Virulence evolution in a virus obeys a trade-off

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The evolution of virulence was studied in a virus subjected to alternating episodes of vertical and horizontal transmission. Bacteriophage f1 was used as the parasite because it establishes a debilitating but non-fatal infection that can be transmitted vertically (from a host to its progeny) as well as horizontally (infection of new hosts). Horizontal transmission was required of all phage at specific intervals, but was prevented otherwise. Each episode of horizontal transmission was followed by an interval of obligate vertical transmission, followed by an interval of obligate horizontal transmission etc. The duration of vertical transmission was eight times longer per episode in one treatment than in the other, thus varying the relative intensity of selection against virulence while maintaining selection for some level of virus production. Viral lines with the higher enforced rate of infectious transmission evolved higher virulence and higher rates of virus production. These results support the trade-off model for the evolution of virulence.

Keywords: microbial evolution; virulence; bacteriophage; trade-off; infectious disease; experimental

1. INTRODUCTION

A striking contrast among infectious organisms is the tremendous variation in virulence or harm they inflict on their hosts. Human infections of Ebola virus, rabies virus and HIV are almost invariably fatal, whereas infections caused by cytomegalovirus, herpes virus and cold viruses are often asymptomatic. The range of virulence observed across different types of infections is thus profound. Although there has long been a general appreciation of these extremes, only recently has there been any serious attempt to explain the evolution of this variation.

Virulence is necessarily a consequence of the interaction between host and parasite, and in principle evolution in either partner could affect its magnitude. The evolution of host resistance—which is obviously beneficial to the host—could explain why formerly lethal parasites no longer cause harm; the evolution of resistance to myxoma viruses by rabbits has in fact been witnessed, resulting in an order of magnitude improvement in host survival (Fenner & Ratcliffe 1965; Fenner & Myers 1978). However, in the last two decades far more consideration has been given to parasites than to hosts in the evolution of virulence. The fast generation time and large population sizes of many parasites allow much faster evolution of virulence through parasite dynamics than through host dynamics. Furthermore, virulence can be bad for the parasite as well as for the host, and so a wide range of virulence levels is compatible with the evolution of just the parasite (Topley 1942; Levin & Svanborg-Eden 1990; Ewald 1994; Bull 1994; Frank 1992, 1996).

(a) *A trade-off*

A fundamental property underlying many perspectives on the evolution of virulence is a link or ‘trade-off’ between the virulence of an infection and the reproductive capacity of the parasite (Anderson & May 1982; May & Anderson 1983; Ewald 1987; reviewed in Bull 1994; Frank 1996). The most commonly assumed mechanism for this trade-off is that virulence is an unavoidable consequence of parasite reproduction in the host—that the host is necessarily debilitated by parasite antigens, metabolic by-products and the use of host tissues—and hence that higher parasite reproduction results in higher virulence. A parasite’s fitness improves with increases in its reproductive capacity, but is diminished by high virulence because virulence debilitates the host’s ability to transmit the parasite. Highest parasite fitness is thus achieved as a compromise, the exact optimum depending on the shape of the trade-off surface. Trade-off models can explain the evolution of different levels of virulence through differences in factors that affect the parasite optimum.

Unequivocal demonstrations of trade-offs between virulence and parasite productivity are still uncommon, but evidence is growing in support of them. Comparisons across parasites evolved in nature are consistent with trade-offs (Herre 1993; Ebert 1994; Ewald 1994) and a handful of selection experiments have reported trade-offs (Bull *et al.* 1991; Diffley *et al.* 1987; Dearsly *et al.* 1990; Ebert & Mangin 1997; Turner *et al.* 1998). However, the two studies employing the most natural selective conditions failed to observe the expected response to selection even though a trade-off was evident (Ebert & Mangin 1997; Turner *et al.* 1998). Thus, although the field is progressively accumulating evidence of trade-offs, understanding the evolution of virulence continues to pose challenges. The present study offers a further investigation of the trade-off model in the evolution of virulence.

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2. BASIC DESIGN AND ANTICIPATED RESULTS

The experimental goal was to vary selection against virulence while periodically requiring parasite transmission to new hosts. A trade-off would manifest as lower virulence accompanied by lower reproductive capacity in those populations selected more strongly against virulence. Using a bacteriophage as a parasite and a bacterium as a host, we propagated parasites through episodes of strict vertical transmission alternating with episodes of strict horizontal transmission. For vertical propagation, infected hosts were serially transferred for n days without the opportunity for new infection (in this study $n=1$ or eight days). Horizontal transmission was then enforced by recovering parasite progeny produced by these n th day-infected hosts and using the parasite progeny to infect naive hosts for the next cycle.

This regimen exerts selection on parasite virulence and reproductive capacity. A simple model of this process reveals that the optimal level of virulence is sensitive to the experimental variable n and to any trade-off between parasite virulence and reproductive capacity. Consider a single bacterium infected at the beginning of a cycle. For the bacteriophage system employed here, the infected host is capable of dividing and the infection is transmitted vertically to both daughter cells. Thus, during the first day of propagation this single infected host produces a number of descendants, G , through strict vertical transmission (horizontal infection cannot occur during this growth phase). By selecting for maintenance of the infection, any hosts that become 'cured' die and are simply omitted from G . On the second day (if $n>1$), a similar G -fold expansion occurs for each of those G descendants, and so on through to day n , yielding a cumulative number of descendants equal to G^n . At the end of the n th day any previously produced free parasites are removed. Each surviving infected host is allowed to produce new progeny for a short time and these are then used to initiate the next cycle by horizontal infection. Assuming that each infected host produces F infectious progeny during this interval, the net expansion of this parasite lineage is thus proportional to $G^n F$. (In practice, cultures are not allowed to expand indefinitely, but are diluted daily from cultures grown to saturation. The dilution factor limits the magnitude of G , but the dilutions otherwise do not affect the relative comparison among parasite genotypes.)

Both G and F are properties of the infected host— G is actually the number of infected hosts, but its relative magnitude reflects the burden imposed by the parasite. Evolution thus acts on the virus to the extent that parasite genotypes affect G and F differentially. Treating each parasite genotype (i) as an independent competitor (no frequency-dependent selection), the product $G_i^n F_i$ is a relative measure of its expansion over the course of one cycle. Arguing from local stability analyses of gene-frequency equations (e.g. Charnov 1982), if $G^n F$ is maximized on the genetic trade-off surface of the parasite, no alternative parasite genotype that lies on or inside the trade-off surface can invade the population. A graphical solution for $n=1$ and $n=8$ is given in figure 1. The figure shows the intuitive result that a higher G is favoured for $n=8$ than for $n=1$, but also shows that the

Figure 1. Graphical solution to the evolution of virulence in the study design. The heavy curve intersecting both axes is a hypothetical trade-off surface between avirulence (G) and fecundity (F). The thin hyperbola represents a fitness isocline for propagation with $n=1$, and the dashed curve represents a fitness isocline for $n=8$. The point of intersection shown between a fitness isocline and the trade-off surface is the value of (F, G) favoured by natural selection. Provided that the trade-off surface monotonically decreases in F as G increases, the $n=8$ regimen will always favour greater G (greater avirulence) and lower fecundity than the $n=1$ regimen; the magnitude of this difference depends critically on the shape of the trade-off.

shape of the trade-off surface is critical in determining the magnitude of difference in selected outcomes. Although the shape of the trade-off is empirically unknown, the trade-off model supposes that F is monotonically decreasing over G , on the general grounds that limited resources prevent increases in maximal cell growth rate without detracting from phage production. The analysis here further shows that the favoured value of G strictly increases with n . Virulence, by definition, increases as G drops.

Selection fluctuates in a zig-zag fashion in this model, because the two traits being traded off are selected at different times of the life cycle: during the n days of strict vertical transmission selection favours low virulence (high G); then, during the brief but obligatory window of horizontal transmission selection favours high fecundity, which entails selection for high virulence because of the trade-off. A facultative response would be optimal for the phage, with F at zero until day n , rising to its maximum on that final day; but constitutive phage production appears to be the rule for this model system. Because of the fluctuating selection, however, it is essential to assay the response to selection at the same point of the cycle across all lines.

(a) *Phage f1 as a model parasite*

These studies were undertaken with the filamentous phage f1 (similar to the common cloning vector M13).

Filamentous phages are atypical of most known phages in that they establish a permanent infection, during which they reproduce without killing the host. The wild-type phage particle is a long filament consisting of a protein coat surrounding a circular, single-stranded DNA genome of 6407 bases that codes for 11 genes (Model & Russell 1988; Marvin & Hohn 1969). The length of the filament is determined solely by the size of the genome, a property which facilitates the cloning and packaging of foreign DNA. Bacterial hosts include *Escherichia coli* and *Salmonella typhimurium*, but natural infections are functionally limited to F-piliated cells (i.e. cells carrying the conjugal plasmid F), even though the phage undergoes a normal life cycle when its genome is artificially introduced into cells lacking F. Upon entering the cell the single-stranded phage genome is converted by host enzymes into a double-stranded replicative form (RF). This RF is transcribed, and ensuing gene expression results in an increase in the number of RF molecules and then in phage production. All transcription and DNA replication is catalysed by host enzymes, except that a site-specific single-stranded nick in the RF DNA, required to initiate DNA synthesis of the genomic DNA strand, is made by the f1 gene *II* product. One consequence of phage gene expression is that the F-pili are permanently disassembled; the infected cell is therefore resistant to superinfection by extracellular filamentous phages. Bacterial division is slowed during infection to about one-third of the rate of uninfected cells.

The pool of RF molecules increases to 30–50 copies per cell in the first hour of infection, but drops to a long-term equilibrium of 5–15 copies per cell within 2 h (Lerner & Model 1981). Single-stranded progeny genomes are produced continuously, but are immediately sequestered by the phage gene *V* product and transported to the membrane-associated packaging machinery. The latter secretes mature phage particles through the cell envelope without causing cell lysis. In this regard, filamentous phages resemble those animal viruses that bud out through the cell membrane. Although the phage genome is initially replicated at a high rate, attainment of equilibrium means that each RF molecule is replicated at the same rate as the bacterial chromosome. Phage-free daughter cells segregate at a rate of 0.01–0.001 per cell division (Lerner & Model 1981) and would normally be available for reinfection, but in the experimental system used they are killed by an antibiotic.

Given the small size and the relatively low copy number of f1 genomes and the high fidelity of *E. coli* replication machinery (10^{-10} errors per base), the within-host genetic variation among copies of the f1 genome is probably small when started from a single infection. Within-host genetic variation could be high, however, if multiple phages infect the same cell during a phase of horizontal transmission.

3. MATERIALS AND METHODS

Bacteriological and molecular methods followed Bull *et al.* (1991) and Bull & Molineux (1992), except where noted.

(a) *Strains and methods of handling*

Selection experiments were carried out using an F-piliated, kanamycin- and neomycin-sensitive strain of *E. coli* [IJ338: *E. coli*

K12 (*pro-lac*) *supD* Tn10 *hsdS*/F' *traD36 proA⁺B⁺ lacZ*:M15] as the host. Assays to detect superinfection used a strain (IJ482) almost identical to IJ338, except that the F' was *tra⁺ lac⁺*.

The phage used is a genetically engineered derivative of the filamentous coliphage f1. The *Pst*I fragment of plasmid pUC4K, containing a gene conferring kanamycin and neomycin resistance (Kn^r, Nm^r) was cloned into the unique *Pst*I site of CGF3 (Terwilliger *et al.* 1988) to create JB5. Infection by JB5 allows host cells to grow in media containing Kn or Nm, whereas uninfected cells die. A second phage (JB17) was created in a similar fashion, except that the insert contained a Kn^r, Nm^s gene (Ferretti *et al.* 1986).

The latent period of f1 is 15–20' and preliminary studies showed that at least 15' is required to confer Kn^r to the infected host. Infection of log-phase bacterial cells was therefore carried out for 20' prior to the addition of antibiotic, to ensure that virtually all successful (horizontal) infections derived directly from the phage added to the culture, rather than from their progeny; hence there was but a single cycle of horizontal transmission at the start of each culture.

Filamentous phages do not kill their host cells and plaques are usually turbid. Phage with low virulence may form plaques that are too turbid to score accurately, but JB5- or JB17-infected cells form Kn^r colonies in top agar at normal efficiency after 24–48 h of incubation. Phage titres were therefore measured as colony-forming units (CFU) of infected cells on LB medium, to which Kn was added 2.5 h after plating. This regimen allows phage infection and expression of Kn^r, but kills all uninfected cells.

(b) *Assays: virulence, phage reproductive capacity (fecundity) and superinfection*

Phage used for all assays was collected from the final (*n*th) day of a selection during the last hour of growth. Many assays used these heterogeneous *n*th-day phage stocks directly (referred to as 'whole cultures'), whereas other assays used clonal isolates of these day-*n* stocks that were obtained as Kn^r colonies after plating bacteria infected with the stock.

- (i) Phage virulence was measured as the density of infected hosts after 24 h growth at 37 °C. After infection, 10 µl of infected hosts were transferred to 2 ml of LB+Kn and grown at 37 °C for 24 h (i.e. the duration of each growth phase in selections). At this time, cell densities were determined by plating a known dilution on to LB+Kn plates. Colony-forming units (CFUs) were counted after 18–24 h of incubation at 37 °C. Virulence assays of phages from the L₁ and L₈ lines (see below) were conducted on several occasions and for several cultures (147 assays on whole cultures or clonal isolates were performed over a period of 26 days). For samples assayed multiple times on the same day, as well as on different days, a significant between-day heterogeneity in virulence was observed, indicating that unmeasured variables systematically contributed to differences between days ($p < 0.001$, $F_{16,92} = 14.5$). Virulence comparisons were thus restricted to assays performed on the same day.
- (ii) Fecundity (reproductive capacity of the phage) was measured as the concentration of phage produced by infected hosts during 1 h growth in fresh LB+Kn. The cells used in this assay had been infected and grown to saturation in LB+Kn for 24 h before being washed and resuspended in fresh medium. Phage produced per

infected cell was calculated as the ratio of the phage density in the culture supernatant to the density of infected cells, but statistical analyses were conducted with absolute titres (phage per ml) to avoid the statistical complications that arise from ratios of variables. Fecundities were assayed on several occasions (240 titres were conducted, including repeated assays of the same stock, over a 23 day period); they too were significantly heterogeneous between days ($p < 0.001$; $F_{14,51} = 12.2$). Comparisons of phage fecundity are thus restricted to assays performed on the same day.

- (iii) Superinfection. f1 has been reported to resist superinfection (Model & Russel 1988; Marvin & Hohn 1969), but as the interpretation of our experiments is sensitive to even moderate levels of superinfection, its absence was confirmed. Two distinguishable types of bacteria were separately infected with distinguishable phages and then grown in mixed culture. Assaying changes in the phage associated with each cell type provides a measure of superinfection. For one combination, IJ338 (Lac⁻) was infected with JB5 (Kn^r, Nm^r) and IJ482 (Lac⁺) infected with phage JBI7 (Kn^r, Nm^s). Reciprocal infections were also performed. After 20 min, 10 µl of each infected cell type were mixed in a single tube with 2 ml of LB+Kn and grown for 24 h. At both zero time and 24 h, infected cells were plated on LB+Kn plates containing IPTG and X-gal (to determine the Lac phenotype); colonies were then stabbed on to LB+Nm. The combination of the Lac and drug-resistant phenotypes indicates whether superinfection occurred. In each of two independent assays of 100–200 Kn^r colonies were scored and no superinfection was detected.

(c) Selection

(i) *The L₁ and L₈ lines: long-term selection (24 days)*

Two millilitres of log-phase cells of IJ338 were infected for 20 min with JB5 (Kn^r) at a multiplicity of infection (MOI) of about one; 50 µl of the mixture were then transferred to each of two tubes containing 2 ml of LB+Kn, cultures referred to henceforth as the L₁ and L₈ selection lines. Uninfected host cells, including those that were infected but had segregated the phage, were killed by the antibiotic, and as JB5-infected hosts are resistant to superinfection no additional infections could occur. A selection cycle consisted of (i) infection of new hosts; (ii) growth of infected hosts in LB+Kn for n days ($n=1$ day in the L₁ line and $n=8$ days in the L₈ line); and (iii) recovery of phage produced only in the last hour of growth for a subsequent infection phase. Every 24 h, cells were washed with LB+Kn and diluted 10³-fold into a new 2 ml culture of LB+Kn for the next cycle of growth. On the n th day of a cycle cells were washed, resuspended in 2 ml of fresh medium, and grown for 1 h to recover phage for the initiation of a new cycle (2 ml of uninfected cells were infected with retrieved phage at an MOI typically 0.1 or less and a minimum transfer size of 10⁵). The washes ensured that most of the phage recovered was produced during the preceding hour of growth.

Each line was selected over a 24 day period—24 cycles of an L₁ line and three cycles of an L₈ line. The 24 day selection experiments were replicated three times (selections I, II and III), each starting with the same stock of JB5. A core facility helped provide the complete phage genome sequence from three isolates: JB5, and clonal isolates from an L₁ and an L₈ line.

(ii) *The S₁ and S₈ lines: short-term selection (eight days)*

Beginning with a presumed mixture of phage genotypes, we expected a more rapid response to selection than would be attained in the 24 day selections, because those long-term experiments started with a clonal phage stock. Mixtures of phages from the L₁ and L₈ lines in selection I were thus subjected to virulence selection in 16 different eight-day experiments (two replicates, eight independent initial conditions). Each S₁ selection was propagated for a total of eight ($n=1$ day) cycles, starting with a 1:99 L₁:L₈ mixture of phages, and each S₈ selection was propagated as a single ($n=8$ days) cycle, starting with a 99:1 mixture. Each line thus started from a population with a presumed favoured genotype at a frequency of 1%. Some selections were started with a mixture of clonal isolates from L₁ and L₈, whereas others were started from a mixture of phages from the L₁ and L₈ whole cultures. The rationale for conducting some selections from isolates and others from whole cultures was simply due to a concern that the response of heterogeneous cultures might differ from that of clonal isolates.

4. RESULTS

(a) *Virulence of L₁ and L₈ lines*

Consistent with the trade-off model, virulence at the end of the selection period was invariably higher for L₁ lines than for L₈ lines (the model of no difference in virulence is rejected by a binomial test at $p < 10^{-5}$; figure 2a). Clonal isolates behaved similarly to whole cultures, and so only the latter are shown. Cells infected with either L₁ or L₈ phage exhibited lower virulence than did the ancestral phage JB5 (data not shown), suggesting that both selected lines evolved toward lower virulence relative to their ancestor.

(b) *Fecundity of L₁ and L₈ lines*

A higher fecundity of L₁ phage than L₈ phage was expected under the trade-off model and was observed in all but three comparisons (two of which showed no difference). The hypothesis of no difference is easily rejected in favour of the expected result ($p < 10^{-6}$, binomial test; figure 2b). Titres shown in the figure are not corrected for the lower 24-hour cell densities of L₁ than L₈ lines, so the per-cell fecundity of L₁ phage is even higher than indicated in this comparison. Both L₁ and L₈ phage produced fewer progeny (per cell per unit time) than did their ancestor JB5. Further support for the trade-off model is evident when comparing variation in avirulence and fecundity together. The trade-off itself is observed as a striking negative relationship between the two variables (figure 3).

(c) *S₁ and S₈ lines*

The expected direction of virulence and fecundity evolution in these short-term S lines is simply that S₁ should evolve higher virulence and higher fecundity than its ancestor, and S₈ should do the opposite. Most of the 16 lines show this expected result, and the data collectively support the trade-off model, rejecting the null model at $p < 0.005$ (figure 4). Note that this statistical test is conservative because it does not use the per-host fecundity of the phages.

Figure 2. Observed values of avirulence and fecundity in the long-term selected phage. Frequency histograms of the differences between phages from whole cultures of the L_1 and L_8 lines: (a) the measure of avirulence, $\ln[\text{cell density}]$, and (b) the measure of parasite reproductive capacity, $\ln[\text{phage titre}]$. Each difference is measured between a pair of L_1 and L_8 assay values, both phage samples originating from the same selection experiment (I, II, III). Values falling into the shaded area of the graph are inconsistent with a trade-off model. The graphs illustrate that the majority of L_8 phage are less virulent and have lower reproductive capacity than L_1 phage.

(d) Sequences

Complete genome sequences were obtained from JB5 and one isolate from each of the L_1 and L_8 lines of selection I. Relative to JB5, only two substitutions were detected in the L_1 isolate (an A→G substitution at position 957; an Asn→Asp change in gene V_1 and a T→G

Figure 3. The trade-off between phage avirulence and reproductive capacity, measured as $\ln[\text{infected cell density}]$ and $\ln[\text{phage titre}]$, respectively. Closed and open symbols represent, respectively, values from L_1 and L_8 cultures (13 points each). A trade-off is indicated because the 13 L_1 fecundities (all of which exceed the 13 L_8 fecundities) are associated with 13 of the 14 lowest cell densities ($p < 10^{-5}$ for the model of random association of cell density with fecundity). Eleven of these L_1 values are from selection I phage, one from selection II, and one from selection III (and similarly for L_8 phage). Raw L_1 and L_8 values were standardized by subtracting the mean for all assay values obtained on the same day. Thus, if three L_1 and three L_8 values (with an observed average M) were obtained, each of the raw values was standardized by subtracting M , thereby removing the day-to-day heterogeneity in assay conditions that was found to be statistically significant. Note that $\ln[\text{phage titre}]$ values are concentrations of phage in cell supernatants and are not corrected for the density of cells producing those phages. The fact that cell densities were lower for L_1 than for L_8 means that the trade-off is even more extreme than shown here.

substitution at position 5692 in the intergenic region containing the replication origins). In the L_8 isolate the same substitution at position 957 was found.

5. DISCUSSION

Constraints are considered fundamental, often ubiquitous features in life-history evolution. This study investigated a constraint thought to be important in parasite evolution: a trade-off between virulence and reproductive rate. This trade-off model assumes that the evolution of increased rates of parasite reproduction must entail higher levels of virulence. Conversely, as a by-product of selection favouring a reduction in virulence, a parasite will necessarily evolve a lower reproductive rate as it evolves lower virulence. If such a trade-off exists, then the level of virulence for a disease may itself be capable of evolving in response to features of the host's ecology that influence parasite transmission.

We observed a trade-off between virulence and reproductive rate (fecundity) across phage strains experimentally evolved under different combinations of vertical and

Figure 4. (a) Assays on S_1 phages (circles) and S_8 phages (squares) from 16 different selection experiments. Each S_1 lineage was started with a 1:99 mixture of L_1 : L_8 phage and propagated through eight cycles of $n=1$ day. The S_8 lineage was started from the reciprocal mixture and propagated for one cycle of $n=8$ days. Solid symbols represent experiments initiated from whole cultures, open symbols represent experiments initiated from pairs of clonal isolates. The expectation is that virulence and phage titre should both increase for S_1 and both decrease for S_8 . Set 2 replicated the selections and initial conditions of set 1. (b) A summary of which S_1 and S_8 selections show increases and which show decreases in virulence and fecundity over their ancestors. Numbers within symbols indicate the set, and letters indicate lines initiated from clonal isolates (a, b, or c) or whole cultures (w). The expectation under the trade-off model is that the assays marked with circles should fall into the upper right quadrant, and those marked with squares should fall into the lower left quadrant. Under the null model that differences in virulence or fecundity are purely stochastic and independent between assays, any quadrant is equally likely; the chance that nine out of 15 points fall into the correct quadrant is thus slightly less than 0.005 (binomial test). All eight S_1 selections show the predicted increase in virulence, which is the only evolved increase in virulence observed in this study (both L_1 and L_8 lines decreased in virulence from their ancestor).

horizontal transmission. Furthermore, the divergence in virulence and in fecundity between the selected lines was in the same direction as expected from the trade-off model. Responses consistent with the trade-off model were obtained in both long- and short-term experiments, although an evolved increase in virulence was obtained only in the latter.

Several other experimental studies have found a trade-off between virulence and parasite transmission. Three studies used extreme conditions, in which a transmission stage of the normal life cycle was bypassed entirely during artificial propagation (Diffley *et al.* 1987; Dearsly *et al.* 1990; Bull *et al.* 1991). In these cases the parasites adapted to the novel conditions in a manner consistent with the trade-off model, but the extreme novelty of propagation methods left open the question of whether a trade-off existed across more realistic variations in transmission. Two studies have since offered designs more in line with those described here. An experimental study of the gut parasites of water fleas manipulated levels of horizontal and vertical transmission and observed a

trade-off across parasite strains, but virulence evolution was contrary to expectation: the highest virulence evolved in the treatment with the highest level of vertical transmission (Ebert & Mangin 1997). The explanation for this unexpected result was that the line with greatest vertical transmission inadvertently experienced the highest level of within-host competition, and that within-host competition drove the evolution of high virulence. Intra-host competition is thought to enhance selection for parasite reproduction and thus lead to a high-virulence optimum on the trade-off.

A second study provided different opportunities for horizontal and vertical transmission to a conjugative plasmid (Turner *et al.* 1998). No evolutionary change in virulence was observed, despite noting genetic variation that obeyed a trade-off. The level of within-host competition was presumably low for this plasmid, in which case our study using phage f1 falls closer to that of the plasmid than to the water flea. Like Turner *et al.* we observed the expected trade-off, but we further observed that the selected lines responded in the expected direction.

Patterns consistent with a trade-off have also been reported for ecological comparisons of virulence with host life history (reviewed by Ewald 1987, 1991, 1994; Bull 1994; Frank 1996). One drawback of ecological comparisons is that they can contain confounding factors, yet they may also be more likely to reveal evolutionary optima than short-term experimental studies. Short-term selections may respond to a limited repertoire of available mutations that do not reveal the true boundary of a fitness surface.

A detailed ecological study of nematode parasites of fig wasps observed a striking interspecific correlation between nematode virulence and opportunities for horizontal transmission (Herre 1993). For some wasp species a fig inflorescence was typically pollinated by a single individual wasp, so that the only parasites entering the inflorescence were carried by her, and the only parasites leaving were carried by her offspring. These parasites thus experienced predominantly vertical transmission. In other wasp species (which pollinated different species of figs) a single inflorescence was pollinated by multiple wasps, allowing parasites from different host lineages to mix before dispersal (horizontal transmission). The virulence caused by worms increased with the potential for horizontal transmission, consistent with the trade-off model. However, Herre argued instead that virulence had evolved in response to different levels of within-host competition. Horizontal transmission was thus confounded by opportunities for within-host competition, but this time in the opposite direction, as suggested for the water flea (Ebert & Mangin 1997). Despite this difference, the two studies point to the likely importance of within-host competition in shaping the evolution of virulence. In assessing all of these studies, trade-offs between parasite virulence and reproductive capacity appear to have generality, but the evolution of virulence itself remains poorly understood.

The response to selection obtained in this study may be viewed as weak or strong, depending on one's perspective. Ewald (1991, 1994, 1996 and references therein) suggested that profound short-term changes in the virulence of human pathogens may result from particular changes in cultural practices that affect parasite transmission. The results observed here are weak by those standards. Yet there is no *a priori* reason to expect a large response: if the trade-off is shallow, any response will also be shallow. The response observed here is typical of what can be expected of evolution along a trade-off surface. A remaining question is whether a much longer selection period would have produced further divergence.

Our assay of phage fecundity was measured as phage particles capable of establishing an infection during a defined interval. Fecundity may thus be affected by (i) changes in phage output from infected cells; (ii) changes in the rate of attachment to cells, (iii) changes in the ability to establish an infection once attached to the cell, etc. The trade-off model may be construed to apply to all of these mechanisms, and indeed a measure of fecundity that did not account for the complete 'viability' of phage particles could be evolutionarily meaningless. Infection rates were measured for L₁ and L₈ phages, and no difference was found (data not shown).

This study used a single, convenient measure of virulence: the saturation density of infected hosts. The literature on virulence encompasses a wide range of virulence definitions, from parasite-induced host mortality (e.g. Anderson & May 1982) to parasite-induced decreases in host fitness (Turner *et al.* 1998); medical uses of the term would include host morbidity, which has no straightforward fitness interpretation. Our measure of virulence is compatible with that of Turner *et al.* Nonetheless, there are countless other ways of measuring virulence that are also compatible with its definition: growth rate, survival, and competitive ability of infected hosts. Each of these measures could be assayed under a variety of conditions: nutrient-rich or nutrient-poor environments, the presence or absence of toxins, the presence or absence of competing species, short-term or long-term selection, etc. There are no set criteria for choosing a measure of virulence, and indeed there is no guarantee that a trade-off observed with one measure will obtain for another measure. Nonetheless, the foundation of the evolutionary approach rests on trade-offs being fundamental, and presumably independent of mechanism.

Only one nucleotide difference distinguished the L₁ and L₈ genomes—a substitution in the (–) strand origin of replication. Phages containing certain deletions in this region form turbid plaques and appear to experience delayed progeny production (Kim *et al.* 1981). However, the entire Kn^r cassette of JB5 was inserted into the (–) strand origin (near its 5' end) and did not obviously reduce progeny yield. Furthermore, the L₁ line exhibited higher progeny production than did the L₈ line, which lacked mutations in the intergenic region. The nucleotide data thus do not offer much insight into the mechanistic basis of virulence evolution except in showing that a single nucleotide substitution accounts for the difference between L₁ and L₈. There is considerable precedent for this latter feature of our results: single-base changes have been found to underlie major changes in virulence in many viruses (as the basis of viral attenuation; reviewed in Bull (1994)).

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