



Mixed-genotype infections of *Trichoplusia ni* larvae with *Autographa californica* multicapsid nucleopolyhedrovirus: Speed of action and persistence of a recombinant in serial passage

Mark P. Zwart^{a,b,*}, Wopke van der Werf^c, Liljana Georgievska^b, Monique M. van Oers^b, Just M. Vlak^b, Jenny S. Cory^d

^a Quantitative Veterinary Epidemiology Group, Wageningen University, The Netherlands

^b Laboratory of Virology, Wageningen University, The Netherlands

^c Centre for Crop Systems Analysis, Wageningen University, The Netherlands

^d Department of Biological Sciences, Simon Fraser University, Canada

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ABSTRACT

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 might be simultaneous release of a wild-type baculovirus, so as to facilitate rapid displacement of the recombinant baculovirus by a wild-type. However, at what ratio must the two baculoviruses be released? An optimum release ratio must ensure both fast action, and the eventual competitive displacement of the recombinant virus and 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 (coding for ecdysteroid UDP-glucosyltransferase) deleted. Time to death increased with the proportion wild-type virus in the inoculum mixture, although a 1:10 ratio (wild-type: recombinant) resulted in equally rapid insecticidal action as vEGTDEL alone. Five serial passages of three different occlusion body (OB) mixtures of the two viruses were also performed. OBs from 10 larval cadavers were pooled and used to initiate the following passage. Although the wild-type baculovirus was maintained over five passages, it did not go to fixation in most replicates of the serial passage experiment (SPE), and there was no good 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 single and multiple larvae are relevant because of potential modulating effects at the between-host level.

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1. 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 and Myers, 2003) and have a restricted host range (Federici, 1997). A major drawback of baculoviruses in insect pest control is their slow speed of action (Moscardi, 1999). Crop damage after application of a baculovirus spray can therefore 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 engi-

neer 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 impacts on non-target organisms and other environmental ramifications.

Many studies have addressed different aspects of recombinant baculovirus fitness. The trend in these studies is that fitness of recombinant viruses is either not distinguishable from that of the parental wild-type virus (Bianchi et al., 2000b; Sun et al., 2004a) or is reduced (Cory et al., 1994, 2004; Sun et al., 2005; Zhou et al., 2005). Lower virus yield associated with shorter survival time of infected insects (Cory et al., 2004) suggests that recombinant baculoviruses may be less fit than wild-type viruses at the

* Corresponding author. Address: Quantitative Veterinary Epidemiology Group, Wageningen University, P.O. Box 338, 6709AH Wageningen, Gelderland, The Netherlands. Fax: +31 317 485006.

E-mail addresses: mark.zwart@wur.nl, markzwart@gmail.com (M.P. Zwart).

between-host level. These patterns in speed of kill and virus yield 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-killed insect from plant surfaces (Hoover et al., 1995) and thereby 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. (2009b) 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. The *egt* gene encodes the ecdysteroid UDP-glucosyltransferase enzyme, which inactivates ecdysteroids and thereby modulates host development in a manner that results in a higher occlusion body (OB) yield (O'Reilly and Miller, 1989; Cory et al., 2004; Zwart et al., 2009b). In some hosts 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 and Miller, 1989; Cory et al., 2004).

The available evidence therefore 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 the competitive displacement of the fast-acting recombinant, a wild-type virus strain could be added to the recombinant virus inoculum. 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 agro-ecosystems?

Other strategies for mitigating the persistence of recombinant baculoviruses have been previously suggested. For example, Hamblin et al. (1990) suggested the use of 'co-occluded' OBs containing both a wild-type virus and the AcMNPV recombinant Ac-E10 that lacks the *polyhedrin* gene and is therefore incapable of generating OBs autonomously. Wood et al. (1993) and Hughes and Wood (1996) suggest the use of only Ac-E10 virions (i.e., pre-occluded 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, and therefore the virus will be quickly lost from the environment. The approach we study here – using 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 occluded baculoviruses (i.e., those retaining the *polyhedrin* gene) is also relevant to evaluating the approaches suggested by Hamblin et al. (1990) and Hughes and Wood (1996).

Zwart et al. (2009b) found that when *Trichoplusia ni* (Hübner) larvae were challenged with an LD₈₀ (a dose that produces 80% mortality) of AcMNPV at a wild-type to recombinant ratio of 1:100, most larvae were solely infected by the recombinant virus. It is plausible that at this dose a simple sampling effect is responsible for the absence of the wild-type virus in the host; i.e., the founder number for the infection is well below 100, and only 1 in 100 founders is of the wild-type virus (see Zwart et al., 2009a). On the other hand, with the same dose but 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., 2009b). Thus, at an LD₈₀ dose and a wild-type to recombinant ratio of 1:10, the wild-type virus will be present in the majority of infected insects. This

inoculum fulfills one of the requirements for a recombinant virus application strategy based on co-packaging of a wild-type virus in a biological control product, but we also need to know: (i) What is the speed of action at this dose and ratio? (ii) Will the wild-type virus go to fixation and displace the faster-acting recombinant virus?

We first determined time to death for different ratios of wild-type to recombinant viruses in bioassays at LD₈₀. As a wild-type virus, we used the parental virus of vEGTDEL, AcMNPV Wt L1 (Lee and Miller, 1978). An LD₈₀ 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) was performed starting with different ratios of the wild-type and recombinant viruses, to test whether the wild-type virus would competitively displace the recombinant completely and go to fixation. Alternatively, the recombinant might competitively displace 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 represents a stable equilibrium (e.g., Gotelli, 2001). 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., 2009b). 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. In our study we therefore chose to perform a SPE with an inoculum preparation from 10 larval cadavers. This experimental design focuses on the competitive process at the between-host level. The two situations – passaging in a single larva or passaging in 10 larvae – probably represent extremes compared to what may happen in the field. This approach complements previously reported studies, as the ensemble of previous results and those reported here bracket the relevant range of the number of OB sources for insect larvae.

2. Materials and methods

2.1. Insects and viruses

T. ni larvae were reared as previously reported (Zwart et al., 2009b; 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° or 16 °C, for 1 or 2 days, in order to keep the insects in a regular rearing scheme. Larvae, pupae and moths were kept with a 16-h photoperiod and at 27 °C.

AcMNPV Wt L1 and vEGTDEL (O'Reilly and Miller, 1991) were amplified in 30 fourth-instar *T. ni* larvae (L4) inoculated at a high dose (approx. 100 × LD₉₉). 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 (2500g for 15 min) and washed thrice, and then stored in 50% (v/v) glycerol. Note that all virus ratios – whether quantified OBs or quantitative real-time PCR genotype ratios – are given as the ratio of Wt L1 to vEGTDEL.

2.2. Time to death bioassays

We obtained developmentally synchronous *T. ni* L5 (final instar) by selecting L5 insects which had slipped their head capsules within a 12-h interval. The chosen larvae were subsequently reared on diet at 27 °C for another 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 hemocytometer (20 counts per virus). The OBs were serially diluted in water to give suspensions of 10^6 OBs ml⁻¹. Two microliters of the OB suspension was applied to small diet plugs placed in 12-well plates. This should give a dose of 2000 OBs/larvae, which is equivalent to an LD₅₀ 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: vEGTDEL), 1:100, 1:10, 1:1, 10:1, 100:1, 1:1000, and Wt L1 alone. For each of the nine virus treatments, 36 larvae were used. For the non-virus controls, 24 additional larvae were fed diet plugs treated with 2 µl of water. After 12 h at 27 °C, those larvae that 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 larvae to pupation, but more diet was added if necessary. Mortality was recorded every 12 h until all larvae had died or pupated. The larval cadavers 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, we determined the mean time to death, which was calculated from the estimated survival curve (Kaplan–Meier estimate). Only larvae that died were included in the analysis; all surviving larvae pupated by the end of the experiment. A Jonckheere–Terpstra test was used to determine if an increase in the proportion of 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 *post hoc* multiple-comparisons test was used to test for significant differences in mean time to death.

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 to death was calculated with Kaplan–Meier survival analysis, and significant differences between treatments tested for with a pair-wise *t*-test. All statistical analyses were performed in SPSS 15.0 (SPSS Inc., Chicago, IL).

2.3. OB yield per cadaver

OB yields were individually determined for all larvae that died in the first bioassay replicate. Larval cadavers were macerated in 500 µl milliQ water. OBs were counted in duplicate in a 1:100 dilution of each sample, using a hemocytometer. Analysis of variance (ANOVA) was performed on square-root transformed OB yield. Between-treatment variation (χ) was partitioned into level, linear and quadratic components:

$$y = b_0 + x + b_2 x^2$$

where y is the square-root transformed OB yield, and x is the base 10 logarithm of the genotype ratio of the OB mixture. Four and –4 were assigned as the x -values for the Wt L1 and vEGTDEL treatments, respectively. Coefficients for the level, linear and quadratic

components were calculated using non-linear regression. Tukey's *post hoc* multiple-comparisons test was used to test for significant differences between treatments in OB yield.

2.4. 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 purified OBs was determined by counting in a hemocytometer (at least two counts per replicate). The next round of passage was initiated with a dose of 2000 OBs. As a control, Wt L1 and vEGTDEL were passaged from a single larva.

2.5. 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. (2009b), with all qPCR reactions being performed twice. In summary, the forward primer 5'-GTCGTTCGTTGAAGCGTTTGCC-3' and the reverse primer 5'-TCGGCCAAACCGTAGCCAGG-3' were used to detect the Wt L1 virus, and the forward primer 5'-CGTTACGGTCGTCAAGCCCAA CTGTTT-3' and the reverse primer 5'-TCGAATTACGGTCTCTGT GAATTGATGGC-3' were used to detect vEGTDEL. Separate PCR reactions were used to detect the Wt L1 and vEGTDEL, with SYBR Green I used as a fluorophore (Quantitect SYBR Green Master Mix, Qiagen, Hilden, Germany). Template concentration was then determined by comparative analysis, using RotorGene 6.0 software (Corbett Research, Sydney, Australia), and the virus ratio (Wt L1 to vEGTDEL) could then be calculated.

2.6. Calculation of selection rates

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 k th passage and $z(0)$ the initial value of z . The proportion of recombinant is $1 - z(k)$. The selection rate w represents 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 baculovirus, 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 or greater than 1000:1 (Zwart et al., 2009b), 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 the 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 parameter w was estimated by taking the antilogarithm of the slope of the regression. Based on the chain rule of mathematical calculus:

$$SE(f(x)) = \frac{df}{dx} SE(x)$$

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

$$SE(w) = SE(\exp(b)) = \frac{d}{db} \exp(b) \cdot SE(b) = \exp(b) \cdot SE(b) \\ = w \cdot SE(b)$$

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

3. Results

3.1. Mean time to death for OB mixtures

Bioassays were performed in order to understand how virus mixtures affected time to death (Fig. 1). A Jonckheere–Terpstra test indicated that mean time to 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 had a significantly slower speed of action than the vEGTDEL alone (Fig. 1). Different virus ratios caused similar levels of mortality (Kruskal–Wallis test; $\chi_8^2 = 2.134$, $P = 0.977$), and there was no significant effect of the proportion of Wt L1 virus on mortality (Jonckheere–Terpstra test; Stand. $JT = -0.42$, $N = 27$, $P = 0.966$).

Trends in the data (Fig. 1) suggest that the mixtures with 1:1000 and 1:100 ratios may have faster speeds of action than the vEGTDEL virus alone, although differences in time to death 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. In this bioassay, there was no significant difference in time to death after challenge with vEGTDEL or a 1:100 mixture (pair-wise *t*-test, $t_4 = -0.911$, $P = 0.414$); in fact, mean time to death was shorter upon challenge with pure vEGTDEL. Thus, overall, the speed of action of pure recombinant virus and mixtures with a small amount of wild-type virus was not significantly different.

OB yield was determined for all larval cadavers from the first replicate of the bioassay (Fig. 2). A one-way ANOVA indicated that there were differences in OB yield between inoculum mixtures ($F_{8,239} = 2.541$, $P = 0.011$), although there were no significant differences between inoculum mixtures when pair-wise comparisons were made with a *post hoc* test (Fig. 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. Non-linear regression resulted in the following coefficients \pm standard error: $b_0 = 10.186 \pm 1.055$; $b_1 = 0.296 \pm 0.457$; $b_2 = -0.108 \pm 0.044$. The quadratic component of the between-treatment variation (b_2) was negative, indicating a downwards curving function. The maximum for the quadratic function for OB

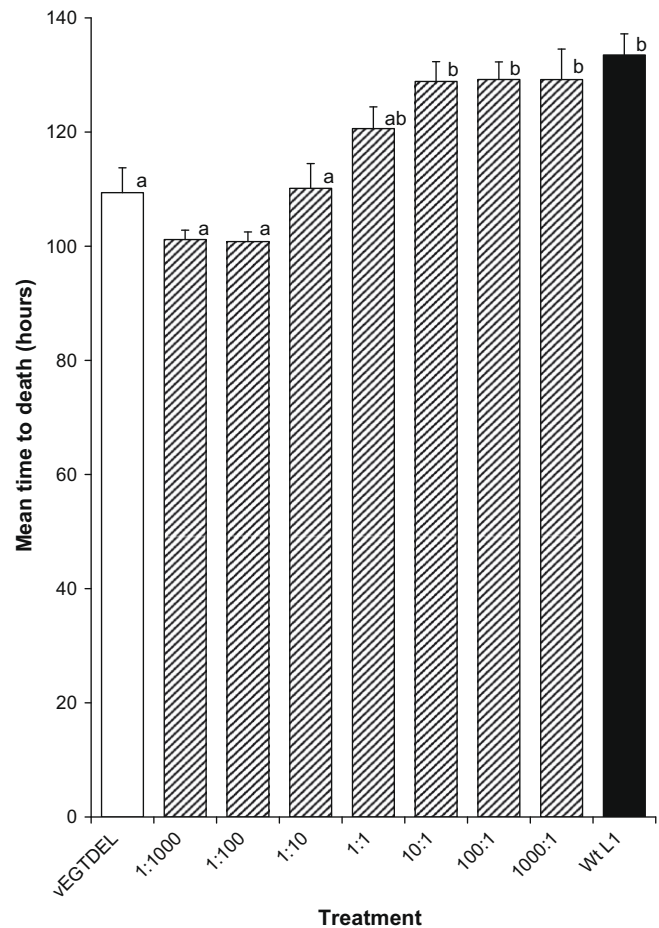


Fig. 1. Time to death for *Trichoplusia ni* fifth instars (L5) after challenge with different ratios of wild-type and recombinant AcMNPV. The mean of three replicate determinations of mean time to death (in hours) is on the y-axis (with standard errors), with different bioassay occlusion body (OB) inoculum mixtures (hatched 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-comparisons 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 less than 0.01.

yield occurred at a log genotype ratio of 1.37 ± 0.99 (estimate \pm standard error), corresponding to a maximum yield between the 1:1 and 10:1 treatments. The yield occurring at this maximum was 117% that of Wt L1 alone, and 171% that of vEGTDEL alone.

3.2. Serial passage experiment

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 (Fig. 3). Note that Zwart et al. (2009b) 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 were 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, whereas 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 rates (w) were not significantly different from 1 for

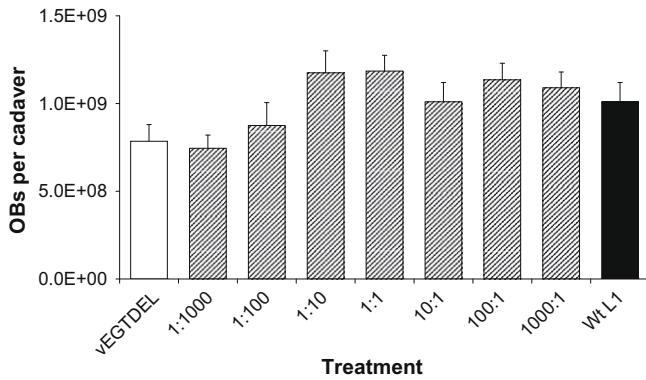


Fig. 2. Virus yield for *Trichoplusia ni* fifth instars (L5) after challenge with different ratios of wild-type and recombinant AcMNPV. Mean occlusion body (OB) yield per cadaver is on the y-axis (with standard errors), with different bioassay OB mixtures (hatched bars), vEGTDEL alone (white bar), and Wt L1 alone (black bar), on the x-axis. All ratios for OB mixtures are given as Wt L1 to vEGTDEL. There were no significant differences between different treatments (mixtures) ratios when pairwise comparisons were made with Tukey's *post hoc* multiple comparison test. ANOVA demonstrated significant linear and quadratic components in the response of yield to genotype ratio.

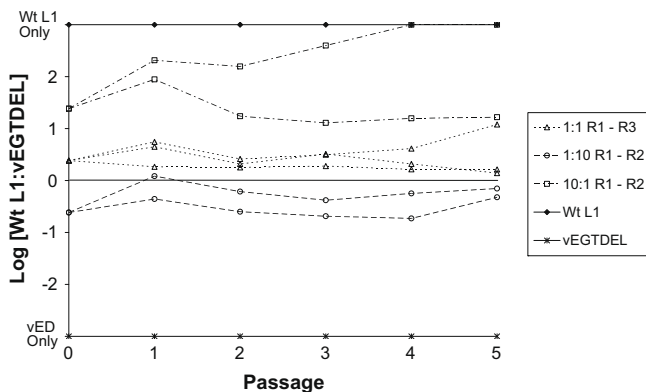


Fig. 3. qPCR data of serial passage experiments. Within each treatment different replicates are denoted as R1-R2 or R1-R3. On the x-axis is the passage number, and on the y-axis is the base 10 logarithm of qPCR ratio (Wt L1 to vEGTDEL) measured in 10 pooled larval cadavers used for passaging.

any inoculum ratio (for 10:1 ratio: $w = 1.65 \pm 0.65$; for 1:1 ratio: $w = 1.00 \pm 0.08$; for 1:10 ratio: $w = 1.05 \pm 0.11$; mean \pm SE given).

4. Discussion

We investigated baculovirus mixed infections using vEGTDEL, an AcMNPV-derived recombinant baculovirus, and 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 action is an indicator for biological control efficacy (Cory et al., 1994; Sun et al., 2004b), whereas fixation of the wild-type baculovirus in serial passage is an indicator for ecological safety. A 1:10 mixture gave a time to death similar to vEGTDEL alone, with a mean difference of only 2 h, which was not a statistically significant difference (Fig. 1). Zwart et al. (2009b) 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 action with the maintenance of

the wild-type virus in infected larvae. However, will the wild-type virus 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 it was maintained in all seven, the wild-type virus went to fixation in only one replicate (Fig. 3). Selection rate constants were not significantly greater than 1 at any of the initial inoculum ratios. We conclude that while the wild-type virus was maintained when an inoculum mixture of 1:10 is used, there is not sufficient 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 a SPE in a single larva (Zwart et al., 2009b). In contrast, in the SPE with inoculum from pooled larvae, reported here, there was no sign of selection for the wild-type virus. The main difference in the experimental design is the number of larvae used for passaging. How could the use of multiple larvae producing the OB inoculum nullify the selection for wild-type virus observed in single-larval passages? The SPEs in single and multiple larvae represent highly different experimental designs: when passaging in a 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 suggest that intermediate mixtures may produce greater OB yield in *T. ni* L5 larvae (Zwart et al., 2009b). OB yield data obtained in our study (Fig. 2) had a significant, negative (i.e., downward curving) quadratic component in the between-treatment variation, suggesting that intermediate mixtures produce a higher OB yield. These observations may explain why there was 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., 2009b), 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 that have addressed this topic.

We have conducted our experiments with a fixed dose (LD_{80}) in L5 larvae, which are more resistant than earlier-instar larvae (e.g., Bianchi et al., 2000b; Hoover et al., 2002; Cory et al., 2004). This means that the infection probability per OB is lowest in L5 larvae, and that when larvae of all instars are exposed to the same OB dose, the average number of viral founders of infection will be smallest in L5 larvae and higher in all other instars. Co-infection with the wild-type virus should therefore be expected in all treated instars when the dose is high enough to give a high frequency of co-infection in the least susceptible instar. This makes L5 larvae very suitable for evaluating the persistence of a recombinant virus: if the recombinant virus is not lost due to stochastic processes in L5 larvae, it will probably not be lost due to stochastic processes in earlier-instar larvae. For effective biological control, however, baculoviruses must be applied against earlier instars (e.g., Bianchi et al., 2002).

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 a laboratory setting – the OB ratio can be optimized for achieving fast speed of action and the maintenance of the wild-type virus. For the wild-type virus to displace

the recombinant virus, it must first be maintained in the virus population, but 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 fast-acting recombinant of AcMNPV in serial passages, even though the same recombinant was competitively displaced by wild-type AcMNPV in previous passage experiments in which inoculum was transferred from larva to larva, without inoculum pooling between source 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 and multiple larvae – must therefore be given due 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 and 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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