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Mixed infections and the competitive fitness of faster-acting genetically modified viruses

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Abstract

Faster-acting recombinant baculoviruses have shown potential for improved suppression of insect pests, but their ecological impact on target and nontarget hosts and naturally occurring pathogens needs to be assessed. Previous studies have focused on the fitness of recombinants at the between-hosts level. However, the population structure of the transmission stages will also be decided by within-host selection. Here we have experimentally quantified the within-host competitive fitness of a fast-acting recombinant *Autographa californica* multi-capsid nucleopolyhedrovirus missing the endogenous *egt* gene (vEGTDEL), by means of direct competition in single- and serial-passage experiments with its parental virus. Quantitative real-time PCR was employed to determine the ratio of these two viruses in passaged mixtures. We found that vEGTDEL had reduced within-host fitness: per passage the ratio of wild type to vEGTDEL was on average enhanced by a factor of 1.53 (single passage) and 1.68 (serial passage). There is also frequency-dependence: the higher the frequency of vEGTDEL, the stronger the selection against it is. Additionally, the virus ratio is a predictor of time to host death and virus yield. Our results show that *egt* is important to within-host fitness and allow for a more complete assessment of the ecological impact of recombinant baculovirus release.

Introduction

Insect pathogens offer viable alternatives to synthetic chemical pesticides for the suppression of insect pests. One particular pathogen, the bacterium *Bacillus thuringiensis* (or one or more of its toxins), is widely used in spray applications or by the incorporation of its toxins into a variety of crop plants (Christou et al. 2006). Representatives of other groups of entomopathogens, such as viruses and fungi, have a long history of use and the occasional widespread success (Moscardi 1999; see also Scholte et al. 2005). However, in general they have not achieved their potential as insecticides. One reason for

this lack of take-up is that they are perceived as slow acting, at least in comparison to synthetic chemicals and insect growth regulators. This has led to the development of faster-acting genetically modified pathogens, particularly in the insect baculoviruses (Inceoglu et al. 2006).

Baculoviruses are highly pathogenic for certain insect species and can have a major impact on the population dynamics of their host (Cory and Myers 2003). It is therefore important to be able to predict whether the release of genetically modified baculoviruses will displace native wild type strains and alter the dynamics of their host or hosts. To date, this issue has received some theoretical treatment (Dushoff and Dwyer 2001; Bonsall et al.

2005) and contained field experiments have compared the horizontal transmission of recombinant and natural viruses (Cory et al. 1994; Hails et al. 2002). These studies focus on between-host transmission. However, the interactions within the host can be equally important in parasite ecology and evolution and yet these have received little attention. Within-host selection will to a large extent determine how many and what type of transmission stages are produced, and this will influence key traits, such as virulence, that are fundamental to the epizootiology of pathogens in natural populations (e.g. De Roode et al. 2005; Ben-Ami et al. 2008). Studies on multiple infection and within-host competition of parasites have tended to focus on how they shape the evolution of parasite virulence in relation to disease intervention strategies such as vaccination and the avoidance of drug resistance (e.g. De Roode et al. 2004). Within-host selection, however, is also of direct relevance to the risk assessment and environmental release of recombinant pathogens, such as those used for insect pest control.

The *Baculoviridae* are a large family of arthropod viruses characterized by their rod-shaped nucleocapsids and relatively large dsDNA genomes (Theilmann et al. 2005). Most known baculoviruses are infectious to Lepidopteran insect larvae, and cause lethal host infections. Due to their life-history traits, baculoviruses are considered obligate host-killing parasites (Ebert and Weisser 1997). The infection cycle is biphasic; after the initial infection, viral spread to different tissues within an individual host is mediated by single virions, known as budded virus (BV). BV has limited persistence outside of the host body. At the end of the infection cycle, the host insect dies and the cadaver releases what is typically a very large number of horizontal transmission stages, referred to as polyhedra for the Alphabaculoviruses (Federici 1997; Jehle et al. 2006). Polyhedra are proteinaceous occlusions containing a large number of virions. They can remain infectious for long periods of time under the right conditions, for example in the soil (Thomas et al. 1972; England et al. 1998). Thus horizontal transmission only occurs upon host death, and the horizontal transmission stage has prolonged persistence outside of the host. These characteristics are crucial to understanding baculovirus ecology and evolution.

A number of genetically modified baculoviruses with improved characteristics for pest control have been created (Inceoglu et al. 2006). Many of these recombinant viruses kill the target pest more rapidly than the original wild type virus, leading to reduced crop damage in the field (Cory et al. 1994; Sun et al. 2004). There are several reports in which the fitness components of wild-type viruses and their derived recombinants have been experimentally determined in the laboratory. A faster speed of

kill usually leads to reduced larval cadaver weight and virus yield (e.g. Burden et al. 2000; Cory et al. 2004), as the host insect has less time to develop and the virus in turn has less host tissue available to convert to transmission stages. Virus virulence, defined here as the likelihood of fatal infection, is not linked to speed of kill. Pathogenicity is therefore often unchanged in recombinant baculoviruses, compared to their parent wild type (e.g. Hernández-Crespo et al. 1999; Burden et al. 2000; Sun et al. 2004). While understanding the effects of genetic modification on traits that affect efficacy and between-host transmission is crucial for predicting the environmental impact of recombinant insecticides, the population structure of the transmission stages will to a large extent be determined by within-host selection. In particular, we need to ask, how will these fast-acting recombinant viruses fare when they are in direct competition with a wild-type virus in the same host?

As natural baculovirus isolates are typically composed of numerous genotypes (Lee and Miller 1978; Smith and Crook 1988; Cory et al. 2005), co-infections appear to be the norm in the field under natural conditions. Moreover, different baculovirus genotypes can be co-occluded in the same polyhedron if the host is infected with multiple genotypes (Hamblin et al. 1990; Bull et al. 2001). This probably contributes to the simultaneous transmission of multiple genotypes, therefore making understanding the competitive process at the within-host level of selection all the more important.

To address the issue of the within-host competitive fitness of a fast-acting virus genotype, we chose to study a baculovirus lacking the *egt* gene. This gene encodes the enzyme ecdysteroid UDP glucosyltransferase, which inactivates ecdysteroids by catalysing their conjugation with glucose (O'Reilly and Miller 1989). Inactivation of ecdysteroids results in the delay or complete block of molting. Deletion of the *egt* gene is a commonly used approach for constructing recombinant baculoviruses with improved biological control properties. Moreover, it is often combined with the incorporation of an exogenous gene, such as an insect selective toxin which further increases speed of kill (Inceoglu et al. 2006). An *egt* deletion mutant (in future called vEGTDEL) was derived from the AcMNPV Wt L1 strain by deletion of a part of the *egt* ORF (O'Reilly and Miller 1989; see also Fig. 2B). The virus kills host larvae faster than the wild-type virus and shows reduced yield in single genotype infections (Cory et al. 2004).

In order to evaluate the within-host competitiveness of vEGTDEL, direct competition experiments with its parental wild-type virus were performed (Elena and Lenski 2003). A calibrated quantitative real-time PCR (qPCR) assay (e.g. Zwart et al. 2008b) for the Wt L1 and vEGTDEL genotypes was developed to determine the ratio of

the two genotypes in individual larval cadavers. Single-passage experiments with a range of mixtures of the Wt L1 and vEGTDEL polyhedra used as inoculum were performed. Serial-passage experiments with a 1:1 mixture of polyhedra were also performed. Both sorts of experiments were carried out as: (i) different baculovirus genotypes can be co-occluded in the same polyhedron (Hamblin et al. 1990; Bull et al. 2001). The initial inoculum used in these experiments was a mixture of single genotype polyhedra. Therefore, using these two set ups allows us to explore if co-occlusion, presumably occurring after the first passage in the serial-passage experiment, has any effect on the persistence of the genotypes. (ii) The serial passage setup is inherently more complex, due to, for example, co-occlusion and differences between larval cohorts. As such, it offers a good test of the results measured in single-passage experiments.

Methods

Insects and viruses

Trichoplusia ni larvae were reared as described for *Spodoptera exigua* (Smits et al. 1986). Briefly, insects were maintained at 27°C, with a 16-h photoperiod. Larvae were reared communally on artificial diet. The cornmeal in the diet formulation described by Smits et al. (1986) was replaced by an equal amount of wheat germ. Adults were kept in breeding cages and fed a 5% sucrose solution. Paper towels were put in the breeding cages for egg laying. The towels with eggs were then surfaced sterilized as described by Smits et al. (1986).

The Wt L1 virus and vEGTDEL were amplified separately by inoculating 30 fourth instar *T. ni* with a high dose (approx. 100 × LD₉₉), and collecting larval cadavers upon death and storing them at -20°C. Polyhedra were purified from cadavers as described by Zwart et al. (2008b).

Electron microscopy

Electron microscopy of thin sections of polyhedra of Wt L1 and vEGTDEL viruses was performed as described by Zwart et al. (2008b). The number of nucleocapsids per occlusion derived virus (ODV) was counted for approximately 100 suitably oriented ODV for each genotype. The Kolmogorov–Smirnov test (KST) was used to test for differences between the two viruses in the number of nucleocapsids per ODV. KST tests for differences in the whole probability density function of nucleocapsids per ODV, not just different means.

More than 100 randomly selected polyhedron cross sections were digitally photographed for each virus. The number of ODV per polyhedron cross section was

counted and the surface area was measured using AnalySIS software (Olympus Soft Imaging Solutions, Münster, Germany). The Mann–Whitney *U*-test was used to test for differences in mean cross-sectional area and mean number of ODV per polyhedron cross-section between the Wt L1 and vEGTDEL viruses.

qPCR

A SYBR Green I-based qPCR assay to quantify Wt L1:vEGTDEL ratios was developed. For specific detection of the Wt L1 virus, the forward primer 5'-GTCGTCGTG GAAGCGTTTGCC-3' and the reverse primer 5'-TCGGC CAAACCGTAGCCAGG-3' were used. For detection of the vEGTDEL, the forward primer 5'-CGTTACGGTCGT CAAGCCCAAAGTGTG-3' and the reverse primer 5'-TCGAATTACGCGTTCTGTGAATTTGATGGC-3' were used. Note that the vEGTDEL primers can in principle also detect the Wt L1 virus, but will give a much larger product (2 kbp, see Fig. 2B). As vEGTDEL contains no unique DNA sequences, the only way to discriminate between the two viruses with a PCR-based assay was to use primers for the amplicon spanning the junction of the deletion in the *egt* ORF, and to keep elongation periods in the PCR program as short as possible (10 s). When PCR was performed on DNA from pure vEGTDEL, only the short PCR product corresponding to vEGTDEL could be observed on an agarose gel. No PCR product was observed when the Wt L1 virus was used (data not shown). During qPCR runs, the 'melting curve peak' of the short PCR product (corresponding to vEGTDEL amplification) was consistently observed. No peaks at higher dissociation temperatures – which would indicate amplification of the Wt template – were observed in any sample tested with the vEGTDEL primers. Thus, the PCR for vEGTDEL appeared to be specific to this genotype.

Wt L1 and vEGTDEL PCR products from a regular PCR reaction were electrophorized in 1% agarose gels, excised from the gel and subsequently purified using the DNA Extraction Kit (Fermentas, Burlington, Canada). The purified DNA was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA), and its identity checked by PCR and restriction enzyme analysis. Plasmid DNA concentration was quantified by photospectrometry and gel electrophoresis. A dilution with 10⁷ plasmids μL⁻¹ containing the respective fragment (Wt L1, vEGTDEL) was used as a calibrator sample in qPCR reactions. Quantification of virus genotype ratios by qPCR was performed as described elsewhere (Zwart et al. 2008b), with minor modifications: (i) an annealing temperature of 58°C instead of 60°C was used and (ii) an elongation time (72°C) of 10 s was used.

Prior to analysis of samples, mixtures of polyhedra were used to calibrate the qPCR assay, as described

(Zwart et al. 2008b). Briefly, pure Wt L1 and vEGTDEL polyhedra, and a range of mixtures (1:10⁴–10⁴:1) with 10-fold intervals were quantified by qPCR. The polyhedra input ratio was corrected for differences in mean number of ODV per polyhedron, by multiplication of the polyhedra ratio by the ODV ratio (see the section ‘Polyhedra morphology of the Wt L1 and vEGTDEL viruses’; ODV per polyhedron Wt L1:vEGTDEL = 1.830). Regression analysis on log transformed genotype ratios was performed to determine the accuracy of the PCR assay. The calibration experiment was performed twice. The regression line obtained from the combined data of the PCR calibration experiments was used to correct the measured ratios and obtain actual genotype ratios, for all subsequent samples analysed.

For all experimental samples the genotype ratio (R) was calculated, which could subsequently be used to determine the proportion of Wt L1 virus in the sample (f_{wt}):

$$\log(R) = \log\left(\frac{f_{wt}}{1-f_{wt}}\right) = \frac{\log(c_{wt}/c_{ed}) - b}{m} \quad (1)$$

Here c_{wt} and c_{ed} are the respective measured copy numbers obtained from the qPCR assay, and m and b are the slope and y -intercept of the qPCR calibration regression line, respectively.

Single-passage mixed-infection of larvae

Newly molted fifth instar *T. ni* larvae were selected by head capsule slippage within a 12 h time frame. The larvae were then kept on artificial diet at 27°C for 24 h before being challenged with mixtures of polyhedra. Polyhedra of the Wt L1 virus and vEGTDEL were quantified by counting with a haemocytometer (20 counts per virus). The polyhedra were then diluted in water to give a final concentration of 10⁶ polyhedra mL⁻¹. Wt L1 and vEGTDEL were mixed in ratios of 1:100, 1:10, 1:1, 10:1 and 100:1. Two microlitres of uncontaminated water, pure virus suspension or one of the mixtures was then pipetted onto a small plug of artificial diet in a 12-well tissue culture plate and allowed to dry briefly, giving a virus dose of 2000 polyhedra per larva (LD₈₀). Twenty-four larvae were taken for the nonvirus control, and 36 larvae for each treatment. The larvae were then added to wells individually, and kept at 27°C for a further 12 h. Only those larvae which had consumed all the diet were individually transferred to new 12-well plates with fresh, uncontaminated artificial diet. Mortality was recorded every 12 h until all larvae had died or pupated, and larval cadavers collected and individually stored in 1.5 mL Eppendorf tubes at -20°C.

Fourteen larvae from each inoculum mixture, and five larvae from the pure Wt L1 and vEGTDEL treatments, were randomly selected for qPCR analysis. Larval weight was determined by weighing the tubes containing larval cadavers, and subtracting the weight of the tube. Larvae were macerated in 500 µL milliQ water. A 1:100 dilution of the resulting virus suspension was counted twice with a haemocytometer to determine polyhedra yield. The larval remains were filtered through cheesecloth no. 80 (muslin), and DNA was then extracted as described elsewhere (Zwart et al. 2008b). DNA was diluted 1:200 in milliQ water prior to qPCR analysis.

Serial-passage mixed-infection experiments

For passaging from individual larvae, a 1:1 starting mixture of polyhedra was used. Per replicate, 10 larvae were challenged as described for the single-passage infection experiment, with the same procedure and virus dose. A randomly selected, individually stored larval cadaver was used for polyhedra purification. Each larva was macerated and the remains filtered through cheesecloth. Polyhedra concentrations were quantified by counting in a haemocytometer (at least 2 counts per replicate). These polyhedra were used to initiate the next round of infection. Fifteen replicates were performed: five replicates in an initial experiment and ten replicates in a subsequent experiment. Five serial passages were performed, and the data from the two experiments were combined for analysis. As controls for the passaging experiment, a single replicate of the Wt L1 virus and vEGTDEL was passaged in individual larvae. DNA extraction and qPCR were performed as described above for each replicate and control at every time point.

Estimating the within-host fitness of vEGTDEL

In order to analyse the qPCR data from the single-passage experiment, a Generalized Linear Model (GLM, SAS Institute Inc., Cary, NC, USA) was used, with the natural logarithm of the virus inoculum ratio [$\ln(c_{wt}/c_{ed})$] as the independent variable, and the observed proportion of Wt L1 virus (f_{wt}) – calculated from the qPCR-measured virus ratio (Equation 1) – as the dependent variable. A logit link function and a binomial error structure were used. Using this link function, the model effectively establishes the relationship between log-transformed output and input ratios, while accommodating the entire range of output values. The scale parameter was estimated from dividing the square root of the deviance by the degrees of freedom.

For analysis of the serial-passage experiments, the deterministic model for selection between competing species proposed by de Wit was used (De Wit 1960; Georgievskaya et al. 2005; see also Godfray et al. 1997). This

model assumes a fixed selection rate constant, resulting in geometric increases of the genotype ratio over generations. Given that the log of the virus genotype ratio was used for analysis, this model can be stated as:

$$\log(R_t) = \log(R_0) + t \cdot \log(w) \quad (2)$$

Here w is the selection rate constant, which in this analysis is a constant factor by which the virus genotype ratio is multiplied in each passage. In addition, t is the passage number, R_0 is the genotype ratio at passage 0, and R_t is the log genotype ratio at passage t . Using the observed mean R -values over passages 1 through 5, w was calculated by linear regression.

Estimating the overall fitness of vEGTDEL with respect to yield

A more elaborate model for competition between species (De Wit 1960) was used to understand the competitive process better, and to explore the combined effect of differences in fitness at the within and between-host levels (using absolute yield of transmission stages as a surrogate for between-host transmission). For each virus, the yield of only that particular virus generated (y_{wt} , y_{ed}) when there is a given proportion of that virus in the inoculum (f_{wt} , f_{ed} ; $f_{wt} + f_{ed} = 1$) is then:

$$y_{wt} = m_{wt} \frac{k_{wt} f_{wt}}{k_{wt} f_{wt} + f_{ed}} \quad (3)$$

and

$$y_{ed} = m_{ed} \frac{k_{ed} f_{ed}}{k_{ed} f_{ed} + f_{wt}} \quad (4)$$

Here m_{wt} and m_{ed} are the respective yields of the virus in single genotype infection, and k_{wt} and k_{ed} are crowding coefficients: constants that describes how the two viruses perform in direct competition with each other. For individual larvae, the yield of polyhedra for each genotype was calculated by partitioning the total yield (determined by counting polyhedra in a haemocytometer) using the qPCR-determined genotype ratio. For single genotype treatments, the mean yield of all larval cadavers collected was determined and assumed to be pure, as no contamination was detected in a subset of these samples. Nonlinear regression (SPSS 12.0, SPSS Inc., Chicago, IL, USA) was then used to estimate m_{wt} and k_{wt} , and subsequently m_{ed} and k_{ed} .

Results

Polyhedra morphology of the Wt L1 and vEGTDEL viruses

From phase-contrast microscopy, it was suspected that there were differences in polyhedron size between the Wt

L1 and vEGTDEL viruses. To define the starting material for experiments, EM analysis of polyhedra of the two genotypes was performed. The mean polyhedron cross-sectional area of the Wt L1 virus was significantly larger than that of the vEGTDEL (Fig. 1A; Mann–Whitney U -test: $Z = -5.747$, $n = 208$, $P < 0.001$). The difference in cross-sectional areas was approx 1.5-fold. The polyhedra of the Wt L1 virus also had significantly more virions per cross section than vEGTDEL polyhedra (Fig. 1B, Mann–Whitney U -test: $Z = -3.400$, $n = 208$, $P = 0.001$), also approx. 1.5-fold. It was assumed that ODV are isotropically distributed in polyhedra, allowing conversion of the difference in ODV per polyhedron cross-sectional area to the actual difference in ODV per polyhedron. The estimated ODV per polyhedron ratio of Wt L1:vEGTDEL is then the observed ODV per polyhedron cross section ratio (=1.496), raised to the power 3/2 (=1.830) to translate from two to three dimensions.

In previous studies on the original wild type AcMNPV isolate and bacmid-derived viruses there was a zero-truncated Poisson-like distribution of the number of nucleocapsids per virion (Zwart et al. 2008b). A similar result was obtained here for the Wt L1 and vEGTDEL virions (Fig. 1C). The distribution was similar for both genotypes (Kolmogorov–Smirnov test: $Z = 0.529$, $n = 217$, $P = 0.942$).

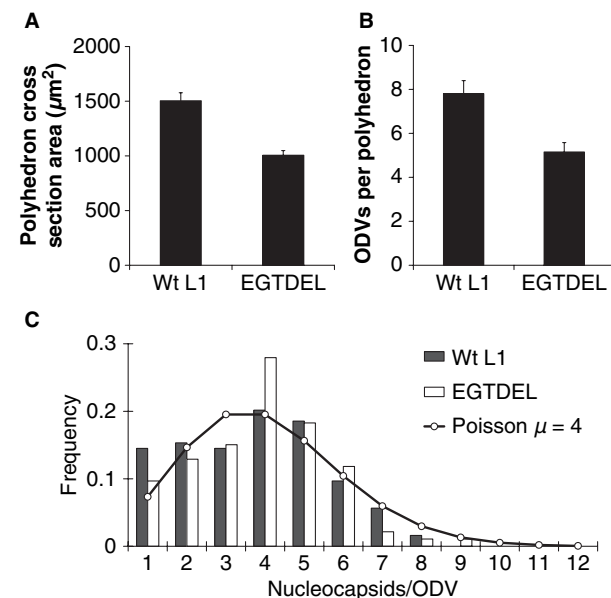


Figure 1 The morphology of polyhedra of the Wt L1 and vEGTDEL viruses. (A) The mean polyhedron cross section area in square micrometers, with standard error. (B) The mean number of ODV per polyhedron, with standard error. (C) The frequency distribution of nucleocapsids per ODV, and a Poisson distribution with a μ (mean) of 4 for comparative purposes.

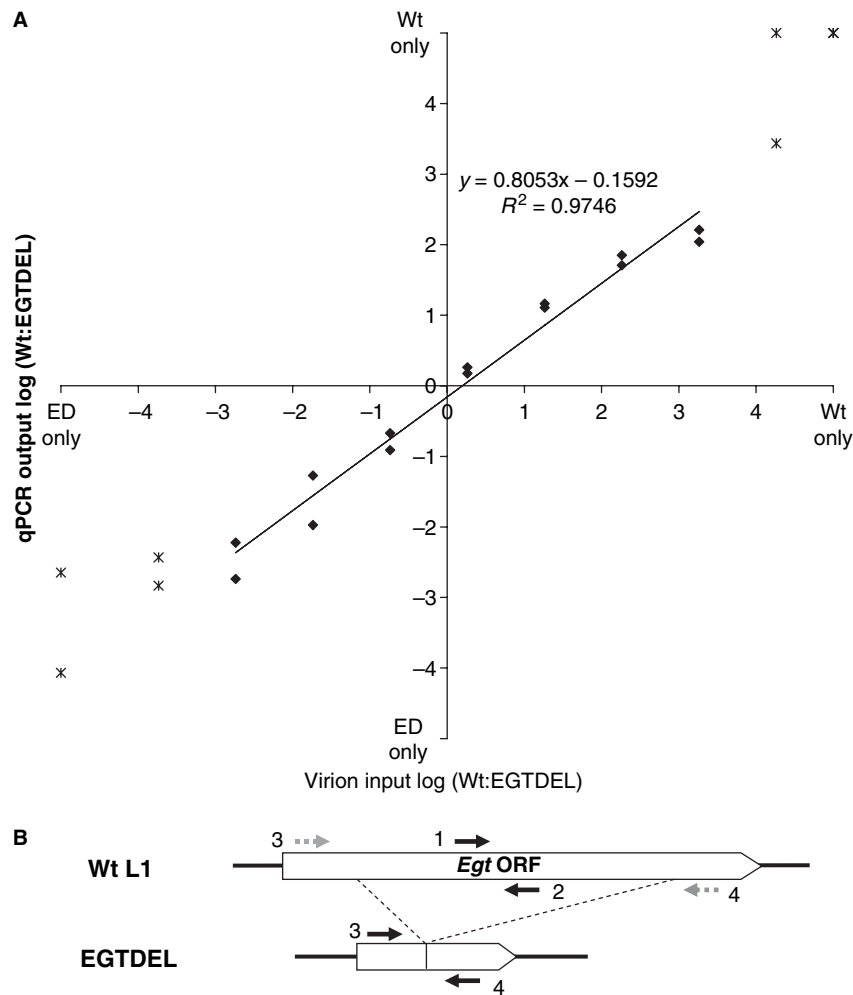


Figure 2 (A) qPCR calibration. On the x-axis is the log input ratio (Wt L1:vEGTDEL), the virion ratio. This is the polyhedra ratio corrected for the difference in ODV content per polyhedron between the two genotypes, by multiplication by 1.830. On the y-axis is the log transformed ratio measured by qPCR (Wt L1:vEGTDEL), for two replicate experiments. A regression line is shown, with equation and r^2 -value. The black diamonds were included in the regression analysis, whereas the crosses were not because the assay was not satisfactory in this range. (B) The location of the primer sets for identification of the Wt L1 and vEGTDEL viruses. PCR primers are denoted by numbered arrows: 1 and 2 are the forward and reverse primers for the Wt virus; 3 and 4 the forward and reverse primers for vEGTDEL detection. Note that although primers 3 and 4 can in principle anneal and amplify a product from the Wt L1 DNA, whether or not this template will be amplified depends on the thermal cycling program used.

qPCR calibration

The qPCR assay performed satisfactorily over six orders of magnitude (1000:1–1:1000; Fig. 2). The regression equation (Fig. 2) allowed us to make a small correction to measured qPCR-values in order to obtain the true genotype ratios. The Wt L1 primers consistently gave a very late fluorescent signal with vEGTDEL alone. Over 10 passages in larvae, the signal remained in the same range (<1:5000; Wt:vEGTDEL), indicating that it is probably background and not indicating presence of Wt virus.

Estimating the within-host fitness of vEGTDEL from single-passage experiments

The ratio of Wt L1:vEGTDEL in individual larval cadavers was determined by means of qPCR (Fig. 3). These data suggest that there may be selection for the Wt L1 genotype, as the ratio appears to be shifted towards the Wt L1 virus for many samples. In order to test if there is selection for the Wt L1 virus, a GLM was used (see the section 'Estimating the within-host fitness of vEGTDEL'). The y -intercept was significantly greater than 0 (0.4221 ± 0.2037 ; $\chi^2 = 4.29$, d.f. = 1, $P = 0.0383$), which

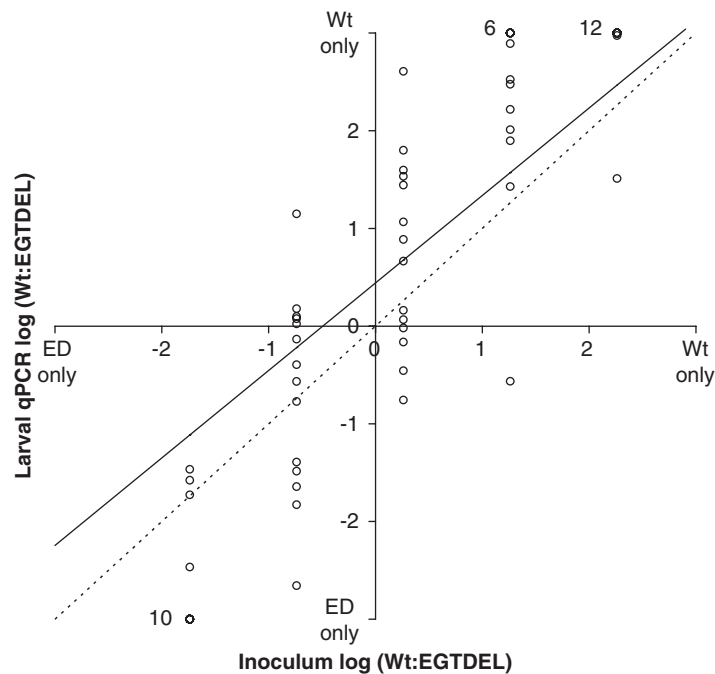


Figure 3 qPCR data from 1:100 through 100:1 polyhedra inoculum mixtures. The log of the ODV ratio in the inoculum (Wt L1: vEGTDEL) on the x-axis. The log ratio Wt L1: vEGTDEL measured in individual larvae by qPCR is on the y-axis, which extends to \pm infinity at log ± 3 . Circles are measurements in individual larvae ($n = 14$ per treatment). The number of data points at \pm infinity is denoted by the numbers to the left of the point. The dotted line denotes a 1:1 relationship between inoculum and qPCR ratio measured. The solid line is the regression line from the GLM.

is evidence for selection for the Wt L1 genotype. Transforming the intercept from a log to a natural scale gives a selection rate constant (i.e. w -value, see equation 2) of $10^{0.4221} = 1.53$. The slope of the regression line was less than one, albeit not significantly (0.8956 ± 0.1253). This does, however, suggest that selection for Wt L1 and against vEGTDEL may be strongest when the frequency of the Wt L1 virus is low.

Comparison of virus ratio to time of death, weight and polyhedra yield

Data on the time to death, cadaver weight and polyhedra yield were available for the individual larval cadavers from which DNA was analysed by qPCR (Fig. 4). A Jonckheere-Terpstra test was used to determine if qPCR ratio was related to time of death, cadaver weight, yield, and yield per milligram cadaver weight. The Jonckheere-Terpstra test assesses whether population medians are ordered when treatments are ordered, and is applicable given its nonparametric nature and because the independent variable (qPCR ratio) is measured accurately on an ordinal scale. A significant positive relationship was found for both time to death (Stand. $JT = 5.243$, $n = 70$, $P < 0.001$), yield (Stand. $JT = 1.994$, $n = 70$, $P = 0.046$) and yield per milligram cadaver weight (Stand.

$JT = 2.205$, $n = 70$, $P = 0.027$), but not for cadaver weight (Stand. $JT = 0.861$, $n = 70$, $P = 0.389$).

Estimating the within-host fitness of vEGTDEL from serial-passage experiments

The ratio of virus genotypes (R) was determined for each replicate after every passage, for the serial passaging from individual and pooled larvae (Fig. 5A). In four out of 15 replicates, the Wt L1 genotype went to fixation, whereas vEGTDEL did not go to fixation in a single replicate. Moreover, the log virus ratio did not drop below approximately -0.3 (approx. 35% Wt virus) in any of the replicates. This is strong evidence of positive selection for the Wt L1 virus in these experiments.

A selection rate constant (w) of 1.68 ± 0.35 was calculated for the serial-passage data using equation (2) (see Methods Section). Calculation of the selection rate constant by time series analysis led to highly similar estimates (data not shown). The estimate of the selection rate constant from the serial-passage data is quite similar to the result of the single-passage experiment ($w = 1.53$). Our data therefore provide consistent evidence for reduced within-host competitive fitness of vEGTDEL, with an approx. 1.6-fold increase of the Wt:vEGTDEL ratio per passage.

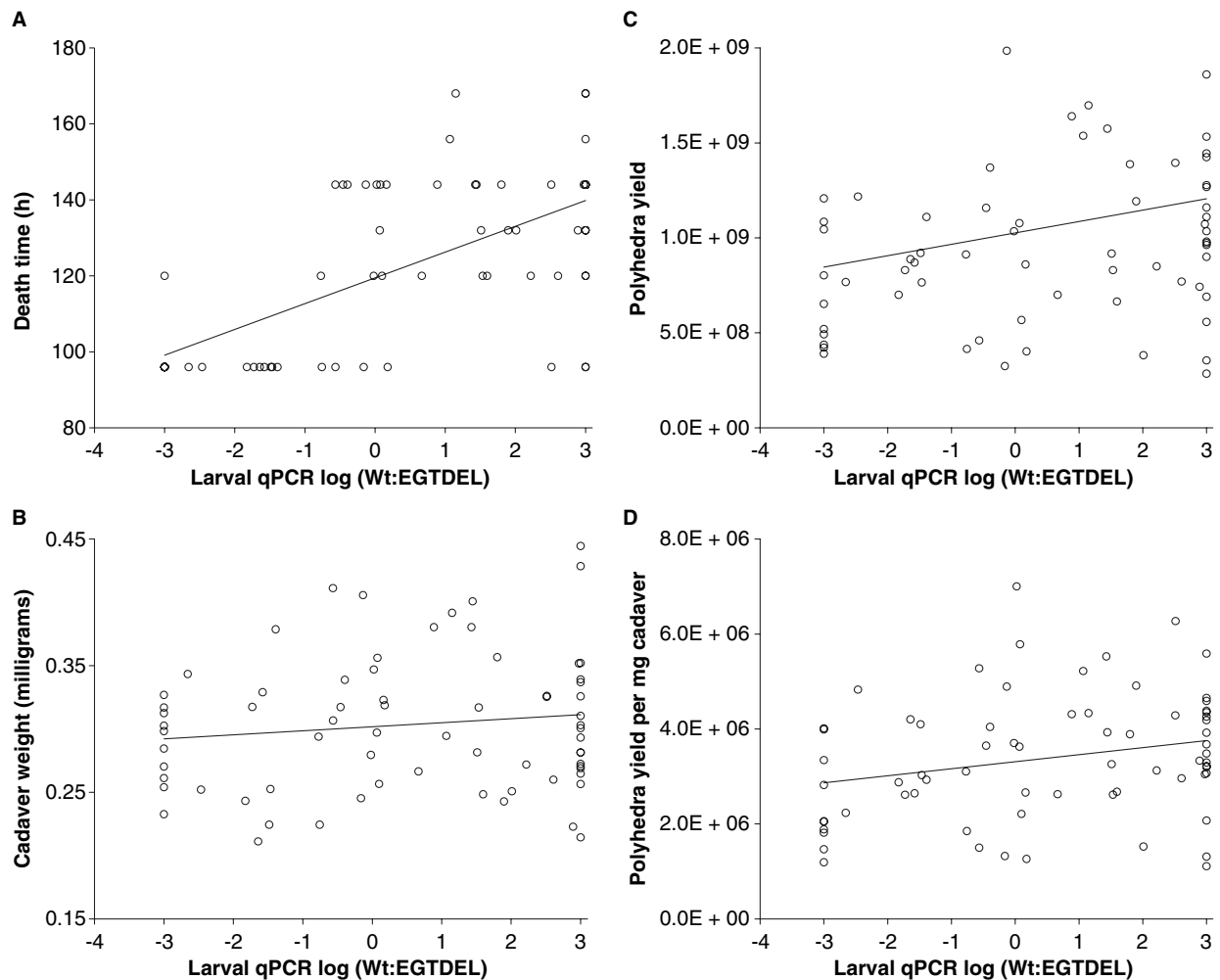


Figure 4 Relationship of virus genotype ratio and other infection parameters. On the x-axis is the log virus ratio (Wt L1:EGTDEL), for all panels. On the y-axis is (A) time to death in hours (B) larval cadaver weight in grams (C) polyhedra yield and (D) polyhedra yield per unit cadaver weight. Note that although a linear regression line is plotted for convenience, the data were analysed with the Jonckheere-Terpstra test.

Integrating within-host fitness and yield

It has been previously reported that vEGTDEL generates a lower polyhedra yield (O'Reilly and Miller 1989). In our experiments we found reduced within-host fitness of this virus. To understand the combined effect of these two observations, the more elaborate competition model of De Wit (1960) was used (see the section 'Estimating the overall fitness of vEGTDEL with respect to yield'). This model adequately described the data (Fig. 6A), and model predictions for the yield in single genotype infection (m_i) were comparable to experimental data (Fig. 6B). The k -values obtained confirm that the Wt virus is more competitive ($k_{wt} = 2.994$) than vEGTDEL ($k_{egtDEL} = 1.052$). Since the k -values are not reciprocals the interaction is not neutral; some mixtures generate a greater total yield

than if each virus fraction were to simply be as productive as in a single genotype infection. If the model is used to predict how the virus genotype ratio would be modulated by passage in larvae, it becomes apparent that there is indeed frequency-dependent selection (Fig. 7). Selection for the wild-type virus is stronger when the frequency of the Wt virus is low (<0.5). These results confirm and further detail those presented in sections 'Comparison of virus ratio to time of death, weight and polyhedra yield' and 'Estimating the within-host fitness of vEGTDEL from serial-passage experiments'.

Discussion

We have experimentally quantified the within-host competitive fitness of a fast-acting recombinant baculovirus

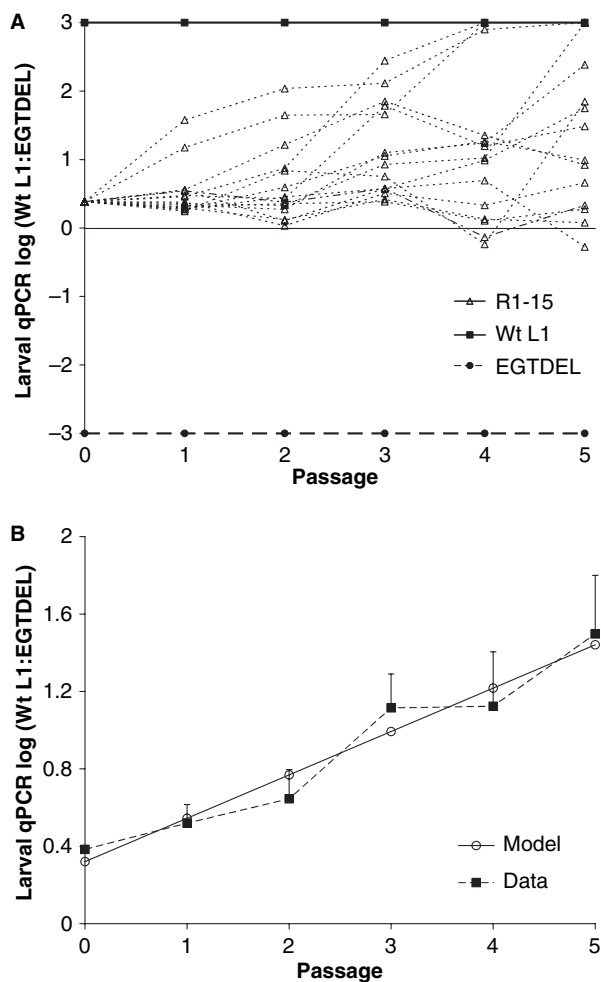


Figure 5 (A) qPCR data of the serial-passage experiments in individual larvae. (B) Model fitting of the mean log ratio of individual larvae, with standard error denoted by bars, and the model estimate (see equation 2).

by means of direct competition in single- and serial-passage experiments. This setup was tailored for selection based on differences in within-host competitive fitness. For single-passage experiments, the frequency of the recombinant virus was determined by qPCR in a randomly selected larval cadaver. For serial-passage experiments, a fixed number of polyhedra from a single randomly selected larval cadaver was used to infect larvae for the following passage. This ensured that the probability of passage to the next generation was dependent on the frequency at which a genotype was present in individual larvae, and independent of, for example, the absolute yield and time of kill.

A qPCR-based assay for determining the Wt L1:EGTDEL ratio in larval cadavers was developed and validated (Fig. 2). A previously reported qPCR-based assay

performed better (Zwart et al. 2008b), but did not have the same constraints for primer design as in the case of Wt L1 and vEGTDEL; in the former case 'tag sequences' were deliberately inserted into the genomes of the viruses used in a well defined locus. Using the qPCR assay developed here, considerable variation between individual larvae in the genotype ratio was observed (Fig. 3). This variation probably arises as a consequence of a genetic bottleneck at the level of colonization of the insect host, with a small number of virions infecting the host. Moreover, given that there is a small number of founders of infection, stochastic effects during virus amplification are expected, as (i) stochastic components act on virus spread within the host (e.g. how long will it take before a BV infects a new cell?), and (ii) BV production varies between individual cells and cell types.

Through single- and serial-passage experiments, we show that vEGTDEL has lower within-host fitness relative to the parent Wt L1 genotype. A selection rate constant of 1.53 was estimated based on a single-passage experiment with different inoculum mixtures (Fig. 3). From a serial-passage experiment, a selection rate constant of 1.68 was estimated (Fig. 5). In the serial-passage experiments, the proportion of wild-type virus never fell very low (0.35) despite high variation between replicates (Fig. 5). This observation confirms positive selection for the Wt L1 virus. It has been previously shown that deletion of the *egt* gene affects other fitness components (O'Reilly and Miller 1989; Cory et al. 2004). These components – virus yield particularly – mainly concern fitness at the between-host level. Our data show that vEGTDEL also has reduced fitness at the within-host level, something not shown in earlier studies as far as we are aware. Given that vEGTDEL is capable of autonomous infection of a host insect, we had not anticipated such a marked fitness reduction at the within-host level. The fact that single- and serial-passage experiments lead to similar estimates of the selection rate constant suggests that co-occlusion of virions with different genotypes in a single polyhedron does not have major effects on competition within the host.

In order to put our results in a broader context, we also considered how the genotype ratio in larvae was related to host time of death, cadaver weight, total virus yield and virus yield per unit cadaver weight (Fig. 4). We observed that time of death, polyhedra yield and yield per unit cadaver weight could be predicted from the qPCR ratio. Cory et al. (2004) previously reported differences in these parameters in single genotype infections, but how they would be affected by co-infection was not considered. The relationship between genotype ratio and yield per unit cadaver weight is especially intriguing, since it suggests that *egt* primarily modulates how larvae develop

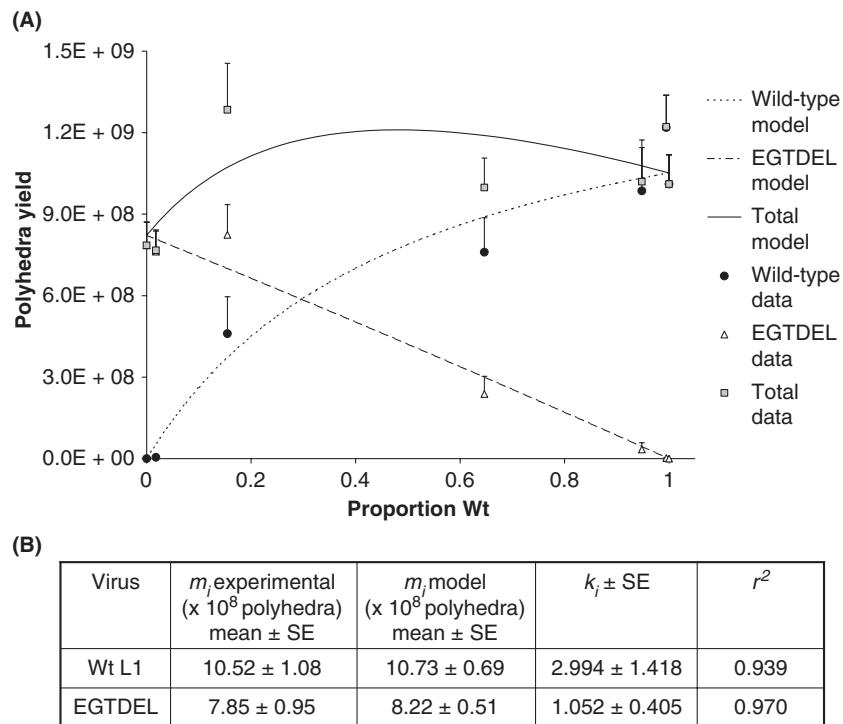


Figure 6 The combined effects of differences in yield and within-host fitness, fitted to the model proposed by De Wit (1960). (A) On the x-axis is the proportion Wt virus in the inoculum; on the y-axis the polyhedra yield. The individual data points are the mean yield for each virus and the total yield, with error bars denoting the standard error of the mean. The dotted lines are the fitted model (see equation 3) for yield of each virus. The solid line is total yield as predicted by the model, which is the sum of the yields for two viruses. (B) Model fitting results: independent experimental data and model predictions for single genotype infection yield (m_i experimental and model), model estimates for the crowding co-efficient k_i , and r^2 -values obtained from the nonlinear regression.

and not simply how large they become. A significant relationship between virus genotype ratio and weight was not found, whereas this has been reported for single genotype infections (Cory et al. 2004).

Using the genotype ratio and yield data from the single-passage experiment, the polyhedra yield per virus could be estimated (Fig. 6). The simplest model that can be employed to describe these data is probably the competition model proposed by De Wit (1960), and we found that the model fitted the data well. The crowding coefficient k_i was greater than 1 for the Wt L1 virus ($k_{wt} = 2.994$), while it was very close to 1 for vEGTDEL ($k_{egt} = 1.052$). This combination of k -values not only confirms within-host selection for the Wt L1 virus and against vEGTDEL (Fig. 6), but it also provides evidence for frequency-dependent selection. Equation 3 illustrates that as the ratio Wt L1:vEGTDEL becomes very small, the change in the ratio per passage will approach $k_{wt}m_{wt}/m_{ed}(=3.91)$. On the other hand, when the ratio Wt L1:vEGTDEL is very large, the change in ratio per passage will approach $m_{wt}/k_{ed}m_{ed}(=1.24)$. Hence, selection against vEGTDEL is highest when the frequency of

vEGTDEL is high. Selection against vEGTDEL becomes weaker if the frequency of vEGTDEL is lower (Fig. 7). The model also shows that the proportion wild-type versus wild-type yield (and therefore also total yield) relationship is not linear. The hump-shaped total yield curve (Fig. 6A) suggests that intermediate mixtures give higher yields, but it cannot be ruled out that low frequencies of Wt L1 virus are sufficient to influence host development and increase yield to 'wild-type virus only' levels.

EGT's effect on speed of kill (O'Reilly and Miller 1989) makes it easy to propose an explanation for how deletion of this gene leads to reduced fitness with respect to yield: larvae die sooner and have less time to grow, and hence there are simply less host resources available for the virus to convert to transmission stages. Understanding why *egt* also influences fitness at the within-host level with mixed infection of Wt and vEGTDEL clearly requires another explanation, as all genotypes present in an individual host will be equally affected by how host development progresses and the time of host death. O'Reilly et al. (1995) observed that wild-type and vEGTDEL virus infection proceeded in a markedly different manner when the

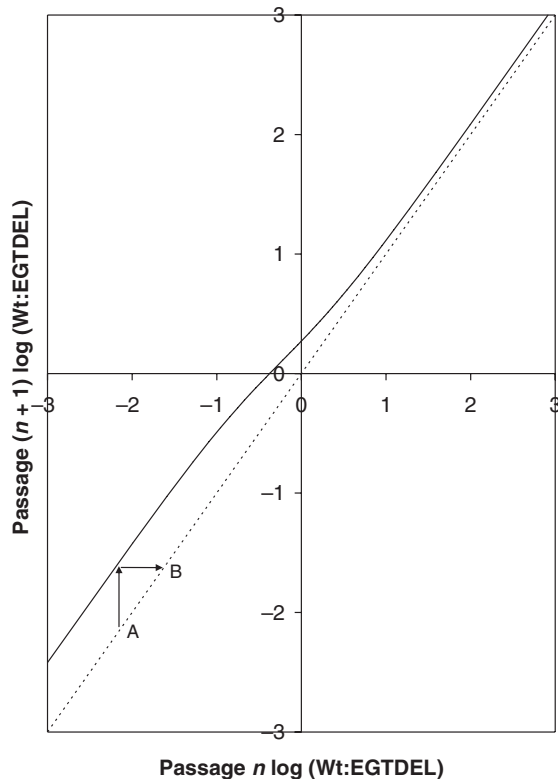


Figure 7 On the x-axis is the log genotype ratio (Wt L1:EGTDEL) at passage n and on the y-axis the log genotype ratio in the virus progeny after one passage of infection ($n + 1$). The dotted line (1:1) indicates no fitness differences [1:1 ratio passage $n:(n + 1)$]. The solid black line is the prediction from the 'de Wit' competition model for the change in log genotype ratio over a single passage of infection. Note that this analysis takes into account differences due to within-host competition and total yield generated. As this line lies above the 1:1 line at all ratios, the Wt virus continually has a selective advantage. For example, if the genotype ratio is Point A, within one passage it will be displaced to Point B, as read along the x-axis. Note the frequency-dependence: the lower the ratio Wt L1:EGTDEL, the stronger selection for the Wild-type virus is.

viruses were engineered to express prothoracicotropic hormone (PTTH): whereas wild-type infection proceeded normally, the vEGTDEL virus had a 100-fold reduction in pathogenicity. O'Reilly et al. (1995) suggested that ecdysteroids can adversely influence viral replication, as PTTH induces ecdysteroid production and vEGTDEL has lost the ability to inactivate ecdysteroids. Similarly, Keeley and Vinson (1975) reported that injection of ecdysone into larvae reduced an NPV's speed of kill and larval mortality. A plethora of mechanisms linking viral replication and ecdysteroids can be proposed, given the importance of the latter in regulating host development. The presence of EGT probably has an effect *in trans* on replication. Consequently, the fitness of the wild-type virus will only

be higher than that of vEGTDEL if (i) cells are predominantly infected by a single BV, such as early in the infection process (Zwart et al. 2008a; see also Bull et al. 2001) and (ii) the positive effects of the EGT protein on replication are local and not systemic. Of course our results do not provide direct evidence for these assertions, but a coherent and parsimonious explanation of our observations demands them.

In terms of the consequences of releasing vEGTDEL as an insecticide, our data suggest: (i) when larvae are co-infected by the vEGTDEL and wild type AcMNPV viruses, vEGTDEL will be outcompeted at this level of selection i.e. within the host. This is a relatively slow process; displacement of vEGTDEL will typically take multiple passages. Importantly, we find no evidence that there is any equilibrium frequency at which vEGTDEL is stably maintained; vEGTDEL is continuously outcompeted until it disappears from the population altogether (e.g. Fig. 7). (ii) We found evidence for frequency-dependent selection for the Wt L1 virus (Fig. 7), when including the between-hosts level (absolute yield of transmission stages). The frequency-dependent selection observed makes it very difficult for vEGTDEL to go to fixation, since selection against vEGTDEL becomes stronger as its frequency becomes higher. (iii) The frequency of mixed infections varies with virus ratio in the inoculum, with the most extreme ratios tested (1:100 and 100:1) giving the lowest frequencies of mixed infections (Fig. 3). Single genotype infections with extreme virus ratios probably come about due to the small number of virions causing infection. For example, if infection is on average initiated by 10 virions, then the Wt L1 will not be represented in most infected larvae when an inoculum with a 1:100 virus ratio is used. Our results therefore suggest that the Wt L1 virus will not be displaced by genetic drift unless the frequency of vEGTDEL in the polyhedra ingested by larvae is very high (i.e. a ratio Wt L1:vEGTDEL $\leq 1:100$).

Care must be taken in extrapolating conclusions based on our data to other recombinant viruses. It has been reported that the engineered expression of a heterologous marker (β -gal) protein leads to reduced fitness (Huang et al. 1991). Similarly, it has been reported that the presence of a toxin (venom of *Euplectrus comstockii*) can adversely affect baculovirus replication *in vivo* (Coudron et al. 1995). On the other hand, the presence of a toxin alone (AaIT) has also been reported not to affect baculovirus competitive fitness *in vivo* (Milks et al. 2001). The different conclusions we reach in this study may be attributed to (i) AaIT not having an effect on viral fitness, as the authors suggest, (ii) differences in experimental setup: the competitive process was studied by considering when a passaged replicate went to fixation. Using this methodology

alone, our evidence for selection of the wild type virus would have been weak.

What the combined effect of *egt* deletion and toxin expression would be on competitive fitness remains to be seen. Given the likely mechanisms bringing about effects on fitness, interactions which lead to restored fitness do not seem probable. Therefore, those viruses which have both *egt* deleted and expressing a toxin expression are likely to see either (i) a similar reduction in within-host fitness as the vEGTDEL or (ii) further reductions in competitive fitness. Thus, from a biological safety perspective, it may be prudent to use recombinants with *egt* deleted – next to further modification enhancing biological control efficacy – simply because of the effects on fitness.

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Literature cited

- Ben-Ami, F., L. Mouto, and D. Ebert. 2008. The effect of multiple infections in the expression and evolution of virulence in a *Daphnia*-endoparasite system. *Evolution* **62**:1700–1711.
- Bonsall, M. B., D. R. O'Reilly, J. S. Cory, and R. S. Hails. 2005. Persistence and coexistence of engineered nucleopolyhedroviruses. *Theoretical Population Biology* **67**:217–230.
- Bull, J. C., H. C. J. Godfray, and D. R. O'Reilly. 2001. Persistence of an occlusion-negative recombinant nucleopolyhedrovirus in *Trichoplusia ni* indicates high multiplicity of cellular infection. *Applied and Environmental Microbiology* **67**:5204–5209.
- Burden, J. P., R. S. Hails, J. D. Windass, M. M. Suner, and J. S. Cory. 2000. Pathogenicity, virulence and productivity of a baculovirus expressing the itch mite toxin Txp-1 in second and fourth instar *Trichoplusia ni*. *Journal of Invertebrate Pathology* **75**:226–236.
- Christou, P., T. Capell, A. Kohli, J. A. Gatehouse, and A. M. R. Gatehouse. 2006. Recent developments and future prospects in insect pest control in transgenic crops. *Trends in Plant Science* **11**:302–308.
- Cory, J. S., and J. H. Myers. 2003. The ecology and evolution of insect baculoviruses. *Annual Review of Ecology, Evolution and Systematics* **34**:239–272.
- Cory, J. S., M. L. Hirst, T. Williams, R. S. Hails, D. Goulson, B. M. Green, T. M. Carty *et al.* 1994. Field trial of a genetically improved baculovirus insecticide. *Nature* **370**:138–140.
- Cory, J. S., E. E. Clarke, M. L. Brown, R. S. Hails, and D. R. O'Reilly. 2004. Microparasite manipulation of an insect: the influence of the *egt* gene on the interaction between a baculovirus and its lepidopteran host. *Functional Ecology* **18**:443–450.
- Cory, J. S., B. M. Green, R. K. Paul, and F. Hunter-Fujita. 2005. Genotypic and phenotypic diversity of a baculovirus population within an individual insect host. *Journal of Invertebrate Pathology* **89**:101–111.
- Coudron, T. A., W. C. Rice, M. R. Ellersieck, and R. E. Pinnell. 1995. Mediated pathogenicity of the baculovirus HzSNPV by the venom from *Euplectrus comstockii* (Hymenoptera, Eulophidae). *Biological Control* **5**:92–98.
- De Roode, J. C., R. Culleton, A. S. Bell, and A. F. Read. 2004. Competitive release of drug resistance following drug treatment of genetically diverse *Plasmodium chabaudi* infections. *Malaria Journal* **3**:33.
- De Roode, J. C., M. E. H. Helsinki, M. A. Anwar, and A. F. Read. 2005. Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *American Naturalist* **166**:531–542.
- De Wit, C. T. 1960. On Competition. *Versl. Landbouwk. Onderzoek*. **66**:1–82.
- Dushoff, J., and G. Dwyer. 2001. Evaluating the risks of engineered viruses: modelling pathogen competition. *Ecological Applications* **11**:1602–1609.
- Ebert, D., and W. W. Weisser. 1997. Optimal killing for obligate killers: the evolution of life histories and virulence of semelparous parasites. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **264**:985–991.
- Elena, S. F., and R. E. Lenski. 2003. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nature Reviews Genetics* **4**:457–469.
- England, L. S., S. B. Holmes, and J. T. Trevors. 1998. Persistence of viruses and DNA in soil. *World Journal of Microbiology and Biotechnology* **14**:163–169.
- Federici, B. A. 1997. *Baculovirus Pathogenesis* in L. K. Miller (ed.), *The Baculoviruses*, pp. 33–59. Plenum Press, New York.
- Georgievska, L., R. Velders, X. Dai, F. J. J. A. Bianchi, W. van der Werf, and J. 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.
- Godfray, H. C. J., D. R. O'Reilly, and C. J. Briggs. 1997. A model of nucleopolyhedrovirus (NPV) population genetics applied to co-occlusion and the spread of the few polyhedra (FP) phenotype. *Proceedings of the*

- Royal Society of London. Series B: Biological Sciences **264**:315–322.
- Hails, R. S., P. Hernández-Crespo, S. M. Sait, C. A. Donnelly, B. M. Green, and J. S. Cory. 2002. Transmission patterns of natural and recombinant baculoviruses. *Ecology* **83**:906–916.
- Hamblin, M., N. A. M. Vanbeek, P. R. Hughes, and H. A. Wood. 1990. Cocclusion and Persistence of a Baculovirus Mutant Lacking the Polyhedrin Gene. *Applied and Environmental Microbiology* **56**:3057–3062.
- Hernández-Crespo, P., R. S. Hails, S. M. Sait, B. M. Green, T. M. Carty, and J. S. Cory. 1999. Response of hosts of varying susceptibility to a recombinant baculovirus insecticide in the field. *Biological Control* **16**:119–127.
- Huang, Y. S., K. L. Bobseine, R. W. Setzer, and C. Y. Kawani-shi. 1991. Selection kinetics during serial cell culture passage of mixtures of wild type *Autographa californica* nuclear polyhedrosis virus and its recombinant AC360-Beta-gal. *Journal of General Virology* **72**:2653–2660.
- Inceoglu, A. B., S. G. Kamita, and B. D. Hammock. 2006. Genetically modified baculoviruses: a historical overview and future outlook. *Advances in Virus Research* **68**: 323–360.
- Jehle, J. A., G. W. Blissard, B. C. Bonning, J. S. Cory, E. A. Herniou, G. F. Rohrmann, D. A. Theilmann *et al.* 2006. On the classification and nomenclature of baculoviruses: a proposal for revision. *Archives of Virology* **151**:1257–1266.
- Keeley, L. L., and S. B. Vinson. 1975. Beta-ecdysone effects on development of nucleopolyhedrosis in *Heliothis* species. *Journal of Invertebrate Pathology* **26**:121–123.
- Lee, H. H., and L. K. Miller. 1978. Isolation of genotypic variants of *Autographa californica* nuclear polyhedrosis virus. *Journal of Virology* **27**:754–767.
- Milks, M. L., M. K. Leptich, and D. A. Theilmann. 2001. Recombinant and wild-type nucleopolyhedroviruses are equally fit in mixed infections. *Environmental Entomology* **30**:972–981.
- Moscardi, F.. 1999. Assessment of the application of baculoviruses for control of Lepidoptera. *Annual Review of Entomology* **44**:257–289.
- O'Reilly, D. R., and L. K. Miller. 1989. A baculovirus blocks insect molting by producing ecdysteroid UDP glucosyl transferase. *Science* **245**:1110–1112.
- O'Reilly, D. R., T. J. Kelly, E. P. Masler, B. S. Thyagaraja, R. M. Robson, T. C. Shaw, and L. K. Miller. 1995. Overexpression of *Bombyx mori* prothoracicotropic hormone using baculovirus vectors. *Insect Biochemistry and Molecular Biology* **25**:475–485.
- Scholte, E. J., K. Ng'habi, J. Kihonda, W. Takken, K. Paaij-mans, S. Abdulla, G. F. Killeen *et al.* 2005. An entomopathogenic fungus for control of adult African malaria mosquitoes. *Science* **308**:1641–1642.
- Smith, I. R. L., and N. E. Crook. 1988. In vivo isolation of baculovirus genotypes. *Virology* **166**:240–244.
- Smits, P. H., M. van de Vrie, and J. M. Vlak. 1986. Oviposition of Beet Armyworm (Lepidoptera, Noctuidae) on greenhouse crops. *Environmental Entomology* **15**:1189–1191.
- Sun, X. L., H. L. Wang, X. C. Sun, X. W. Chen, C. M. Peng, D. M. Pan, J. A. Jehle *et al.* 2004. Biological activity and field efficacy of a genetically modified *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus expressing an insect-selective toxin from a chimeric promoter. *Biological Control* **29**:124–137.
- Theilmann, D. A., G. W. Bissard, B. Bonning, J. Jehle, D. R. O'Reilly, G. F. Rohrmann, S. Thiem *et al.* 2005. Baculoviridae. In C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger and L. A. Ball (eds), *Virus Taxonomy—Eighth Report of the International Committee on Taxonomy of Viruses*, pp. 1129–1185. Springer, New York.
- Thomas, E. D., C. F. Reichelderfer, and A. M. Heimpel. 1972. Accumulation and persistence of a nuclear polyhedrosis virus of the cabbage looper in the field. *Journal of Invertebrate Pathology* **20**:157–164.
- Zwart, M. P., E. Erro, M. M. van Oers, J. A. G. M. de Visser, and J. M. Vlak. 2008a. Low multiplicity of infection in vivo results in purifying selection against baculovirus deletion mutants. *Journal of General Virology* **89**:1220–1224.
- Zwart, M. P., M. M. van Oers, J. S. Cory, J. W. M. van Lent, W. van der Werf, and J. M. Vlak. 2008b. Development of a quantitative real-time PCR to determine genotype frequencies for studies in baculovirus population biology. *Journal of Virological Methods* **148**: 146–154.