

## Phenotypic variation between naturally co-existing genotypes of a Lepidopteran baculovirus

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### ABSTRACT

There is increasing evidence that populations of microparasites vary genetically at a variety of spatial scales, including within host individuals. Understanding this fine-scale structuring of microparasite populations requires descriptions of the relative fitness of individual genotypes isolated from natural mixed infections. Here we examine whether differences between virus genotypes isolated from a single host individual translate into phenotypic differences, and discuss the processes by which such variation might be maintained. Twenty-five genotypic variants of a nucleopolyhedrovirus (Baculoviridae) have been identified and purified from a single pine beauty moth (*Panolis flammea*) larva. The phenotypes of four genotypes were compared. Genotypes differed in three phenotypic traits, each predicted to be an important component of fitness: pathogenicity, speed of kill and yield. Variation in pathogenicity was described by seven-fold differences in LD<sub>50</sub> and by differences in the slopes of the fitted dose-response curves. Mean speed of kill of the genotypes differed by up to 36 h. Two genotypes produced 65% higher yields, over and above any differences predicted by a significant intra-genotypic relationship between yield and speed of kill. Inter-genotypic trade-offs between virus phenotypic traits, which could promote the co-existence of genotypes, were not found. Mechanisms that may promote the co-existence of competing virus genotypes are discussed.

*Keywords:* genotype, mixed infection, nucleopolyhedrovirus, *Panolis flammea*, phenotype, trade-off.

### INTRODUCTION

The development of molecular techniques for distinguishing between microparasite strains has shown that microparasite populations can be surprisingly diverse (Lord *et al.*, 1999). However, we know little about the source and maintenance of this genotypic diversity, and how it changes in time and space. A bias towards theoretical explorations of interactions between co-infecting pathogen genotypes (Ewald, 1995; Taylor *et al.*, 1997) is starting to be redressed by descriptions of mixed-genotype infections in populations of diverse micro-parasites and pathogens, including rodent malaria parasites (Taylor *et al.*, 1997, 1998), rat

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nematodes (Fisher and Viney, 1998) and trematodes (Barral *et al.*, 1996), trypanosomes in bumblebees (Wu, 1994; Imhoof and Schmid-Hempel, 1998) and tsetse flies (MacLeod *et al.*, 1999), and baculoviruses in Lepidoptera (Muñoz *et al.*, 1998). Mixed-genotype infections are also known in plant pathogen systems (e.g. Ennos, 1991; Meijer and Leuchtman, 1999) and in human diseases (Gupta and Anderson, 1999) such as malaria (Paul *et al.*, 1999), hepatitis B (Santos *et al.*, 1998) and C (Tuveri *et al.*, 1997), and human papillomavirus (Tucker *et al.*, 1993). Further empirical studies of similar host-pathogen systems will help reveal patterns of diversity and co-existence, test the predictions of host-pathogen models and elucidate the mechanisms that promote within-host genotypic diversity in natural populations.

An important step in the analysis of genotypic variation is to determine whether it is phenotypically neutral, or whether different genotypes have distinct phenotypic characteristics. It is phenotypic differences between genotypes that cause differences in fitness and promote selection for the genotype with highest fitness. Phenotypic differences between parasite isolates (by 'isolate' we refer to a parasite sample collected from a single or small group of hosts in the same location and at the same time; Cory *et al.*, 1997) have been described in several systems [e.g. *Daphnia* (Ebert, 1994), bovine tropical theleriosis (Miled *et al.*, 1994), baculoviruses (see below)]. Within-isolate phenotypic variation has also been described [e.g. *Drosophila* C virus (Gravot *et al.*, 2000) and baculoviruses (see below)], but it is often unclear whether distinct parasite genotypes were isolated from a single host individual in these studies. The individual host represents a distinct patch of resource and is, therefore, a critical scale at which to study parasite interactions. Qualitative phenotypic differences between co-infecting parasite genotypes have been described in vertebrate systems, exemplified by antigenic differences between strains of malaria (Gupta *et al.*, 1994) and bovine tropical theleriosis (Miled *et al.*, 1994). To our knowledge, only one study of the parasites of animal hosts has described variation in quantitative phenotypic traits between parasite genotypes isolated from a single host individual (Imhoof and Schmid-Hempel, 1998). In this case, bumblebee trypanosome genotypes were cloned and chosen for co-infection experiments on the basis of 'fast' and 'slow' rates of proliferation *in vitro*. No consistent phenotypic differences were observed between genotypes *in vivo*.

Baculoviruses are arthropod DNA viruses that primarily infect insects, particularly the Lepidoptera (Miller, 1997). Vertical transmission has been noted in these viruses (Kukan, 1999), but the most common mode of transmission is horizontal, after the death of the infected host. Baculoviruses provide a useful model system for the study of pathogen variability because their hosts are often abundant or easy to rear in the laboratory. Baculoviruses persist outside the host, embedded within occlusion bodies, allowing easy storage and manipulation of virus stocks. Baseline descriptions of the phenotypes of many baculoviruses have been made due to their importance as biopesticides (Fuxa, 1991; Moscardi, 1999). Genotypic variation in baculovirus populations has been detected at several ecological scales, from between geographical regions (e.g. Gettig and McCarthy, 1982; Cherry and Summers, 1985; Shapiro *et al.*, 1991; Vickers *et al.*, 1991; Laitinen *et al.*, 1996) to within virus isolates (e.g. Lee and Miller, 1978; Knell and Summers, 1981; Smith and Crook, 1988; Maeda *et al.*, 1990; Muñoz *et al.*, 1999). Differences in pathogenicity have also been demonstrated between geographical isolates (Allaway and Payne, 1983; Hatfield and Entwistle, 1988; Ebling and Kaupp, 1995) and between genotypes derived from the same isolate by *in vitro* techniques (Lynn *et al.*, 1993; Stiles and Himmerich, 1998). Only very recently have baculovirus genotypes derived from a single isolate, using *in vivo* techniques,

been compared (Paul, 1997; Muñoz *et al.*, 1999). Despite this, the evolutionary and ecological processes leading to the existence and persistence of genotypic variation in baculovirus populations have received little attention (Cory *et al.*, 1997).

The pine beauty moth, *Panolis flammea* (Lepidoptera: Noctuidae), is a native herbivore of Scots pine (*Pinus sylvestris*) in Scotland and central Europe, but has become an economically important pest in lodgepole pine (*Pinus contorta*) plantations in the UK (Evans *et al.*, 1991). Larvae, killed by *P. flammea* nucleopolyhedrovirus (*Pf*NPV), were collected during an outbreak of this pest in 1976. Molecular characterization of the virus revealed a high level of genotypic variation. Twenty-five distinct virus genotypes were isolated from a single cadaver by *in vivo* cloning (J.S. Cory and B.M. Green, unpublished data; Paul, 1997). The isolation of pure virus genotypes allowed the examination and comparison of viral phenotypes found in natural mixed infections.

Three commonly studied nucleopolyhedrovirus life-history traits are pathogenicity, speed of kill and yield. These parameters can be estimated using laboratory experiments, and each is predicted to be an important component of virus fitness. Pathogenicity is here defined as the capacity of the nucleopolyhedrovirus to kill the host and is traditionally described by the LD<sub>50</sub>, the dose that kills 50% of the hosts challenged with virus. Speed of kill describes the time elapsing between initial infection and death of the host. Faster speed of kill is assumed to increase virus fitness due to the benefit it confers when the virus competes with other viral genotypes or microparasite species within the host (Frank, 1996). Yield describes the number of occlusion bodies released upon death of the host. Nucleopolyhedroviruses invade a wide variety of host tissues during the infection cycle, and yields may be in excess of 10<sup>9</sup> occlusion bodies in a late instar noctuid larva. Here we describe an experiment in which we wished to ascertain whether genotypic variation translates into phenotypic differences that could impact on host-pathogen dynamics and virus co-existence. We also examined relationships between the three phenotypic traits, using fitted trait values for the four virus genotypes tested.

## METHODS

### Insect stock cultures

Adult *P. flammea* were sourced from five populations in northern Scotland, either by light trapping adults (one population) or by collecting larvae from trees and storing them at ambient temperature as overwintering pupae (four populations). Adults from different populations were mated in separate cages and all resulting eggs were collected. Larvae were reared outdoors on bagged *P. contorta* saplings in May 1999. Third-instar larvae were harvested for the experiment.

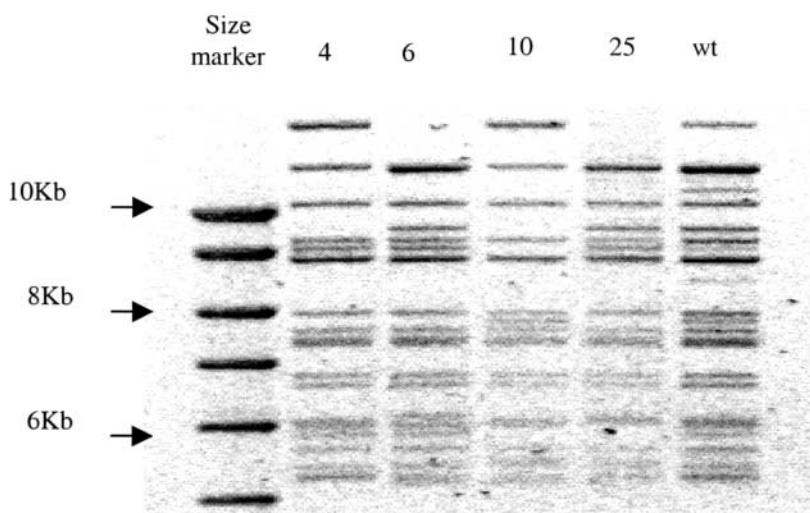
### Virus stock and dilutions

Four pure *Pf*NPV genotypes were selected to be bioassayed in this experiment (genotypes *Pf*4, *Pf*6, *Pf*10 and *Pf*25), on the basis of phenotypic variation during passage through *Mamestra brassicae*, an alternative host species (Paul, 1997). These genotypes showed distinct variation in their DNA profiles when digested with the *Hind*III restriction endonuclease (Fig. 1). Electron microscopy has demonstrated no difference between these genotypes in the size of occlusion bodies (OBs) or the number of virus genomes each

occlusion body contains (Paul, 1997). Each genotype was serially diluted in sterilized water to produce suspensions with concentrations of 1, 1.5, 2, 2.5, 3 and  $3.5 \log_{10}(\text{OBs}) \cdot \mu\text{l}^{-1}$ . This resulted in  $4 \times 6 = 24$  dosing treatments. Thirty larvae were allocated at random to each treatment, except at the lowest doses ( $1$  and  $1.5 \log_{10}(\text{OBs}) \cdot \mu\text{l}^{-1}$ ), in which 50 larvae were used.

#### Dosing and monitoring the larvae

All larvae were weighed before dosing. The larvae were starved for 24 h, then placed individually in the wells of microtitre plates, each containing a small fragment of young, decontaminated, *P. contorta* needle coated with a  $1 \mu\text{l}$  dose of virus solution. Thirty control larvae were fed in an identical fashion, but with no virus inoculum. The larvae were checked three times, at 12 h intervals, and transferred to their individual rearing containers if the dose had been eaten. Time of transfer was noted as the start of the infection cycle. Handling deaths and larvae that had not eaten the inoculum within 36 h were discarded. The larvae were then reared individually and fed *ad libitum* with new shoots of *P. contorta*. The shoots were decontaminated in  $0.1 \text{ mmol} \cdot \text{l}^{-1}$  sodium carbonate for 1 h to remove any resident virus. Frass was regularly removed from the containers. Larvae in containers were stored in random positions in a rearing cabinet at  $16^\circ\text{C}$  with a 16:8 L:D photoperiod. Larvae were checked every 12 h until the wave of viral deaths ended (day 28). Death was recorded when the larvae did not respond to tactile stimulus (using sterile cocktail sticks). Viral death generally coincides with liquefaction of the cadaver; where possible, whole cadavers



**Fig. 1.** Gel electrophoretic descriptions of the genomes of *PfNPV* genotypes *Pf4*, *Pf6*, *Pf10* and *Pf25* after digestion with the DNA restriction enzyme *Hind*III. Lane 1 is a standard DNA size marker. Lane 5 (wt) shows the profile for the mixture of >25 virus genotypes found in the original mixture. The presence and absence of bands in each lane demonstrate the presence or absence of *Hind*III restriction sites on the virus genomes. The 'wt' lane contains bands representing every DNA fragment from the four selected genotypes and unique bands representing other genotypes.

were collected, transferred to Eppendorf tubes and stored at -20°C to measure virus yield.

### Cause of death and yield measurements

Each dead larva was macerated in 1 ml of sterilized water and vortexed to mix. Cause of death was confirmed by viewing a subsample of the macerate at 1000 $\times$  under oil immersion. Three microlitres of each suspension were then diluted 100-fold to allow virus yield counts on a haemocytometer: five counts per haemocytometer and two subsamples per suspension were measured to reduce counting and dilution errors. Only those cadavers that could be transferred whole from the rearing containers were used for yield analysis.

### Statistical analysis

All data were analysed with model simplification and generalized linear models, using GLIM (McCullagh and Nelder, 1989). The statistical models included virus genotype as a categorical variable and initial larval weight and virus log<sub>10</sub> dose as covariates. Further yield analysis included speed of kill as a covariate. Source population was included as a blocking factor in all statistical analyses but was never a significant determinant of virus phenotype. Larval source, therefore, was ignored in all analyses to simplify descriptions of the statistical models. The validity of the error structures and transformations used was checked using standard model diagnostics.

## RESULTS

### Pathogenicity

Larval mortality due to virus was analysed using a binary error structure and logit transformation. All larvae dying of causes other than virus were removed from the dataset. The LD<sub>50</sub> values suggested that genotypes *Pf4* and *Pf6* were more infective than genotypes *Pf10* and *Pf25* (Table 1). The LD<sub>50</sub> of genotype *Pf10* was nearly seven times greater than that of genotype *Pf6*. The LD<sub>50</sub> may, however, be an oversimplified description of the pathogenicity of nucleopolyhedroviruses. Analysis of covariance demonstrated that the relative pathogenicity of virus genotypes changed with varying dose (significant interaction between genotype and log<sub>10</sub> virus dose:  $\chi^2_3 = 8.6$ ,  $P = 0.035$ ) (Fig. 2). *A posteriori* grouping showed that the dose-response regression coefficients for genotypes *Pf4* and *Pf10* were similar, and greater than the shared coefficient of genotypes *Pf6* and *Pf25* ( $\chi^2_2 = 2.2$ , n.s.). Genotype *Pf4*, in particular, was relatively poorly pathogenic at low doses and relatively highly pathogenic at high doses (Fig. 2). None of the control larvae were killed by virus.

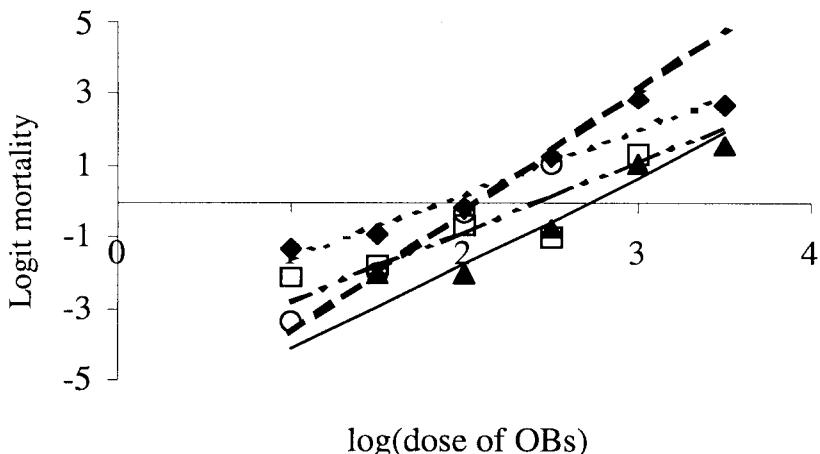
### Speed of kill

Speed of kill data, measured over 12 h intervals, were log-transformed for analysis. Genotypes differed in their speed of kill ( $F_{3,223} = 2.8$ ,  $P = 0.04$ ; Fig. 3). Genotype *Pf4* killed significantly faster than the other three genotypes, which shared a similar speed of

**Table 1.** Virus (*Pf*NPV) doses required to kill 50% of third-instar *Panolis flammea* larvae

Genotype	Virus dose ( $\log_{10}(\text{OBs}) \cdot \mu\text{l}^{-1}$ )		
	LCL	$\text{LD}_{50}$	UCL
<i>Pf</i> 4	1.913	2.088	2.260
<i>Pf</i> 6	1.652	1.893	2.139
<i>Pf</i> 10	2.515	2.726	2.981
<i>Pf</i> 25	2.169	2.409	2.672

Note:  $\text{LD}_{50}$  estimates [with lower (LCL) and upper (UCL) 95% confidence limits] are  $\log_{10}$  doses of virus occlusion bodies.



**Fig. 2.** Dose–mortality curves for four genotypes of *Pf*NPV infecting third-instar *P. flammea* larvae. Curves were fitted via logit-transformation of the binary mortality variable and analysis of covariance. Open circles describe actual mortality and the dashed line fitted mortality due to *Pf*NPV genotype *Pf*4 ( $\text{logit}(\text{mortality}) = -7.18 + 3.44(\log_{10}\text{dose})$ ); solid diamonds and dotted line genotype *Pf*6 ( $\text{logit}(\text{mortality}) = -3.36 + 1.79(\log_{10}\text{dose})$ ); solid triangles and solid lines genotype *Pf*10 ( $\text{logit}(\text{mortality}) = -6.59 + 2.24(\log_{10}\text{dose})$ ); open squares and dot-and-dash line genotype *Pf*25 ( $\text{logit}(\text{mortality}) = -4.70 + 1.95(\log_{10}\text{dose})$ ). Treatments with zero or total mortality could not be represented on the logit scale.

kill ( $F_{2,223} = 0.24$ , n.s.). Larvae were killed more quickly with increasing virus dose ( $F_{1,223} = 32.65$ ,  $P < 0.001$ ; Fig. 3); this is likely to have been due to multiple sites of infection being initiated at higher doses.

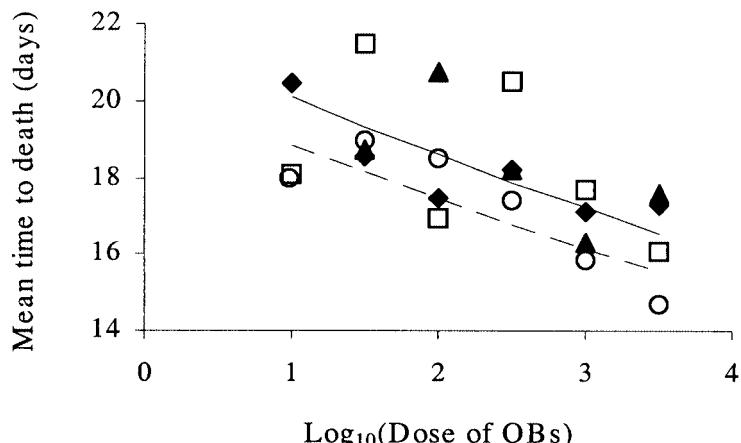
#### Virus yield

Yield data were log-transformed for analysis. Virus yield differed significantly between genotypes ( $F_{3,196} = 5.85$ ,  $P < 0.001$ ). Genotypes *Pf*6 and *Pf*10 had similar yields, as did

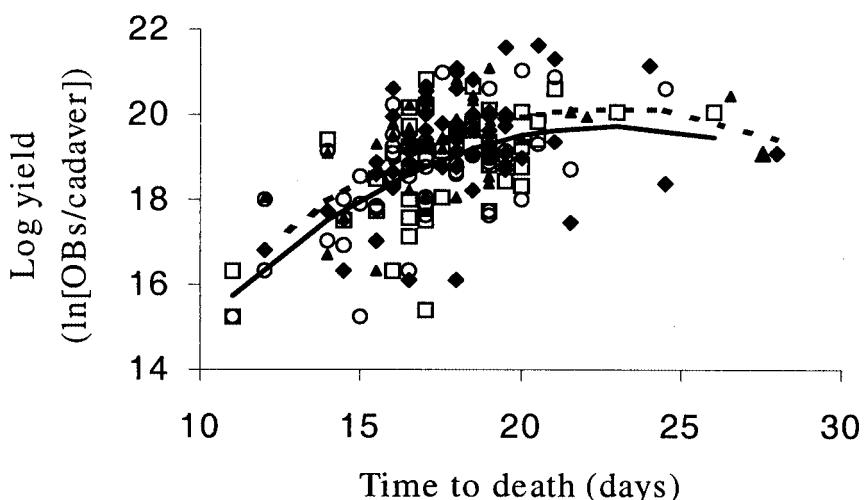
genotypes *Pf4* and *Pf25* ( $F_{2,196} = 0.90$ , n.s.). The former pair produced higher yields than the latter pair. Yield decreased with increasing dose ( $F_{1,196} = 10.45$ ,  $P < 0.001$ ), but increased with increasing initial larval weight ( $F_{1,196} = 16.19$ ,  $P < 0.001$ ). There was no interaction between genotype and  $\log_{10}$  virus dose ( $F_{3,193} = 1.72$ , n.s.). We predicted that the longer a virus takes to kill its host, the larger that host will grow, and therefore the greater the yield of virus. This was examined by introducing speed of kill as a new covariate in the analysis. Speed of kill contributed to the statistical model both as a linear ( $F_{1,195} = 50.74$ ,  $P < 0.001$ ) and a quadratic ( $F_{1,194} = 36.52$ ,  $P < 0.001$ ) covariate. Thus virus yield increased with slower speeds of kill, but this effect levelled off and yield even began to decrease at very slow speeds of kill (Fig. 4). Introducing speed of kill removed the effect of dose on virus yield ( $F_{1,194} = 0.78$ , n.s.) due to correlations between dose and speed of kill. It had no impact, however, on yield differences between genotypes. Genotypes still differed in yield, independently of speed of kill ( $F_{3,195} = 4.76$ ,  $P = 0.003$ ). The groupings of genotypes *Pf6* and *Pf10* versus *Pf4* and *Pf25* still held ( $F_{2,195} = 0.09$ , n.s.; Fig. 4); genotypes *Pf6* and *Pf10* produced yields approximately 65% greater than genotypes *Pf4* and *Pf25*.

#### Trade-offs between viral traits

The description of several viral traits for a group of virus genotypes allowed an analysis of relationships and trade-offs between these traits. Intra-genotypic relationships between dose, speed of kill and virus yield were accounted for by calculating mean speed of kill and mean yield of an infection resulting from the ingestion of a standard dose of 100 occlusion bodies of each virus genotype. Mean speed of kill and yield at this dose were calculated from the analysis of covariance models relating these variables to virus genotype and virus



**Fig. 3.** Dose-speed of kill curves for four genotypes of *Pf*NPV infecting third-instar *P. flammlea* larvae. Curves fitted by reciprocal transformation of time-to-death and analysis of covariance. Open circles describe mean time to death for each dose of genotype *Pf4*; solid diamonds genotype *Pf6*; solid triangles genotype *Pf10*; open squares genotype *Pf25*. Dashed line describes the fitted response curve for genotype *Pf4* (speed of kill =  $\exp\{3.01 - 0.08(\log_{10}\text{dose})\}$ ); solid line the grouping of genotypes *Pf6*, *Pf10* and *Pf25* (speed of kill =  $\exp\{3.08 - 0.08(\log_{10}\text{dose})\}$ ).

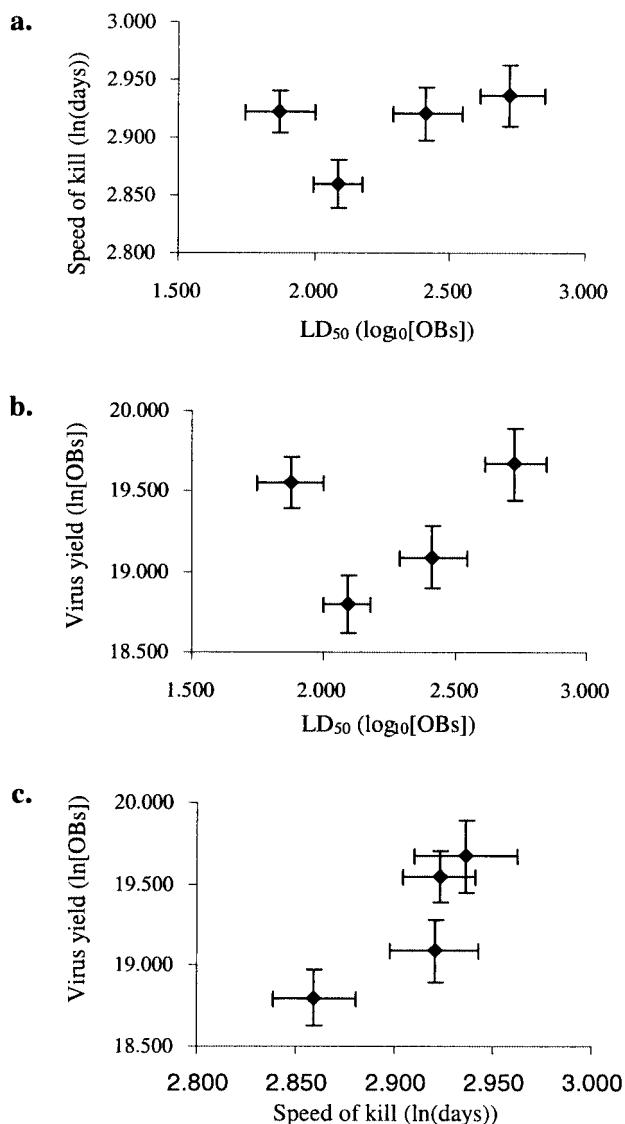


**Fig. 4.** Yield–speed of kill curves for four genotypes of *Pf*NPV infecting third-instar *P. flammea* larvae. Yield is log-transformed to improve presentation and statistical validity. Curves fitted by analysis of covariance. Open circles describe actual yields of genotype *Pf*4 infections; solid diamonds genotype *Pf*6; solid triangles genotype *Pf*10; open squares genotype *Pf*25. Solid line represents the fitted values for genotypes *Pf*4 and *Pf*25 combined ( $\ln(\text{yield}) = 3.71 + 0.18(\text{larval weight}) + 1.29(\text{speed of kill}) - 0.03(\text{speed of kill})^2$ ); dotted line genotypes *Pf*6 and *Pf*10 ( $\ln(\text{yield}) = 4.21 + 0.18(\text{larval weight}) + 1.29(\text{speed of kill}) - 0.03(\text{speed of kill})^2$ ).

dose. Average yield was also based on the average larval weight in the experiment. The LD<sub>50</sub> correlated very poorly with both speed of kill (Pearson's correlation coefficient,  $r = 0.450$ ,  $P = 0.550$ ; Fig. 5a) and virus yield ( $r = 0.287$ ,  $P = 0.713$ ; Fig. 5b). Speed of kill and virus yield, which correlated strongly at the within-genotype scale (Fig. 4), appeared to correlate between genotypes also (Fig. 5c), but this relationship was not significant ( $r = 0.868$ ,  $P = 0.132$ ). It should be noted, however, that with only four data points, statistical power is low, requiring a correlation of at least 0.95 to be significant at  $\alpha = 0.05$ .

## DISCUSSION

The results of this study reveal some subtle, and some striking, differences between the phenotypes of the four virus genotypes. The most striking differences were in measures of pathogenicity, which differed nearly seven-fold. The pathogenicity analysis also highlighted differences in the gradient of the dose–response curve. The mechanisms behind such dose-effects are unknown and deserve further study. Infection is a complex process and may involve the action of several genes, as evidenced by studies of the host range in the *Autographa californica* nucleopolyhedrovirus (Griffiths *et al.*, 1999). Differences in absolute and dose-dependent pathogenicity mean that competition between virus genotypes for transmission to new hosts will be determined by the relative doses of each encountered under natural conditions. After a virus epizootic, where high densities of virus-killed cadavers are likely to be found, the dose ingested by a larva is likely to be high, possibly obscuring differences in pathogenicity between virus genotypes. At low densities of



**Fig. 5.** Relationships between three phenotypic traits of four genotypes of *Pf*NPV. Virus doses, yields and speeds of kill log-transformed to improve presentation. The LD<sub>50</sub> values calculated from mortality analyses. Mean yields and speeds of kill based on predictions from analysis of covariance models, calculated for a standard dose of 100 occlusion bodies (OBs).

host and pathogen, the dose encountered by a foraging host will depend on the spatial distribution and aggregation of the virus but will be lower than during an epizootic. This will favour genotypes with higher pathogenicity at low doses.

Pathogenicity, measured here as host mortality, does not account for the impact on nucleopolyhedrovirus fitness of sublethal or vertically transmitted infections. Vertical

transmission (of overt infection from adult to offspring) is very infrequent in the *Pf*NPV system in the field (< 0.5%; J.S. Cory, unpublished). Sublethal infections of *Pf*NPV have not yet been studied, but have been identified in other insect virus systems (Smith, 1976) and may be important in maintaining the presence of the virus in low-density host populations (Anderson and May, 1981). It is possible that occlusion bodies that fail to infect a host upon ingestion may be transmitted to new hosts via frass or sloughed gut lining, but these occlusion bodies are likely to be less resistant to weathering due to degradation in the alkaline conditions of the host's midgut. Vasconcelos (1996) demonstrated horizontal transmission of nucleopolyhedrovirus (NPV) between *M. brassicae* hosts via frass, but only during late stages of infection, just prior to host death. Although vertical transmission and occult infections may donate little fitness to NPV genotypes in competition with genotypes causing fatal infections, their persistence in NPV populations can only increase the genotypic and phenotypic complexity of the nucleopolyhedrovirus–host interaction.

Differences in speed of kill were less dramatic: genotype *Pf*4 killed significantly faster than the other genotypes, but mean speed of kill differed by less than 36 h between the fastest and slowest killing genotypes. Again, the importance of such a difference will depend on natural conditions. Faster speed of kill may improve a variant's competitive ability during a mixed infection or competition with other natural enemy species, and may allow a greater number of rounds of infection during a single host generation. The observation of two peaks of NPV infection within a larval generation in natural gypsy moth (*Lymantria dispar*) populations (Woods and Elkinton, 1987) provides circumstantial evidence for multiple rounds of infection. Differences in the length of the larval development season may also have an important impact on the relative success of fast- and slow-killing viruses.

Yield analysis demonstrated that genotypes *Pf*6 and *Pf*10 produced 65% more occlusion bodies than genotypes *Pf*4 or *Pf*25, for any given speed of kill. The within-genotype relationship between virus yield and speed of kill showed the value of delaying host death when not competing for host resources, but also provided evidence for diminishing returns with slower speeds of kill. This may have been due to a limit to host growth during the infection cycle, or to 'weak' infections that took a long time to kill the host. Virus yield depended not only on speed of kill, but also on the weight of the larva at the time of infection; together, these variables describe the dimensions of the host resource to be converted into occlusion bodies at the end of the infection cycle.

The existence of phenotypic variation between sympatric and co-infecting virus genotypes, as found in this study, has important implications for the maintenance of genotypic variation in *Pf*NPV populations (and populations of microparasites in general). Selection pressures acting on the virus and host populations will promote the dominance of the most fit virus genotype. Why, then, do we see such extreme genotypic diversity at the level of the individual host? Pathogen diversity in vertebrate systems is thought to be driven by the specificity of the host immune response (Gupta and Anderson, 1999); this is less likely to be true in invertebrate systems where immune responses are less complex. We believe there are three features of nucleopolyhedrovirus–host interactions that may promote genotypic variation, despite phenotypic variation between virus genotypes:

1. *Trade-offs between phenotypic traits.* Virus fitness is determined by a suite of phenotypic traits and interactions with host and environment. Relationships between phenotypic traits that correlate positively with virus fitness may allow different phenotypes to co-exist with equal fitness. No such relationships between pathogenicity, yield and speed of kill

were found in this study, although our sample size (four genotypes) was low. There is increasing evidence for an important trade-off between speed of kill and virus yield in other nucleopolyhedrovirus–host systems, based on the effects of gene insertion (Burden *et al.*, 2000; Hernández-Crespo *et al.*, 2001) or deletion (Wilson *et al.*, 2000) on virus phenotypes. Comparing further *Pf*NPV genotypes may clarify any correlations, in particular between speed of kill and yield. This is a promising approach to the study of trade-offs in pathogen–host systems, especially when the intra-genotypic correlations between phenotypic traits can be accounted for.

2. *Differential selection of virus genotypes.* The co-existence of virus genotypes may be promoted by differential selection of genotypes in different ecological conditions. *Panolis flammea* feeds on two main host plants in the UK (Watt *et al.*, 1991), shows geographical genotypic variation (Wainhouse and Jukes, 1997) and undergoes population outbreaks (Watt *et al.*, 1989). *Pf*NPV is not host-specific (Paul, 1997) and may infect different, sympatric host species. If the fitness of virus genotypes differs according to such varying ecological conditions, the competitive outcome of mixed infections will be altered. Subsequent remixing of differentially selected virus subpopulations – for example, dispersal between host populations by predators (Entwistle *et al.*, 1993; Vasconcelos *et al.*, 1996) or natural movement of virus within the host habitat (D'Amico and Elkinton, 1995; Richards *et al.*, 1999) – could result in high local genotypic variation.

3. *Micro-evolutionary dynamics in mixed infections.* The dynamics of mixed-genotype pathogen infections are poorly understood. Co-infection may result in important changes in the infection cycle that may in turn influence the maintenance of pathogen diversity. There is at least the potential for synergistic effects of co-infection on parasite fitness (Knell, 1996; Taylor *et al.*, 1998). In NPV infections, such a mutualistic interaction may be limited to the infection stage, since co-infecting viruses must eventually compete for limited host resources. There is also the potential for parasitic interactions between virus genotypes. Multiple virus genomes are packaged within each occlusion body. These occlusion bodies are formed in infected cell nuclei; therefore, multiple infection of a nucleus may lead to genotypic variation within occlusion bodies. This may allow parasitic genotypes to be masked within occlusion bodies formed by more fit genotypes (Godfray *et al.*, 1997), similar to the masking of deleterious recessive alleles in diploid population genetics. As an extreme example, virus genotypes producing few or defective occlusion bodies have been identified in cell cultures (Beames and Summers, 1989) and natural mixed-genotype virus isolates (Muñoz *et al.*, 1999). Co-infection may also promote the sharing of genetic information between genotypes, increasing the rates of genotypic change above natural mutation rates. The observed level of genotypic variation may, therefore, be a result of a dynamic process of selection against unfit genotypes and the frequent formation of new ones. Recombination between two or more co-infecting virus genotypes has been described both in cell culture (e.g. Summers *et al.*, 1980; Croizier and Ribeiro, 1992) and in *in vivo* mixed infections (Muñoz *et al.*, 1997).

These processes are not mutually exclusive and each deserves further study. Here we have demonstrated variation in important phenotypic traits between naturally co-infecting pathogen genotypes isolated from a very diverse pathogen population. Our laboratory experiments rear larvae under ideal conditions; more natural conditions may exacerbate the absolute phenotypic differences observed here, or result in differences in relative infection properties according to host condition. Future work in this system will examine the

interactions between genotypes in controlled co-infection experiments. Descriptions of the relative fitness of different virus genotypes within the ecological complexity of the *Pinus-Panolis*-nucleopolyhedrovirus system will also help to explain the existence and persistence of genotypic variation in this and other host-pathogen and host-parasite systems.

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