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Mixed genotype transmission bodies and virions contribute to the maintenance of diversity in an insect virus

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An insect nucleopolyhedrovirus naturally survives as a mixture of at least nine genotypes. Infection by multiple genotypes results in the production of virus occlusion bodies (OBs) with greater pathogenicity than those of any genotype alone. We tested the hypothesis that each OB contains a genotypically diverse population of virions. Few insects died following inoculation with an experimental two-genotype mixture at a dose of one OB per insect, but a high proportion of multiple infections were observed (50%), which differed significantly from the frequencies predicted by a non-associated transmission model in which genotypes are segregated into distinct OBs. By contrast, insects that consumed multiple OBs experienced higher mortality and infection frequencies did not differ significantly from those of the non-associated model. Inoculation with genotypically complex wild-type OBs indicated that genotypes tend to be transmitted in association, rather than as independent entities, irrespective of dose. To examine the hypothesis that virions may themselves be genotypically heterogeneous, cell culture plaques derived from individual virions were analysed to reveal that one-third of virions was of mixed genotype, irrespective of the genotypic composition of the OBs. We conclude that co-occlusion of genotypically distinct virions in each OB is an adaptive mechanism that favours the maintenance of virus diversity during insect-to-insect transmission.

Keywords: baculovirus; co-occlusion; genotype diversity; occlusion body; transmission; virion

1. INTRODUCTION

The fitness of all parasites is determined by their transmission. For virus pathogens (microparasites) of multicellular organisms, transmission occurs in two phases. Between-host transmission involves abandoning an infected host and entering a susceptible one, followed by replication and cell-to-cell transmission in the susceptible tissues or organs of the newly infected host. Each of these steps is subject to overcoming host-mediated control measures, the success of which will depend to a large extent on the genetic resources available to the invading pathogen and their frequency in the pathogen population.

Genetic diversity in macro- and microparasite populations is a universal requirement owing to continuously evolving host defence capabilities (**Read & Taylor 2001**). Virus populations are renowned for their diversity, and many viruses adopt infection strategies to ensure that part of that diversity is present in individual infected hosts (**Hurst 2000**). Within-host diversity may be achieved when inoculum comprises a diverse pool of virus genomes (**Lord *et al.* 1997**; **Bonneau *et al.* 2001**; **Cory *et al.* 2005**; **Herring *et al.* 2005**; **Jerzak *et al.* 2005**), when genotypically distinct viruses cooperate to achieve infection, such as the facilitation observed in homologous or heterologous 'helper viruses' (**Eckner 1973**; **Stenger 1998**), or when viruses are capable of generating *de novo* diversity during the replication process in the newly infected host (**Domingo *et al.* 1998**; **Chang *et al.* 2002**; **López-Bueno *et al.* 2003**). Genotypic diversity within infected hosts can have dramatic repercussions for both host and virus, including alterations in virulence (harm suffered by the host) (**Sedarati *et al.* 1988**; **Brown *et al.* 2002**; **Schjørring & Koella 2002**; **Alizon 2008**), within-host population dynamics (**Miralles *et al.* 2001**; **Rauch *et al.* 2008**), virus fitness (**Hodgson *et al.* 2004**; **Arends *et al.* 2005**; **Čičin-Šain *et al.* 2005**; **Simón *et al.* 2006**), and if frequent, may favour the evolution of cheating genotypes (**Turner & Chao 1999**; **Frank 2000**; **Grande-Pérez *et al.* 2005**).

Nucleopolyhedroviruses (NPVs) are important mortality factors in the population dynamics of a number of insects (**Cory & Myers 2003**), and they have unique insecticidal properties that make them useful as the basis for biological control agents (**Moscardi 1999**). NPVs are structurally complex with one double-stranded circular DNA genome packaged into each nucleocapsid. Individual nucleocapsids produced in the early stages of infection bud out of the infected cell. This budded virion (BV) phenotype is responsible for systemic transmission to other cells. Later in the cell infection cycle,

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nucleocapsids that remain in the nucleus are enveloped singly or in multiples to form virions that are occluded in a protein matrix, the occlusion body (OB), which is responsible for insect-to-insect transmission. Large numbers of virions are occluded into each OB. When contaminated foliage is consumed by a susceptible larva, the OB dissolves in the alkaline midgut releasing numerous occlusion-derived virions (ODVs). Each ODV is capable of infecting a host midgut cell. This primary ODV infection initiates the secondary systemic BV infection that results in the death of the insect several days later (**Theilmann et al. 2005**).

Recent studies on a Nicaraguan isolate (SfNIC) of the multiply-enveloped NPV from the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), have revealed that this virus exists as a mixture of at least nine genotypes, eight of which have deletions of 4.8–16.4 kb in length in one region of the genome (**Simón et al. 2005**). The only complete genotype, SfNIC-B, is the majority genotype. Each individual genotype has a characteristic pathogenicity, as measured by dose-mortality metrics (**Robertson et al. 2007**), which is lower than that of the complete population. Two of the deleted genotypes (SfNIC-C and SfNIC-D) can produce OBs, but they lack genes for essential *per os* transmission factors; these two genotypes survive in the population by complementation with the complete genotype in co-infected cells.

Co-infection appears to be common during the systemic phase of disease; on average cells are infected by an estimated 4.3 BV particles (**Godfray et al. 1997**; **Bull et al. 2001**; **Simón et al. 2006**). In consequence, OBs produced in cells infected by defective and complete genotypes are infectious *per os*, whereas OBs produced in cells infected by defective genotypes alone are not infectious. Moreover, serial passage experiments involving sequential rounds of insect-to-insect *per os* transmission have demonstrated that experimental mixtures, comprising different ratios of complete and defective genotypes, rapidly converge to a common equilibrium that maximizes their transmissibility and that closely reflects their relative proportions in the wild-type population (**Simón et al. 2006**; **Clavijo et al. 2009**).

Co-infection by multiple genotypes appears to be adaptive in this virus as it increases the transmissibility of inoculum. The particular characteristic of NPVs of being able to produce OBs that contain multiple virions and, for the multiply-enveloped NPVs, that these virus particles may contain multiple genomes, calls into question the role of the virion assembly and the occlusion process in maintaining virus diversity. The question as to whether genotypic diversity exists within OBs and whether this diversity is maintained by selection for genotypically heterogeneous OBs, or selection against genotypic discrimination during the occlusion process, is at present unclear.

To our knowledge, the possibility of co-occlusion of multiple genotypes into a single OB has not been examined in detail. Using a mixture of wild-type and non-occluded mutant genotypes, **Hamblin et al. (1990)** provided anecdotal evidence of a physical association between genotypes although a formal statistical framework was lacking. Recently, **Zwart et al. (2009)** applied an independent action hypothesis to test whether a single pathogen individual can lead to host death. For this, a model was developed to predict the frequency of single and dual genotypes in mixed infections with the use of recombinant genotypes. Differences in host susceptibility resulted, in some cases, in higher than expected frequencies of dual genotype infections compared with model-generated predictions. These studies have highlighted that independence of action is unlikely to be a broadly applicable paradigm in baculovirus ecology.

Without doubt, co-occlusion of genotypically distinct virions within each OB is one mechanism that can improve the likelihood that multiple virus genotypes are involved in primary infection of the host midgut cells. An alternative model involves segregation of differing genotypes into distinct OBs, either by selective crystallization of the OB polyhedrin protein around virions according to their genotypic identity, or by physical separation of genotypes in virus factories in different areas of the cell nucleus. The importance of answering this question is clear, both for the validation of the current model of NPV replication and for elucidating a mechanism by which a virus can maintain genetic diversity during inter-host transmission. To test the co-occlusion hypothesis, we analysed the within-host genetic diversity originating from experimental mixtures of SfNIC genotypes as a function of the inoculum dose. Specifically, we examined the hypothesis that the consumption of a single OB frequently results in the transmission of multiple genotypes because the OBs themselves are genotypically heterogeneous. Following the consumption of numerous OBs, multiple genotypes would be transmitted similarly, whether they were physically associated or segregated into distinct OBs (the non-associated model). Finally, we confirmed the co-occlusion hypothesis by showing that a portion of the ODVs themselves are genotypically heterogeneous.

2. MATERIAL AND METHODS

(a) *Insects and viruses*

Larvae of *S. frugiperda* were obtained from a laboratory colony maintained in stable conditions (25 ± 1°C; 75 ± 5% R.H.; 16 h L: 8 h D photoperiod) and reared on a semi-synthetic diet (**Greene et al. 1976**). Three different virus inocula were used in this study. The first of these was a wild-type population of the *S. frugiperda* nucleopolyhedrovirus (SfMNPV) collected in Nicaragua (SfNIC-wt) (**Escribano et al. 1999**). SfNIC-wt is composed of at least nine genotypes, named SfNIC-A to SfNIC-I, of which SfNIC-B is the only complete genotype. SfNIC-C presents a 16.4 kb deletion with respect to SfNIC-B and cannot be transmitted *per os* unless its OBs were produced in a cell co-infected with a *per os* infective genotype. SfNIC-wt OBs were produced by inoculating fourth instar larvae using the droplet feeding method (**Hughes & Wood 1981**). The other virus inocula used in the study comprised experimental mixtures of SfNIC-B and SfNIC-C genotypes and an artificially constructed SfNIC population (SfNIC-lab), described below. OB concentrations were determined by counting using an improved Neubauer haemocytometer (Hawksley, Lancing, UK) under phase-contrast microscopy. Viral DNA extractions were performed as described by **Simón et al. (2008)**. PCR analyses were performed to verify the identity and composition of each inoculum. For cell culture studies, the Sf-9 ATCC cell line was cultured at 28°C

in TC-100 medium supplemented with 10 per cent foetal bovine serum (FBS, Gibco).

(b) *Construction of experimental genotype mixtures*

Purified OBs of the SfNIC-B and SfNIC-C genotypes were counted and adjusted to a concentration of 5×10^8 OBs ml^{-1}. ODVs were released by treating each OB suspension with an equal volume of 0.1 M Na_2CO_3 and five volumes of sterile distilled water followed by incubation at 60°C for 10 min. Undissolved OBs were pelleted at 2655*g* for 5 min and discarded. The ODV-containing supernatants were then mixed in two different proportions (50% SfNIC-B + 50% SfNIC-C or 75% SfNIC-B + 25% SfNIC-C) and used to inject (8 µl per larva) groups of 50 *S. frugiperda* fourth instars. Injected larvae were reared individually until death. The OBs obtained from these larvae, named [50B + 50C] and [75B + 25C], respectively, were purified and used in inoculation experiments (described below).

An artificial 'wild-type' population was produced by mixing purified OBs of [75B + 25C] with the OBs of the other six genotypes in proportions similar to those found in the natural SfNIC isolate (Simón *et al.* 2004). The overall composition of the mixture, in terms of percentages of OBs of each genotype, was 80 per cent [75B + 25C], 0.5 per cent SfNIC-A, 3 per cent SfNIC-E, 9 per cent SfNIC-F, 0.6 per cent SfNIC-G, 0.2 per cent SfNIC-H and 5 per cent SfNIC-I. SfNIC-D was not included in the mixture as this genotype encompasses the same deletion as SfNIC-C. This OB mixture was fed to fourth instars using the droplet feeding method and the resulting OBs were named SfNIC-lab.

(c) *Quantification and discrimination of genotypes*

The relative proportions of each genotype in the [50B + 50C], SfNIC-wt and SfNIC-lab inocula were estimated by measuring the relative intensity of a genotype-specific fragment amplified by PCR with respect to a PCR fragment common to all genotypes. This methodology uses 15 different primers to identify SfNIC individual genotypes (see table 1 in Simón *et al.* 2008). Two of these primers (Sfgp41.1 and Sfgp41.2) were used to amplify a fragment within a genomic region common to all genotypes, and these were mixed with genotype-specific primers. Consequently, each reaction mixture generated two different sized amplicons; a larger product of *ca* 760 bp common to all genotypes (the reference amplicon), and a specific product with a length of *ca* 650 bp.

ODVs released from SfNIC-lab and SfNIC-wt were subjected to a varying number of PCR cycles (15–30), depending on the relative proportion of each genotype in the wild-type population (Simón *et al.* 2008). Each reaction was stopped before reaching saturation. PCR products were separated by 1 per cent agarose gel electrophoresis, and the relative proportions of the two products were compared by densitometric analysis using the QUANTITYONE image analysis program (BioRad, Alcobendas, Spain).

(d) *Transmission assays to determine occlusion body genotypic structure*

For transmission assays with [50B + 50C] OBs, one group of 300 and three groups of 50 *S. frugiperda* second instars were orally inoculated with a mixture of purified OB suspension and sucrose solution (10% sucrose; 0.1% Fluorella blue) during a 10 min period at concentrations of 9.0×10^2, 8.3×10^3, 2.5×10^4 and 7.5×10^4 OBs ml^{-1}, respectively. These concentrations were estimated to result in mortalities of 5, 30, 40 and 50 per cent, respectively. For assays involving SfNIC-wt OBs, groups of second instars were orally inoculated with one of two different concentrations: 9.0×10^2 OBs ml^{-1} ($n = 500$ insects) representing the 5 per cent lethal concentration (LC_5), or 7.1×10^5 OBs ml^{-1} ($n = 50$ insects) representing an LC_{50} concentration. For assays involving SfNIC-lab OBs, a group of 50 second instars were inoculated with 7.1×10^5 OBs ml^{-1}, representing an LC_{50} concentration. In all assays, inoculated larvae were maintained individually on a semisynthetic diet until death. Control larvae were treated identically with solutions not containing virus. OB samples from insects that died of NPV infection were collected for semiquantitative PCR analysis of genotypic composition as described above.

Ingested volume data were used to convert LC values into the corresponding lethal dose (LD) values. To determine the mean volume ingested by *S. frugiperda* larvae during transmission assays, the radioactivity of larvae that drank a ^{32}P-labelled sucrose solution (10% sucrose; 0.1% Fluorella blue) during a 10 min period was measured using a scintillation counter (Wallac 1450 MicroBeta Trilux, Wallac Oy, Finland). Scintillation counts were then correlated with the ingested volume by comparison with a volume-scintillation count calibration curve. A total of 60 larvae were individually assayed and the mean volume value was determined.

(e) *Determination of occlusion-derived virion genotypic composition*

To determine the genotypic composition of ODVs, a suspension of [50B + 50C] OBs (1.0×10^8 OB ml^{-1}) was treated with Na_2CO_3 to release ODVs and the resulting suspension was centrifuged to sediment undissolved OBs as described above. Sf9 cells were inoculated with one of five serially diluted concentrations of ODV suspension (10^{-1}–10^{-5}). For this, a 2 ml volume of cells (5×10^5cells ml^{-1}) was transferred to the wells of six-well tissue culture plates and cells were allowed to attach for 1 h. The medium was removed and 200 µl of each ODV dilution was added to each well in triplicate. The plates were incubated at room temperature for 1 h on an orbital shaker at 20*g* to disperse ODVs. Following incubation, ODV inoculum was removed and 2 ml of sterile 2 per cent SeaPlaque agarose (Rockland, USA) mixed with TC-100 (10% FBS) at 37°C was added to the plates, which were then incubated at 28°C for 5–6 days. Formed plaques were identified and only wells that contained 30 or fewer plaques were subjected to genotypic analysis. Between 69 and 81 plaques were subjected to genotypic analysis in each repetition. The procedure was repeated for SfNIC-wt OBs, using the same OB concentration and ODV dilution steps. Between 12 and 21 SfNIC-wt plaques were subjected to genotypic analysis in each repetition. The entire experiment was performed three times.

The identity of each genotype in the [50B + 50C] or SfNIC-wt ODVs was determined by measuring the presence of a genotype-specific fragment amplified by PCR. Thirteen different primers described by Simón *et al.* (2008) were employed to amplify a product with a length of approximately 650 bp that was specific to each genotype. Genotypes C and D are nearly identical and could not be differentiated using the primers sets used here, and were therefore considered as a single genotype (C). BVs obtained from individual cell culture plaques were subjected to 30 amplification cycles. PCR products were separated by

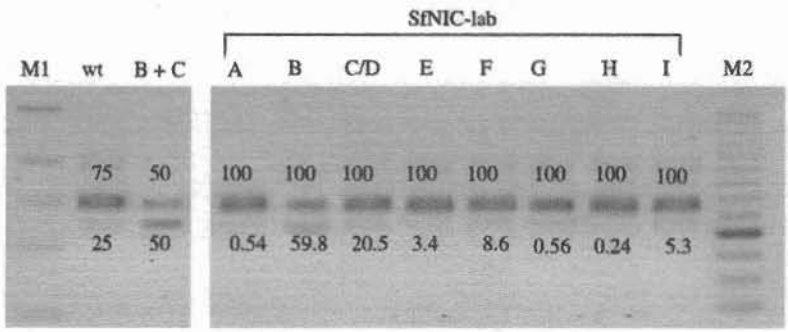

Figure 1. Semiquantitative PCR of the inocula prepared for the bioassays ((wt) is SfNIC-wt, B + C is 50B + 50C and SfNIC-lab). Amplification of SfNIC-wt DNA extracted from OBs confirmed the proportion of genotypes with *per os* transmission factor genes (75%) and those lacking these genes (25%) that survive in the natural population by complementation with complete genotypes in co-infected cells. Amplification of 50B + 50C DNA from OBs confirmed the presence of each genotype in the expected proportion. The SfNIC-lab inoculum was amplified with all pairs of primers to calculate the relative proportions of genotypes present. Letters A–I represent their corresponding genotypes (SfNIC-A–SfNIC-I). Relative density values are shown above or below the corresponding amplicon. Reactions were performed in triplicate and pooled prior to densitometric analysis. Molecular markers were M1 1 kb DNA ladder (Stratgene, La Jolla, CA, USA) and M2 was 100 bp DNA ladder (Invitrogen Corp., Carlsbad, CA, USA).

1 per cent agarose gel electrophoresis and the presence of genotype-specific products was determined using the QUANTITYONE image analysis program (BioRad).

(f) *Statistical analysis*

Results were analysed under the assumption that larval consumption of OBs followed a Poisson distribution under which OBs are ingested at random and do not adhere to one another. Under a model in which each OB contains a single genotype (the non-association hypothesis), the probability of a genotype occurring in a given larva will be the product of the probability of a larva being infected and the frequency of that genotype in the population. Multiple infections will be dependent on the ingestion of more than one OB. By contrast, if OBs contain multiple genotypes (the co-occlusion hypothesis), multiple infection may occur even in the case of ingestion of a single OB. Differences in the within-host diversity under these two hypotheses will be most evident at low doses.

The probability that an insect consumes no OBs is given by the zero term of the Poisson distribution ($P_{(0)}$) based on the proportion of insects that did not become infected at a particular dose: $P_{(0)} = e^{-\lambda}$, where λ is the mean number of ingested OBs given by $-\log_e$(proportion of surviving insects that consumed each inoculum). Similarly, the probability of consuming one OB is $P_{(1)} = \lambda \cdot e^{-\lambda}$ and the probability of consuming more than one OB is $P_{(>1)} = 1 - (P_{(0)} + P_{(1)})$ or $1 - e^{-\lambda}(1 + \lambda)$.

For experiments with only two genotypes, the relative proportions of the genotypes predicted by the non-associated model based on the Poisson generated values were compared with the observed frequency of genotypes in individual insects. For the analysis of the multiple genotype infections in insects that consumed SfNIC-wt OBs, we used a similar argument to determine the probability of a genotype being absent. In both cases expected relative frequencies were compared to the observed frequencies by a log-likelihood ratio test (G-test). For large sample sizes (300–500), the Poisson distribution provides a good approximation for rare events, although small expected values (less than 5) are prone to inflate the significance of log-likelihood tests and the precision of p-values should therefore be viewed with caution. Consequently, G-test results were confirmed by an exact binomial test in which n was the number of insects that died of infection, k was the number of insects with multiple infections and p was the probability of multiple infections in any particular insect, calculated from the expected number of multiple infections under the non-association hypothesis divided by n. The one-tailed exact binomial probability (P) was then calculated by the application of the binomial formula: $P = (n!p^k(1-p)^{n-k})/k!(n-k)!$.

3. RESULTS

The composition of OBs comprising SfNIC-wt, [50B + 50C] and the artificial wild-type mixture SfNIC-lab was verified by densitometric analysis of PCR products (figure 1). The LC_{80} values of SfNIC-wt and [50B + 50C] OBs were similar at 7.1×10^5 OBs ml^{-1}. The LC_5 values of SfNIC-wt and [50B + 50C] OBs were also similar at 9.0×10^2 OBs ml^{-1}. The mean ($\pm$s.d.) volume ingested by *S. frugiperda* second instars was 0.051 ± 0.003 µl. The ingested volume value was used to convert LC values into LD values, which ranged from the LD_5 value of SfNIC-wt and [50B + 50C] OBs estimated at 0.046 OBs/larva to the LD_{80} value that was estimated at 36.2 OBs/larva. None of the control insects died from virus disease in any experiment. Assuming that at least one OB is required to kill a larva, an LD_5 of 0.046 OBs/larva implies that the few larvae that died of the polyhedrosis disease consumed an average of only one OB (table 1), suggesting that OBs are transmitted as physically independent particles that do not adhere to one another. This has been confirmed by scanning electron microscopy of the OB preparations (data not shown). The mean number of OBs required to kill approximately 30 per cent of insects (LD_{30}) was 1.51 OBs per killed larva, increasing to 2.90 OBs per killed larva at the LD_{40}. For higher doses, the number of OB increased greatly (table 1).

[50B + 50C] OBs administered at the calculated 5, 30, 40 and 50 per cent LDs resulted in 2.7, 28, 44 and

Table 1. Estimated consumption of OBs and observed mortality of insects that ingested different concentrations of inoculum.

LC of inoculum (%)	OBs per µl in inoculum	mean number OBs consumed/larva[a]	observed mortality (%)	OBs consumed/infected larva[b]
5	0.9	0.046	2.7–5.4[c]	1.7–0.85[c]
30	8.3	0.42	28	1.51
40	25	1.28	44	2.90
50	75	3.82	54	7.08
80	710	36.20	82	44.2

[a]The mean number OBs consumed/larva was calculated assuming an ingested volume of 0.051 µl in second instars.
[b]OBs consumed/infected larva was calculated as OBs consumed per larva/proportion of infected insects.
[c]Mortality and estimated number OBs consumed/infected larva varied with type of inoculum ([50B + 50C]; SfNIC-wt). To become infected, a larva must consume a minimum of one OB.

54 per cent mortality of inoculated insects, respectively. At the LD_5 dose, eight larvae died, of which four presented a multiple infection (presence of both SfNIC-B and SfNIC-C genotypes), a number significantly higher than that expected if each OB contains a single genotype (table 2, [50B + 50C] OBs). The other four dead larvae were infected by a single genotype, three of them by SfNIC-B and one by SfNIC-C. The OBs obtained from these eight infected cadavers were used for subsequent inoculation of groups of fourth instars by *per os* administration of OBs and intrahaemocelic injection of alkali-released ODVs. All eight ODV inocula were able to kill larvae, but only seven OB inocula were able to kill larvae *per os*. The OBs in which only the SfNIC-C genotype was detected did not kill any of the larvae inoculated *per os*, confirming that no other virus genotype was present (data not shown).

Inoculation with [50B + 50C] OBs at the LD_{30} dose (0.41 OBs/larva) resulted in the death of 14 larvae: seven insects presented a multiple infection (genotypes SfNIC-B and SfNIC-C) and seven presented a single infection involving either SfNIC-B or SfNIC-C, which also differed significantly from the independent transmission expected value (table 2, [50B + 50C] OBs). By contrast, inoculation with the LD_{40} (1.28 OBs/larva) or LD_{50} (3.83 OBs/larva) doses both resulted in frequencies of single and multiple infections similar to that generated by a non-associated transmission model.

SfNIC-wt OBs administered at the estimated LD_5 and LD_{80} resulted in 5.4 and 82 per cent mortality, respectively (table 2, SfNIC-wt OBs). Of the 27 insects that died following the consumption of an LD_5 dose, in 18 a single genotype was present and in nine, more than one genotype was present. The prevalence of multiple infections was significantly greater than expected if OBs comprised a single genotype. In addition, of the nine insects in which more than one genotype was found, seven of them presented at least three and up to seven distinct genotypes (table 3). More than one genotype was present in all 41 insects that succumbed to polyhedrosis disease following inoculation with the LD_{80} dose. Genotypic analysis of these 41 cadavers revealed that 27 insects contained all the genotypes present in the population (table 3), whereas at least one of the three least frequent genotypes (SfNIC-A, SfNIC-G or SfNIC-H) was not detected in 14 insects (17%), and in one insect two infrequent genotypes were absent (SfNIC-A and SfNIC-G).

In larvae inoculated with SfNIC-lab OBs at the LD_{80} dose, 36 out of 50 insects (72%) died of virus disease; all virus-killed larvae contained both SfNIC-B and SfNIC-C, which were the only genotypes for which the OBs had been produced in insects by injection of ODVs. Additional genotypes (SfNIC-E, SfNIC-F or SfNIC-I) were detected in only five insects (table 3). These results were in stark contrast to those observed following the ingestion of SfNIC-wt OBs.

Cell culture plaque purification revealed that single genotype ODVs were common but mixed genotype ODVs were also present at a frequency of over 30 per cent. Of a total of 225 plaques obtained from [50B + 50C] ODVs, 60 comprised genotype B, 81 comprised genotype C and 84 comprised a mixture of both genotypes (table 4). Similarly, analysis of plaques derived from SfNIC-wt ODVs revealed that 33 plaques comprised single genotypes (predominantly genotype C), 17 plaques comprised mixtures of two genotypes (predominantly genotypes B + C) and a single plaque comprised genotypes B + C + F. The proportion of mixed genotype plaques was similar between [50B + 50C] (84 out of 225) and SfNIC-wt (17 out of 50) ODVs (*G*-test $\chi^2_1 = 0.194$, $p = 0.659$). The presence of multiple genotypes in ODV-derived plaques provides explicit evidence both for co-occlusion of genotypes in NPV OBs and for the co-envelopment of genotypically distinct nucleocapsids in approximately one-third of the ODV population.

4. DISCUSSION

The goal of the present study was to examine the hypothesis of the physical association of multiple genotypes in a single OB for the maintenance of virus diversity. Previous studies have demonstrated that during the systemic phase of infection, single cells in an insect can be co-infected by multiple BV particles, each carrying a single genotype (**Godfray *et al.* 1997**; **Bull *et al.* 2001**). To maintain genetic diversity during between-host transmission, it is necessary that the larva acquire different genotypes at the moment of primary infection. As the number of OBs required to kill a larva is usually low, the occurrence of multiple genotype infection would be improbable for highly susceptible larvae that can be infected by a single or very few OBs. Under these conditions, maintaining diversity within a larva could be compromised if genotypes are segregated among different OBs, except perhaps during the course of an epizootic, when unusually high densities of OBs may be present briefly in the environment.

If OBs are composed of single genotypes, the genotypic diversity in the larva would be dependent on the number of OBs that contributed to the primary infection in the host midgut. Accordingly, for low doses, it can be expected that multiple infections will become rare events. Conversely, the occlusion of numerous genotypically distinct virions in a single structure could increase

Table 2. Frequencies of single and multiple infections in insects inoculated with differing doses of I. [50B + 50C] OBs or II. SfNIC-wt OBs. (Observed frequencies were compared with the non-associated transmission model expected values by the *G*-test and the exact binomial test. $^{**}P < 0.01$, $^{***}P < 0.001$.)

	number of insects		observed (expected) frequencies of infection			
inoculum and dose	inoculated	dead	single	multiple	*G*-test (χ^2)[a]	binomial test, *P*
[50B + 50C] OBs						
LD_5	300	8	4 (7.89)	4 (0.11)	23.31***	2.4×10^{-6}
LD_{30}	50	14	7 (11.83)	7 (2.17)	9.05**	0.003
LD_{40}	50	22	13 (16.23)	9 (5.77)	2.23	0.143
LD_{50}	50	27	18 (17.86)	9 (9.14)	0.003	1.000
SfNIC-wt OBs						
LD_5	500	27	18 (26.26)	9 (0.74)	31.37**	6.6×10^{-43}
LD_{80}	50	41	0 (15.43)	41 (25.57)	38.72***	7.4×10^{-9}

[a]*G*-test, d.f. = 1.

Table 3. Frequency and composition of single and mixed genotype infections identified in insects that died following inoculation with SfNIC-wt or SfNIC-lab OBs at LD_5 and LD_{80} doses. (Detection of genotypes was performed by PCR on OBs obtained from virus-killed larvae.)

			single infections		multiple infections	
virus	dose	no. dead/treated insects	genotype	frequency	genotype	frequency
SfNIC-wt	LD_5	27/500	B	10	B, E	1
			C	2	F, I	1
			E	2	B, C, I	1
			F	2	A, C, F	1
			I	2	C, E, F	1
					B, H, I	1
					B, E, G, I	1
					A, B, E, F	1
					B, C, F, H, I	1
SfNIC-wt	LD_{80}	41/50	—	—	all (A–I)	27
					B, C, E, F, G, H, I	7
					B, C, E, F, H, I	1
					A, B, C, E, F, H, I	3
					A, B, C, E, F, G, I	3
SfNIC-lab	LD_{80}	36/50	—	—	B, C	31
					B, C, E	2
					B, C, F	2
					B, C, I	1

the probability that various genotypes establish infection when few OBs are ingested. Multiple infections will not be rare even at low doses.

We tested this hypothesis assuming the probability of infection to be similar for each OB, independent of the genotype(s) it contains, and that the probability of infection can be approximated by a Poisson distribution for rare events (low doses). The first ($P_{(0)}$) term of the Poison distribution, based on the proportion of insects that did not become infected, was used to calculate the probabilities of ingestion of single or multiple OBs. The fitting of a Poisson distribution to the experimental data was tested (table 2), confirming the validity of the co-occlusion model at low doses. In addition, consumption of a single OB appears to be sufficient to kill a larva, indicating that the virus is well adapted to transmission in low virus density conditions. However, it is important to point out that when multiple genotypes are present in the inoculum, ingestion of multiple OBs is not necessarily reflected in genotypic variability in the infected host because a fraction of the insects may have consumed multiple OBs of a single genotype. Nonetheless, this in no way alters the findings of our study, in which higher than expected frequencies of multiple infection were observed in insects that consumed very low numbers of OBs.

An experiment was performed using only two genotypes because it simplified the analysis. We previously demonstrated that *per os* infection with mixtures of SfNIC-B OBs and SfNIC-C OBs resulted in only the SfNIC-B genotype in the virus progeny, whereas *per os* infection with OBs produced in insects injected with ODV mixtures comprising both genotypes were infectious *per os* and resulted in the presence of both genotypes in the progeny OBs (López-Ferber *et al.* 2003). This rescue experiment confirmed that cells had been infected

Table 4. Genotypic composition of ODV plaques derived from I. [50B + 50C] or II. SfNIC-wt ODVs as determined by PCR amplification using genotype-specific primers.

	frequencies			
	Rep. 1	Rep. 2	Rep. 3	total
[50B + 50C] ODVs				
genotype B alone	22	21	17	60
genotype C alone	26	29	26	81
B + C mixture	27	31	26	84
total	75	81	69	225
SfNIC-wt ODVs				
single genotype[a]	8	11	14	33
mixed genotype[b]	4	6	7	17
total	12	17	21	50

[a]Single genotype plaques comprised genotypes A ($n = 1$), B ($n = 9$), C ($n = 19$), F ($n = 1$) and I ($n = 3$).
[b]Mixed genotype plaques comprised B + C ($n = 11$), B + E ($n = 2$), B + F ($n = 1$), B + G ($n = 1$), C + F ($n = 1$) and B + C + F ($n = 1$).

by multiple genotypes. The presence of SfNIC-C was also observed in infections derived from low dose inoculation. We strongly suspected that this was owing to co-occlusion of both genotypes in each OB. In the present study, low doses of [50B + 50C] OBs resulted in a much higher than expected frequency of multiple infections (50% in the experiment involving an LD_5 dose). With an LD_5 of 0.05 OBs, the probability of a larva ingesting more than one OB is less than 0.001. A similar result occurred in insects inoculated with the LD_{30} dose. However, as expected, increasing the number of OBs ingested by a larva resulted in a reduction in the differences between observed frequencies and expected frequencies based on non-associated transmission.

The diversity present in the natural SfNIC-wt population allowed us to address the question, what is the probability of a given genotype being present in an insect as a function of its frequency in the population and the dose ingested? Again, ingestion of the LD_5 dose resulted in a significantly higher frequency than expected of multiple infections. Moreover, of nine insects with multiple infections, seven presented more than two genotypes. When larvae were inoculated at the LD_{80} dose, 38.3 OBs/larva, all or most genotypes were present in each and every insect tested. Even under these high-dose conditions, the probability of such high diversity is very low when genotypes are segregated into different OBs, indicating again that genotypes tend to be acquired together (table 3).

Finally, we constructed a virus population (SfNIC-lab) comprising OBs of each genotype in approximately the same proportion as that of the SfNIC-wt population. Of these OBs, only genotypes B and C had been produced by injection of ODVs into insects to produce the [75B + 25C] inoculum. Interestingly, only genotypes B and C were present in all infected insects and other genotypes were rarely present. What could be responsible for this difference between the SfNIC-wt and SfNIC-lab populations? It appears that the transmission of the co-occluded genotypes B and C was markedly more efficient than that of any of the other single genotype OBs present in the inoculum, that is, consumption of SfNIC-lab [B + C] + [A] + [E] + [F] + [H] + [I] OBs appears not to be equivalent to the SfNIC-wt population structure [B + C + A + E + F + H + I] OBs, presumably because all genotypes are probably co-occluded in the latter, whereas only B and C were co-occluded in the former.

These results provide clear evidence for the association of genotypes within OBs. Two kinds of association are possible for multiply-enveloped NPVs: virions can be composed of single genotypes, with many genetically distinct virions occluded in the same OB, or the virion envelope can wrap nucleocapsids that contain dissimilar genotypes. Results of the genotypic analysis of individual ODV-derived cell culture plaques revealed that each type of association exists in OBs; approximately one-third of ODVs comprise mixed genotypes and two-thirds of ODVs comprise single genotypes. Crucially, however, the observation of mixed genotype virions provides conclusive support for the co-occlusion hypothesis.

In this respect, the lower genotypic diversity detected in the granuloviruses (**Jehle *et al.* 2003**; **Eberle *et al.* 2009**), members of the genus *Betabaculovirus*, may be related to the morphological structure of the members of this genus, in which each OB occludes a single virus genome, thereby eliminating the possibility of physical association of different genotypes.

Genetic diversity is common in NPV populations (**Graham *et al.* 2004**; **Cory *et al.* 2005**; **Erlandson *et al.* 2006**; **Ogembo *et al.* 2007**), and provides clear advantages for virus transmission under varying ecological conditions (Hodgson *et al.* **2001**, **2004**; **Cooper *et al.* 2003**; Simón *et al.* **2004**, **2006**; **Hitchman *et al.* 2007**). It seems reasonable to accept that a system of multiple occlusion is adaptive in facilitating continuous transmission of high variability of the virus population when the pathogen density is low, leading to an improved likelihood of virus survival. However, this strategy also allows for the survival of defective genotypes that could reduce the fitness of complete genotypes in the population, although in the SfNIC population this is not the case, as defective genotypes actually potentiate virus transmissibility.

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