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Dynamics of deletion genotypes in an experimental insect virus population

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Defective viruses, that are deficient in certain essential genes, are maintained in the population by transcomplementation, exploiting the gene products of complete genotypes in co-infected cells. This process becomes prevalent only when cells are frequently infected by several virus particles, and only then will the fitness of defective viruses be subjected to frequency-dependent selection. Deletion variants that are not infectious *per os* are present in a multicapsid nucleopolyhedrovirus (SfMNPV, Baculoviridae) that infects the fall armyworm, *Spodoptera frugiperda*. These variants enhance the pathogenicity and, therefore, the likelihood of transmission of the virus when co-infecting cells with complete genotypes, resulting in occlusion bodies (OBs) that may contain both genotypes co-occluded. Mixtures of complete (B) and defective (C) variants in ratios of 90% B+10% C, 50% B+50% C and 10% B+90% C were used to inoculate by injection *S. frugiperda* larvae. Viral OBs extracted from diseased insects were subjected to four or five successive rounds of *per os* infection. Following successive passages, genotype frequencies in all three experimental populations converged to a single equilibrium frequency comprising ~20% of deletion genotype C and ~80% of complete genotype B. This mirrors the relative proportions of deletion (22%) and complete (78%) genotypes observed in the wild-type SfMNPV population. The pathogenicity of experimental populations at the final passage was not significantly different from that of the wild-type isolate. In contrast, OBs of all genotype mixtures were significantly more pathogenic than OBs of genotype B alone. A population genetics model, in which virus populations were assigned linear frequency-dependent transmissibility values, was in remarkably close agreement to empirical data. Clearly, non-infectious deletion variants can profoundly affect the likelihood of transmission and the genetic structure and stability of virus populations.

Keywords: *Spodoptera frugiperda*; nuclepolyhedrovirus; serial passages; deletion genotypes; virus dynamics

1. INTRODUCTION

Virus populations are genetically heterogeneous. The forces that foster and sustain genotypic diversity in virus populations are complex and appear to arise from processes acting on parasite reproduction within a host, that favour certain genotypes, and processes acting on between host transmission, that may favour other variants (Ebert & Hamilton 1996).

One of the most intriguing aspects of virus population biology is the persistence of deletion mutants that lack genes essential for replication, particle assembly or transmission. These variants survive by co-infecting cells infected by complete genotypes. The deletion variant sequesters the transcription products of the complete genotype to compensate for the missing genes, like the structural proteins, enzymes or replication regulating factors. The shorter genome of deletion variants may provide a selective advantage as they can replicate faster than complete genotypes (Roux *et al.* 1991). The prevalence at which deletion variants persist in virus populations, therefore, represents a trade-off between replicative advantage within co-infected host cells and opportunities for between-host transmission limited by the need to co-infect with complete genotypes. This trade-off will be subject to negative frequency-dependent selection because the prevalence of hosts infected by complete genotypes declines as deletion variants become increasingly common. Deletion mutants have usually been considered as deleterious to complete genotypes in virus populations because they compete for viral transcription products (Roux *et al.* 1991), are incapable of independent replication (Frank 2000) and can reduce the transmissibility of the virus population (Muñoz *et al.* 1998).

Recently, a high prevalence of deletion variants was detected in a nucleopolyhedrovirus (NPV) population that infects the fall armyworm, *Spodoptera frugiperda*. Some of the deletion mutants were incapable of transmission *per os* when alone, but were able to replicate in isolation in cell culture or if injected into a suitable host larva (Simón *et al.* 2004). None of the isolated genotypes,

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either complete or deletion variants, attained the degree of pathogenicity of the original, natural virus population. However, when mixed with complete genotypes, both of the deletion variants tested to date was observed to overcome the attenuated pathogenicity of single NPV genotypes. This positive interaction between deletion and complete genotypes was only observed when the variants were co-occluded in occlusion bodies (OBs) and could thereby co-infect host cells (López-Ferber *et al.* 2003; Simón *et al.* 2005).

NPVs are DNA viruses that infect arthropods, especially insects of the family Lepidoptera. NPVs produce two types of virion for transmission within and between hosts. Occlusion-derived virions (ODVs) infect gut cells following consumption of contaminated foliage. Nucleocapsids produced early in the infection process individually bud through the basement membrane and disperse to infect other cells (Volkman 1997). Several budded virions of different genotypic composition may infect each host cell. Defective genotypes can thereby co-infect and take advantage of the gene products of complete genotypes. Later in infection, nucleocapsids are retained in the nucleus where they are wrapped by a membrane, either singly (SNPVs) or in groups of several nucleocapsids (MNPVs). These enveloped virions are occluded in OBs that permit virus survival in the environment (Volkman 1997). Following death of the host, progeny OBs are released onto the host plant to achieve transmission between hosts (Goulson 1997).

The present study examines, empirically and theoretically, the consequences of the interaction of complete and deletion genotypes on the genetic structure of an experimental virus population subjected to sequential cycles of transmission. Such studies provide important clues to the factors that favour the persistence and determine the prevalence of deletion mutants in virus populations.

2. MATERIAL AND METHODS

(a) *Insects and viruses*

S. frugiperda larvae were reared individually on semi-synthetic diet (Greene *et al.* 1976) at 25 °C, 70% humidity and 16 h light : 8 h dark photoperiod. A wild-type population of *S. frugiperda* nucleopolyhedrovirus (SfMNPV) has been characterized by Escribano *et al.* (1999). This isolate originated from a group of diseased larvae collected on one occasion by P. Castillo in Nicaragua in the late 1980s and had been passaged several times in laboratory colonies of *S. frugiperda* in Nicaragua and Honduras (R. D. Cave 1998, personal communication). Individual genotypes were isolated from that population by passage in *S. frugiperda* IPBL-Sf-9 cells, as described previously (López-Ferber *et al.* 2003). Nine genotypes that differed in their restriction profile were identified (SfNIC-A to -I) and have been characterized in detail (Simón *et al.* 2005). All nine genotypes produce OBs. The prototype complete genome is represented by SfNIC-B. The pathogenicity of SfNIC-B alone is 28% of that of the SfNIC population based on relative potency calculated by logit regression of concentration–mortality responses. The genotype SfNIC-C, presents a 16 Kb deletion and is not infectious *per os* but is capable of replication following injection or when co-occluded with a genotype that is infectious *per os*. Viruses were propagated by infecting fourth instar larvae by injection with ODVs, or *per os* with OBs. ODVs were obtained by dissolution of purified OBs of different genotypes as described previously (López-Ferber *et al.* 2003). Four instar larvae were injected with ODV aliquots of 8 µl derived from a suspension of 1×10^9 OBs ml^{-1}. *Per os* infection was performed using the droplet feeding technique (Hughes *et al.* 1986).

(b) *Experimental virus populations*

Three virus populations that differed in the relative proportions of genotypes B and C were produced by mixing the appropriate quantities of purified OBs obtained from single genotype infections. OBs were dissolved in alkaline buffer (1 vol. OBs : 1 vol. 0.5 M Na_2CO_3 : 5 vol. water) to release ODVs, which were mixed and injected into larvae as described above. When mixtures of complete and deletion genotypes were injected, the proportion of each genotype in the OBs purified from dead infected larvae corresponded to the proportions injected, indicating no significant replicative advantage in the deletion genotype (López-Ferber *et al.* 2003). Population 1 comprised 90% of B genomes and 10% of C genomes. Population 2 comprised equal numbers of genomes B and C. Population 3 comprised 10% of B and 90% of C genomes. The OBs obtained from these larvae were considered to be passage 0. The OBs were purified and used to infect larvae *per os*. A total of 25 newly moulted fourth instar *S. frugiperda* larvae were infected with the obtained OBs at 1×10^9 OBs ml^{-1}, representing an 80% lethal concentration (LC_{80}). The corpses of all the insects that died of NPV disease were pooled (18–22 insects per passage), OBs were extracted and purified. These OBs were considered to be passage 1 and were used as inoculum to infect the next group of larvae. The virus populations were followed for four (first replicate) or five (second replicate) rounds (passages) of *per os* infection. The relative proportions of each genotype at each passage were assessed as described below. The complete experiment (3 virus populations + controls) was performed twice.

(c) *Bioassays*

The 50% lethal concentrations (LC_{50}) of SfNIC-B and the co-occluded genotypic variants mixtures were determined using the droplet feeding method (Hughes *et al.* 1986). Second instar *S. frugiperda* were starved for 8–12 h at 23 °C and were then allowed to drink from an aqueous suspension containing 10% (w/v) sucrose, 0.001% (w/v) fluorella blue and OBs at one of five different concentrations (1.2×10^6, 2.4×10^5, 4.8×10^4, 9.6×10^3, 1.92×10^3, 1.27×10^2 OBs ml^{-1}). Control insects were fed sucrose and fluorella blue solution alone. The concentration range used for each virus genotype was previously determined to kill between 5 and 95% of experimental larvae. Larvae that ingested the suspension within 10 min were transferred to individual wells of a 25-well tissue culture plate with semi-synthetic formaldehyde-free diet. Bioassays with 25 larvae per virus concentration plus 25 control larvae were performed three times. Insects were incubated at 23 °C and mortality was recorded every 12 h until they had either died or pupated. Logit analysis of virus induced mortality was performed in generalized linear interactive modelling (GLIM, Royal Statistical Society, London) with a binomial error structure specified (Crawley 1993).

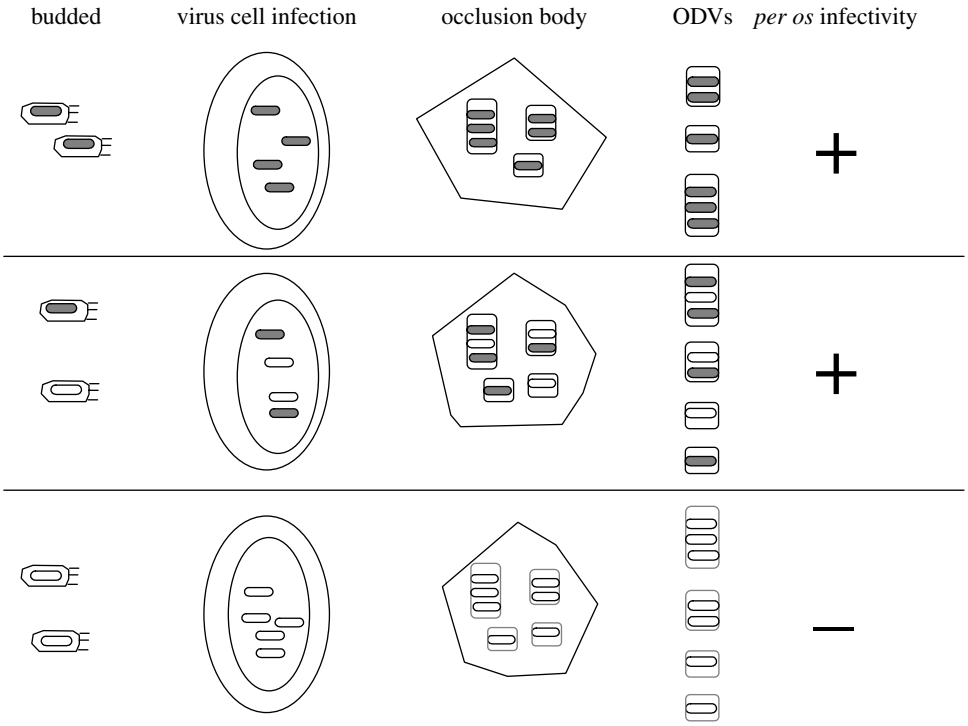

Figure 1. The different types of infection in insect cells. Cells are infected with budded virions that contain a single genome, either complete (grey nucleocapsids) or defective (white nucleocapsids). A cell can be infected with only one type of genome or with both types. Defective genomes cannot encode proteins required for the infectivity of ODVs. If only defective genomes infect a cell, the ODVs produced lack a required protein (represented by pale ODV envelopes). As a result, they are not infectious *per os*. All ODVs produced in mixed infections carry the required proteins in the envelope, independently of the genomes contained in the nucleocapsid, and consequently they are infectious *per os*.

(d) *Quantification of the relative proportions of each genotype*

The relative proportion of each genotype in OBs originating from all the infected insects that died at each passage was analysed by polymerase chain reaction (PCR) using primers that differentially amplify the two genotypes. The lengths of the amplified fragments were calculated to be 750 and 650 bp for variants B and C, respectively. PCR reactions were stopped in the linear phase of amplification (25 cycles) determined in preliminary assays (López-Ferber *et al.* 2003). PCR products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide and photographed on a UV transilluminator. The relative proportions of each genotype were then estimated by densitometric analysis of the intensities of each PCR product using Scion Image PC (Scion Corp., Frederick, MD, USA). The PCR reactions and measuring of the product intensities were performed three times.

(e) *Model development*

Both B and C genotypes produce OBs that contain virions. Virions produced in a pure B infection are infectious *per os* whereas virions produced in a pure C infection are not infectious *per os*. When a cell is infected by both B and C genomes, all the virions produced will be infectious, independent of their genotype composition. Following the conceptual framework developed by Godfray *et al.* (1997) and Bull *et al.* (2003), we assume that multiple genomes infect the host, and that their frequencies at the beginning of the infection correspond to their frequencies in the primary infected midgut cells. This represents a selection factor acting at the beginning of the infection. Accordingly, the frequency of each genotype in the inoculum that initiates the infection is based only on those midgut cells that produce virus progeny which subsequently colonize the haemolymph. As such, the frequencies of genotypes causing systemic infection may differ from their frequencies in the OBs ingested. This assumption clearly differentiates our model development from those previously published. In contrast, the frequency of each genotype once systemic infection is achieved does not change: there is no replication advantage, as observed in deletion mutants of *Autographa californica* NPV (AcMNPV) that lack the major occlusion protein, polyhedrin (*occ*-) (Bull *et al.* 2001). Each cell in the larvae can be infected by multiple virus particles, the multiplicity of infection being μ. To achieve transmission from insect to insect, variant C relies on the availability in the cell of viral products required for ODV infectivity that are encoded by the genome of variant B. Consequently, infection of a cell only by variant C genomes leads to OBs containing non-infectious virions, i.e. to be transmitted, variant C must co-infect cells with variant B.

Let p be the frequency of variant C inside an insect at generation t. The frequency of the C genotype in the $t+1$ round would depend on both the original C frequency and on the probability of a cell being infected by both variants B and C in the previous round. Co-infection follows a Poisson distribution with a mean multiplicity of infection μ. Each of the discrete combinations (multiplicity n) results in infectious ODVs if genotype B is present or non-infectious ODVs if genotype C is present but B is absent (figure 1). The frequency of each genotype in progeny OBs would correspond to their frequency in the infected cell. A fraction of those ODVs will not be able to infect the next generation. This fraction corresponds to the probability of a cell being

Table 1. Observed potencies and expected frequencies of pure and mixed genotype infections in the cells of the insect following ingestion of OBs containing different proportions of genotype C. Multiplicity of infection taken as $\mu=4.3$ (Potency values (s) from López-Ferber *et al.* (2003), multiplicity value (μ) from Bull *et al.* (2003)).

	frequency of genotype C in OBs						
	0.00	0.10	0.25	0.50	0.75	0.90	1.00
potency (s)	1.00	1.28	2.91	2.10	1.51	1.61	0.00
proportion of cells infected by							
pure genotype B	0.00	0.0073	0.0262	0.1028	0.3257	0.6254	1.00
pure genotype C	1.00	0.6254	0.3257	0.1028	0.0262	0.0073	0.00
mixed B+C	0.00	0.3220	0.6028	0.7490	0.6028	0.3220	0.00

infected by only C genotypes, that is p^n for each value of n, i.e. for each multiplicity of infection. The fraction of C genotypes that would be transmitted would therefore be $1-p^n-(1-p)^n$ for each value of n that corresponds to the mixed infections.

At the whole insect level, the total proportion of transmissible C genotypes would be

$$\sum_n \Pi(n,\mu)(1-[(1-p)^n+p^n]), \tag{2.1}$$

where the first term corresponds to the density function of the Poisson distribution, and the second term indicates the relative contribution of mixed infections. The probability of transmission will be directly related to the pathogenicity of virions within OBs, i.e. their *per capita* ability to infect the host, indicated by their relative potency (table 1). The likelihood of transmission of OBs will depend on the genetic composition of the virions infecting the cell in which they were produced, and is represented by a transmissibility factor, s. OBs derived from cells infected by pure genotype B will have $s=1$, or $s=0$ for OBs from cells infected only by genotype C. OBs from mixed genotype infections will have $s>1$. Accordingly, the factor s is introduced in equation (2.1) to reflect the contribution of each infection. In the simplest model, where only three different values of s apply, it becomes

$$\sum_n \Pi(n,\mu)([s_B(1-p)^n]+s_{BC}[1-(1-p)^n-p^n]+s_C p^n), \tag{2.2}$$

where s_B, s_{BC} and s_C are the fitness of pure B infections, mixed infections and pure C infections, respectively. In more complicated models, the value of s will depend on the actual proportion of B to C in a given cell. Equation (2.2) would then be developed for each term of the polynomial

$$\sum_n \Pi(n,\mu)\left[s_{m/n}\binom{m}{n}p^m(1-p)^{n-m}\right], \tag{2.3}$$

where m is the number of C genomes in a cell infected by a total of n genomes. In a first approach, the value of s can be estimated from the global fitness values (relative potency of SfMNPV relative to genotype B alone) in the wild-type population, 2.55 (López-Ferber *et al.* 2003) or from the relative potency of mixed genotype OBs (table 1). The global fitness or potency can be considered as the sum of the individual contributions of each of the possible types of infection. In *Trichoplusia ni* larvae infected by AcMNPV, μ was calculated to have a value of 4.3 (Bull *et al.* 2001). Under such conditions, the probability of $n>10$ is less than 0.005. Accordingly, we have limited our calculations to n values equal to or less than 10.

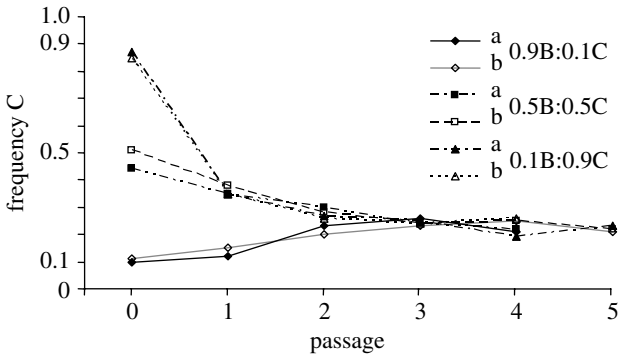

Figure 2. Evolution of frequencies of C genotypes in co-occluded mixtures during successive passages in *Spodoptera frugiperda* larvae. Three experimental populations involving genotypes B and C in proportional ratios of 0.1 B : 0.9 C, 0.5 B : 0.5 C and 0.9 B : 0.1 C were analysed for up to five generations. The results of two independent experiments (a and b) are represented.

3. RESULTS

No significant differences were observed in the relative proportion of each genotype present in the primary inocula injected and the progeny OBs (passage 0, P0) extracted from larvae in either replicate (see electronic supplementary material, fig. 5). These OBs were used as the inoculum for four or five successive rounds of *per os* infection. Over the passages, genotype frequencies in all six experimental populations converged to a common ratio comprising approximately 20% of deletion genotype C and 80% of complete genotype B and maintained these proportions in a fifth passage (figure 2 and see electronic supplementary material, fig. 6). This mirrors the relative proportions of deletion (22%) and complete (78%) genotypes previously observed in the wild-type SfMNPV population (López-Ferber *et al.* 2003).

Once the proportion between genotypes was stabilized, at the fourth passage, bioassays were performed for each experimental population. The pathogenicity of the mixtures (table 2, *III*, d–f) was not significantly different from that of the wild-type isolate (table 2, *I*, a), or an experimental mixture comprising 80% B+20% C genotypes in co-occluded OBs (table 2, *II*, c), whereas OBs of the genotypic mixtures were significantly more pathogenic than OBs of pure genotype B (table 2, *I*, b). This confirms previous findings that co-occluded mixtures of B+C genotypes restores the pathogenicity of the virus population to that observed in the wild-type isolate (SfMNPV-NIC; López-Ferber *et al.* 2003).

Table 2. Logit regression analysis of virus induced mortality of *Spodoptera frugiperda* larvae inoculated with (*I*) OBs originating from wild-type SfMNPV-NIC isolate or variant B (complete genotype) (*II*) OBs containing a mixture of 80% B and 20% C genotype co-occluded virions and (*III*) OB mixtures of B and C variant co-occluded virions in different proportions after four serial passages *in vivo* (Logit regression of number of responding insects against $\log_e$(virus concentration) was performed in GLIM (Numerical Algorithms Group, Oxford, UK) and given in terms of $\log_e$ odds ratio: $\log_e(p/q) = a + bx$. All bioassays were performed in triplicate and pooled for analysis. Fitting genotype composition (labelled a–f) as a factor with six levels resulted in a significant increase in model deviance when removed from the full model ($\chi^2_5 = 38.9$, $p < 0.001$) as did $\log_e$(virus concentration) ($\chi^2_1 = 897$, $p < 0.001$). A test for non-parallelism (interaction term) was not significant ($\chi^2_5 = 7.69$, $p = 0.174$) allowing regressions to be fitted with a common slope of 0.7126 ± 0.0287 (s.e.). Scaling was not necessary (residual deviance of minimal significant model/residual d.f. = 0.942). (*I*) LC_{50} values and 95% C.L. were calculated according to Collett (1991) using the Fieller macro present in the GLIM program. *p* values were calculated by *t*-test of the differences between regression intercepts compared to that of wild-type isolate. Relative potency was calculated as the ratio of the number of OBs of each genotype (or mixture of genotypes) required to produce the same mortality response. As such, relative potencies indicate the relative pathogenicity of each genotype (or mixture of genotypes) compared to that of wild-type. All bioassays were performed simultaneously in second instar *S. frugiperda* (25 insects per concentration, performed three times) using the droplet feeding technique. (*II*) OBs from the B and C variants were mixed in the ratio of 80 B : 20 C, the ODVs were extracted and injected into the haemolymph of fourth instar *S. frugiperda*. Progeny OBs containing this percentage of co-occluded B and C variants were purified from the dead larvae, and used for the bioassays (*III*) OBs from the B and C variants were mixed in the proportions indicated (passage 0), the ODVs were extracted and injected into the haemolymph of fourth instar *S. frugiperda*. Progeny OBs containing different proportions of co-occluded B and C variants were purified from the dead larvae, and the virus populations were followed for four rounds (passages) of *per os* infection. The OBs obtained after four passages were used for bioassay. Mixtures of B and C variants are indicated in the proportions in which they were injected; the proportion of B and C variants in the co-occluded mixtures after four serial passages is given in parentheses. The proportions of genomic DNA were estimated by densitometric estimates from semi-quantitative PCR amplification (see electronic supplementary material, fig. 5)).

	inoculum		LC_{50} (OBs $ml^{-1} \times 10^4$) (range of 95% C.L.)	intercept ± s.e.	relative potency	*p*
	(I)					
a	wild type isolate		5.31 (3.70–7.61)	−7.755 ± 0.3385	1.00	—
b	variant B		22.19 (15.48–32.13)	−8.772 ± 0.3616	0.24	<0.001
	(II) co-occluded mixed genotypes (%) passage 0					
	variant B	variant C				
c	80	20	6.36 (4.48–9.11)	−7.885 ± 0.3391	0.83	0.479
	(III) co-occluded mixed genotypes (%) passage 4					
	variant B	variant C				
	initial P0 (final P4)	initial P0 (final P4)				
d	90 (79)	10 (21)	6.29 (4.44–8.93)	−7.876 ± 0.3388	0.85	0.509
e	50 (78)	50 (22)	6.23 (4.35–8.84)	−7.864 ± 0.3395	0.85	0.549
f	10 (74)	90 (26)	7.92 (5.58–11.24)	−8.038 ± 0.3439	0.67	0.132

As in the original model of Bull *et al.* (2001), if no other factors act, the model predicts a steady decrease in deletion genotype frequency upon successive rounds of replication (figure 3*a*). To attain equilibrium, selection must be incorporated into the model. Various distributions can be applied to *s*. The simplest assumption is given in equation (2.2), in which s_B and s_C indicate the potency of each pure genotype, 1 and 0, respectively, and s_{BC} is determined empirically from the potency of the wild-type isolate, $s_{BC} = 2.55$. Another approach is to analyse the influence of different *s* values on the final potency of B and C co-infections, for a range of C frequencies. From equation (2.2), once the mean multiplicity of infection (μ) is fixed, the potency depends only on the s_{BC} value. It is, therefore, possible to compare the goodness of fit between these predicted potencies and the experimentally observed potencies. A single minimum is obtained for s_{BC} of 3.0 (figure 3*b*). Using $s_{BC} = 3.0$, the expected frequencies of C converge to an equilibrium after 10 passages (figure 3*c*). The value of the equilibrium frequency depends on the value assigned to μ. Higher multiplicities result in higher equilibrium frequencies of the deletion genotype in the population. For the value of $\mu = 4.3$, deduced by Bull *et al.* (2003), the equilibrium frequency is 0.31. The equilibrium falls to 0.21 if we consider $\mu = 3$.

Improving the biological realism of the model is achieved by assuming that not all mixed infections show the same potency. Extreme values of *s* can be calculated from the relative potency of co-occluded genotype OBs with 90% B + 10% C as $s = 1.28$ and 10% B + 90% C as $s = 1.61$ (table 1), determined previously by López-Ferber *et al.* (2003). Assuming a multiplicity of $\mu = 4.3$, for the primary inoculum comprising 90% B + 10% C, 64.4% of infected cells will not receive any C genomes and will contain only B genomes i.e. they will produce OBs with a transmissibility of $s = 1.0$. Likewise, 35.6% of infected cells will receive one or more B genomes and a single C genome (the proportion of cells receiving more than one C genome is negligible). These cells will produce OBs that increase the overall potency of the mixture to $s = 1.28$, because their intrinsic potency is 1.78 (calculated from equation 2.2). Conversely, in co-occluded mixtures of 10% B + 90% C, 64.4% of the cells will be infected only by C genomes, producing non-infectious OBs. However, from equation (2.2), the transmissibility of OBs produced in the 35.6% of cells that become infected by C genomes and a single B genome is $s = 4.52$ (again, the probability of >1 B genome infecting a cell is unimportant). A linear interpolation between those two extreme *s* values can be used in equation (2.3). This results in the predicted

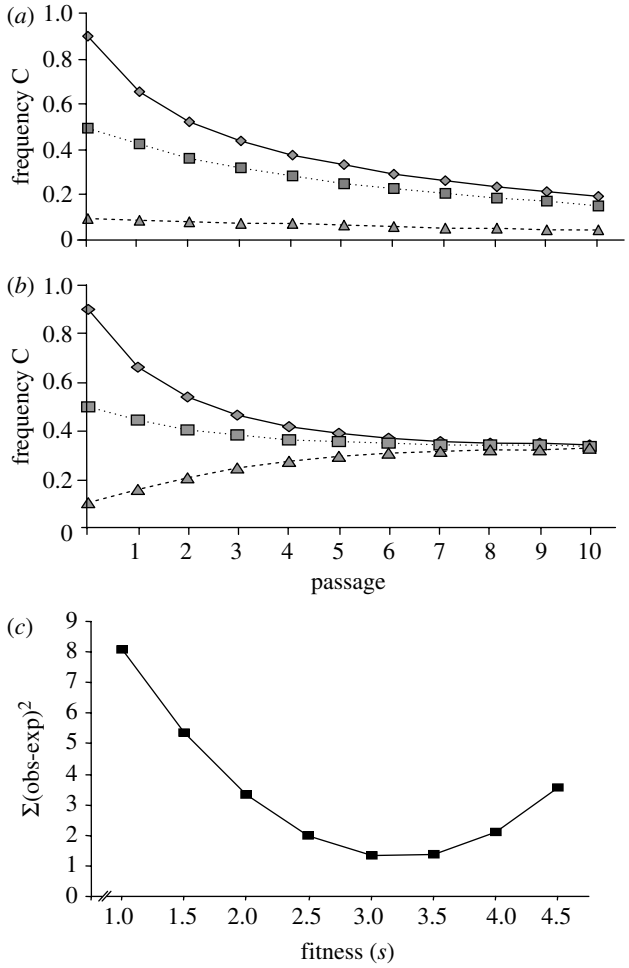

Figure 3. (*a*) Evolution of frequencies of defective genotypes (C) in the absence of selection. Initial conditions are 0.9 deletion genotype C+0.1 complete genotype B (diamonds and solid line); 0.5 C+0.5 B (squares and dotted line); 0.1 C and 0.9 B (triangles and dashed line). (*b*) Goodness of fit between observed and expected fitness values for $s_B=1$; $s_C=0$ and the indicated values of s_{BC}. (*c*) Predicted dynamics of defective genotype frequencies for $s_{BC}=3$.

convergence of the population to a single stable equilibrium at a C frequency of 28% (figure 4). The predicted rate of convergence from extreme frequencies in mixtures was somewhat slower than the rate observed empirically, but the goodness of fit to experimental observations obtained using these *s* values ($\Sigma(\text{obs}-\text{exp})^2=0.86$) is better than when using the previous model.

4. DISCUSSION

Experimental SfMNPV populations composed of only two genotypes converge upon successive passages towards a single equilibrium frequency, independent of their starting frequencies. The equilibrium coincides with the frequencies of deletion genotypes observed in the natural population from which they were derived. This equilibrium is reached in only four passages in *S. frugiperda* larvae. Mathematical models based on virus biology and the transmissibility of single and mixed genotype OBs were developed to investigate the factors that drive the population to the observed equilibrium.

Mixtures of co-occluded variants in all proportions restored the pathogenicity of the inoculum to that of the SfMNPV-NIC isolate, although a mixture containing a

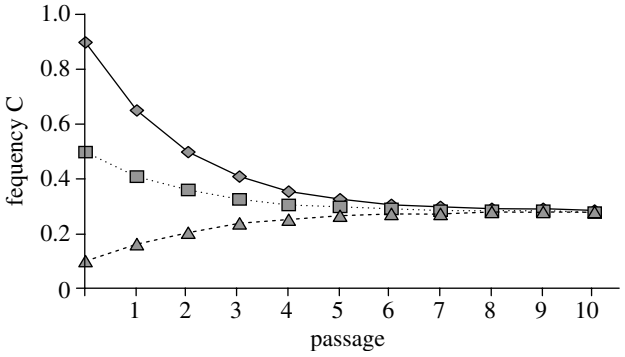

Figure 4. Evolution of frequencies of defective genotypes (C) when s_{BC} values change linearly in function of the proportion of C genomes infecting a cell. Initial conditions are 0.9 deletion genotype C+0.1 complete genotype B (diamonds and solid line); 0.5 C+0.5 B (squares and dotted line); 0.1 C and 0.9 B (triangles and dashed line).

prevalence of defective genotypes of ~25%, approximately the proportion observed in the wild-type population, resulted in the closest match to the pathogenicity observed in the natural population (López-Ferber *et al.* 2003). In another study, the pathogenicity of mixed genotype infections was observed to be greater than that of clonal infections in the NPV of *Panolis flammea* (Hodgson *et al.* 2003). The virulence (speed of kill) of single genotypes was modulated by the species of host food plant whereas the highest and lowest levels of virulence were attenuated in mixed genotype infections (Hodgson *et al.* 2002). Mixed genotype infections also influenced mortality patterns and the yield of progeny OBs from infected hosts (Hodgson *et al.* 2003). Clearly, interactions between genotypes in mixed infections can strongly affect the likelihood of virus transmission.

Previous quantitative studies of baculovirus biology have employed an AcMNPV genotype that could not produce the principal OB constituent protein, which is therefore non-transmissible *per os*. This variant replicates at the same rate at the wild type clone from which it was derived (Bull *et al.* 2001), as does a 'few polyhedra' (FP) mutant that was generated spontaneously in cell culture. The FP mutant is impaired in the production of OBs, but has a budded virus production advantage that favours its proliferation in cell culture (Bull *et al.* 2003). Comparison of the final yield of deletion genotype C and the complete genotype B when infection is initiated by injection lends support to our assumption that in the SfMNPV system, both genotypes replicate at similar rates.

A remarkably close agreement was obtained to empirical data using the final model in which experimental populations converged to a common equilibrium at passage 4, whereas convergence of the model populations assigned linear frequency-dependent fitness values occurred around passage 5 (figure 4). The best fit to the empirical results was obtained by linear interpolation, suggesting that the action of C genotypes is additive rather than multiplicative.

Two different processes intervene in the observed phenomenon: first, the absence of *per os* infectivity of OBs produced in cells infected only by C genomes, and second, the greater transmissibility of OBs produced in cells infected by both genotypes compared to cells infected by genotype B alone. Evidently, the first of these

phenomena tends to eliminate the deletion genotype from the viral population, in a frequency-dependent manner, whereas the second favours the persistence of this variant whatever its frequency in the population. For this reason, once an equilibrium has been attained, perturbation of the system, for example by artificial elevation of the frequency of deletion variants, should result in a rapid return to the equilibrium point.

The absence of *per os* infectivity of C variants is due to the disruption of at least one of the genes located in the deleted region of the genome. We have previously demonstrated that the C variant is defective in two genes responsible for *per os* infectivity: *pif-1* and *pif-2* (Simón *et al.* 2005). Viruses deficient in *pif* are not infectious by ingestion, but these viruses can acquire infectivity if they replicate in cells co-infected by complete genotypes or cells into which the *pif* gene has been introduced on a plasmid vector (Kikhno *et al.* 2002). Similarly, by exploiting gene products transcribed from the complete genotype B in co-infected cells, and co-occluding in the same OBs, ODVs comprising variant C alone can be assumed to be equally infectious as ODVs comprising variant B alone or genotypic mixtures. Consequently, the selection step that occurs in the insect midgut depends on the origin of the OBs in the inoculum consumed by the host. This is because ODVs that originated in cells infected by the deletion genotype alone fail to infect epithelial cells, although the precise molecular basis for this failure has yet to be revealed (Kikhno *et al.* 2002).

The key parameter at the centre of this issue is the multiplicity of infection (μ). Variation in μ alters the probability of cells being infected by pure B or pure C genotypes, which in turn modifies the proportion of progeny OBs that cannot achieve transmission (OBs comprising pure C genomes) and will therefore be eliminated from the population. Changes in μ will affect the final equilibrium point but an equilibrium state will always be reached. The value of $\mu = 4.3$, previously calculated by Bull *et al.* (2003), indicated close agreement with our empirical results. We cannot deduce, however, that this value is broadly representative of multiplicities of infection in baculovirus infections of insects. The wrapping of multiple genomes in a single virion is unique to NPVs. Multiple encapsidation also facilitates the transmission of defective genomes, although in theory non-infectious defective variants could persist in any virus population in which co-infection of cells by multiple genotypes is a common phenomenon. Frequency-dependent selection acting on complementation of deleted genes by the complete genotype will only influence virus transmissibility at high multiplicities of infection wherein the majority of cells are infected by multiple genotypes. In contrast, at low multiplicities, co-infection would be rare and interactions between genotypes would be limited to extracellular events (Wilke *et al.* 2004). For example, frequency-dependent fitness was observed at a multiplicity of 5 in a population comprising cooperating and defecting bacteriophages, whereas fitness was not found to be frequency dependent at a very low multiplicity (0.002; Turner & Chao 2003). Furthermore, Wilke *et al.* (2004) recently demonstrated that low-fitness clones of RNA viruses could stably co-exist with normal fitness genotypes over a wide range of conditions even when multiplicities vary widely from one round of infection to the next.

Previously, naturally occurring deletion mutants in a commercial preparation of *S. exigua* nucleopolyhedrovirus (SeMNPV) were classified as parasitic genotypes, as they reduced the pathogenicity of OBs when mixed with complete genotypes (Muñoz & Caballero 2000). However, for these studies, genotypic variants were isolated by *per os* infection of insects by end-point dilution. This process inevitably eliminated non-infectious variants from the experimental population, unlike the *in vitro* cloning procedure that we employed to isolate the SfMNPV-NIC variants.

It has been suggested that non-infectious genotypes, like genotype C, would contribute to the population fitness by a 'gain of function' mechanism, having a gene that the other genotype, B, does not carry (Frank 2003). It would be surprising, however, that such a mechanism persists over time without the generation by recombination of a new virus that would integrate the novel function. Such a virus would become predominant by selection. Another hypothesis is that the effect is obtained by diluting a viral factor produced by the complete genotype that is essential, but detrimental if overproduced. Additional experiments are required to determine which of these hypotheses, if either, is correct. Whatever the mechanism responsible for the observed effects, it is evident that, contrary to current thought, non-infectious deletion variants can profoundly affect the likelihood of transmission and the genetic structure and stability of virus populations.

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