of *egt*-negative variants of HaSNPV are likely to be affected by lower virus yield, but not by behavioural effects of *egt* gene deletion. By 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. In addition, larvae infected with both the wild-type virus and the toxin recombinant behaved as larvae infected with the toxin recombinant only, which might be a positive factor for the risk assessment of such toxin recombinants in the environment.

## Introduction

Baculoviruses are a large and diverse group of insect pathogens. They cause natural epizootics in insect populations, especially in forest species (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 (McCutchen et al., 1991;

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Tomalski & Miller, 1991; Chejanovsky et al., 1995; Hughes et al., 1997; Chen et al., 2000). There is concern, however, about the ecological safety of such recombinant baculoviruses, in particular the possible effects of genetic modification on non-target hosts and the likelihood and consequences of long-term environmental persistence (Cory & Hails, 1997; Hernandez-Crespo et al., 2001; Hails et al., 2002; Cory & Myers, 2003). It is therefore pertinent to study factors that are likely to affect the persistence and transmission of wild-type and genetically modified viruses, including the possibility of mixed infections.

The persistence of natural and recombinant virus genotypes depends on their competitive interactions at the individual and the ecosystem level. Persistence of baculoviruses in the host population is primarily determined by their ability to be transmitted to new susceptible hosts, which in turn is related to how virus infection influences host behaviour (Hoover et al., 1995). There is ample evidence that baculoviruses induce modified behaviour in their hosts, including enhanced locomotory activity and a tendency to climb to elevated positions 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 by disintegration of infected larvae due to enzymes encoded by the viral cathepsin and chitinase genes (Hawtin et al., 1997). Thus, the transmission stages of the virus, occlusion bodies (OBs), can be spread over large areas of the plant when it rains, 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). A recent report (Raymond et al., 2005) shows negative phototaxis in baculovirus-infected larvae of the winter moth, *Operophtera brumata* (L.). 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 between generations in this univoltine species and hence foster transmission in the longer term. Alternatively, behavioural changes in infected insects could also hamper virus transmission, and thereby be adaptive to the host. An upward movement of infected individuals, away from healthy conspecifics, could reduce virus transmission. All in all, the effects of baculoviruses on host behaviour appear diverse and differ from one system to another. Likewise, the consequences of altered behaviour for transmission are likely to be system-specific.

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a polyphagous insect 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* Berliner (Bt) toxins (Liang et al., 1998, 2008). However, chemical pesticides are still used on non-genetically modified varieties (Men et al., 2005). Resistance to Bt has already been demonstrated in field populations of *Plutella xylostella* (L.) (Huang, 2006) and glasshouse populations of *Trichoplusia ni* (Hübner) (Janmaat & Myers, 2005). There is, therefore, a continued need for alternative sustainable control options. One such opportunity is offered by insect baculoviruses, although resistance to baculoviruses can develop under intensive use (Asser-Kaiser et al., 2007). *Helicoverpa 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, HaSNPV-G4 (Chen et al., 2001) and HaSNPV-C1 (Zhang et al., 2005). 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 primarily on the behavioural effects of an *egt*-negative strain of HaSNPV (HaSNPV-LM2) in comparison with the wild-type virus, HaSNPV-G4, in both single and mixed infections. In addition, in one of the experiments we include an AaIT-positive HaSNPV (HaSNPV-4A; Sun et al., 2004), which has a markedly shorter time to kill. Previous studies with recombinant baculoviruses expressing AaIT in other systems, have demonstrated that they cause paralysis, resulting in infected larvae dropping off the plant (Hoover et al., 1995; Hernandez-Crespo et al., 2001; Sun et al., 2004). 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 the detection of behavioural effects, which – in the case of a gene deletion – might be subtle. There are no differences in median lethal dose among the three virus genotypes HaSNPV-G4, HaSNPV-4A, and HaSNPV-LM2 (Sun et al., 2004; Georgievska et al., 2010b)

Studies were conducted with the L4 stage of *H. armigera*. Fourth instars 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 and decreased virus yield in infected L3 larvae of *H. armigera* (Sun et al., 2005; Georgievska et al., 2010b).

Two preliminary questions were addressed in the laboratory: does deletion of the *egt* gene modify survival time and virus yield in the fourth instar of *H. armigera*? And how is survival time and virus yield affected when larvae are challenged with a mixture of HaSNPV-G4 (wild-type) and HaSNPV-LM2 (*egt*-negative), compared to each virus alone?

These experiments were followed up by glasshouse and field studies to address the following questions. Does infection with HaSNPV-G4 modify the behaviour of *H. armigera* and, if so, how is this behaviour altered when the virus is genetically modified by gene deletion (HaSNPV-LM2) or gene deletion and insertion (HaSNPV-4A)? How do mixed wild-type and recombinant virus infections influence the behaviour (movement and location) of the larvae, as compared to single virus infections?

## 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 *A. australis* (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 infected cell culture supernatant into fourth-instar *H. armigera* and harvesting OBs upon death. HaSNPV-4A was amplified by oral infection of third-instar *H. armigera* with a dose causing more than 99% mortality.

Occlusion bodies were isolated by macerating virus-killed cadavers in sterile water and filtering the homogenate through two layers of muslin. OBs were purified by centrifugation at 11 200 ***g*** for 60 min on a 30–60% continuous sucrose gradient. Virus inoculum was prepared by serially diluting the OB stock solutions with sterile water. The OB concentration was determined by three independent counts using the improved Neubauer counting chamber under phase contrast microscopy at ×400 magnification. OB stocks were maintained at 20 °C until further use.

### Insect culture

*Helicoverpa 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). Larvae were reared individually in 1-ml plastic containers to prevent cannibalism. They were fed on artificial bean diet, modified after Patana (1985) and were kept at constant temperature of 27 ± 3 °C, 70% r.h., and L14:D10 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 and eggs were collected on paper strips hanging on the sides. Eggs were surface-sterilized in 4% formaldehyde for 15 min, rinsed in tap water and air-dried. *Helicoverpa armigera* used in the field experiment (experiment 4) were obtained from the insect culture reared at China Cotton Research Institute, Anyang, China (CCRI). The larvae used in experiments 1 and 3 were reared on artificial bean diet, whereas those used in experiments 2 and 4 were reared on cotton leaves, to adapt them to the plant.

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

For each treatment, 48 newly moulted fourth instars were collected from the culture, starved for 16 h at 25 °C, and then inoculated with HaSNPV-wt, HaSNPV-LM2, or a 1:1 mixture of HaSNPV-wt and HaSNPV-LM2, using the droplet feeding method and a 10-min inoculation time (Hughes & Wood, 1981). Larvae were challenged with an $LD_{90}$ dose for this instar, 42 000 OBs/larva (Sun et al., 2004). 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 120–200 μm, pore diameter 70 nm) and centrifuged at 6 000 ***g*** for 5 min. Supernatant was removed and 500 μl of sterile water was added to disperse the OBs. The concentration of the OBs was determined with an improved Neubauer counting chamber using phase contrast microscopy.

#### Behavioural observations

Three experiments were conducted to determine the effects of single and mixed infections on larval behaviour. 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 with virus and one larva was released per plant, after which their position was recorded three times per day until the moult into the fifth instar or virus-induced death.

The distance travelled by each larva was measured by marking its position with a small sticker placed either on the plant (leaf or stem) or on the soil at each observation time. Larvae were observed and positions marked daily at 08:00, 14:00, and 20:00 hours, until infected insects died. The total distance travelled (in cm) was estimated as the shortest distance that the larva could have moved between the two points while still remaining on the plant. The total distance covered was estimated by summing up the daily totals over a maximum of 5 (experiment 3) or 8 days (experiments 2 and 4), or as long as an infected individual lived. During the 5 or 8 days of each experiment, larvae would typically moult once, so the observations spanned two instars.

In addition, the position of the larvae along the vertical axis of the plant was recorded. These records were made according to 'level', with level 0 representing ground level, i.e., underneath the plant, level 1 representing branches 1 and 2 (the lowermost and oldest branches), level 2 representing branches 3 and 4, and so forth (Figure 1). Obviously, the number of levels, as defined, would increase as the plants grew. In experiment 1, the larvae were released at level 3 or 4 (middle section of the plant).

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

Cotton plants (var. Zhongmiansuo no. 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, L14:D10 h photoperiod, and 60–70% r.h. Fourth-instar *H. armigera* were infected individually by feeding them a 5-mm-diameter cotton leaf disc, coated with 200 000 OBs (5 × $LD_{99}$ for this instar) (Sun et al., 2004), ensuring virtually 100% infection, and near certainty that larvae challenged with mixed inoculum express symptoms of a mixed virus infection (Zwart et al., 2009a). 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/disc). Larvae were kept individually for 24 h in 24-well plates at 27 °C until release.

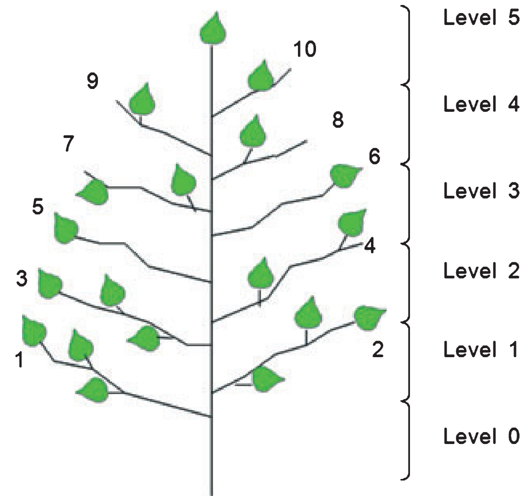


**Figure 1** Diagram of a cotton plant, showing the coding system for recording the position of the larvae: level 0 (ground), level 1 (branches 1 and 2), level 2 (branches 3 and 4), level 3 (branches 5 and 6), and level 4 (branches 7 and 8). As the plants grow, extra levels are added to the coding system, with two branches in each level.

Larvae that had completely consumed the leaf discs were released haphazardly on the middle section of the plant on 23 April 2004, when the plants were 16 weeks old and producing bolls. 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 *Helicoverpa armigera* on cotton plants in a glasshouse after challenge with wild-type HaSNPV, HaSNPV-LM2, HaSNPV-4A, or with mixtures of wild-type and recombinant viruses

Cotton plants (var. Zhongmiansuo no. 35) were sown on 13 August 2007 and grown in a glasshouse (UNIFARM) at 28 ± 3 °C, L14:D10 photoperiod, and 60–70% r.h. 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. Each treatment was replicated 10 times.

The fourth instars used in the second glasshouse experiment were infected using the droplet feeding method. Newly moulted fourth-instar *H. armigera* were individually separated to prevent cannibalism and starved for about 16 h at 24 °C. The virus solution consisted of an aqueous suspension of 10% (wt/vol) sucrose, 0.001% (wt/vol) Fluorella blue (food dye) and resulted in a dose of approximately 42 000 OBs/larva, based on 4.5 μl ingested volume (Sun et al., 2004). Larvae showing blue colouration in the foregut after 10 min were transferred to 24-well plates

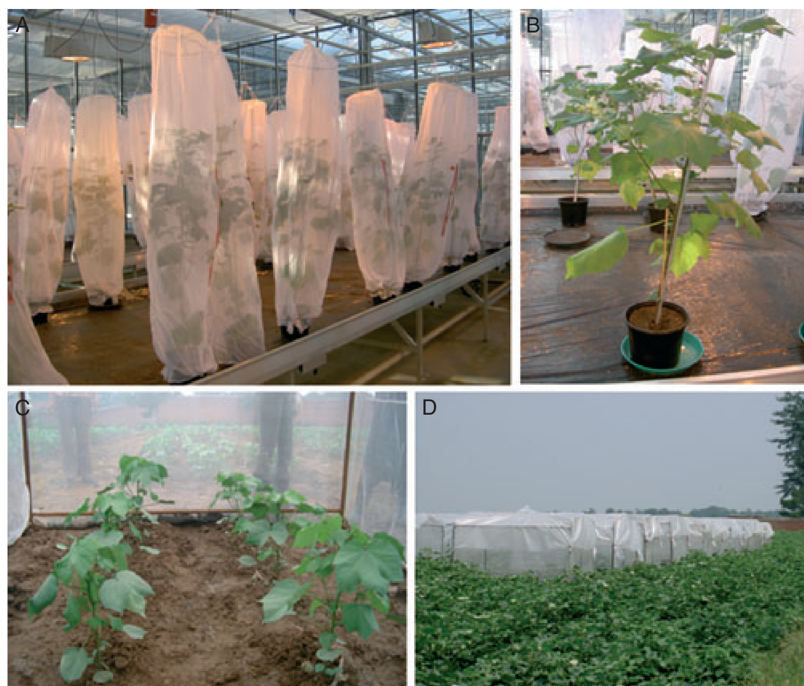


**Figure 2** (A) Individually netted cotton plants in the glasshouse (experiment 3); (B) individual cotton plant in experiment 3; (C) field cages in experiment 4; and (D) six cotton plants per cage in experiment 4.

containing cubes of artificial diet and kept at a constant temperature of 27 °C. After 1 day, on 13 October 2007, one larva was released on each cotton plant. At the time of the experiment the plants were 8 weeks old, 50–60 cm high, with 10–15 full-grown leaves and flowers (Figure 2A, B).

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

A field experiment was carried out at the CCRI, Anyang, Henan Province. Cotton plants (var. Zhongmiansuo no. 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 cover (Figure 2C, D). The sides of the cages were buried 10 cm into the soil to prevent larvae from escaping.

When plants were in the fruiting and flowering stage on 5 September 2005, one fourth instar 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. There were five cages for each treatment. The method of infection and the viral dose were the same as in experiment 2. The position of the larvae on each plant in each cage was recorded three times daily, at 07:00, 13:00, and 19:00 hours, for 7 days post-release. In the statistical analysis, we assumed that the movement record of each single larva constituted an independent data item.

#### Statistical analysis

Median survival time ($ST_{50}$) values of fourth instars in experiment 1 were estimated with the Kaplan–Meier Product Limit Estimator, using survival analysis procedure 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 (2003). Daily distances (cm) travelled by larvae in experiments 2–4 were $\sqrt{x}$ transformed to normalize residuals. Differences in distance moved among treatments were analysed by univariate repeated-measures 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, 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, 2003). Letter codes for indicating significance of pairwise differences were assigned on the basis of the SPSS output, according to the method of Piepho (2004). In experiments 2–4, each cotton plant was treated as a replicate.

## Results

#### Experiment 1: Survival time and virus yield of infections 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 treatments according to the log rank test in the survival analysis ($\chi^2$ = 74.95, d.f. = 2,

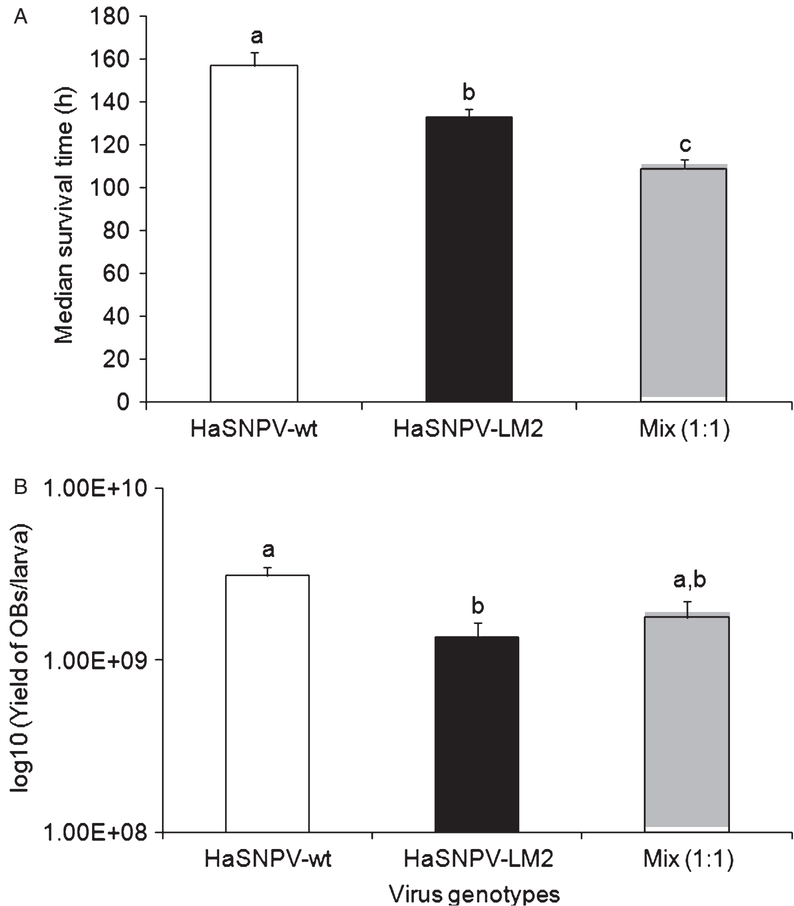


**Figure 3** (A) Median survival time (+ SE) of fourth instar *Helicoverpa armigera* infected with wild type HaSNPV, recombinant HaSNPV-LM2 (−*egt*), and a 1:1 mixture of the two viruses. (B) Yield (+ SE) (logarithm of the number of occlusion bodies per larva) for the various virus treatments. Treatments sharing a letter above the bar are not significantly different (Mann–Whitney U-test for pairwise comparisons: P>0.05).

P<0.0001). Forty-eight larvae challenged with the 1:1 mixture of wild-type HaSNPV and HaSNPV-LM2 died significantly faster (by 24 and 48 h, respectively) than either of the single virus treatments (Figure 3A). As expected, larvae infected with HaSNPV-LM2 died more rapidly than larvae infected with HaSNPV-wt. There were significant differences in virus yield among treatments (ANOVA: $F_{2,37} = 6.43$, 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, d.f. = 28, P = 0.002). However, there were no significant differences in virus yield between the wild-type and mixed infected larvae (t = 1.87, d.f. = 23, P = 0.073) or between recombinant and mixed infected larvae (t = 1.61, d.f. = 23, P = 0.12) (Figure 3B).

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

There was a significant difference in the total distance travelled among virus treatments ($F_{3,27} = 5.0$, P = 0.007), mostly representing the shorter life span of the infected larvae (Figure 4A). Larvae infected with the recombinant virus HaSNPV-LM2 moved less than half the distance of the control larvae (t = 2.15, d.f. = 15, P = 0.048), and approximately 60% of the distance moved by wild-type-infected larvae (t = 2.91, d.f. = 15, P = 0.011). The distance moved by larvae challenged with the virus genotype mix did not differ significantly from any of the other treatments and was most similar to the distance moved by larvae infected with the wild type virus.

There was also a significant time effect on daily distance travelled (repeated-measures ANOVA: time, $F_{3,81} = 5.0$, P = 0.003). The interaction between time and treatment was not significant ($F_{9,81} = 0.9$, P = 0.476) (Table 1). On day 2 after infection, HaSNPV-wt infected larvae moved more than larvae infected with HaSNPV-LM2 (t = 3.71, d.f. = 15, P<0.05). There were no significant differences in movement among virus treatments on any of the other days. The positions of the larvae on the plant did not differ among virus treatments (Table 2). Also there was no observed difference among treatments on a daily basis, when the position of live larvae was monitored (Figure 5A).

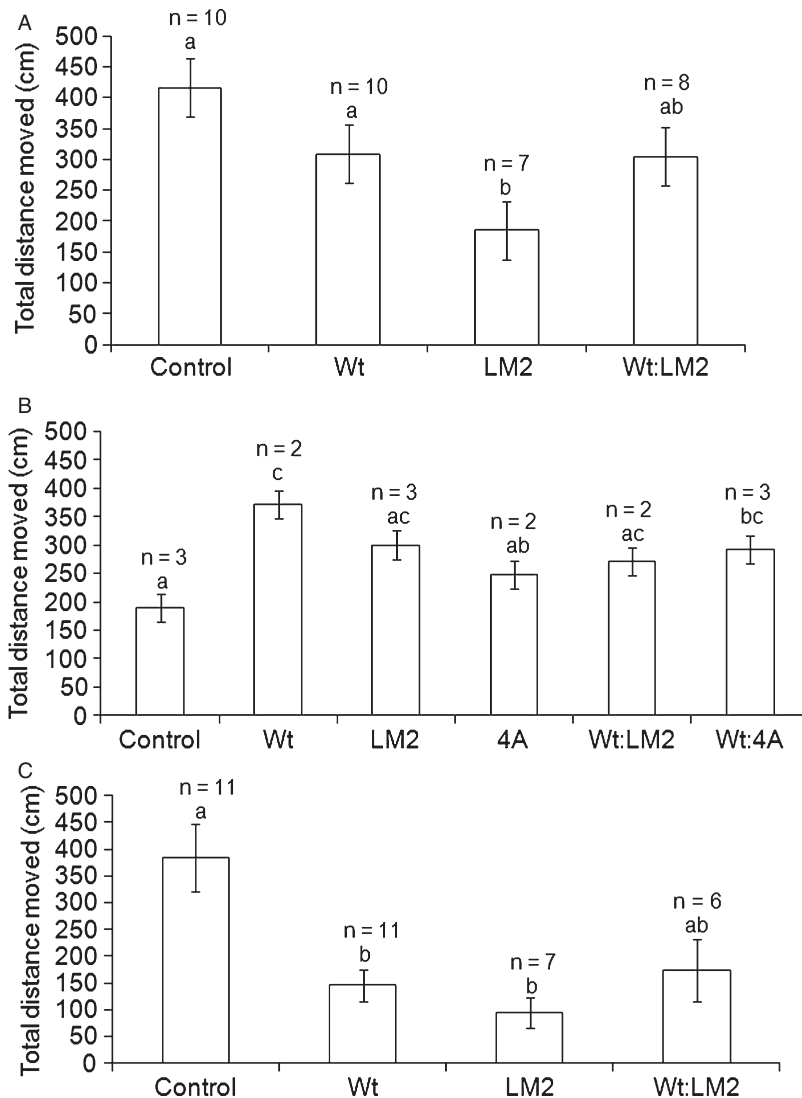


**Figure 4** Mean (± SE) total distance travelled in 4 days (cm) by *Helicoverpa armigera* larvae infected with different genotypes or genotype mixtures of HaSNPV in two glasshouse experiments (A: experiment 2; B: experiment 3) and one field experiment (C: experiment 4). Movement distances per day were √x transformed before ANOVA. Significance of pairwise differences is indicated by lettering over the bar. Treatments sharing a letter are not significantly different (Mann–Whitney U-test for pairwise comparisons: P>0.05). n, number of larvae per treatment.

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

Virus treatment had a significant impact on the total distance moved ($F_{5,9}$ = 3.52, P = 0.048), although this differed from the pattern seen in experiment 2 (Figure 4B). Surprisingly, the uninfected control larvae moved least, although this distance only differed significantly from insects infected with the wild-type and the wild-type: HaSNPV-4A mixture. Insects infected with the single HaSNPV-4A moved less than insects infected with the wild-type virus.

There was a significant effect of time on daily distance travelled (repeated-measures ANOVA: time, $F_{3,27}$ = 4.05, P = 0.017), whereas the interaction between time and treatment was not significant ($F_{15,27}$ = 0.98, P = 0.499) (Table 1). Differences between treatments were first seen on the 2nd day post-infection when larvae infected with HaSNPV-wt moved more than those infected with HaSNPV-LM2 (t = 2.47, d.f. = 13, P = 0.028). At the 3rd day post-infection, larvae infected with HaSNPV-LM2 moved more than insects with the mixture of HaSNPV-wt and HaSNPV-4A (t = 2.81, d.f. = 8, P = 0.023). No further pairwise comparisons within days were significant.

A large proportion of the 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 also 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)

**Table 1** Repeated-measures ANOVA of the effects of three HaSNPV variants (HaSNPV-wt, HaSNPV-LM2, and HaSNPV-mix) on distance travelled of fourth instar *Helicoverpa armigera* in three experiments

| Source of variation | MS | F | d.f. | 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 |

**Table 2** Final position (level) of dead *Helicoverpa armigera* larvae

| | Glasshouse | | Field |
|---|---|---|---|
| | Experiment 2 | Experiment 3 | Experiment 4 |
| HaSNPV-wt | 4.1 ± 0.9a | 6.3 ± 0.3a | 4.9 ± 0.3a |
| HaSNPV-LM2 | 3.4 ± 0.8a | 6.2 ± 0.7a | 3.3 ± 0.8a |
| Mix Wt:LM2 | 4.7 ± 0.9a | 4.6 ± 1.0ab | 3.6 ± 0.6a |
| HaSNPV-4A | | 2.1 ± 0.8b | |
| Mix Wt:4A | | 3.3 ± 0.0b | |

Position was recorded in terms of level, where level 0 is ground level, and there are two branches in each level (cf. Figure 1).
Mean values followed by the same letter within an experiment do not differ significantly (Mann–Whitney U-test for pairwise comparisons: P>0.05).

(Table 2). The position of cadavers infected with HaSNPV-wt and HaSNPV-LM2 were found significantly higher up the plant than those infected with HaSNPV-4A ($U_{3,7}$ = 2.5, z = −1.90, P = 0.05 and $U_{7,4}$ = 2.5, z = −2.3, P = 0.02, respectively). However, there was no significant difference in the final position of cadavers infected with a mixture of HaSNPV-wt and HaSNPV-LM2 and HaSNPV-wt and HaSNPV-4A and those infected with HaSNPV-4A ($U_{7,6}$ = 10.0, z = −1.63, P = 0.138 and $U_{7,4}$ = 5.0, z = −1.5, P = 0.171, respectively) (Table 2).

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

Although 30 larvae were released in each treatment, there was substantial natural mortality in the field experiment. Therefore in the analysis we only used those larvae that we were able to locate every day, until they died of virus infection. Larvae that could not be recovered because they had left the plant, or died of causes other than virosis (e.g., natural enemies which could not be excluded from the cage completely) were excluded from analysis. Thus we included in the analysis: six control larvae, 11 larvae in the wild type treatment, seven larvae in the LM2 treatment, and six larvae with mixed infections, giving a total of 30 larvae.

Total distance moved by the control larvae on the plant was substantially and significantly greater than the distance covered by larvae infected with HaSNPV-wt or HaSNPV-LM2 (over twice as long; Figure 4C). The insects with mixed infections of wild-type and HaSNPV-LM2 covered an intermediate distance, not significantly different from the other treatments (Figure 4C). Overall the pattern was similar to that seen in the first greenhouse experiment.

There was a significant difference in daily distance travelled between times (repeated-measures ANOVA: $F_{4,104}$ = 7.57, P = 0.001). There was no significant interaction between time and treatment ($F_{12,104}$ = 1.3, P = 0.176) and there were no significant differences among virus treatments in distance travelled by larvae ($F_{3,26}$= 1.3, P = 0.27; Table 1). There were significant differences in the position of the larvae on the plant at different days post-infection (Figure 5C) but no significant differences were observed in the final position of cadavers (Table 2).

## Discussion

We found varying effects of virus infection on the behaviour of fourth instars of *H. armigera*. In the first glasshouse experiment and the field study, the overall movement of infected larvae was generally less than that of healthy larvae, which is the result that we might expect given the effects of virus infection on vigour and life span. However, in the second glasshouse study, virus-infected larvae moved more than healthy larvae. 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 overall movement and a change in location of the cadavers, compared with larvae infected with the wild-type virus. The effect of a mixture of wild-type and HaSNPV-LM2 was

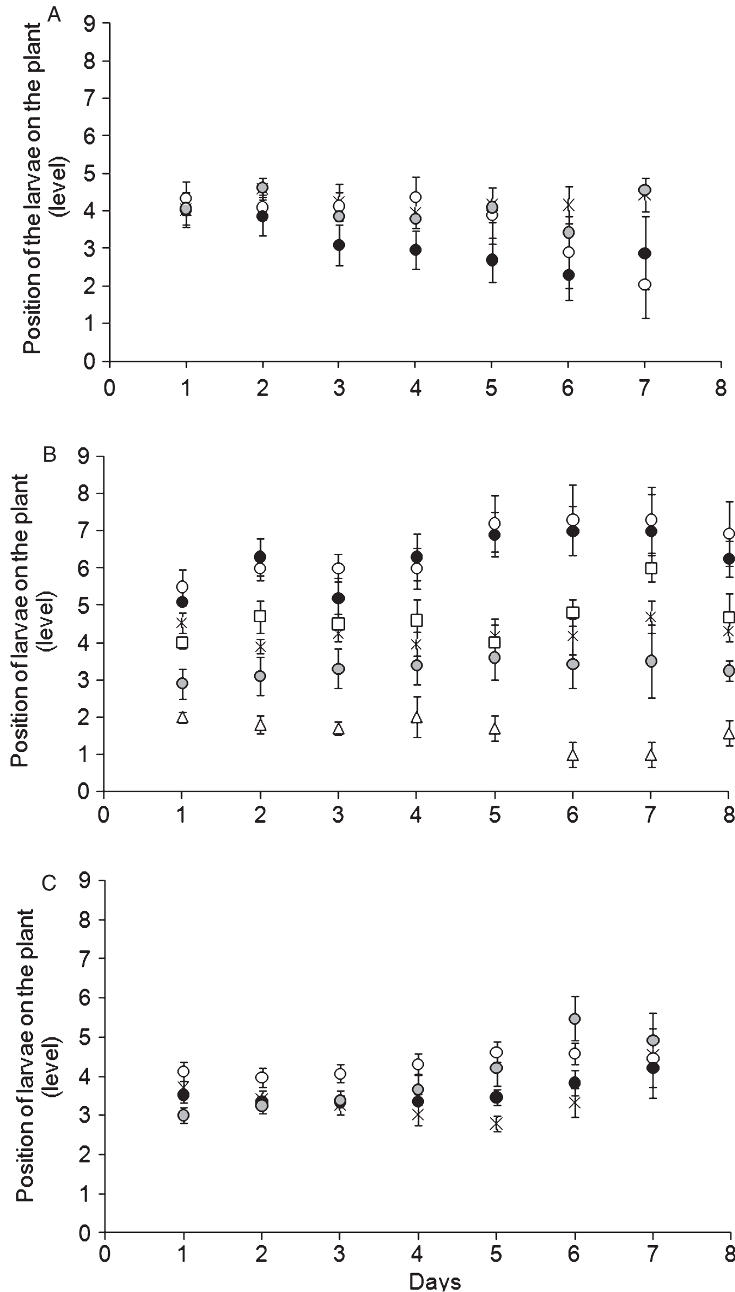


**Figure 5** Daily position (average level ± SE; see Figure 1 for description of levels) of *Helicoverpa armigera* larvae on cotton plants in different virus treatments in two glasshouse experiments (A: experiment 2; B: experiment 3) and one field experiment (C: experiment 4). Asterisk, control; open circles, HaSNPV-wt; black circles, HaSNPV-LM2; grey circles, mix HaSNPV-wt+ HaSNPV-LM2; triangles, HaSNPV-4A, and squares, mix HaSNPV-wt+ HaSNPV-4A.

most similar to pure wild-type infection, except for speed of kill in the laboratory bioassay, where it was more rapid than either single genotype. Larvae infected with the mixture of HaSNPV-wt and HaSNPV-4A behaved the same as the single HaSNPV-4A infection, in terms of assuming a lower position on plants at death compared with those from single infections of these two viruses and total mobility.

Differences in the 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 (40 °C) during the day and low (15 °C) during the night, whereas in the glasshouse temperature fluctuations between night and day were much more moderate, and environmental conditions may have been more conducive for activity.

In agreement with the data for *Mamestra brassicae* (L.), larvae infected with the unmodified HaSNPV-G4 moved upwards (Vasconcelos et al., 1996) (Figure 5A–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, and is in agreement with results in other systems, such as *T. ni* (Cory et al., 1994) and *Heliothis virescens* (Fabricius) (Hoover et al., 1995; McCutchen & Hammock, 1994). The mobility of virus-infected larvae in the field and the position where they die on the host plants will affect the distribution of the virus and thus the likelihood of secondary transmission. 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. Larvae infected with HaSNPV-4A (−*egt*, AaIT), and perhaps more importantly, also larvae infected with a mixture of HaSNPV-4A and the wild type, showed the characteristic symptoms of paralysis before death and exhibited a different behaviour from HaSNPV-wt infected larvae. This knockdown behaviour induced by the recombinant virus reduces horizontal transmission (Hails et al., 2002; Zhou et al., 2005; Georgievska et al., 2010a), but on the other hand it also increases deposition of virus in the soil and thus enhances virus persistence in agro-ecosystems. This might be beneficial in terms of longer-term control by a microbial agent, but may be considered a negative attribute with respect to biosafety of using baculovirus recombinants. Transmission could also be reduced as a result of reduced yields of OBs from larvae infected with HaSNPV-4A (Sun et al., 2005). However, survival time of these larvae will also be shortened, compared with larvae infected with wild-type virus, meaning that the recombinant virus can recycle more rapidly. Thus there are features that are both positive and negative in terms of ecological risk assessment and further studies would be needed to elucidate the longer-term consequences in the field.

In the introduction, we note that the effects of baculoviruses on the behaviour of their insect hosts are likely to be 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.

Based on these findings, we predict that transmission of HaSNPV variants with an *egt* deletion is likely to be similar to that of the wild type, whereas that of variants expressing a toxin gene will be reduced. Results of experiments under glasshouse and field conditions were in accordance with this prediction (Georgievska et al., 2010a). Transmission of virus recombinants also depends on the competitive interaction between wild-type and genetically modified virus within the host insect. Results of Zwart et al. (2009b, 2010) indicate that the competitive fitness of *egt*-deletion variants within individual insect hosts is reduced. The overall fitness of genetically modified baculoviruses depends on the interaction of within-host and between-host competitive processes, and remains difficult to predict. Milks et al. (2001) reported unaltered within-host fitness, when a recombinant AcMNPV expressing the insect-specific toxin AaIT was in direct competition with its parental wild-type virus. Models can help to assess these interactions (Bianchi et al., 2002; Bonsall et al., 2005; Sun et al., 2006; Zwart et al., 2009b, 2010). A critical parameter that may be needed in models for competitive fitness of baculovirus genotypes at agro-ecosystem level is the transmission of virus genotypes from hosts that die from mixed infections. No information is available on this topic, and hence, modelling studies remain explorative rather than predictive tools for the moment. In models at the agro-ecosystem level, the possibility of spatial separation between infected and healthy hosts is a factor that needs to be taken into account.

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