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Population genetic structure determines speed of kill and occlusion body production in *Spodoptera frugiperda* multiple nucleopolyhedrovirus

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Abstract

A Nicaraguan isolate of *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfNIC) survives as a complex mixture of genotypes (named A to I). The speed of kill, time-mortality distribution, and occlusion body (OB) production of single genotypes (A, B and F) and co-occluded mixtures of genotypes, in a 75% + 25% ratio, were compared to determine the contribution of each genotype to the transmissibility of the viral population. Pure genotypes differed markedly in their speed of kill in second instar *S. frugiperda*. The speed of kill of SfNIC was attenuated compared to that of the dominant genotype B, indicating that interactions involving two or more genotypes likely determine host killing traits in the virus population. Genotypes A, F and defective genotype C, had no significant effects on the distribution of insect deaths over time when present as minority components in mixtures comprising 75% of genotype B. Similarly, the mortality pattern over time of insects infected by genotype F, the fastest-killing genotype tested, was not affected by the presence of genotypes A or C. Semi-quantitative PCR studies indicated that the genetic composition did not differ significantly between SfNIC-infected insects that died soon (67 h) or late (139 h) after inoculation, suggesting that stability in genotypic composition is important for virus survival. Median OB production per insect was correlated with mean time to death so that attenuated speed of kill of SfNIC resulted in high OB yields. We conclude that (i) minority genotypes play a functional role in determining the timing of mortality of infected hosts and (ii) the genotypic structure of the virus population is stably maintained to maximize the likelihood of survival.

Keywords: Genotypes; Baculovirus; Occlusion body production; SfMNPV; Speed of kill; Transmissibility

1. Introduction

Studies on genotypic heterogeneity within baculovirus populations by *in vitro* (Lee and Miller, 1978; Maruniak et al., 1984) or *in vivo* (Smith and Crook, 1988; Muñoz et al., 1998) techniques have revealed that natural populations often comprise a number of different genotypes that

may differ in the presence of certain genes (Durantel et al., 1998; Simón et al., 2004; Cory et al., 2005). Certain genotypic variants may also be more prevalent than others in wild-type populations (Muñoz et al., 1998; Hitchman et al., 2007). The fact that minority genotypes are not eliminated from the population suggests that this heterogeneity is important for virus survival (Possee and Rohrmann, 1997).

An insect that consumes a nucleopolyhedrovirus (NPV, genus *Nucleopolyhedrovirus*, family Baculoviridae) occlusion body (OB) ingests multiple virions, and for the multi-

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ply enveloped NPVs, a single virion may contain multiple genomes (Theilmann et al., 2005). Multiple encapsidation is a way of overcoming the host midgut epithelial cell sloughing response to viral infection, because some of the nucleocapsids from multicapsid virions are immediately repackaged and exported from the epithelial cell as budded virus to initiate systemic infection (Washburn et al., 1999). This infection strategy significantly increases the probability of successfully establishing an infection following the ingestion of OBs (Washburn et al., 2003). This has important consequences for the genetic structure of NPV populations as genotypically different virions can be occluded in a single OB, so that an individual insect is likely to be infected by multiple genotypes. Following the primary infection, it has been demonstrated that, on average, each cell in an insect is infected by multiple virions (Bull et al., 2001), so that the genotypic diversity in each cell of the secondary infection likely reflects the diversity present in the primary infected cell(s).

A Nicaraguan isolate of a multiple NPV (SfNIC) of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) has been selected for formulation and field evaluation for control of *S. frugiperda* by Mesoamerican maize growers (Castillejos et al., 2002). The SfNIC isolate survives as a complex mixture of at least nine genotypes SfNIC-A to SfNIC-I (hereafter referred to as genotypes A to I) (Simón et al., 2004). Genotype B was the predominant genotype in the SfNIC population and its genome size was calculated to be around 129 kbp (Simón et al., 2005a). All the other genotypes displayed one or more deletions compared to genotype B. Genotypes F, G, and I had a deletion of around 7 kb, genotypes A, E, and H had a deletion of around 10 kb, and genotypes C and D had a deletion of around 15 kb (Simón et al., 2004, 2005c).

Deletion genotypes C, D and G are not able to infect *S. frugiperda* larvae by ingestion (Simón et al., 2004). The absence of oral infectivity of genotypes C and D is related to the deletion of the *pif* and/or *pif-2* genes that are essential for cell binding and penetration during the process of infection of insect midgut cells (Kikhno et al., 2002; Pijlman et al., 2003; Simón et al., 2005b). None of the *per os* infectious genotypes is as pathogenic (*sensu* Thomas and Elkington, 2004) as the wild-type population, suggesting that interactions among genotypes result in altered pathogenicity, as measured by 50% lethal concentration assays (LC₅₀). This hypothesis was briefly confirmed in a previous study, where it was shown that the mixtures B+A, B+C, B+D, B+F showed increased pathogenicity compared to single genotypes alone (López-Ferber et al., 2003; Simón et al., 2005c). However, only the combination of B+C and B+D restored the pathogenicity of the mixture to that of the natural population when mixed in approximately the same proportion (75% + 25%) as observed in the wild population.

Phenotypic characteristics, such as speed of kill, differ between genotypes so that genotypic composition will affect the efficacy of the pathogen as a biological insecti-

cide. Our aim in this study was to compare virus-induced mortality patterns over time and OB production of single genotypes and mixtures of genotypes in order to determine the contribution of each genotype to these phenotypic traits and their likely influence on the survival of the virus population.

2. Materials and methods

2.1. Insects and viruses

Larvae of *S. frugiperda* were obtained from a laboratory colony continuously maintained in a growth chamber at 25 ± 1 °C, $75 \pm 5\%$ RH and a photoperiod of 16 h light and 8 h dark on semisynthetic diet (Greene et al., 1976). A Nicaraguan isolate of *S. frugiperda* multiple nucleopolyhedrovirus (SfMNPV-NIC) was originally isolated from diseased *S. frugiperda* larvae infesting maize plants in Nicaragua (Escribano et al., 1999). Viral occlusion bodies (OBs) of this isolate (SfNIC) were produced by inoculating fourth instars by the droplet feeding method (Hughes and Wood, 1981). OBs were extracted from dead diseased larvae by homogenization in water and purified by filtration and differential centrifugation. OBs were stored at 4 °C in distilled water until used. OB concentration was determined using an improved Neubauer hemocytometer (Hawksley, Lancing, UK) under phase-contrast microscopy.

The SfNIC genotypic variants used in this study had been cloned by plaque purification as previously described. Nine different genotypes were purified (Simón et al., 2004). Genotypes SfNIC-C, -D and -G, that are not infectious *per os*, were produced by injection of occlusion derived virions (ODVs) in larvae, whereas the infective genotypes were amplified by oral inoculation of larvae.

For extraction of viral DNA, virions were released from a suspension comprising $\sim 10^9$ OBs by addition of 100 μ l of 0.5 M Na₂CO₃ and 50 μ l of 10% w/v SDS in a final volume of 500 μ l and were incubated at 60 °C for 10 min. Undissolved OBs were pelleted by low-speed centrifugation (2700g, 5 min). The supernatant containing the ODVs, was treated with 25 μ l of proteinase K (20 mg/ml) for 15 min at 50 °C. Viral genomic DNA was extracted twice with TE buffer-saturated phenol and once with chloroform, and precipitated by addition of sodium acetate, pH 5.2, to a final concentration of 0.3 M, and 500 μ l of ice-cold ethanol (final concentration 70%). This was centrifuged at 3800g for 10 min, washed with 70% ethanol and dissolved in 0.1 \times TE buffer. DNA concentration was estimated by absorbance at 260 nm.

2.2. Production of genotype mixtures

Genotype mixtures were produced by injecting larvae with mixtures of the following combinations of genotypes: B+A, B+C, B+F, A+C, F+C, A+F and F+A as previously described (López-Ferber et al., 2003; Simón et al.,

2005c). OBs of each genotype were mixed in the proportion 75%+25%, since the ratio 75%B+25%C is close to the proportion observed in the SfNIC population and this proportion restored the pathogenicity of genotype B, in terms of LC₅₀ value, to that of the wild isolate (López-Ferber et al., 2003). The final concentration of the OB suspensions was 1×10^9 OBs/ml. ODVs were released from the above OB mixtures by alkali disruption with a dissociation buffer (1 vol. OBs:1 vol. 0.5 M Na₂CO₃:5 vol. H₂O). Undissolved OBs and other particulate materials were pelleted by low speed centrifugation at 2700g for 5 min. For each genotype mixture, an 8 µl volume of the ODV-containing supernatant was injected into each of 50 *S. frugiperda* fourth instars. This dose was equivalent to approximately 64 pfu by end point dilution in cell culture (O. Simón, unpublished data), and resulted in ~90% mortality in all cases. Injected larvae were individually maintained on diet at constant environmental conditions (see above) until death. Extraction of OBs containing mixtures of genotypes, OB purification and DNA extraction were then performed as described above. In all cases, the relative proportions of each genotype in mixed genotype OB experimental preparations were confirmed by semi-quantitative PCR.

2.3. Relative proportion of genotypes in mixtures and the SfNIC population

The relative proportions of each genotype in the SfNIC population and in mixed genotype OBs were estimated by measuring the relative intensity of a genomic fragment amplified by PCR. The length of the fragment is characteristic for each genotype and was previously used to differentiate infective genotypes (A, B, F) and defectives (C, D)

(Simón et al., 2005c). Fifteen different primers were designed, two of them (Sfgp41.1 and Sfgp41.2) amplified a ~760 bp fragment in a genomic region common to all the genotypes. These two primers were mixed with specific primers to each genotype that amplified a ~650 bp fragment (Table 1). DNA extraction of genotype mixtures was followed by 15–30 cycles of PCR depending on the relative proportion of each genotype in the wild-type population. Each reaction was stopped before reaching amplicon saturation. The relative proportion of individual genotypes was estimated by measuring the intensities of the two PCR products using the Scion Image PC program (Scion Corp., Frederick, MD, USA). Previous studies demonstrated that the proportion of genotypes in each of the genotypes mixtures present in OBs was virtually the same as the proportion in which they were injected into host larvae, indicating the absence of an asymmetric amplification in favor of one genotype (López-Ferber et al., 2003; Simón et al., 2005c).

2.4. Mortality over time of single and mixed genotypes

The mean time to death (MTD) of the pure and mixed genotypes was determined by inoculation of second instar *S. frugiperda* following the droplet feeding method (Hughes and Wood, 1981). Larvae were starved for 8–12 h at 25 °C and were then allowed to drink from an aqueous suspension containing 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue and an OB concentration that previous studies had indicated would result in 70–80% insect mortality (Simón et al., 2004, 2005c). This was 1.2×10^6 OBs/ml for all single genotypes and genotype mixtures, with the exception of the SfNIC isolate and the B+C mixture which were inoculated with a concentration of 2.4×10^5 OBs/ml.

Table 1
Summary of locations (nucleotide, nt) and direction of the primers in the 17.8 kb SfNIC sequenced region

Primer	Sequence	Dir	nt	Reference	Genotype
Sfgp41.1	CGACAATGTCATCGTCTTCG	Forward	NL (1)	Simón et al., 2005a	All
Sfgp41.2	ATATGTTAGTGGTGGCGGAC	Reverse	NL (1)	Simón et al., 2005a	All
Sf14.1	TCGATGACCCATTCTCTGCC	Forward	NL (2)	Simón et al., 2005a	H
Sfcath1	GCAGAATCGTTACGACTA	Forward	3–20	Simón et al., 2005c	C, D, E, I
Sfcath2	GCCGCGTTTAGTAACAGCAA	Forward	210–229	Simón et al., 2005c	A
Sf19.1	CGTGGTGTATCTGCGCGAA	Forward	1487–1506	Simón et al., 2005c	F
Sf21.1	AACAATAGTCGCGTTGCCGG	Forward	3381–3400	Simón et al., 2005c	G
Sf28.3	GATTAGAACACGTTCTTGCC	Reverse	7757–7738	Simón et al., 2005c	F
Sf30.8	TACTATGATTGAAAAGGATG	Reverse	8742–8723	Simón et al., 2005c	I
Sf30.7	CTACCACCTATCAACAATAC	Reverse	8875–8856	Simón et al., 2005c	G
Sf30.6	TCATAACGCACCAACCATGC	Reverse	11154–11135	Simón et al., 2005c	A
Sf32.1	TGGTGGAGCTGCATGATTGT	Reverse	11895–11876	Simón et al., 2005c	H
Sf35.4	CGATACGATCAATGTGCTGG	Reverse	14303–14282	Simón et al., 2005c	E
Sfpif.4	GTGTTCAAGTGGGCACGGCG	Forward	16247–16266	Simón et al., 2005c	B
		Reverse			C, D
Sf100a	GTCTACTTGGATTTCGTCGAAGGTGA	Reverse	16986–17010	Simón et al., 2005c	B

Nt, nucleotide positions according to the sequence of a 17.8 kb region reported by Simón et al. (2005c).

NL, not located in the sequenced region.

(1) In the ORF homologue to Se80 (gp41 gene), between the SfMNPV HindIII-H and -K genome fragments.

(2) In the ORF homologue to Se8, left extremity of the SfMNPV PstI-G genome fragment.

These primers were used in the semi-quantitative PCR in order to estimate the relative proportion of each genotype in the wild-type population and mixtures of *Spodoptera frugiperda* multiple nucleopolyhedrovirus.

Larvae that ingested the suspension within 10 min were transferred to individual wells of a 25-well tissue-culture plate with a diet plug. Experiments were performed with 25 larvae per virus treatment and 25 larvae as negative control. The experiment was performed three times on separate days. Larvae were reared at 25 ± 1 °C and, starting at 48 h post-inoculation, the number of surviving insects was recorded every 8 h until insects had either died or pupated. Time mortality data were subjected to Weibull survival analysis without censoring using the Generalized Linear Interactive Modeling program GLIM (Numerical Algorithms Group, 1993). Survival models, such as the Weibull, are preferred for analysis of time to death data as the variance in age at death tends to increase with the mean. Only individuals that died from NPV disease, confirmed by the microscopic observation of OBs, were included in these analyses. The suitability of Weibull models was determined by examination of Kaplan–Meier probability plots (Aitkin et al., 1989). The cumulative time mortality distribution of single genotypes and mixtures was analyzed graphically.

The proportion of each genotype in SfNIC OBs from larvae dying at early (67 h post-inoculation, hpi) and late (139 hpi) times following inoculation was estimated by pooling groups of ten larvae that died at each timepoint. OBs were semi-purified and subjected to semi-quantitative PCR, and densitometric analysis as described above.

2.5. Productivity of single and mixed genotypes

The OB production of single and mixed genotypes was determined. All the larvae that died of NPV disease in the speed of kill experiment (at least 20 for each virus treatment and each replication, ~60 larvae per virus treatment) were collected and stored at -20 °C until used for OB counting. Each larva was homogenized in distilled water. This OB suspension was diluted in distilled water. The production from each larva was estimated by OB counting in triplicate using a Neubauer hemocytometer. The results were analyzed by Kruskal Wallis and Mann Whitney non-parametric statistics. *P* values were subjected to false discovery rate adjustment for multiple pairwise comparisons (Benjamini and Hochberg, 1995).

3. Results

3.1. Relative proportion of genotypes in mixtures and the SfNIC population

The proportion of each genotype in the SfNIC population was estimated by densitometric analysis of genotype-specific PCR amplified fragments (Fig. 1). The complete genotype B was the most prevalent genotype in the natural isolate representing approximately 60% of genotypes, followed by defective genotypes C/D with a combined prevalence of 18%, very close to values reported previously (López-Ferber et al., 2003). The pro-

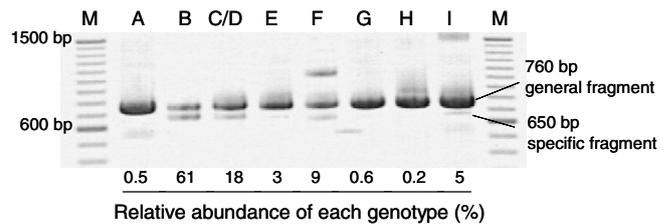


Fig. 1. Semi-quantitative PCR analysis of the relative proportion of each genotype in the Nicaraguan isolate of *Spodoptera frugiperda* MNPV (SfNIC). PCR was performed using SfNIC DNA and specific primers for each genotype (Table 1). The molecular marker (M) used was 100 bp ladder (Gibco BRL), molecular sizes are to the left and specific PCR fragment sizes to the right. The 760 bp amplicon was amplified from a common genomic region present in all genotypes. The 650 bp amplicon represented the characteristic fragment of each genotype. Figures below amplicons indicate the relative proportion of each product compared to the reference amplicon (taken as 100) estimated by densitometric analysis.

portion of these genotypes was estimated by the intensity of the PCR fragments measured after 15 PCR cycles. Genotype F was present at a prevalence of ~9% as estimated after 20 cycles of PCR, followed by genotype E (~3%), and genotype I (~5%), both measured after 25 cycles of PCR. Finally, genotypes A, G and H each represented <1% of the SfNIC population, all measured after 30 cycles of PCR. The SfNIC isolate is thus composed mainly of complete genotype B and defective genotypes C/D, together comprising almost 80% of the population. The relative abundance of each genotype in the mixtures did not differ significantly from the composition of the ODV suspensions used to inject insects (data not shown). The identity of each mixture was additionally checked by comparison of restriction endonuclease profiles following treatment with PstI. Restriction profiles were identical to those described previously (Simón et al., 2004), except for the appearance of sub-molar bands indicating the presence of the respective minority genotype (data not shown).

3.2. Mortality over time of single and mixed genotypes

To determine the speed of kill of the 11 virus treatments, the mean time to death (MTD) was estimated for virus concentrations that resulted in approximately 70–80% insect mortality (Table 2). Single and mixed genotype inocula killed the larvae with the typical signs and symptoms of NPV disease within a mean interval of 78–135 hpi. As the mortality patterns over time of genotype A alone and the mixture of 75% A + 25% C differed from those of all other genotypes and mixtures, these two treatments were analyzed separately. The speed of kill of SfNIC (118 h, 70% mortality) was similar to that of the slowest acting variant, genotype A (127 h, 73% mortality), based on non-overlap of 95% C.I. However, pure genotypes B (90 h, 70% mortality) and F (82 h, 69% mortality) had markedly faster speeds-of-kill than SfNIC. No mortality was observed in the controls.

Table 2

Mean time to death (MTD) of *Spodoptera frugiperda* larvae inoculated with SfNIC wild-type or individual genotypes and genotype mixtures originating from the SfNIC isolate of SfMNPV

Inoculum ^a	Number of dead insects ^b	MTD ^c (h)	95% C.I.	
			Low	High
SfNIC WT	49	118	110	125
Genotype A	55	127	124	130
Genotype B	51	90	84	96
Genotype F	51	82	77	87
75% B + 25% A	52	87	82	93
75% B + 25% C	50	92	87	98
75% B + 25% F	58	89	84	94
75% A + 25% C	60	135	132	138
75% F + 25% C	59	79	75	84
75% A + 25% F	58	84	80	89
75% F + 25% A	58	78	74	83

^a Larvae of *S. frugiperda* were inoculated in the second instar. Genotype C alone is not infectious *per os* but can infect when co-occluded with a genotype that expresses the *pif* or *pif-2* gene.

^b Total number of insects that died from polyhedrosis disease in each treatment.

^c MTD values were estimated by Weibull analysis (Crawley 1993). MTD values for genotype A alone or a mixture of 75% A + 25% C were fitted with a shape parameter (α) of 12.218, whereas all other genotypes and combinations thereof, including SfNIC WT, were fitted with a shape parameter of 4.388 (see explanation in text).

The mixtures in which genotype B was the dominant genotype in the mixture (75% prevalence) did not result in modified speed of kill compared to genotype B alone. The estimated MTD values of the genotype mixtures B+A (69% mortality), B+C (69% mortality) and B+F (77% mortality) were 87, 92 and 89 h, respectively, which were similar to that of genotype B alone. Similarly, the speed of kill of genotype F was not significantly affected by the presence of minority genotypes. The MTD of the mixtures F+C (79% mortality) and F+A (75% mortality) were 79 and 78 h, respectively, which were similar to that of genotype F alone (82 h). Finally, the MTD of the mixture A+C (79% mortality) was 135 h, which was similar to that of pure genotype A. In contrast, the MTD value of the mixture A+F (78% mortality) was 84 h, which was a significantly shorter speed of kill compared to genotype A alone (127 h), but was similar to that of genotype F alone (82 h).

Examination of the patterns of mortality over time generally supported the MTD findings and provided additional information. The mortality dynamics of insects infected by genotype A alone or in a mixture with genotype C were clearly different from those of all other genotypes and mixtures tested (Fig. 2A). The majority of insects infected by genotype A died at 115–131 hpi. In A+C mixed infections, the mortality peak was delayed by approximately 16 h compared to genotype A alone. A corresponding effect of C genotype was not observed for other mixed infections involving B+C (Fig. 2B) or F+C (Fig. 2C).

The time-mortality distribution of genotype B was clearly reflected in mortality patterns over time when pres-

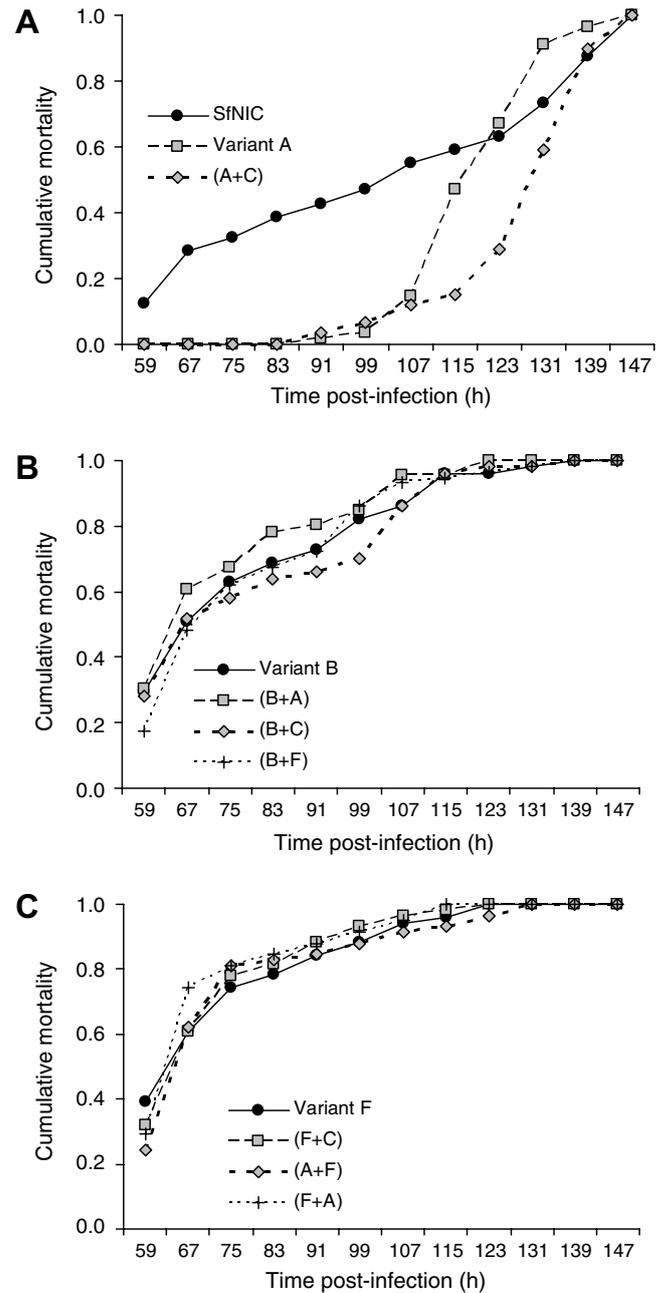


Fig. 2. Cumulative mortality of *Spodoptera frugiperda* larvae inoculated in the second instar with single genotypes or co-occluded mixtures (75% + 25%). (A) SfNIC wild-type isolate, genotype A alone and the mixture A+C, (B) genotype B alone and mixtures comprising B+A, B+C, B+F, and (C) genotype F alone and mixtures F+C, A+F and F+A. Proportional mortality was calculated based on those insects that became infected following consumption of an LD₇₀₋₈₀ dose of OBs (replicates = 3); non-infected survivors were excluded from the analysis.

ent as the dominant component in mixtures with genotypes A, C or F (Fig. 2B). In fact, the distinct mortality pattern of genotype A was completely abolished in the presence of the majority genotype B.

Finally, genotype F and the mixtures F+C, F+A and A+F were the fastest killing treatments; most insects died at 59–75 hpi (Fig. 2C). The patterns of mortality were

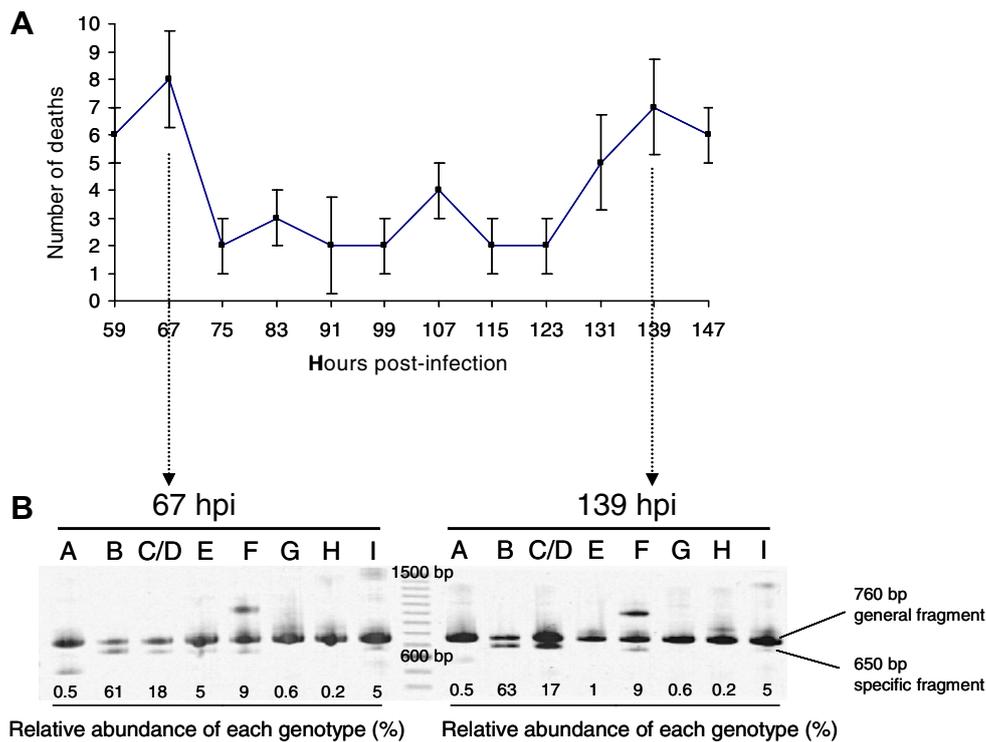


Fig. 3. Relative proportion of each genotype in the SfNIC isolate along the time-mortality distribution. (A) Time mortality distribution of *Spodoptera frugiperda* killed by SfNIC isolate after inoculation in the second instar. Vertical bars indicate S.D. (B) Semi-quantitative PCR analysis was performed using DNA extracted from SfNIC OBs from pooled groups of ten larvae that died at each of the two peaks of mortality (at 67 and 139 hpi) and the specific primers for each genotype (Table 1). The molecular marker (M) used was 100 bp ladder (Gibco BRL). The 760 bp amplicon amplifies in a common region present in all the genotypes. The 650 bp amplicon represented the characteristic fragments of each genotype. The intensity of each fragment was measured using the Scion Image Program. Figures below amplicons indicate the relative proportion of each product compared to the reference amplicon (taken as 100) estimated by densitometric analysis.

the same for these three genotype mixtures indicating that genotype F determines phenotype even when present as a minority component (mixture 75% A + 25% F). For treatments involving single genotypes and genotype mixtures, variability in insect deaths over time were similar to that observed in the SfNIC isolate (Fig. 3A), but for clarity error bars are not shown in Fig. 2A–C.

Evidently, the genotypic composition of experimental inocula had a major influence of the speed of kill phenotype. This led us to examine the relative proportion of each genotype at each end of the time-mortality distribution in insects infected with the SfNIC population (Fig. 3A). The distribution of the numbers of insects that died from SfNIC infection over time appeared to be bimodal in nature with mortality peaks at 67 and 139 hpi. The prevalence of each genotype was approximately the same in insects that died early or late in the time-mortality distribution, and this did not change with respect to that observed in the original wild-type population (Fig. 3B). Genotype B was present as the predominant genotype (60%), whereas genotypes A and F appeared as minority genotypes (1% and 9%, respectively) in insects sampled at both peaks of mortality. A slight reduction in the relative prevalence of genotype E was observed from 5% at 67 hpi to 1% at 139 hpi. We conclude that the genotypic composition of the wild popula-

tion is stably maintained over the time-mortality distribution.

3.3. Productivity of single and mixed genotypes

The OB production of each genotype and mixtures was determined by counting the OBs obtained from dead diseased insects that had been inoculated in the second instar. Results were not normally distributed and are presented as medians with non-parametric statistics. OB production differed significantly with inoculum (Kruskal–Wallis $\chi^2 = 271$, d.f. = 10, $P < 0.001$). The most productive infections were those of genotype A and A+C (Fig. 4) followed by the SfNIC wild-type. Compared to SfNIC, median OB production was greatly reduced in insects infected by genotype B alone or in mixtures with A, C or F, presumably due to the rapid speed of kill of these treatments. The mixture of B+C resulted in significantly increased OB production compared to variant B alone. OB production was lowest in insects infected by genotype F alone and the presence of other genotypes as minority components (C and A) or as the majority component (genotype A) did not significantly influence OB yields per insect. There was a highly significant logarithmic correlation between speed of kill and median OB yield per insect (Fig. 5).

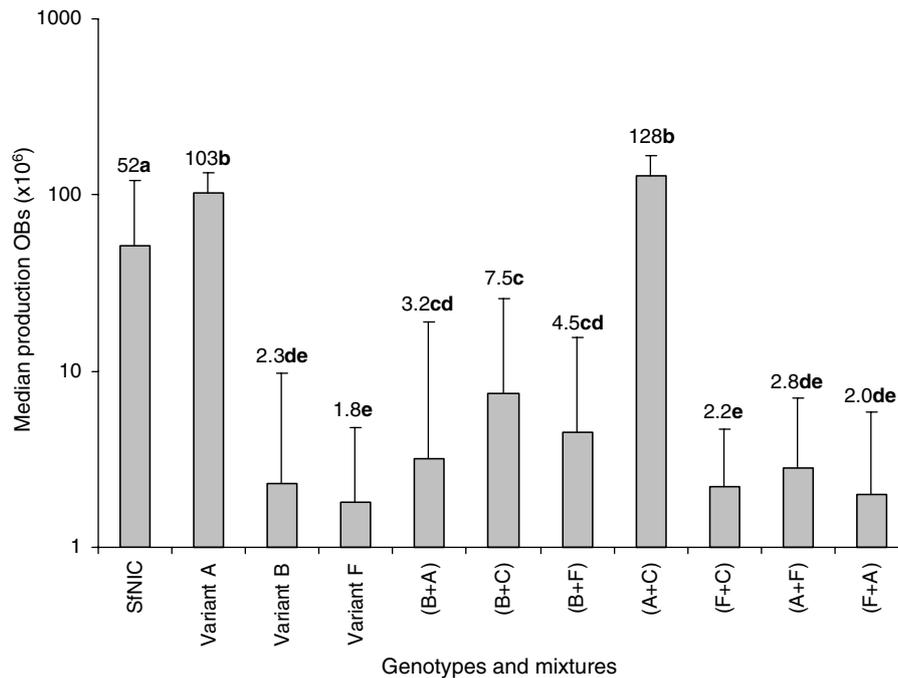


Fig. 4. Median OB production per insect ($\times 10^6$) for larvae that died from a Nicaraguan isolate of *Spodoptera frugiperda* multiple nucleopolyhedrovirus SfMNPV (SfNIC) wild-type, single genotype or mixed genotype infections. Sample sizes are given in Table 2. Figures above bars indicate values of the medians. Values followed by identical letters did not differ significantly for pairwise comparisons (Mann–Whitney *U*-test followed by Benjamini and Hochberg (1995) false discovery rate adjustment of *P* values for multiple comparisons). Vertical lines indicate the upper half of the interquartile range.

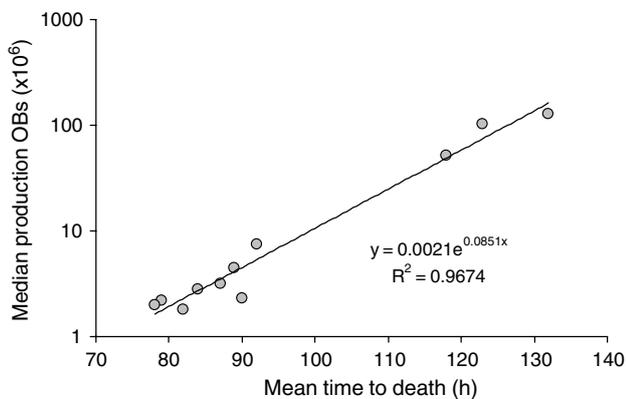


Fig. 5. Logarithmic relationship between mean time to death and median OB production in SfNIC wild-type, three single genotypes and seven genotype mixtures in *Spodoptera frugiperda* larvae inoculated in the second instar.

4. Discussion

The SfNIC isolate represents a virus population composed of at least nine different genotypes, present in various proportions. Individual genotypic variants differed markedly in their speed of kill. It was not possible to determine the MTD of genotypes C or D alone due to their lack of *per os* infectivity. However, by producing OBs containing a mixture of deletion genotype C and other genotypes that present the *pif* or *pif-2* genes that are required for *per os* infection, we were able to examine the outcome of interactions among these genotypes on speed of kill, mortality over time and OB production.

Examination of cumulative mortality patterns over time (Fig. 2A–C) and MTD values allows us to draw four clear conclusions. First, it was evident that the distribution of host mortality over time in insects infected by genotype A or the mixture A+C was quite different from that of any other genotype or combination of genotypes (Fig. 2A). Second, the speed of kill of SfNIC was much slower than that of the dominant genotype B, indicating that minority genotypes attenuate the influence of B in the SfNIC population. However, minority genotypes A, C or F had no significant influence on the killing phenotype of genotype B (Fig. 2B), suggesting that an interaction involving a minority genotype that was not included in this study, such as genotype E (MTD = 140 h) or genotype H (MTD = 136 h) (Simón et al., 2004), or interactions involving various genotypes, are likely responsible for the attenuated speed of kill of SfNIC. It is important to note that the MTD values presented here for SfNIC and genotype B are derived from 8 h observation intervals and are somewhat lower than those reported previously based on 12 h observation intervals (Simón et al., 2004). Third, in spite of its unusually slow speed of kill, genotype A had no significant influence on host killing by genotypes B or F, even when F was present as a minority component (Fig. 2C). This provides a good example as to why determination of the factors that favor the persistence of rare variants such as genotypes A, E, G, H or I, in NPV populations remains a major challenge in understanding NPV ecology. Finally, genotype F was the fastest killing genotype tested with a MTD value of 82 h. Its killing behavior was not influenced

in mixtures with genotypes A or C, whereas the mixture of B+F was statistically similar to genotype B alone.

In nature, genotypic interactions are likely to be more complex in NPV populations than those considered in the present study. It was clear, however, that the identity and prevalence of virus genotypes infecting an insect influence the speed of kill. In consequence, we hypothesized that a difference may be expected in the relative abundance of each genotype in progeny OBs between larvae that died sooner or later after inoculation. Surprisingly, the genetic composition of the SfNIC infection did not differ significantly between those insects that died early or late in infection (Fig. 3). This further supports the concept that is not the major genotype that determines virus killing traits, but the presence of specific combinations of genotypes. Indeed, experimental virus populations comprising non-natural frequencies of defective or complete genotypes, rapidly converge to a common equilibrium value. Once this equilibrium has been reached, it is stably maintained at the population level (Simón et al., 2006), and during the infection process involving cell-to-cell transmission within individual hosts (this study). This likely explains the apparent genetic stability of the isolate when subjected to multiple rounds of replication and transmission in field and laboratory experiments.

Other phenotypic characteristics likely to affect transmissibility, such as total OB production, differed significantly between genotypes. It was not possible to obtain a consistent measure of productivity in terms of OBs/mg larval weight as larvae were very small at the time of death and could not be individually weighed without risking loss of OBs. The tradeoff between time to kill and total OB production is well established in baculoviruses (Hernández-Crespo et al., 2001; Hodgson et al., 2001, 2004; Cory et al., 2004). Thus, fast-killing genotypes produce fewer OBs than their slower-acting counterparts, probably due to the small size of larvae that died soon after inoculation with fast-killing genotypes. The total OB production observed in insects infected by the SfNIC population was more than twenty-fold greater than that of insects infected by dominant genotype B alone. As a positive relationship exists between OB density and transmission (Dwyer et al., 2002; D'Amico et al., 2005), this result underlines the advantages of attenuated speed of kill in the transmissibility of the SfNIC population.

Unlike the diversity reported in variants of *Pannolis flammea* NPV, that was scattered throughout the virus genome (Cory et al., 2005), the observable diversity among SfNIC genotypes is located in a single region of the genome (Simón et al., 2005c). Notably, genotypes with shorter deletions, such as genotype F, killed faster and were therefore less productive than genotypes with larger deletions, such as genotype A (Simón et al., 2005c). Genotype F lacks ORFs 3–8, whereas genotype A lacks ORFs 2–10 located in a 17.8 kb genomic region

(Simón et al., 2005c). One clear feature seen at the genomic level is that the fastest-killing genotypes retain the ORF homolog to Se30 in their genome whereas this ORF is absent in the slower-killing genotypes. This ORF, of unknown function, may therefore play an important role in determining the transmissibility of these viruses, although additional genes in the deleted region could also be involved. Further studies are in process to examine the importance of these genes, and particularly the ORF homologue to Se30 in determining virus phenotype.

From a biocontrol viewpoint, Hodgson et al. (2002a) have pointed out that it is vital to retain genotypic diversity in NPV insecticidal products because (i) they are more likely to contain genotypes that are adapted to local host ecology, such as a particular foodplant (Hodgson et al., 2002b), (ii) mutualistic interactions between genotypes tend to result in higher pathogenicity and OB production (Hodgson et al., 2004), and (iii) certain genotypes may be better adapted for persistence in the environment. Indeed, Murillo et al. (2007) recently provided evidence that certain variants of *Spodoptera exigua* multiple NPV were more likely to be isolated from greenhouse soils with high pH, whereas other variants were more commonly isolated from soils of lower pH.

There are two particular areas that require additional scrutiny to better understand the results of our study. First, an examination of within-host dynamics in the abundance and relative proportions of interacting genotypes may reveal significant changes during the course of infection that affect the duration of the disease and the timing of host death. As mentioned above, the proportions of genotypes B and C changed rapidly when experimental mixtures were subjected to serial passage in larvae, although changes in genotypic mixtures that occurred during the progression of disease in infected individuals was not addressed (Simón et al., 2006). Second, the OBs of single genotypes may differ in size, ODV number or in the distribution of numbers of nucleocapsids among ODVs. Differences in OB composition are likely to influence both the number of OBs required to initiate a lethal infection and the rate at which infected insects die of disease, both for single and mixed genotype OBs.

In conclusion, the results presented here and those obtained previously (Simón et al., 2006), have confirmed that the SfNIC population is genotypically structured to maximize the likelihood of survival. This isolate has attenuated speed of kill compared to its principal genotypic components, which results in improved OB yield and increased likelihood of transmission. The relative frequencies of genotypes are stably maintained in infected insects throughout each infection cycle and during successive cycles of insect-to-insect transmission. This clearly indicates that population genetic structure involving multiple minority genotypes should be actively conserved during the development of NPV-based biological insecticides.

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