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Timing of transmission and the evolution of virulence of an insect virus

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We used the nuclear polyhedrosis virus of the gypsy moth, *Lymantria dispar*, to investigate whether the timing of transmission influences the evolution of virulence. In theory, early transmission should favour rapid replication and increase virulence, while late transmission should favour slower replication and reduce virulence. We tested this prediction by subjecting one set of 10 virus lineages to early transmission (Early viruses) and another set to late transmission (Late viruses). Each lineage of virus underwent nine cycles of transmission. Virulence assays on these lineages indicated that viruses transmitted early were significantly more lethal than those transmitted late. Increased exploitation of the host appears to come at a cost, however. While Early viruses initially produced more progeny, Late viruses were ultimately more productive over the entire duration of the infection. These results illustrate fitness trade-offs associated with the evolution of virulence and indicate that milder viruses can obtain a numerical advantage when mild and harmful strains tend to infect separate hosts.

Keywords: virulence; timing; transmission; trade-off; *Lymantria dispar*; nuclear polyhedrosis virus

1. INTRODUCTION

Over most of the past century, literature on the evolution of virulence has generally presumed that parasites evolve towards benign coexistence with their hosts. Theoretical analyses indicate, on the contrary, that parasites may evolve to intermediate or even high levels of virulence (e.g. Fine 1975; Levin & Pimentel 1981; May & Anderson 1983; Bremermann & Pickering 1983; Ewald 1983; Bonhoeffer & Nowak 1994; Lipsitch *et al.* 1995), but relatively few of the proposed influences on virulence have been tested experimentally. Current theory implies that the evolution of virulence will depend on the trade-off between positive effects of host exploitation on competition among pathogens within hosts and negative effects of this exploitation on transmission between hosts. Although several comparative tests are consistent with this trade-off model (Herre 1993; Ebert 1994; Ewald 1994), empirical research has focused mostly on the effects of vertical versus horizontal transmission (Bouma & Lenski 1988; Bull *et al.* 1991; Turner *et al.* 1998; Messenger *et al.* 1999). The theoretical importance of trade-offs inherent to transmission between hosts draws attention to the need for experimental manipulation of each proposed influence (Ewald 1983; Lipsitch *et al.* 1995).

Theory indicates, for example, that high densities of hosts should favour relatively high virulence (Bremermann & Pickering 1983; Bull 1994), but this association could result from any of several hypothetical factors that may co-occur when host density is high. (i) The extinction rate of virulent lineages may decline because 'herd immunity' is less likely to reduce the pool of susceptibles below a critical transmission threshold for parasite maintenance (Fine 1993). (ii) High host density may reduce the dependence on host mobility for transmission and permit greater host exploitation (Ewald 1994; Lenski & May 1994). (iii) Virulent variants may gain a numerical advantage during epidemic spread because of more frequent transmission (Anderson 1991). (iv) Transmission during the early stage of an infection may be enhanced.

The last of these factors is generally unappreciated. If transmission occurs early during an infection, pathogens that have produced more progeny by this time will be transmitted preferentially. Pathogens that are particularly successful at early transmission may eventually be more damaging to the host as a result of accelerated replication. However, this damage can be thought of as a delayed side effect of selection for an immediate benefit, much as senescence can be considered to have evolved as a consequence of selection for benefits early in life (Williams 1957; Hamilton 1966; Ewald 1994). Conversely, if transmission occurs late during an infection, those pathogens that reproduce slowly may be transmitted in greater frequency because they do not pre-empt their own transmission by causing earlier host death or immobilization. Moreover, if hosts themselves are in a growing phase during infection, the reduced virulence associated with slower rates of pathogen replication may also allow for increased reproduction per host by allowing pathogens to exploit the

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extra host tissue generated by the additional host growth (Ewald 1983; Ebert & Weisser 1997).

In this study, we test the hypothesis that timing of transmission affects the evolution of pathogen virulence using the nuclear polyhedrosis virus (NPV; family: Baculoviridae) of the gypsy moth *Lymantria dispar* (family: Lymantriidae), which is the most economically significant defoliator of hardwood forests in eastern North America (Lewis 1981). We transmitted viruses cyclically from one batch of larvae to the next, early during infection in one treatment and late during infection in the other. By holding constant the number of infection opportunities per cycle, we controlled for any influences of strain extinction (hypothesis 1), requirements for host mobility (hypothesis 2) and differential spread of different strains (hypothesis 3).

The experiment consisted of nine cycles of selection; each cycle comprised inoculation, larval growth, harvest of infected larvae and purification of virus. Two sets of 10 lineages were established. In the Early set of 10 lineages, live larvae were harvested for viral purification on the fifth day after infection, allowing transmission only early during each infection. In the Late set of 10 lineages, live larvae were harvested on the ninth day after infection. Purified viruses were then used for subsequent cycles of infection. All larvae killed by the virus were excluded from the chain of transmission in order to maintain a requirement for transmission between living hosts. Although this requirement does not apply to the natural NPV–*Lymantria* association, it does apply to most host–parasite associations; it was therefore maintained in this experiment to broaden the relevance of the experiment to host–parasite associations in general. After nine cycles of Early or Late selection, we assayed virulence for each lineage by quantifying the percentage of larvae that died in the nine days after inoculation.

We kept viral dosages low to maintain homogeneity within lineages and to minimize 'superinfection', or multiple competing strains within a single host. With high dosages, rapidly replicating viruses would have been present more often within each dose and might have overgrown slowly replicating viruses within each insect. Theory indicates that within-group homogeneity facilitates characteristics such as slow replication, which may increase the success of the group of viruses, even though the slow replicators may be competitively inferior to fast replicators when both are present within a host (Wilson 1980; Ewald 1983; Nowak & May 1994; van Baalen & Sabelis 1995; Taylor *et al.* 1998). Such small dosages also reflect what is thought to occur in many natural host–parasite systems, and in particular, HIV (Ewald 1994; Bull 1994).

2. MATERIAL AND METHODS

(a) *Infection protocol*

The original virus population was *L. dispar* NPV (LdNPV) obtained from the US Forest Service (Hamden, CT, USA), which distributes the virus under the name 'Gypchek'. Gypsy moth egg masses of the New Jersey strain were obtained from the United States Department of Agriculture (USDA) Otis Methods Development Laboratory (Otis Air National Guard Base, MA, USA). To eliminate pathogens on their surface, egg masses were soaked in a 5% formaldehyde solution for 1 h and then rinsed under running water for 1.5 h. Enough egg masses to yield 4000–5000 larvae were then homogenized to reduce the effects of differences between egg samples. Eggs were incubated at 28 °C; larvae were inoculated within a day of hatching.

LdNPV virions are enclosed within a protein coat called a polyhedral inclusion body (PIB) which is visible under light microscopy for measurement and enumeration. PIB density was quantified using a haemocytometer; suspensions were then diluted to 5000 PIBs per ml for infection. We added blue dye (FD&C Blue #2) to this virus suspension, which we distributed in three rings of droplets to create a virus 'gauntlet' through which the larvae crawled. Uninfected first-instar larvae were placed in the centre of these rings. When the larvae, which had not previously been allowed to feed or drink, encountered a drop, they typically ingested *ca.* 1 µl and obtained a dosage of *ca.* 5 PIBs. Approximately 100 of the larvae that had ingested the virus solution (confirmed by dye in their translucent guts) were transferred to cups containing a 1 cm layer of a wheat germ and casein formula (Bell *et al.* 1978). This number was randomly culled to 50 living larvae per replicate after 2 days. On the date specified by the treatment assignment, live larvae were collected and frozen for storage prior to purification. PIBs were separated from the larval corpses using a tissue grinder; the suspension was then filtered through cheesecloth. Viruses were purified from the filtrate through six rounds of centrifugation (2800 rpm for 20 min), using the following sequence of resuspension fluids: distilled water (twice), 1% SDS, 0.5 M NaCl, and distilled water (twice). Each lineage was then stored at 4 °C. PIB size was quantified using computer-aided analysis of images generated by light microscopy, with a haemocytometer as a reference for calibration. For each lineage, 20 randomly selected PIBs were measured.

(b) *Equilibrating dosage across treatments*

We discovered a difference in viral packaging between Early and Late lineages that could have created a bias in viral dosage. During the experiment, average PIB size decreased in the Early but not the Late lineages (Early lineages: Kendall's $\tau = 0.847$, $p < 0.01$; Late lineages: $\tau = 0.238$, n.s.). This decrease in size specific to Early lineages may have resulted from insufficient time being available for the generation of high viral density early in the infection; thus smaller PIBs were produced. Because PIBs were counted as the unit of dosage and PIB diameter is positively correlated with the number of enclosed virions (Mazzone & McCarthy 1981), the size difference was reduced by relaxing selection for one cycle immediately prior to the virulence assays. Specifically, we inoculated insects with extremely large dosages, then harvested the PIBs that were generated for use in the subsequent virulence assay. After this procedure, PIB diameters were significantly enlarged in Early lineages ($t_{18} = 2.79$, $p = 0.01$), but not in Late lineages ($t_{18} = 0.29$, $p = 0.78$) or Gypchek controls ($t_{18} = 0.274$, $p = 0.80$). Two independent measurements of the PIBs harvested from this treatment revealed no significant difference (combined probabilities $\chi^2 = 0.07$) in PIB diameter between Early (mean of 2.69 $\pm$ 0.256 µm s.d.) and Late lineages (mean of 2.89 $\pm$ 0.31 µm s.d.), thus reducing any dosage bias against the predicted outcome.

(c) *Virulence assays*

Percentage mortality by day 9 was used to quantify virulence. Daily mortality counts were taken by counting the total number of living larvae, because larvae killed by virus sometimes disintegrated into indistinguishable virus pools. For each virulence

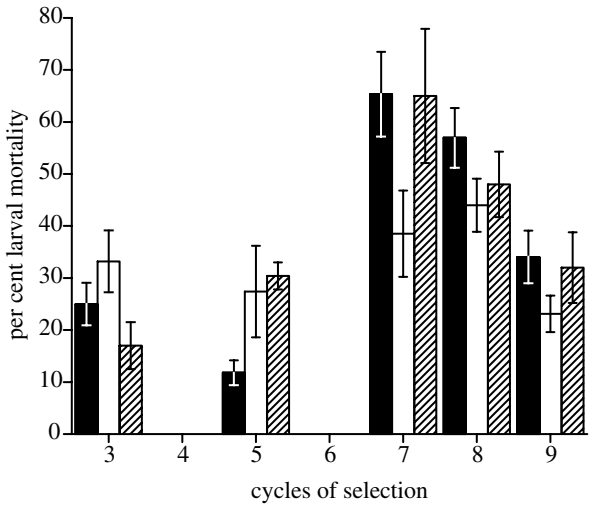

Figure 1. Mean percentage larval mortality caused by NPV over the course of selection. Each column is the average of 10 independent lineages, each replicated at least twice. Error bars are standard errors. Early (filled bars): viruses transmitted early; Late (open bars): viruses transmitted late; Gypchek (hatched bars): ancestral stock.

assay, an additional group of 50 larvae was infected with the original stock of Gypchek as a positive control. Distilled water was used as a negative control in two additional replicates to detect any contamination; almost none was observed. Mortality data were arcsine transformed prior to statistical testing.

3. RESULTS AND DISCUSSION

Over the course of selection, we discovered that the PIBs of Early lineages became smaller than those of Late lineages. PIBs enclose the individual virions and their size is positively correlated with the number enclosed (Mazzone & McCarthy 1981). Because we used PIBs as our unit of dosage (and smaller PIBs would result in fewer virions per infection), we corrected for this size difference by relaxing selection on all isolates from the last three cycles of infection for one additional cycle of infection (see § 2). We did not attempt to correct for PIB sizes after cycles 3 and 5 because Early and Late lineages did not significantly differ in PIB size at that time (data not shown).

Mortality assays on these similarly sized PIBs supported the hypothesis that early transmission caused the evolution of increased virulence (figure 1). Assays conducted at the end of each of the last three cycles of selection indicated that Early lineages had become significantly more lethal than Late lineages (cycle 7: $t_{18} = 2.28$, $p = 0.018$; cycle 8: $t_{18} = 1.97$, $p = 0.033$; cycle 9: $t_{18} = 1.78$, $p = 0.047$). However, we observed considerable variation between cycles in the absolute larval mortality caused by the selected lineages and in the relative virulence of the original Gypchek population (figure 1). To explore the source of this variation, we conducted a larger mortality assay with viruses from cycle 9 using three times as many larvae as in the assays conducted at the end of each cycle (6000 versus 2000). A nested analysis of variance revealed only a marginally significant treatment effect, but highly significant lineage variation within treatments as well as a significant block effect (table 1).

The block effect may best be explained by random genetic differences in the resistances of the larvae used in the assay. More importantly, the significant variation among lineages may reflect founder effects that result from the small numbers of viruses (approximately five PIBs) that were used in the initial infection and subsequently transmitted in each cycle. Restriction fragment length polymorphism (RFLP)-based genetic analyses of these lineages support this interpretation, because each sample from a given lineage produced identical patterns, but nearly every lineage had a unique fingerprint (S. Bhutta, unpublished data). Thus, the small dosages required to maintain homogeneity within hosts and to minimize multiple infections resulted in substantial heterogeneity among lineages. Some of this variance probably originated in the initial inoculates, but genetic drift during the selection experiment may also have contributed to divergence among lineages. Although this heterogeneity within Early and Late lineages probably reduced the magnitude of the treatment effect, we propose that these results may better reflect the biological reality of many pathogens that begin infections with very few individuals. In any case, a combined probabilities test (Sokal & Rohlf 1981) using the results from the last round of infection, together with the larger assay, supported the hypothesis that Early lineages were more lethal than Late lineages by the end of the experiment ($\chi^2_4 = 12.3$, $p < 0.02$)

Most empirical evaluations of the evolution of virulence have employed serial passage experiments, in which parasites are transmitted by the experimenter from one host to another, in a manner that may relax certain constraints on the parasite (Ebert 1998). As a result, serial passage experiments nearly always increase virulence (sometimes confounding the original predictions) and the magnitude of increase may be dramatic (Ebert 1998). The serial passage experiments reported here thus run contrary to the norm, because the virulence of Late lineages did not increase despite serial passaging, but rather tended to decrease. This lack of change in virulence may reflect our particular selection regime, as well as the previous history of the ancestral virus stock. Two components of the selection regime may have hindered a systematic increase in virulence: (i) each infection was founded by relatively few viruses to maintain within-host homogeneity; and (ii) only viruses from living hosts were artificially transmitted. We suspect that relaxing these two constraints would lead to a dramatic increase in the virulence of both Early and Late lineages. The ancestral Gypchek viruses had also already been serially passaged during their production by a division of the US Forest Service (Doane & McManus 1981). The production protocol harvested all viruses from successful infections, which probably generated the genetic heterogeneity of the source population (S. Bhutta, unpublished RFLP evidence). This ancestral genetic diversity probably accounted, in part, for our failure to resolve consistent differences between either group of selected lineages and the Gypchek population. Nonetheless, the presence of less virulent viruses in the serially passaged Gypchek population indicates that selection may have maintained them.

Understanding the lesser virulence of Late strains requires consideration of the fitness benefits associated with mildness. Relative mildness may increase the viral

Table 1. Analysis of variance in virulence among and within transmission groups.
(Nested ANOVA of larval mortality caused by viruses that underwent nine cycles of selection. Mortality data were arcsine transformed. The test for a treatment effect was one-tailed because of our *a priori* directional prediction. The block effect was also nested within lineages.)

source of variation	sum of squares	d.f.	mean square	F	p
treatment	0.454	1	0.454	3.175	0.046
lineage within treatment	2.574	18	0.143	3.654	0.003
experimental block	0.783	20	0.0391	1.730	0.045
error	1.81	80	0.0226		

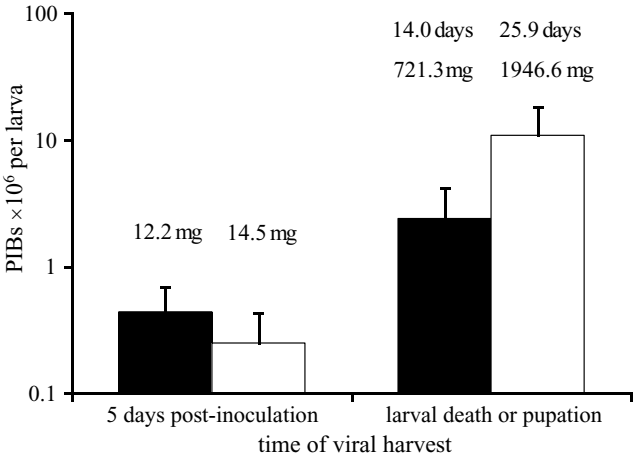

Figure 2. Viral productivity at 5 days after inoculation and after larval death or pupation for one virulent Early-transmitted lineage (filled bars, $n = 19$) and one mild Late-transmitted lineage (open bars, $n = 20$). Error bars are 95% confidence intervals. Numbers above each column indicate mean larval mass at termination (mg) and time to death or pupation (days).

productivity per host because a host infected with a mild strain may grow to a larger size and, hence, eventually yield more host tissues for the virus to exploit (Ewald 1983; Ebert & Weisser 1997). Consistent with this hypothesis, we observed a significant positive correlation between insect mass and viral productivity in multiple independent infections of the genetically variable Gypchek virus population (data not shown). We further evaluated this trend in greater detail by comparing virus production from a highly virulent Early lineage with that from a benign Late lineage. Larvae infected with the benign Late viruses lived nearly twice as long, on average, and grew to nearly three times the mass of those infected with the virulent Early viruses (figure 2). As a result, while Early viruses produced more PIBs by day 5, ($t_{36} = 2.08$, $p = 0.045$), Late viruses were far more productive over the entire lifespan of the larvae ($t_{36} = 2.79$, $p = 0.008$; figure 2).

Our results indicate that the timing of transmission can influence the evolution of virulence by favouring either more rapid or slower production of propagules: rapid production may allow progeny to be transmitted earlier and lead to greater virulence, but slower production may allow the pathogen the use of additional host tissue for more extensive reproduction and greater total fecundity, despite lower virulence. The milder variants should tend to obtain this benefit, however, when the host is not coinfected with harmful strains that would otherwise outcompete them (Wilson 1980; Ewald 1983, 1994; Nowak & May 1994; van Baalen & Sabelis 1995; Taylor *et al.* 1998). Ecological factors, such as the distribution of susceptible hosts, may therefore determine the trajectory of the evolution of virulence. If opportunities for early transmission are frequent, virulence may increase, but the pathogen may forego the use of additional host resources. Conversely, if transmission tends to occur infrequently, selection may favour less virulent pathogens because they produce more progeny.

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