

# Reduced fecundity is the cost of cheating in RNA virus $\phi 6$

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Co-infection by multiple viruses affords opportunities for the evolution of cheating strategies to use intracellular resources. Cheating may be costly, however, when viruses infect cells alone. We previously allowed the RNA bacteriophage  $\phi 6$  to evolve for 250 generations in replicated environments allowing co-infection of *Pseudomonas phaseolicola* bacteria. Derived genotypes showed great capacity to compete during co-infection, but suffered reduced performance in solo infections. Thus, the evolved viruses appear to be cheaters that sacrifice between-host fitness for within-host fitness. It is unknown, however, which stage of the lytic growth cycle is linked to the cost of cheating. Here, we examine the cost through burst assays, where lytic infection can be separated into three discrete phases (analogous to phage life history): dispersal stage, latent period (juvenile stage), and burst (adult stage). We compared growth of a representative cheater and its ancestor in environments where the cost occurs. The cost of cheating was shown to be reduced fecundity, because cheaters feature a significantly smaller burst size (progeny produced per infected cell) when infecting on their own. Interestingly, latent period (average burst time) of the evolved virus was much longer than that of the ancestor, indicating the cost does not follow a life history trade-off between timing of reproduction and lifetime fecundity. Our data suggest that interference competition allows high fitness of derived cheaters in mixed infections, and we discuss preferential encapsidation as one possible mechanism.

**Keywords:** cheating; co-infection; fitness; life history; trade-off; virus

## 1. INTRODUCTION

All interactions between individuals that involve the generation, acquisition and use of a common pool of resources are potentially subject to evolutionary cheating (Crespi 2001; Velicer 2003). Cheaters can benefit by using more, but producing less, fitness-enhancing products than their competitors (Travisano & Velicer 2004). However, cheating strategies may be costly for selfish individuals who are vulnerable when victims are not available. For example, in the slime mould *Dictyostelium discoideum*, where individuals unite to form fruiting bodies composed of somatic stalk and germinative spore cells, cheaters produce only spore and no stalk cells (Buss 1982; Strassmann *et al.* 2000). In the absence of ordinary genotypes, cheating is costly, because stalkless fruiting bodies hinder spore dispersal from nutrient-poor environments (Buss 1982).

In viruses, defective genotypes lacking one or more essential genes can arise spontaneously in laboratory culture, or can co-evolve alongside ordinary viruses in the wild (Roux *et al.* 1991; Qiu & Scholthof 2001; López-Ferber *et al.* 2003). These viruses may cheat by replicating at the expense of co-infecting ordinary (helper) viruses that provide the needed proteins. Hence, the selfish behaviour has led certain virus cheaters to be referred to as defective-interfering (DI) particles (Cole & Baltimore 1973; Roux *et al.* 1991). The fitness of defective viruses is zero, however, when infecting the host alone, because they cannot produce all the needed proteins. Full-length (non-defective) viruses can also cheat during co-infection. For example, umbraviruses cheat by usurping proteins provided by

co-infecting luteoviruses, in order to be vector-transmitted by aphids between host plants (Falk *et al.* 1999). Here, we explore the cost of cheating in full-length genotypes of the RNA virus  $\phi 6$ .

Previously, an experiment allowed populations of bacteriophage (phage)  $\phi 6$  to evolve for 250 generations (50 serial passages) in an environment permitting high multiplicities of infection (MOI; Turner & Chao 1998). MOI is the ratio of infecting viruses to host cells. At elevated MOI, the probability of co-infection (i.e. multiple virus genotypes replicating within the same host cell) is high, producing strong selection for viral genotypes that thrive under such conditions. At the end of the experiment, the fitness of the evolved genotypes was measured relative to the ancestor (stored in the freezer). The results showed that the evolved viruses possessed a large fitness advantage *only* in the presence of co-infection with unlike genotypes (Turner & Chao 1998). One interpretation is that the derived viruses are cheaters, which possess traits that promote the selfish use of limited intracellular resources. We demonstrated the phenotypic (overall fitness) cost of cheating by showing that the cheaters compete poorly when co-infecting cells with identical cheater genotypes (Turner & Chao 1999).

Here, we examine the cost of cheating in phage  $\phi 6$  more closely through classic burst assays that measure components of the lytic infection cycle (Stent 1963). The infection cycle in phage  $\phi 6$  has been well studied (Mindich 1999), and can be partitioned into three phases (figure 1a, Appendix A), comprising the various life-history stages of a virus (Abedon 1989; Bull *et al.* 2004). Phase I (dispersal/diffusion) starts with release of phage from the lysed host, and ends when a phage infects a new host. The rate at

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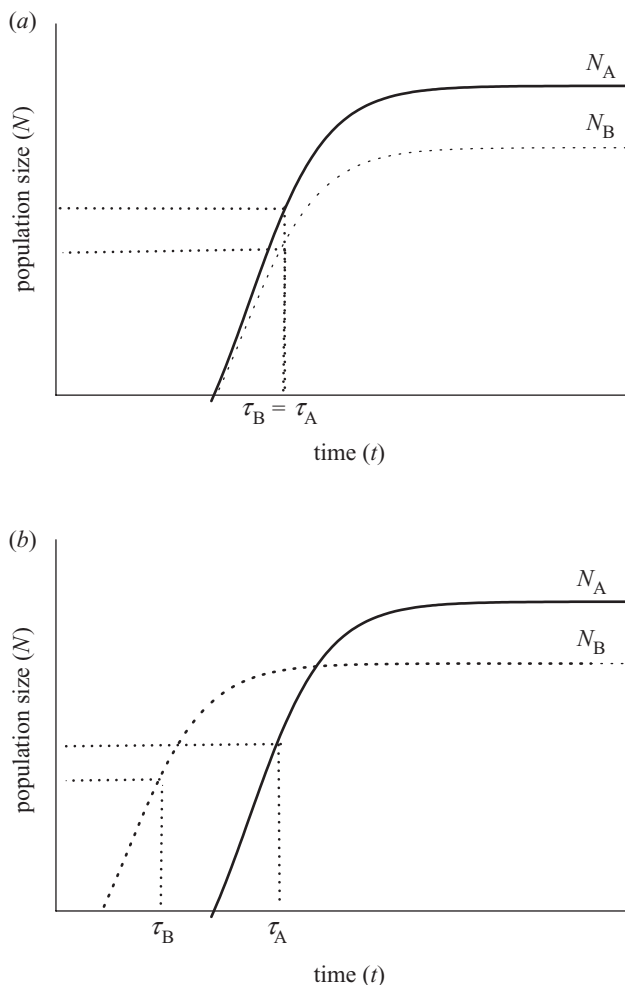


Figure 1. Hypothetical growth curves for phages A and B (see Appendix A) during co-infection. Solid line represents growth of phage A, whereas dashed line is that of phage B. (a) If the phages differ only in burst size ( $N_A > N_B$ ), then A is competitively advantaged because it has produced more progeny at the end of the growth cycle. (b) If B features a shorter latent period than A ( $\tau_B < \tau_A$ ), this could offset B's growth disadvantage because the cell bursts before the A offspring have fully matured ( $N_A < N_B$ ). Dotted lines represent estimation of latent periods.

which phages infect new hosts is termed the attachment rate. Phase II (juvenile) begins with infection and ends when the phage progeny mature inside the host, a time span termed the latent period. Phase III (adult) begins when the virus offspring mature, and ends with cell lysis, when many progeny are released from the infected cell. The total number of progeny released per infected cell is termed the burst size. The growth parameters associated with each phase can be measured easily using standard microbiological techniques (Stent 1963; Chao *et al.* 2002).

Although phage  $\phi 6$  is increasingly used as a model for evolution in segmented RNA viruses (Chao 1994; Turner 2003), growth differences between wild-type and evolved strains of the virus have never been closely analysed. Moreover, examination of juvenile and adult stages of phage life history for the derived cheater virus and its ancestor is useful, because the associated growth parameters (attachment rate, latent period, exponential growth, and burst) yield plausible hypotheses to account

for the phenotypic cost that arises when cheaters infect cells alone. In particular, the most compelling hypothesis to explain the fitness differences of a derived cheater and its ancestor might be the virus equivalent of a life-history trade-off between timing of reproduction and lifetime fecundity (Stearns 1992; Bull *et al.* 2004). Here, selection may favour genotypes that produce relatively few offspring at an early age. Rapid reproducers could gain an advantage in generation number (and hence, offspring number) per unit time, relative to more fecund longer-lived genotypes. This trade-off is, perhaps, more relevant for microbes that often feature expanding populations than for macroscopic organisms that exist close to their carrying capacity.

Low fitness in clonal infections implies that the cheater genotype of  $\phi 6$  features a characteristically small burst size, analogous to poor lifetime fecundity. Although low fecundity would often be disadvantageous, the life-history trade-off hypothesis predicts that the reproductive cost may be overcome by creating offspring at a very young age. In viruses, this trade-off would amount to a small burst size that is offset by a short latent period (i.e. reduced generation time). Thus, a less fecund virus can be very successful in some environments if it initiates cell lysis faster than its competitors. Specifically, a virus with these traits could gain a competitive advantage over co-infecting genotypes, because the virus could initiate cell death before the offspring of competing strains have fully matured (figure 1b). It is generally predicted that, in viruses, a short latent period and small burst size go hand in hand, but few experiments have directly addressed this idea (Doermann 1948; Abedon 1990). Although high MOI might select for viruses with shortened latent periods, these decreased generation times must be sufficiently brief to offset low fecundity. Furthermore, the combined traits would be costly if the environment suddenly changes such that the less fecund virus must infect a cell on its own (see Abedon (1989) and Wang *et al.* (1996) for a discussion of the advantage of shortened latent periods when the environment contains only single infections). In the current study, burst assay data allowed us to examine the cost of cheating in phage  $\phi 6$ , and to determine whether a life-history trade-off governs success of derived cheaters in the presence of co-infection.

## 2. MATERIAL AND METHODS

### (a) Phage and bacteria

Phage  $\phi 6$  (family Cystoviridae) features a genome divided into three RNA segments, and causes lytic infection in certain plant-pathogenic *Pseudomonas* bacteria (Vidaver *et al.* 1973; Mindich *et al.* 1999). Viruses used in this study were originally derived from a single clone of wild-type  $\phi 6$ , hereafter referred to as wt $\phi 6$  (Vidaver *et al.* 1973). Previously, we obtained a spontaneous host-range mutant of wt $\phi 6$ , denoted  $\phi 6h$ . The host-range expansion of  $\phi 6h$  occurs through a mutation on the medium segment. Previous studies show this mutation imposed a 5–7% fitness cost on the virus when it is reared on its original host, *Pseudomonas phaseolicola* (Chao *et al.* 1992; Turner & Chao 1998). Preliminary fitness measurements (see §2c) confirmed that  $\phi 6h$  carries a 5% cost under our experimental conditions; mean fitness of wt $\phi 6$  relative to  $\phi 6h$  was  $1.0548 \pm 0.0258$  s.e.m. ( $n = 18$ ). All fitness

measurements relative to  $\phi 6h$ , reported below, were adjusted to reflect the cost of the  $h$  marker. The evolved cheater clone  $\phi H2$  was described previously by Turner & Chao (1999), and was isolated from a population of  $wt\phi 6$  evolved at high MOI (Turner & Chao 1998).

The *P. phaseolicola* host strain used in the previous evolution study (Turner & Chao 1998), and in all current experiments, was purchased from the American Type Culture Collection (ATCC No. 21781). An additional host strain, *P. pseudoalcaligenes* East River isolate A (ERA), was obtained from the laboratory of L. Mindich (Public Health Research Institute, New York). The phage  $\phi 6h$  forms clear plaques when plated on a mixed lawn containing both *P. phaseolicola* and *P. pseudoalcaligenes* ERA. By contrast, non-host-range phages form turbid plaques on a mixed lawn because they do not kill the *P. pseudoalcaligenes* ERA cells present.

### (b) Culture conditions and media

All phages and bacteria were grown, plated, incubated and diluted at 25 °C in LC medium, a modification of Luria broth (Mindich *et al.* 1976). All bacterial cultures were inoculated by a single bacterial colony placed in 10 ml LC medium in a sterile flask. Culture flasks were grown for 24 h on a platform shaker rotating at 120 r.p.m. inside a 25 °C incubator. During this 24 h period, bacterial cultures attained stationary phase densities. All bacterial stocks were stored in a glycerol-LC (4 : 6, v/v) solution at -20 °C.

Agar concentrations in plates were 1.5 and 0.7% for bottom and top LC agar, respectively. Top agar volume was 3 ml per plate, to which we added 200  $\mu$ l of *P. phaseolicola* culture to produce a bacterial lawn. P/E plates used in some assays contained a mixture of *P. phaseolicola* and *P. pseudoalcaligenes* ERA in a 200 : 1 volumetric ratio, which produces a mixed bacterial lawn with equal proportions of each *Pseudomonas* strain.

Phage lysates were prepared by plating plaque-purified phage with 3 ml top agar and 200  $\mu$ l *P. phaseolicola* overnight culture. After 24 h, the plaques in the top agar were resuspended in 4 ml of LC broth and centrifuged at 3000 r.p.m. for 10 min. Supernatant containing the phage lysate was filtered (0.22  $\mu$ m, Durapore; Millipore, Bedford, MA, USA) to remove bacteria. Phage lysates were stored at -20 °C in a glycerol-LC (4 : 6, v/v) solution.

MOI was manipulated by mixing viruses and *P. phaseolicola* cells at a defined ratio. Assuming Poisson sampling (Sokal & Rohlf 1995), at a given MOI, the proportion of cells infected with 0, 1, and at least 2 viruses is, respectively,  $P(0) = e^{-\text{MOI}}$ ,  $P(1) = (e^{-\text{MOI}} \times \text{MOI})/1$ , and  $P(\geq 2) = 1 - P(0) - P(1)$ . Thus, only 0.1% of all infected cells contain two or more viruses at an MOI of 0.002, whereas at an MOI of 5, 97% of infected cells experience multiple infections.

### (c) Fitness measurements

To measure fitness, a test phage was mixed in a 1 : 1 volumetric ratio with the host-range mutant phage  $\phi 6h$ . The mixture was then allowed 40 min adsorption to *P. phaseolicola* at either MOI = 5 (i.e.  $2 \times 10^{10}$  phage  $\text{ml}^{-1}$  to  $4 \times 10^9$  bacteria  $\text{ml}^{-1}$ ), or MOI = 0.002 (i.e.  $8 \times 10^6$  phage  $\text{ml}^{-1}$  to  $4 \times 10^9$  bacteria  $\text{ml}^{-1}$ ). Before cells had burst, the diluted mixture was plated on LC agar with a *P. phaseolicola* lawn, and then incubated for 23 h. The resulting approximate 500 plaques on the plate were then collected and filtered to remove bacteria (see § 2b for lysate preparation). This lysate was then plated on P/E plates, where the ratio of the two phages was based on the  $h$  marker, which revealed the ratio of test phage to  $\phi 6h$  in the starting mixture ( $R_0$ ) and in the

harvested lysate ( $R_1$ ). Thus, fitness was assayed on a *P. phaseolicola* lawn, but the starting and final ratios were assayed on a mixed lawn of hosts. The number of plaques per plate was maximized at 500, because this minimized plaque overlap and, hence, genetic exchange (segment reassortment) between plaques. Fitness ( $W$ ) is defined as:

$$W = R_1/R_0$$

If  $W \neq 1$ , the test phage differs in fitness from the reference phage ( $\phi 6h$ ). Fitness assays were replicated ( $n = 6$ ) for each virus.

At MOI = 5, a modified fitness assay was conducted to examine the effects of co-infection by multiple identical viruses. A test phage and  $\phi 6h$  were allowed to adsorb to cells at MOI = 5 in separate tubes, and then mixed in a 1 : 1 volumetric ratio immediately before assaying fitness. The standard and modified fitness assays are identical except that intracellular interaction between different genotypes is prevented in the separate adsorption environment. Thus, whereas the standard high MOI assay allows cells to be co-infected by  $\phi H2$  and  $\phi 6h$ , the modified assay provides that cells are multiply infected with either  $\phi H2$  or  $\phi 6h$  genotypes.

### (d) Attachment assays

To measure attachment rate (Stent 1963), a virus strain was mixed with exponentially growing *P. phaseolicola* cells ( $4 \times 10^8$  colony forming units  $\text{ml}^{-1}$ ) at MOI =  $1 \times 10^5$  (i.e.  $6 \times 10^3$  phage to  $6 \times 10^8$  bacteria), in LC medium in a sterile centrifuge tube. The mixture was incubated with shaking (120 r.p.m.) at 25 °C for 40 min. Immediately after mixing and every 10 min thereafter, a sample from the mixture was centrifuged at 5000 r.p.m. for 1 min to pelletize the cells, and 100  $\mu$ l of the supernatant was plated on a *P. phaseolicola* lawn to estimate the plaque-forming units (PFU) of phage in the supernatant. Because bound phage are pelletized with the bacterial cells, only unbound phage remained in the supernatant. At each time-point, we calculated  $R_t$ , the ratio of PFU at time  $t$  relative to the initial PFU. We then obtained the slope of the regression line for the natural logarithm of  $R_t$  regressed versus time. The slope is equal to  $-kC$ , where  $k$  is the attachment rate constant and  $C$  is the concentration of host cells in the mixture.

### (e) Burst assays

Growth parameters were measured in modified assays of classic single-burst experiments (Stent 1963; Chao *et al.* 2002). A virus strain was mixed with exponentially growing *P. phaseolicola* cells at MOI = 5 (i.e.  $1 \times 10^9$  phage to  $2 \times 10^8$  bacteria), in a sterile flask. Viruses were then allowed to undergo a single infectious growth cycle ( $\sim 180$  min) on a platform shaker rotating at 120 r.p.m. inside a 25 °C incubator. Throughout the infection process, samples were plated on a *P. phaseolicola* lawn to estimate titre ( $N$ , phage per millilitre) of the phage population. Samples were obtained a minimum of 10 times during the three phases of the growth cycle, e.g. 0, 40, 50, 70, 90, 100, 110, 160, 170 and 180 min. Maximal growth rate ( $r$ ) is the slope obtained by regressing the natural logarithm ( $\ln$ ) of  $N$  against time during the period of exponential growth. We also computed mean titre (phage per millilitre) during stationary phase ( $N_f$ ). Assuming that very few cells escape infection at MOI = 5, burst size ( $S$ ) is the number of progeny produced per infected cell:  $S = (N_f)/(2 \times 10^8 \text{ bacteria } \text{ml}^{-1})$ . The latent period ( $\tau$ ) is defined as the time at which half the number of virus progeny have been released into the environment. Burst assays for different viral strains were conducted in parallel to eliminate any confounding effects of host cell culture.

### 3. RESULTS

#### (a) Phenotypic cost of cheating

We confirmed a previous observation (Turner & Chao 1998, 1999) that the cheater phage  $\phi$ H2 competed poorly against its ancestor in the absence of co-infection. The results (figure 2) showed that the adjusted mean fitness of wt $\phi$ 6 relative to itself across all environments was  $W = 1.00 (\pm 0.0245 \text{ s.e.m.})$ , and did not differ according to assay environment (one-way ANOVA with mean square error = 0.0124, d.f. = 2,  $F_2 = 0.0459$ ,  $p = 0.955$ ). In marked contrast, fitness of  $\phi$ H2 was very different across the various assay environments (figure 2). Our *a priori* expectation (based on earlier observations) was that the cheater genotype  $\phi$ H2 would be favoured only in mixed infections at MOI = 5. Indeed,  $\phi$ H2 was clearly advantaged in this environment, presumably because of its intracellular competitive advantage gained through cheating (mean  $W = 1.3665 \pm 0.0823 \text{ s.e.m.}$ ;  $t_5 = 4.1527$ , d.f. = 10, one-tail  $p = 0.0010$ ), whereas in an identical environment, which prevents intracellular interaction between viruses (MOI = 5 separate adsorption), as expected,  $\phi$ H2 fared very poorly (mean  $W = 0.7563 \pm 0.0377 \text{ s.e.m.}$ ;  $t_5 = 3.2230$ , d.f. = 10, one-tail  $p = 0.0046$ ).

#### (b) Comparison of life-history parameters

We sought to examine more closely the phenotypic cost of cheating in  $\phi$ H2 by contrasting the life-history parameters of the virus and its ancestor. We first conducted attachment assays to estimate the attachment rate constant ( $k$ ) for each virus. Attachment assays were replicated ( $n = 5$ ) for each clone (table 1). Results showed no significant differences in the attachment rate constants of the two viruses ( $t$ -test:  $t = -0.63$ , d.f. = 8,  $p = 0.55$ ).

We then conducted a series of burst assays (see § 2) that measured latent period ( $\tau$ ), maximal growth rate ( $r$ ) and burst size ( $S$ ) of wt  $\phi$ 6 and  $\phi$ H2, when the viruses are grown separately at MOI = 5. Burst assays were replicated ( $n = 10$ ) for each clone, for a total of 20 independent assays (table 1). We first compared maximal growth rate of wt $\phi$ 6 and  $\phi$ H2. Here, we found that the growth rate was slower in  $\phi$ H2 than in its ancestor ( $t$ -test with  $t_5 = 4.416$ , d.f. = 18,  $p < 0.001$ ). When we compared burst sizes of the two viruses, we found a significantly smaller burst in  $\phi$ H2 ( $t_5 = 3.632$ , d.f. = 18,  $p = 0.0019$ ). These two results are satisfying, because they are consistent with our observations that  $\phi$ H2 was competitively disadvantaged relative to the marked ancestor in environments where fitness should be influenced strongly by a virus' ability to produce progeny (figure 2). Finally, the latent period in  $\phi$ H2 was observed to be much longer than that of the ancestor ( $t_5 = 2.203$ , d.f. = 18,  $p = 0.041$ ). Table 1 summarizes the growth parameters estimated for wt $\phi$ 6 and  $\phi$ H2. For all three life-history parameters we quantified using burst assays, wt $\phi$ 6 and  $\phi$ H2 statistically differed.

#### (c) Analysis of the life-history trade-off

At face value, the burst assay results in table 1 do not support the hypothesized life-history trade-off between early reproduction and lifetime fecundity as a plausible explanation for success of  $\phi$ H2 in different competitive environments. Lack of support is best illustrated when these data are substituted for parameter values to solve the phage life-history equations in Appendix A. According to

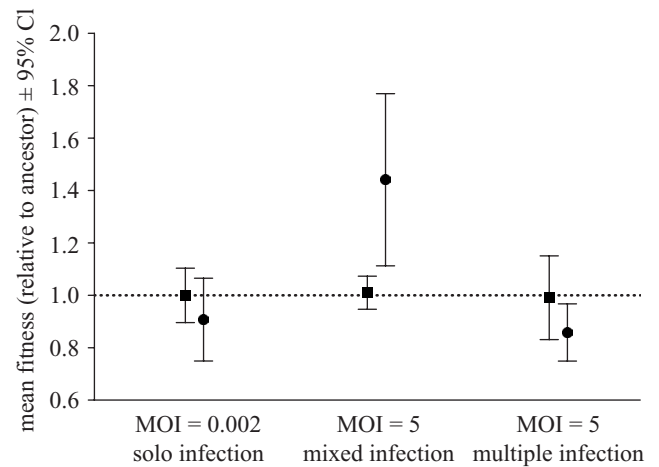


Figure 2. Mean fitness of  $\phi$ H2 (filled circles) and its ancestor wt $\phi$ 6 (filled squares) relative to a common competitor of the ancestral genotype, in three environments. Each point represents the mean ( $\pm 95\%$  CI) of six independent estimates. See text for statistical analyses.

the model, the phage that is less efficient at producing progeny ( $\phi$ H2) must offset this cost by initiating lysis faster than the time needed for the co-infecting competitor wt $\phi$ 6 to complete its growth. Figure 3 depicts simulated growth curves of  $\phi$ H2 and wt $\phi$ 6 at MOI = 5 based on the data for mean titre at each time point during our burst assays. Genotype  $\phi$ H2 is generally disadvantaged because it begins growing later, experiences slower exponential growth, and produces roughly three-quarters of the progeny made by wt $\phi$ 6. This result is a clear contradiction to the trade-off hypothesis, because  $\phi$ H2 does not possess a short latent period that can compensate for its poor fecundity.

### 4. DISCUSSION

Evolutionary cheating in microbes and other organisms can lead to fitness trade-offs (costs) in environments where cheaters rarely encounter 'helper' genotypes (Buss 1982; Maynard Smith 1982; Turner & Chao 1999; Strassmann *et al.* 2000; Velicer *et al.* 2000; Greig & Travisano 2004). Here, we confirmed that phage  $\phi$ 6 genotypes evolved under high MOI, compete strongly when co-infecting host cells with the ancestral genotype, but compete poorly when infecting cells on their own. We also demonstrated that the cost of cheating in phage  $\phi$ 6 is due largely to a substantial reduction in virus fecundity. The attachment rates of the two strains did not differ significantly, indicating that neither strain gained a fitness advantage by infecting cells more rapidly. Burst assays of the derived cheater ( $\phi$ H2) and its ancestor (wt $\phi$ 6) showed that the two strains differed in all three key parameters of Phases II and III of the lytic infection cycle. The exponential growth rate and burst size were both significantly lower in the cheater virus, and the latent period was significantly longer. Thus, the cost of cheating in  $\phi$ 6 was at least twofold: reduced fecundity for individual cheaters in the absence of co-infecting helper genotypes, and an overall reduction in the mean fitness of the infection group when cheaters take over the population at high MOI. This result is consistent with the Prisoner's Dilemma outcome of evolutionary game theory (Maynard Smith 1982; Turner & Chao 1999).

Table 1. Comparison of growth parameters<sup>a</sup> for the ancestral virus wt $\phi$ 6 and the cheating clone  $\phi$ H2.

Parameters	Wt $\phi$ 6	$\phi$ H2
attachment rate constant ( $k$ )	$9.939 \times 10^{-11} (\pm 2.69 \times 10^{-11})$	$9.740 \times 10^{-11} (\pm 2.10 \times 10^{-11})$
latent period ( $\tau$ )	108.90 min ( $\pm 1.552$ )	116.20 min ( $\pm 2.928$ )
growth rate ( $r$ )	$0.085 \ln \text{phage min}^{-1} (\pm 0.0065)$	$0.042 \ln \text{phage min}^{-1} (\pm 0.0074)$
burst size ( $S$ )	210.71 phage ( $\pm 11.47$ )	148.54 phage ( $\pm 12.69$ )

<sup>a</sup> Values for  $k$  are means and s.e.m. based on five independent assays; all other data are from 10 independent burst assays at MOI = 5. See text for statistical analyses.

Our interpretation is that cheater viruses adapt to high MOI by sacrificing between-host fitness for within-host fitness. This result is a classic example of evolution under soft selection, which refers to the difference between the fitness a genotype possesses when alone and the fitness it experiences in the presence of other competing genotypes (Chao *et al.* 2000). Soft-selected viruses can evolve by acquiring traits that promote a competitive advantage over co-infecting genotypes, such as manufacturing fewer, but using more, shared gene products within the cell. However, such adaptive traits are not necessarily beneficial for host exploitation, evidenced by the poor performance of soft-selected viruses when co-infecting victims are absent. In contrast, clonally evolved viruses experience only hard selection, where a genotype adapts by acquiring traits that directly promote host exploitation. Propagation of the same ancestral virus at low MOI provides a good illustration of this phenomenon; the derived viruses greatly increased in fitness relative to their ancestor after 250 generations of hard selection (Turner & Chao 1998, 2003).

In addition, we substituted parameter estimates from burst assays into a model comparing phage growth (see Appendix A) to show that a life-history trade-off hypothesis does not support the success of  $\phi$ 6 cheaters during co-infection. That is, organisms might evolve life-history trade-offs where adaptive traits are beneficial under certain ecological conditions, but costly in alternate environments. Thus, evolved cheater viruses might thrive in high MOI environments by featuring reduced fecundity, combined with an ability to initiate rapid cell lysis. Our data do not support this explanation.

A potential limitation of our design for evaluating the trade-off hypothesis is that we did not measure genotype-specific growth parameters for wt $\phi$ 6 and  $\phi$ H2 in mixed infections at MOI = 5, the habitat where the presumed trade-off promotes high fitness of cheaters. We were prevented from doing such experiments by one important factor. Assuming Poisson sampling (Sokal & Rohlf 1995), 97% of infected cells contain multiple viruses at MOI = 5, but these infection events comprise a large proportion of non-mixed infections. Consider two viruses mixed together at a 1 : 1 ratio ( $p = q = 0.5$ ) and allowed to co-infect cells at MOI = 5. Out of cells infected by *more than one* virus particle, 0.25 ( $q^2$ ) will contain two copies of virus one, 0.25 ( $p^2$ ) will contain two copies of virus two, and only 0.50 ( $2pq$ ) will contain copies of both viruses. (Here we assume that only two virus particles infect a cell, based on previous experiments showing that co-infection in  $\phi$ 6 is limited to an average of 2.3 viruses per cell (Olkkonen & Bamford 1989; Turner *et al.* 1999). Therefore, our above statement that, at MOI = 5, 97% of the infected cells contain two or *more* viruses is more correctly stated as 97% contain two or *three*

viruses. Below, we further discuss the limit to co-infection in  $\phi$ 6.) The varied combinations of mixed infections at MOI = 5 do not prevent detection of large fitness differences for cheaters in mixed infections compared with solo infections (however, they probably contribute to large error bars associated with our fitness estimates; see figure 2). Unfortunately, there is no easy method to study *only* mixed infections in burst assays. Thus, we were forced to evaluate the life-history trade-off comparatively, and this may have caused us to sacrifice some accuracy.

Our results strongly suggest that a life-history trade-off does not explain the success of the representative evolved cheater,  $\phi$ H2, during mixed infections. We believe that a more complex interference mechanism is responsible. Below, we suggest one plausible mechanism. In addition, we discuss the relevance of our findings for the observed limit to co-infection in  $\phi$ 6, and for the importance of intrahost conflict between parasite genotypes in the evolution of virulence.

#### (a) *Interference mechanism*

Certain DI particles contain extra sequences recognized by encapsidation enzymes, and are, thus, preferentially packaged during intracellular replication (Roux *et al.* 1991). Although we observed that  $\phi$ H2, unlike a true DI particle, was able to successfully infect a cell on its own, it too may employ a mechanism for preferential encapsidation. In this scenario,  $\phi$ H2 would compensate for poor capsid production by ensuring that its RNA is preferentially placed within exogenously manufactured procapsids during virus particle formation. The hypothesis would account for the fitness advantage observed for  $\phi$ H2 during co-infection. Because the success of this mechanism relies on a productive co-infecting, it would also explain  $\phi$ H2's low fitness during clonal infection. This mechanism would not depend on how many capsids are produced during co-infection of  $\phi$ H2 and wt $\phi$ 6, but would instead necessitate that  $\phi$ H2 is the virus that is preferably packaged. Our burst assay data support this conjecture, because  $\phi$ H2 replicates significantly slower than its ancestor wt $\phi$ 6. One possibility is that  $\phi$ H2 contains modified or additional genetic material needed for the virus to gain an encapsidation advantage, which presumably slows its maximal growth rate in the absence of other co-infecting genotypes.

Mindich (1999) describes a model for the packaging of  $\phi$ 6 particles within the host cell. In short, phage(s) enter a cell where viral genes code for capsid production and the synthesis of viral RNA. A complete phage particle contains three distinct RNA segments (small, medium and large), which are encapsidated in order of increasing size from small to large. According to the model, this phenomenon occurs because the capsid initially presents a recognition

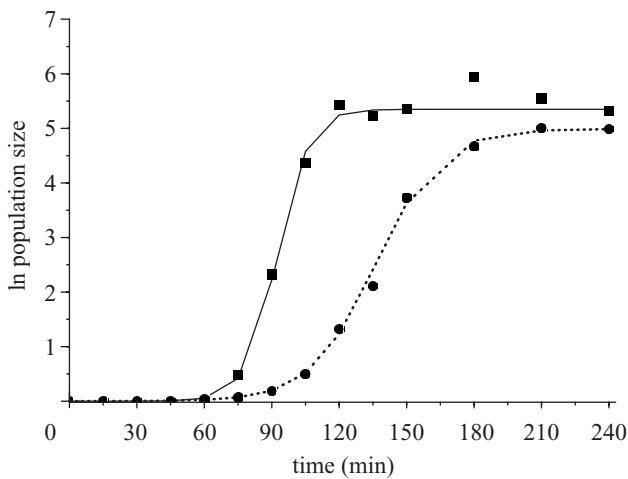


Figure 3. Realized growth curves for the cheater phage  $\phi$ H2 and its ancestor wt $\phi$ 6 based on the mean data (table 1) from replicate burst assays ( $n = 10$ ) conducted at MOI = 5. The filled squares and filled circles are representative data from burst assays for wt $\phi$ 6 and  $\phi$ H2, respectively. The solid line (wt $\phi$ 6) and dashed line ( $\phi$ H2) are generated using equation (A 3) in Appendix A and the mean data from combined burst assays for each virus.

site for the small segment. After the small segment enters the capsid, the conformation changes such that the medium segment is the next recognized, and so on. Thus, a virus featuring one or more segments with greater affinities for capsid entry could gain an encapsidation advantage over co-infecting genotypes. One possibility is that greater affinity may be accomplished through a duplicated region of RNA segment(s), and that this would slow viral replication in the absence of co-infection. We tested this prediction by extracting RNA from  $\phi$ H2 and wt $\phi$ 6, and comparing the sizes of the viral segments using gel electrophoresis. A size difference was not discernable (data not shown), suggesting that preferential encapsidation (if it exists) most probably occurs through novel mutations affecting packaging.

When segmented RNA viruses co-infect the same cell, hybrid progeny can be produced that contain a random reassortment of segments descending from the parent viruses. Although reassortment is a genetic exchange mechanism that can bring together favourable combinations of segments, the mechanism may also tear apart beneficial combinations at its next occurrence. For this reason, one might predict that a selfish strategy (such as the encapsidation hypothesis described above) is unlikely to evolve in phage  $\phi$ 6. That is, selection for selfish viral particles would be opposed by random reassortment that tends to separate coadapted segments, suggesting that segments from selfish viruses would need to be under strong linkage disequilibrium. This idea presumes that any intracellular competitive advantage is due to epistatic interactions among loci on different segments. Our assays estimate fitness by tracking a marker located on the medium segment (see §2). Thus, if the fitness advantage associated with selfishness was due to a mutation on the medium segment, whether or not reassortment occurs, would be

irrelevant. In this sense, luck may have led us to discover the intracellular advantage of  $\phi$ H2, because we happened to be tracking the correct segment in our fitness assays. Furthermore, even if epistasis exists, reassortment may occur at such low rates that its effects are relatively unimportant. Elsewhere, we show that hybrid genotypes, resulting from co-infection, are generated at fairly low rates even at MOI = 5 (Turner *et al.* 1999). Therefore, reassortment might dilute the advantages of positive epistasis governing complex traits that span RNA segments, but it may not be a sufficient force to prevent the evolution of a genome-wide selfish strategy. In a separate study, we explore the effects of virus co-infection on selection against negative epistatic interactions between segments (Froissart *et al.* 2004). The current study suggests that theory and experiments concerning virus co-infection and positive epistasis are equally warranted.

### (b) *Limit to co-infection*

Viral co-infection is an environment where cheating can cause the fitness of an ordinary virus to decrease (von Magnus 1954; Lewontin 1970; Chao 1994). Thus, viruses that can limit or prevent co-infection should possess a selective advantage, and it is not surprising that numerous viruses have evolved mechanisms to do so (Zebowitz & Brown 1968; Simon *et al.* 1990; Singh *et al.* 1997). Here, we describe two viral adaptations of cheaters that are likely to evolve in response to high MOI: shortened latent period and competitive interference. Interestingly, a potential consequence of both adaptations is a reduction in the overall number of viral progeny produced per infection. A shorter latent period causes the host cell to burst early, resulting in the release of fewer progeny compared to single infections (Doermann 1948; Abedon 1990). Also, a reduction in viral progeny is evidenced by interference in selfish DI particles, which can have a negative effect on the group of viruses co-infecting a cell. For instance, the total yield of polioviruses produced by an infected cell is inversely proportional to the frequency of DI particles present (Cole & Baltimore 1973).

We previously showed that when phage  $\phi$ 6 is evolved under co-infection, viruses adapt by enhancing their intracellular fitness at the expense of their ability to exploit the host (Turner & Chao 1998). Although this intracellular advantage allows the viruses to fix in the population, it also reduces the population's overall mean fitness (Turner & Chao 1999). Our results suggest that the competitive advantage was gained through an interference mechanism, but that the fitness consequence is a smaller burst. These results clearly indicate a cost to evolution under intracellular competition in  $\phi$ 6, and suggest that a limit to co-infection should evolve. Olkkonen & Bamford (1989) determined the limit using the amount of incorporated  $^{14}\text{C}$  label as a measure of the number of phage  $\phi$ 6 particles entering a cell. We later explored the limit using a population genetics approach (Turner *et al.* 1999). By comparing the frequency of observed hybrid genotypes to that predicted by a mathematical model, we confirmed the earlier estimate for a limit between two and three phages per cell on average. These data indicate that cell entry is limited to a few  $\phi$ 6 viruses, but more than one particle. Such a limit would simultaneously reduce the cost of co-infection, while maintaining any potential advantage of segment

reassortment that would occur between mixed co-infecting genotypes (e.g. the ability of segment reassortment to combat mutational load; Chao *et al.* 1992, 1997). Elsewhere, however, we suggest that the advantages of reassortment may be surprisingly narrow in  $\phi 6$ , because the genetic exchange mechanism necessitates co-infection, where selection against deleterious mutations can be weakened by a process (virus complementation) analogous to dominance in diploid organisms (Froissart *et al.* 2004).

### (c) Evolution of virulence

Conventional models predict that virulence (parasite damage to the host) should evolve to higher levels whenever individual hosts are co-infected by unrelated genotypes (Hamilton 1972; Bremermann & Pickering 1983; Knolle 1989; Frank 1992, 1996). This view assumes that intrahost competition favours evolution of parasites that produce more progeny within the host than their competitors, which in turn leads to higher parasite densities and greater virulence levels. Elsewhere, we argue that the result emerges because these kin selection models have assumed that only hard selection operates on the co-infecting parasites (Chao *et al.* 2000). If soft selection is allowed to operate, the models can instead predict that virulence evolves to lower values with co-infection (Turner & Chao 1998); however, Frank (1996) shows that soft selection does not necessarily produce this outcome.

In our previous evolution study (Turner & Chao 1998), the phage evolved at low MOI infected a host cell alone and the progeny produced within a cell were closely related, whereas, at high MOI, relatedness was lower because the offspring were created by multiple phage genotypes co-infecting the same cell. Because viral fitness was shown to decrease at high MOI, this confirms that virulence may decrease when co-infection is coupled with intrahost competition. The observation, however, stems from some unique aspects of our study system. Previous models (e.g. Frank 1996) assumed only simple resource competition between parasites, where evolution of increased reproduction (hence, higher virulence) is an excellent mechanism for achieving greater fitness within the host. By contrast, cheaters in our system seem to achieve high fitness by stealing resources produced by co-infecting viruses. West & Buckling (2003) predicted similar results in their model of production of siderophores (iron-scavenging molecules) in bacterial parasites. They showed that related bacteria were more likely to cooperate in siderophore production than non-related bacteria; therefore, related bacteria would produce more offspring and be more virulent than non-related bacteria. The current study extends this interpretation because the evolved cheaters show roughly a one-quarter reduction in lifetime fecundity. Greater relatedness led to increased virulence: the opposite of the outcome predicted by conventional models. To define virulence as damage to the host is difficult in lytic phages because the host always dies in a successful infection. One possibility is to gauge virulence of lytic viruses by the number of viral progeny produced (burst size); alternatively, virulence may be correlated with the rate of the infection process (latent period). In either case, we have confirmed that the cheating clone  $\phi H2$  is relatively less virulent than its ancestor  $wt\phi 6$  because the evolved clone features both a smaller burst size and a longer latent period.

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### APPENDIX A

A logistic model provides a relatively good approximation of the lytic infection cycle. Let  $N_0$  and  $S$  denote, respectively, initial and final (burst) population size of the virus during one round of infection. If  $r$  is maximal growth rate, then the growth of the viral population is

$$dN/dt = rN_0(1 - N_0/S). \quad (\text{A } 1)$$

The solution to this equation is

$$N(t) = S/[1 + (S/N_0 - 1)e^{-rt}]. \quad (\text{A } 2)$$

Initiation of reproduction ( $\varepsilon$ ) marks the end of the virus eclipse period, and enters the equation by slightly modifying it to read

$$N(t) = S/[1 + (S/N_0 - 1)e^{-r(t-\varepsilon)}] \quad (\text{A } 3)$$

where  $t > \varepsilon$ . Thus, growth of the population is delayed until after the eclipse period.

A simple mathematical model shows how a life-history trade-off between latent period (average reproductive age) and burst size (lifetime reproduction) may determine the phenotypic (fitness) success of derived cheaters. The model assumes that viruses adsorb (attach) to cells at the same rate, i.e. Phase I of the infection cycle is equivalent. Consider two viral genotypes, A and B, whose difference in burst size ( $S_A > S_B$ ,  $r_A = r_B$ ,  $\tau_A = \tau_B$ ) translates to competitive superiority of A during co-infection (figure 1a). However, if B features a relatively shorter latent period ( $\tau_B < \tau_A$ ), the trait can afford a competitive advantage because the cell lyses before the offspring of virus A have matured (figure 1b). By this same logic, derived cheaters might be advantaged during co-infection because they mature earlier (feature shorter latent periods), despite featuring lower lifetime fecundity (smaller burst). Precedence for the life-history trade-off comes from other virus studies showing that a short latent period tends to be associated with small burst size (Doermann 1948; Abedon 1990).

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