Parasitism Between Co-Infecting Bacteriophages

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I. SUMMARY

Co-infection of a single host by multiple virus genotypes or species is common in nature, facilitating studies of ecological interactions between viruses at the cellular level. When two or more viruses co-infect the same host cell, this can have profound consequences for the fitness (growth performance) of an individual virus. Co-infection may be advantageous to an individual virus due to increased pathogenesis, enhanced transmission, or the opportunity for genetic exchange (sex) which produces the raw material for natural selection. In contrast, co-infection may be disadvantageous to an individual virus because it increases the likelihood of competition for proteins and other resource products available within the cell. One intriguing cost of co-infection is the recent evidence that intra-cellular interactions between viruses can be antagonistic, where a virus genotype evolves to specialize in parasitizing other co-infecting viruses. Here I review laboratory experiments involving the RNA bacteriophage ϕ6, which demonstrate the evolution of parasitism when viruses are propagated in environments where co-infection is common. Frequency-dependent selection is shown to govern the fitness of these parasitic (cheater) genotypes, because their benefit of cheating depends on the relative abundance of ordinary and cheater genotypes encountered within the host cell. I relate why the evolution of parasitic

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viruses may be relevant for observed limits to the absolute number of phages that can simultaneously infect a single cell. The evolution of parasitic interactions in viruses infecting animal and plant hosts is briefly discussed. I suggest directions for future research on the evolutionary ecology of virus co-infection, especially the need to study phage interactions under natural (non laboratory) conditions.

So, naturalists observe, a flea
Has small fleas that on him prey;
And these have smaller still to bite 'em
And so proceed ad infinitum.
—Jonathan Swift

II. INTRODUCTION

Viruses usurp the biomolecular machinery of living cells in order to replicate their genomes. Depending on the virus, reproduction may occur within a unicellular host organism such as a bacterium or alga, or inside specific cells (tissues) that comprise a multicellular host. When multiple genotypes or species of virus co-infect the same host cell, ecological interactions can occur between viruses within the cellular milieu. Virus ecology (relationships between viruses, their hosts and the abiotic environment; Hurst, 2000) includes these intracellular virus encounters, which can span the entire interaction continuum ranging from mutualism to parasitism (Petney and Andrews, 1998; Hammond *et al*., 1999; Lopez-Ferber *et al*., 2003). Mutualistic interactions lead to enhanced fitness (reproductive success) of the co-infecting viruses, such as their improved reproduction due to an overall weakening of the host or the host's immune system. In contrast, viruses can experience antagonistic encounters within the cell, which decrease the fitness of one or more virus strains in the co-infection group. Lowered fitness can arise due to competition for limiting intra-host resources, interference competition that causes one virus to disrupt the reproduction of another, and apparent competition where one virus stimulates a host response (e.g., immunity) that acts against both strains. Thus, multiple infections can have either positive or negative consequences for the fitness of an individual virus genotype.

Theoretical models predict that within-host ecological interactions between viruses (or other parasites) should profoundly impact selection for traits relating to virulence (damage to the host). May and Nowak (1995) examined the evolution of virulence in light of co-infection, an ecological complexity that was often missing in previous modeling efforts (but see e.g.,

Levin and Pimentel, 1981). Their work shows that competing strains simultaneously infecting the same host are selected to rapidly exploit the host before resources are depleted, causing escalation to greater virulence; similar theoretical outcomes are shown by other authors (Nowak and May, 1994; van Baalen and Sabelis, 1995; Frank, 1996; Mosquera and Adler, 1998). Empirical support comes from human immunodeficiency virus-1, where within-host evolution of competing virus strains contributes to the increased virulence associated with the onset of AIDS (Antia *et al*., 1996). Additional models have examined the evolution of virulence, under differing genetic relatedness of co-infecting parasites. The general prediction is that antagonism between genetically diverse parasites favors rapid exploitation of within-host resources and, hence, increased virulence (Frank, 1992). However, in controlled experiments with viruses (and other parasites) avirulent genotypes can outcompete virulent ones (see Read and Taylor, 2000, for many examples). We argued that this discrepancy between theory and data may be due to incorrect assumptions regarding the nature of competition, because faster rates of host exploitation need not be the only parasite adaptation favored by within-host competition (Chao *et al*., 2000). Rather, traits that involve exploitation or inhibition of competing genotypes (such as production of anti-competitor toxins) can also be selected, potentially resulting in less effective exploitation of the host and reduced virulence. Data supporting this hypothesis include experiments on bacteria and their viruses (Turner and Chao, 1998, 1999), which are the primary focus of this chapter. Overall, the various models generally agree that multiple infections tend to select for antagonistic interactions between unrelated parasites, whether or not this result leads to increased or decreased evolution of virulence. A thorough account of the mathematical theory relating to multiple infections extends beyond the scope of this chapter, but interested readers should consult recent papers and books dealing with the subject (Nowak and May, 2000; Read and Taylor, 2001; Frank, 2002). Despite the large body of theoretical work, relatively few experiments have directly addressed the predictions of the various models.

One powerful method that can be used to test these theoretical predictions is laboratory experiments using model organisms, where evolutionary ecology can be studied under strictly controlled conditions. Natural selection is the differential survival and reproduction of genotypes in a population, due to the challenges imposed by the prevailing environment. Therefore, laboratory environments can be manipulated while still allowing evolution to proceed through natural selection, because the experimental habitats determine which genotypes contribute their genes to subsequent generations. This process should not be confused with artificial selection (such as animal breeding), where the experimenter explicitly chooses which individuals contribute genes to the descendants in order to obtain a population

dominated by genotypes featuring a desired trait (such as increased milk production in dairy cattle). Drosophila fruit flies are well-known as study organisms in experimental evolution (Kohler, 1994), but microbes (bacteria, viruses, fungi) are increasingly used due to their large population sizes, short generation times, and ease with which their environments and genetic systems can be manipulated (Lenski, 2002). Most importantly, microbes can be stored in a freezer for indefinite periods of time, permitting direct comparisons between an ancestral genotype and its evolved descendants.

This chapter will summarize the data from recent laboratory experiments on bacteria and the RNA virus ϕ6, which show that antagonistic interactions can evolve when viruses are propagated under high levels of co-infection. These studies demonstrate how parasite genotypes can be selected to selfishly interfere with the reproduction of other strains during co-infection, at the expense of reduced performance in alternate habitats where multiple infections are less common. This chapter also describes how frequency-dependent selection underlies within-host competition for resources in these experiments, and relate why the data may be relevant for observed limits to the number of viruses that can simultaneously infect a single host cell. Then the evolution of parasitic interactions in viruses that infect animal and plant hosts is briefly discussed. Throughout, ideas for future research are suggested that deal with the consequences of co-infection in viruses, especially in terms of the largely unexplored natural ecology of viruses that infect bacteria.

III. PHAGE BIOLOGY AND INTRACELLULAR CONFLICTS

Natural ecosystems abound with viruses, especially the bacteriophages (literally "eaters of bacteria") that infect bacteria (Paul and Kellogg, 2000). For example, bacteriophage (or simply phage) particle counts ranging from 70,000 to 15 million per milliliter have been measured in the open ocean (Bergh *et al.*, 1989; Wommack and Colwell, 2000), and it is suggested that phages can influence global ecological processes by regulating the population sizes of their less numerous bacterial hosts (Fuhrman, 1999). Aside from the continual ecological influence exerted by their teeming presence in the biosphere, phages were highly influential in the development of basic laboratory research during the past century. In particular, they factored prominently in experiments that laid the groundwork for molecular biology as a separate discipline (Cairns *et al.*, 1992). Examples include bacteria/phage studies that demonstrated that mutations occur spontaneously (at random) and not in direct response to environmental change (Luria and Delbruck, 1943), and experiments that proved a triplet genetic code underlies protein

synthesis (Crick *et al.*, 1961). Because phages possess a rich history as subjects of laboratory study, there is a wealth of information on their basic biology, genetics, and techniques for genome manipulation. For these reasons, phages provide powerful systems for the study of experimental ecology and evolution in laboratory settings (Turner, 2003; Lenski, 2002).

Many phages are lytic, featuring infection cycles that result in death (lysis) of the host cell. The basic reproductive cycle of a lytic virus is shown in Fig. 1. The lytic cycle features three discrete stages: (i) attachment of the virus to a host cell, and injection into the cell of the phage genetic material (whether RNA or DNA); (ii) replication of virus genetic material to create viral enzymes, structural components and other products within the cell; and

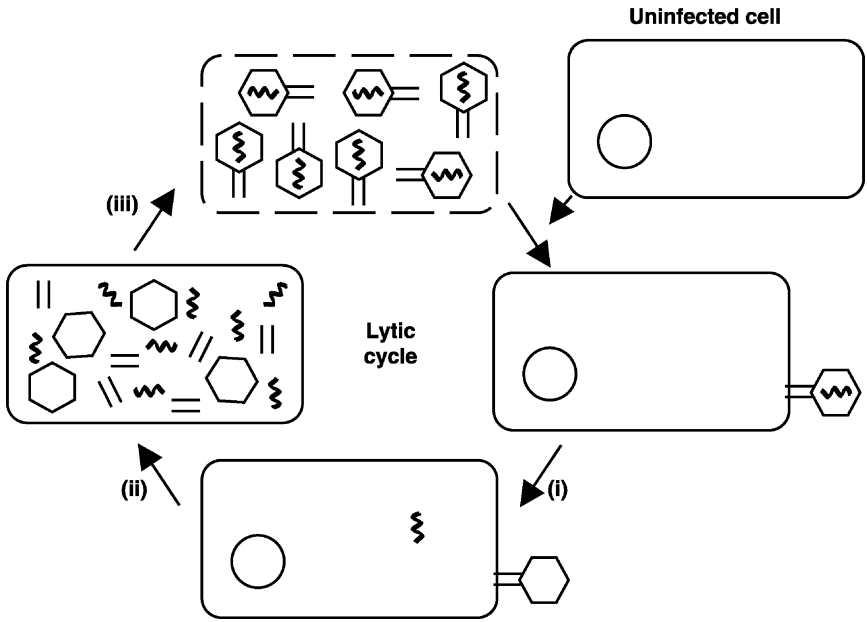

Figure 1 The reproductive cycle of lytic bacteriophages includes intracellular production of sharable resources. (i) The cycle begins with phage attachment to an uninfected host cell, and injection of the virus genetic material (RNA or DNA) into the cell. (ii) The genome is replicated and transcribed, to create virus products which diffuse within the cell to create a sharable resource pool (e.g., replicase enzymes, virus structural proteins). Other phages that simultaneously co-infect the cell may contribute equally to the resource pool, a process analogous to cooperative behavior. In contrast, the co-infecting phages might contribute fewer resources, but specialize in sequestering products from the pool; an antagonistic process analogous to selfish behavior. (iii) The cycle ends with assembly of progeny viruses, which are released into the extra-cellular environment through cell lysis (death).

(iii) assembly of progeny phages that are released into the environment through cell lysis, setting the stage for further rounds of infection.

One key to parasitic interactions between lytic viruses is the intracellular manufacturing of viral products (stage ii in Fig. 1). These products diffuse within the cell to create a common resource pool available to all co-infecting strains. Representative products include replication enzymes, and structural proteins that are used to construct the capsid which surrounds virus genetic material. We have argued that the resource pool tends to prevent an individual virus genotype from exclusive access to any of the gene products it contributes to the pool (Turner and Chao, 1999, 2003). In turn, the resource pool can create a 'conflict-of-interest' between viruses over available resources. Thus, the resource pool should promote selection for virus genotypes that parasitically interact with other co-infecting strains, through traits where one virus interferes with the ability of other viruses to use or acquire essential intracellular products (Nee and Maynard Smith, 1990; Nee, 2000; Brown, 2001).

Viruses do not exhibit behavior *per se*, but terminology borrowed from behavioral ecology is useful (though decidedly anthropomorphic) in describing how viruses utilize products in the resource pool. A virus that makes large (excess) amounts of useful products stands to benefit other co-infecting genotypes; hence, the virus can be defined as a cooperator (Szathmary, 1993; Turner and Chao, 1999). In contrast, a virus might synthesize fewer products but specialize in appropriating a larger share of the products available within the pool, a cheating strategy that exemplifies parasitism (Turner and Chao, 1999). The recent literature suggests that cheating (defection) is a universal phenomenon in biological populations; nature provides numerous examples of the temptation for individuals to cheat for personal reward, in lieu of cooperative behavior that promotes the common good (Axelrod, 1985; Dugatkin, 1997). Viruses also appear subject to the temptation, as shown in the following experiments where evolved genotypes of phage $\phi 6$ resort to cheating strategies to obtain products from the intracellular resource pool.

IV. PARASITISM IN CO-INFECTING RNA PHAGES

Phage $\phi 6$ is a member of the family *Cystoviridae*, bacterial viruses containing RNA genomes that are divided into three smaller segments (termed large, medium and small). Cystoviruses infect certain bacterial pathogens of wild and cultivated plants (Mindich *et al*., 1999), explaining why $\phi 6$ was originally isolated from bacterial infections of bean straw (Vidaver *et al*., 1973). Great care has been taken to identify the infected plant from which each of the nine viruses in the family *Cystoviridae* were isolated (Vidaver *et al*., 1973; Mindich *et al*., 1999), but the far more difficult task of determining the

primary bacterial host for these viruses in the wild remains a goal for future research. Nevertheless, the typical host of $\phi 6$ in the laboratory is *Pseudomonas syringae* pathovar *phaseolicola*, a plant pathogen that causes bean halo-blight disease (Tsiamis *et al*., 2000). The reproductive cycle of $\phi 6$ is typical of a lytic phage (Fig. 1). Phage $\phi 6$ has been the subject of extensive research on molecular virology (Mindich, 1999), and has successfully been used as a model for experimental ecology and evolution (Burch and Chao, 1999; Turner and Chao, 1999; Lythgoe and Chao, 2003), especially in testing theories for the evolution of genetic exchange between co-infecting viruses (Chao, 1990; Chao *et al*., 1997; Turner and Chao, 1998; see review by Turner, 2003).

Parasitism and other competitive interactions can only occur when viruses co-infect cells. Rates of co-infection in $\phi 6$ are controlled by manipulating the multiplicity of infection (moi) or ratio of infecting phages to host cells in laboratory culture (Turner and Chao, 1998). Assuming Poisson sampling (Sokal and Rohlf, 1995), the probability that a cell will be infected with zero viruses is:

$$P(0) = e^{-moi}.$$

Similarly, the probability that a cell is infected by a single virus is:

$$P(1) = (e^{-moi} \times moi)/1.$$

The probability of co-infection (two or more viruses per cell) can be calculated as:

$$P(\geq 2) = 1 - P(0) - P(1).$$

To examine evolutionary ecology of $\phi 6$ in the presence and absence of co-infection, we conducted an experiment that manipulated moi (Turner and Chao, 1998). A single clone of wild type $\phi 6$ was used as an ancestral virus to initiate three high moi (moi = 5) and three low moi (moi = 0.002) populations, which were then allowed to evolve on *P. phaseolicola*. Following the above logic, at moi = 5, co-infection by two or more viruses is common and 97% of cells should experience multiple infections. In contrast, only 0.1% of all infected cells contain two or more viruses at moi = 0.002. Viruses and host cells were mixed together in a test tube at high or low moi, and the resultant virus progeny were then diluted and plated onto agar containing a lawn (superabundance) of host cells, where each virus that hits the lawn grows to form a visible plaque overnight. The plaques were then harvested and filtered to obtain a bacteria-free lysate. Finally, a dilution of the lysate was mixed with a fresh stock of naïve host cells at high or low moi. This propagation cycle was repeated for 50 consecutive days, which is equivalent to 250 generations of viral evolution (1 generation in the tube plus 4 generations on the plate per day) (Turner and Chao, 1998). Thus, moi differences between treatments were imposed every fifth generation.

At the end of the experiment, population samples (stored in the freezer at −20°C) were competed against a common competitor of the ancestral (wild type) genotype to measure changes in fitness (defined as growth performance on *P. phaseolicola*). To do so, an evolved population and a genetically marked ancestor were mixed together, and assayed for their ability to grow on *P. phaseolicola* cells in a 24-hour period. The ratio of phages in the starting mixture (R_0) and after 24 hours (R_1) was monitored by plating on selective agar, where genetically marked and unmarked viruses are easily distinguished by their plaque morphology (Chao, 1990). Fitness (W) was defined as:

$$W = R_1/R_0.$$

Thus, $W \neq 1.0$ indicates that the competitors differ in fitness because they produce dissimilar numbers of progeny during the assay.

Fitness trajectories of high-moi– and low-moi–evolved populations were obtained by plotting the grand mean fitness of each treatment group over time (in 50 generation increments). Figure 2A shows that all of the derived viruses increased in fitness relative to their common ancestor, indicating that beneficial mutations led to fitness improvements in the populations. The low-moi populations increased linearly in fitness over time to achieve the highest end point fitness values. In contrast, the fitness trajectory of high-moi populations was concave; these populations appeared to quickly reach a selective plateau that led to a fitness decline. At face value, these data suggested that co-infection hindered the rate of fitness enhancement. But the odd fitness trajectory observed in the high-moi lineages hinted that a more complex explanation existed.

We realized that the standard assay (Chao, 1990) used to measure fitness in Fig. 2A compares virus growth rates during strictly clonal infections, where viruses do not ecologically interact within the cell (Turner and Chao, 1998). During clonal infections, selection is primarily for a virus that best exploits the host cell. But intracellular interactions can occur during co-infection; thus, the viruses evolved at high moi experienced an ecological habitat radically different from that of the standard assay. Co-infecting viruses may be selected for host exploitation *and* for within-host competition of limited products available in the resource pool. Adaptation to intra-host competition may occur through novel virus traits that detract from the ability of the virus to exploit its host (Lewontin, 1970), and this within-host selection may then create a cost for co-infection.

To examine the importance of ecological differences between high- and low-moi treatments, we conducted additional fitness measurements for the evolved populations at moi = 5 (Turner and Chao, 1998). Figure 2B shows that the high-moi–evolved viruses perform much better relative to the ancestor during mixed infections, in comparison to their fitness during clonal

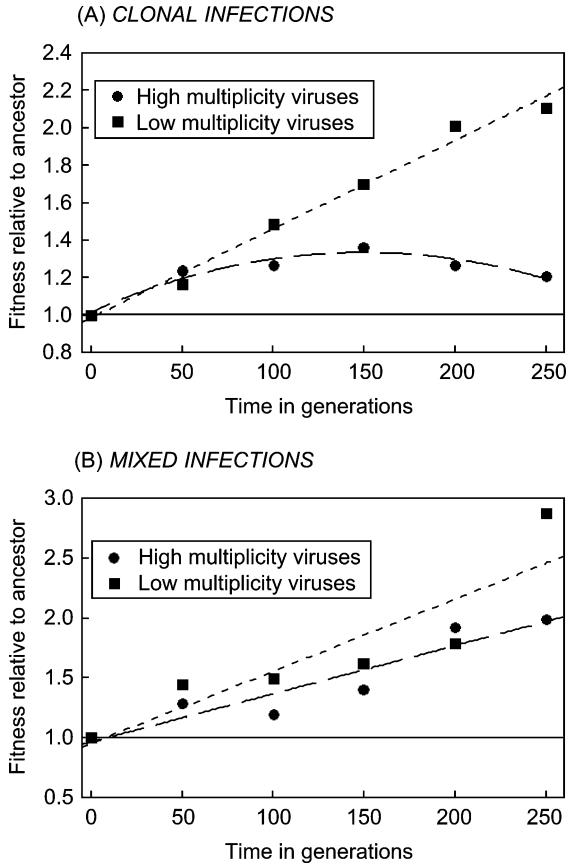

Figure 2 Results of a laboratory evolution experiment, where populations of the RNA phage $\phi 6$ were propagated for 250 generations in the presence and absence of co-infection. Viruses evolved under high multiplicity of infection (moi) experienced frequent co-infection of *Pseudomonas phaseolicola* cells, whereas those grown at low moi infected cells alone. Each point represents the grand mean fitness (relative to the ancestral wild-type virus) of three populations. (A) Traditional assays measure fitness by comparing the growth rates of evolved and ancestral viruses in strictly clonal infections. Here, the low-moi–evolved viruses (filled squares) show rapid fitness improvement through time, but their counterparts evolved at high moi perform very poorly. (B) When the fitness assays were modified to allow for co-infection, it was revealed that the high-moi–evolved viruses perform much better in assay habitats resembling their evolutionary environment. The combined results indicate that phages propagated at high moi evolve traits that are beneficial for co-infection, but which cause a performance tradeoff in environments where mixed infections are disallowed. These data suggest that the high-moi–evolved viruses adapted by acquiring traits to parasitically interact with co-infecting genotypes, at the cost of reduced fitness when infecting cells on their own. Adapted from Turner and Chao, 1998.

infections. These data clearly indicate a fitness trade-off; the viruses evolved traits specifically for co-infection, but detrimental for clonal infection (see also Sevilla *et al.*, 1998). The performance trade-off is quite obvious by late in the experiment when a downturn occurs in the fitness trajectory of high-moi–evolved viruses during clonal infections, compared with their continuing improvement in the ability to compete during mixed infections (compare Figs 2A and 2B).

The combined results can be explained by the evolution of parasitic traits in the phages propagated at high moi. The viruses obviously gain an added advantage in mixed infections, strongly suggesting that they evolved traits to selfishly sequester products that other co-infecting viruses contribute to the resource pool. The drawback to this evolved strategy is that genes for parasitism (or genes at other loci that are closely linked to parasitism genes) are inferior when the viruses compete in alternate environments where co-infection is uncommon and, hence, ecological interactions between viruses are less important. It is important to emphasize that these viruses are able to infect and replicate within cells completely on their own (Fig. 2A). Therefore, the cheating strategy is not simply due to a shortened genome, where the virus has eliminated key genes whose products are provided in trans by co-infecting helper strains (as seen in many plant and animal virus systems; Vogt and Jackson, 1999). Because the benefit of virus traits for parasitism depends on the likelihood of encountering parasitic versus cooperative genotypes during co-infection, frequency-dependent selection should determine the relative fitness of a selfish genotype.

V. FREQUENCY-DEPENDENT SELECTION AND VIRUS PARASITISM

Frequency-dependent selection occurs when the fitness of a genotype is not constant, but depends on the relative frequencies of other genotypes in the population. In inverse frequency-dependent selection, the rarer a genotype is in the population, the greater its fitness. Many biological phenomena give rise to this form of selection. In a classic example involving predation, Popham (1942) observed that aquarium fish prey selectively on one of three color morphs of the aquatic bug, *Sigara distincta*—namely, the morph that was most abundant at a given time. However, the complexity of natural environments often makes it difficult to identify the ecological mechanisms responsible for frequency-dependent selection. Laboratory experiments with microbes have been important in documenting frequency dependence (e.g., Rosenzweig *et al.*, 1994; Turner *et al.*, 1996; Elena *et al.*, 1997; Rozen and Lenski, 2000), and the tractability of these systems offers hope that researchers can decipher the underlying causes.

In environments where co-infection is common, one can predict that the fitness of parasitic viruses should be governed by inverse frequency-dependent selection. This outcome is expected because the selfish viruses should gain their greatest fitness advantage when rare; situations where the parasitic viruses are most likely to co-infect cells with ordinary genotypes and gain a large competitive advantage by sequestering products from the resource pool. In contrast, when cheater viruses are very common, they will most often co-infect cells with other cheaters and experience no benefit.

To examine this idea, we isolated individual cheater clones from the high-moi populations (Turner and Chao, 1999). We then measured the fitness of a cheater virus relative to its ancestor, when the two competitors were mixed at different initial frequencies in competition on *P. phaseolicola*. To ensure that co-infection was common, all of these fitness assays occurred at moi = 5. Results showed that the fitness of a cheater was a decreasing function of its initial frequency in competition (Fig. 3). That is, when the cheaters are rare they mostly co-infect cells with ancestral genotypes and gain a large fitness advantage, whereas when they are common they co-infect cells with other parasitic viruses and are less advantaged on average. Because all of the observed fitness values in this experiment exceed unity ($W > 1.0$), the data indicate that the cheating strategy (i.e., parasitic genotypes) should spread to fixation regardless of initial frequency. In turn, this result explains how the cheaters were able to sweep through the virus populations evolved at high moi, despite the fitness trade-off experienced by these viruses in alternate environments (Figs. 2A and 2B).

Although we demonstrated that the cheaters should be able to completely replace the ancestral genotype (Fig. 3), it is unknown whether the cheaters were the only genotypes present in the high-moi lineages at generation 250. That is, the possibility exists that the majority of cheaters co-existed with a minority population of cooperator viruses during all or a portion of the experiment. Exploring this hypothesis is extremely difficult because there is currently no genetic marker that distinguishes cheaters from other viruses that may be present in small numbers in the population. Future efforts could involve sequence analyses to look for genetic polymorphisms in the high-moi lineages, and to examine the possibility of co-existence between cheaters and helper viruses in these populations.

We conducted additional experiments at moi = 5, where the cheaters competed against viruses that evolved at low moi (i.e., in the virtual absence of co-infection according to the Poisson) (Turner and Chao, 2003). Once again, frequency dependence was observed (Fig. 3). But the decreasing fitness function for parasitic viruses in these assays was observed to cross a value of $W = 1.0$. Thus, the cheaters are shown to be advantaged when rare, but the magnitude of their fitness disadvantage when common is now so large that they are prevented from sweeping through the population. Rather,

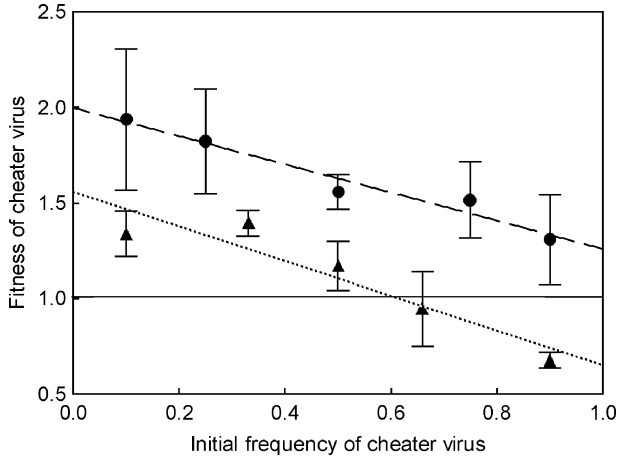

Figure 3 A parasitic virus specializes in sequestering intracellular resources provided by co-infecting ordinary genotypes. Because the benefit of parasitism depends on the relative abundance of parasitic versus ordinary viruses encountered inside the cell, the fitness of a cheater virus is expected to be frequency-dependent. Laboratory experiments prove that when co-infection is common, the fitness of an evolved parasitic phage is governed by inverse frequency-dependent selection; each point represents the mean and standard error of replicate fitness assays, initiated at different initial frequencies of competing viruses. The dashed and dotted lines indicate the best linear fit to each respective dataset. When the cheater phage is competed against its wild type ancestor (filled circles), it is most advantaged when rare because it primarily encounters the ordinary genotype inside the cell; however, the advantage diminishes at higher initial frequencies as the cheaters more often encounter one another during mixed infections. Because all of these data exceed 1.0, parasitic viruses (i.e., the cheating strategy) should always fix when invading a population of wild types. In contrast, when the parasitic phage is competed against an evolved cooperator (filled triangles) the frequency-dependent result crosses 1.0, indicating that each genotype is expected to invade when rare. For this reason, both viruses (strategies) should co-exist in a mixed polymorphism. Adapted from Turner and Chao, 1999, 2003.

if the two genotypes are together in the same population either can increase in frequency when rare, resulting in their co-existence in a mixed polymorphism. The data in Fig. 3 can also be used to predict the equilibrium frequencies of the two genotypes in the mixed population; the linear regression for these data intercept $W = 1.0$ when the initial frequency of cheaters equals 0.62. Therefore, it is predicted that the equilibrium percentage for cheaters in the population should be 62% (Turner and Chao, 2003).

Why are the parasitic viruses able to displace their ancestor but unable to sweep through a population of low-moi–evolved viruses? As previously described, viruses that specialize in overproducing intracellular products

are effectively cooperators, whereas those that specialize in sequestering products are cheaters. The previous experiment allowed the viruses in the low-moi treatment to be grown under strictly clonal conditions (absence of competitive interactions), creating the opportunity for greater cooperation to evolve (Turner and Chao, 1998). One possibility for the polymorphism observed in Fig. 3 is that the low-moi viruses evolved to synthesize extremely large quantities of intracellular products that are made available in the intracellular resource pool. These over-abundant resources could decrease the sensitivity of the low-moi viruses to parasitism. Therefore, the ancestral population might succumb to invasion by cheaters, whereas the derived cooperators could tolerate a subpopulation of selfish individuals.

The mechanism for cheating in the high-moi–evolved genotypes is unknown. One possibility is that during intracellular replication the RNA of cheater genotypes is preferentially packaged into protein capsids of the virus progeny. The cheaters may be rather inefficient at producing capsids (or other key virus products) when they infect cells on their own, as suggested by their reduced competitiveness in clonal infections (Fig. 2A). However, the cheaters may gain a competitive edge during mixed infections if their genetic material experiences biased entry into capsids produced by other co-infecting strains. The encapsidation process in ϕ 6 is well-described (Qiao *et al*., 2003), and a packaging preference might result from a duplication or other change in the *pac* region that governs binding and entry of RNA to capsids. Through this hypothesized mechanism, a selfish virus would reduce the probability that other co-infecting genotypes contribute the expected share of their genes to the progeny. This idea is supported by data from animal virus systems, because certain defective (non full-length) RNA viruses feature gene duplications involved in encapsidation, which provide these cheaters with a replication advantage over their co-infecting helper viruses (Holland, 1991).

VI. HOW MANY VIRUSES SHOULD ENTER A CELL?

Our studies and others suggest that co-infection can be costly to an individual virus, due to the presence of parasitic viruses that selfishly appropriate intracellular products (Turner and Chao, 1998; Hammond *et al*., 1999; Vogt and Jackson, 1999). However, there are clear benefits associated with co-infection, such as enhanced pathogenicity or greater transmissibility of the co-infecting strains (Cohen, 1998; Lopez-Ferber *et al*., 2003). Perhaps the most intriguing benefit of co-infection is that it allows viruses to generate novel variants through genetic exchange otherwise known as sex (Chao *et al*., 1997; Nagy and Simon, 1997), where sex can be broadly defined as any exchange of genetic material between individuals (Michod and Levin,

1988). Under this definition, sex extends beyond the framework of obligately sexually reproducing populations composed of males and females, to include macro- and micro-organisms that asexually reproduce but also feature mechanisms for inter-individual genetic exchange (such as transduction, transformation, and conjugation in bacteria). In any such population, sex promotes linkage equilibrium, free association between alleles at two or more loci. The presumed advantage of sex is that it reduces the frequency of suboptimal allele combinations, and can potentially increase the frequency of superior allele combinations (Fisher, 1930; Muller, 1932; see also Barton and Charlesworth, 1998, and West *et al*., 1999 for reviews of debates on the origin and maintenance of sex). Thus, along with spontaneous mutations, sex generates the population genetic diversity which provides the raw material for evolution by natural selection.

Viruses experience genetic exchange through two basic mechanisms. In DNA viruses, sex is promoted exclusively by recombination (generation of a new nucleotide strand from two or more parental strands). Some RNA viruses also undergo recombination, but others do not. Instead, genetic exchange occurs because the genome is divided into several smaller RNA molecules (segments), allowing formation of hybrid progeny containing a random re-assortment of segments descending from two or more co-infecting parent viruses. For example, ϕ6 and its relatives in the family *Cystoviridae* feature genomes divided into three segments, and co-infection of the same cell by multiple genotypes yields reassortants (Mindich *et al*., 1999). Interestingly, recombination between segments is rare or non-existent in many segmented RNA viruses including ϕ6 and the majority of cystoviruses (Horiuchi, 1975; Mindich *et al*., 1976, 1999), fueling arguments that segment reassortment might have evolved as an alternative to recombination for the purpose of promoting sex in some viruses (Pressing and Reanney, 1984; Chao, 1988).

If sex is advantageous in virus populations (Turner, 2003), one adaptive compromise would be virus evolution which would balance the positive and negative consequences of co-infection; viruses would limit the number of co-infecting particles to reduce intra-host competition, but allow entry by more than one virus to achieve genetic exchange. This hypothesis is intriguing, but has not yet been explicitly tested. Indirect support for an adaptive compromise could be the exclusion mechanism in phage ϕ6 where three viruses (on average) are allowed to enter the cell. Olkkonen and Bamford (1989) used radioactive labeling of viruses to show that a limit to co-infection exists in phage ϕ6. In particular, their experiments used the amount of incorporated carbon-14 label as a measure of the number of ϕ6 phages entering a *P. phaseolicola* cell.

We sought to confirm the estimated limit using a simpler method that is easily adapted to a variety of viruses and culture conditions (Turner *et al*.,

1999). To do so, we devised a method that compares the frequency of hybrids produced by two marked phage strains to that predicted by a mathematical model based on differing limits to co-infection. Wild type ϕ6 may be symbolized as +/+/+, corresponding to segments L (large), M (medium), and S (small). We studied two ϕ6 strains, +/β/+ and β/+/+, with an engineered marker (β) (Onodera *et al*., 1993) on the M and L segments, respectively (Turner *et al*., 1999). The β marker encodes the alpha-subunit of the beta-galactosidase (β-gal) gene from the bacterium *Escherichia coli* (Horwitz *et al*., 1964). The marker is highly stable when inserted into the M or L segment, evidenced by its very low rate of mutational reversion (Turner *et al*., 1999). Marked phages form blue plaques on selective plates containing the chemical X-gal and *P. phaseolicola* carrying a plasmid that encodes the beta-subunit of the β-gal gene (Onodera *et al*., 1993).

To measure the limit to co-infection in ϕ6, we conducted experiments that crossed the *β-gal*–marked strains at moi = 0.02, 1, 2, 3, 4, 5, 10, and 25 on *P. phaseolicola* (Turner *et al*., 1999). As moi increases, hybrid frequency also should increase to a maximum that is determined by the limit of co-infection. A cross between marked strains produces six possible hybrids, but only the +/+/+ reassortants were monitored because they are easily distinguished on selective plates. In theory, if phage fitnesses are equal, a maximum frequency of +/+/+ progeny is reached when cells are infected with an equal number of marked phage [frequency (+/β/+) = frequency (β/+/+) = 0.5]. Here, the probability that progeny will acquire both + segments is obtained by simply multiplying the frequencies of the two segments: $0.5 \times 0.5 = 0.25$. Only if the limit is infinitely large (i.e., no limit) will the ratio of marked viruses within a given cell approach 1:1. Thus, as the limit decreases, so will the maximum frequency of +/+/+ progeny. At a limit of one, no hybrids are formed regardless of moi. To determine the limit in ϕ6, we compared our observed hybrid frequencies to those expected based on a theoretical model (Turner *et al*., 1999).

Our model (Turner *et al*., 1999) predicts the frequency (H) of any one hybrid over a range of multiplicities in crosses between two marked viruses according to the Poisson (Sokal and Rohlf, 1995). Because genetic markers are often deleterious and may be prone to revert, the model incorporates several fitness parameters that adjust H if marked segments experience a replication disadvantage relative to wild type, and if marker reversion (even at low rates) occurs. Figure 4 shows a series of curves generated by the model for expected frequencies of +/+/+ hybrids over a range of multiplicities in crosses between beta-marked phages of ϕ6. Expected values of H asymptote above 0.25 because of the fitness disadvantage suffered by marked phage and segments (Turner *et al*., 1999). Observed data reveal that hybrid frequency increases with moi but reaches a plateau of $H = 0.23$. The observed maximum matches the mathematical model for a limit to co-infection between

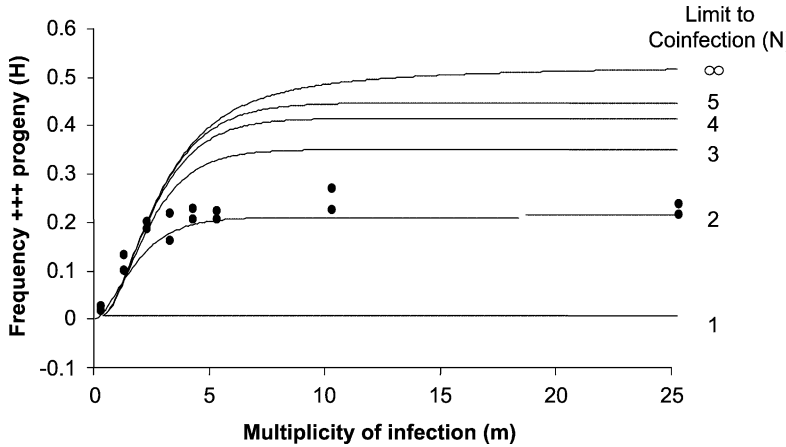

Figure 4 Laboratory experiments reveal that a limit to co-infection exists in phage ϕ6. A comparison was made between expected and observed values of H, the frequency of +/+/+ hybrid reassortants generated in crosses between two marked genotypes (β/+/+ and +/β/+). Theoretical curves were generated by a mathematical model that predicts H over a range of multiplicities of infection (m), assuming a defined limit to co-infection (N). Expected values for H are adjusted if marked segments experience a replication disadvantage relative to wild-type (see text for details). Observed values of H were generated in two replicate experimental crosses between the marked phages at 8 moi; each point represents an independent estimate. Observed H saturates at a value corresponding to a limit of between two and three phages per cell. Adapted from Turner *et al.*, 1999.

two and three phages per cell, and confirms the previous estimate of three phages per cell by Olkkonen and Bamford (1989).

Although the limit is firmly established in phage ϕ6, details of the phenomenon are fodder for future studies. If the limit is an adaptive compromise, mean fitness of a virus population should be maximal when an intermediate number of viruses co-infect a cell. By definition, this requires that variation in the co-infection number be at least partly due to genetic differences among viruses (i.e., due to a genetically determined viral mechanism). In contrast, the phenomenon could be due to ecological interactions between viruses and the host cell. For instance, it might be that too many viruses attaching to a cell breaks down the integrity of the cell wall, thus destroying the cell before virus replication can be completed. If the latter is true, benefits of co-infection may lead to directional selection to increase co-infection number, but the upper threshold for virus entry prevents balancing selection because large numbers of viruses never enter the cell. However, this scenario seems unlikely for ϕ6 because as many as 50 viruses can attach to a cell, but only 3 can enter (Olkkonen and Bamford, 1989). Limits are widely observed in

viruses (e.g., Lu and Henning, 1994; Singh *et al*., 1997), although it is sometimes very difficult to elucidate the underlying mechanism that prevents an overabundance of viruses from entering. When the mechanisms have been unraveled they are demonstrated to be under strict virus control, and this overwhelmingly suggests that the limit in ϕ6 is virus determined as well.

Future experiments on viral limits could involve a comparative approach using ϕ6 and its relatives. For example, one could hold the host bacterium (such as *P. phaseolicola*) constant, and measure the limit for all nine members of the family *Cystoviridae*. These assays would inform whether the limit on a single host is highly conserved within this evolutionarily related group; if not, the results would suggest that the particular ecology of the virus-host cell interaction is very important for defining the limit. For example, some cystoviruses (such as ϕ6) appear to use the type IV pilus of a host bacterium as a site of initial attachment, whereas others enter directly through the cell membrane (Mindich *et al*., 1999). Perhaps the difference in surface area available for attachment also translates to a difference in the number (limit) of viral particles able to infect the cell. As previously mentioned, the natural host for each member of the *Cystoviridae* has yet to be determined. But measurements of the limit for each virus in its primary host would be most informative for elucidating how the natural ecology impacts virus population genetics. For example, in some viruses there may be restrictions to the genetic variation created through reassortment simply because the limit to co-infection lies close to a value of one (on average). The ultimate goal would be to study limits to co-infection when virus growth occurs under more natural conditions, such as in infections of plant pathogenic bacteria that are themselves parasitizing plants grown in a greenhouse.

Because segment reassortment occurs in ϕ6, possibilities for genetic exchange can impact the interpretation of our data in the study where virus lineages were propagated in the laboratory at high moi (Turner and Chao, 1998). In particular, it is a challenge to hypothesize how a selfish *genotype* of the virus can evolve in a high-moi environment where reassortment readily occurs. That is, parasitism might occur through some gene that promotes cheating, and the mutated gene would be tightly linked to other genes residing on the same segment because recombination appears absent in ϕ6. However, the mutated gene should not be tightly linked to genes located on the other two RNA segments, due to the phenomenon of reassortment. For this reason, we have suggested that it may be more appropriate to speak of cheater 'segments' in ϕ6 rather than cheater 'viruses' (Turner and Chao, 2003). The encapsidation mechanism previously proposed would still apply because parasitism might evolve if a cheater gene biased the entry of its resident segment into available capsids, a process analogous to meiotic drive in eukaryotes. An intriguing possibility is that the cheater segments or viruses may feature a limit to co-infection that differs from wild type ϕ6;

for instance, the limit may be increased in the derived viruses due to the benefit that results from co-infection with other genotypes. This idea is highly speculative, and relies on the untested assumption that the limit is at least partially controlled by virus genes.

VII. PARASITISM IN PLANT AND ANIMAL VIRUSES

Viruses of plants, humans, and other animals are very well-studied because they can be infectious agents of agricultural, medical, and veterinary importance. Most often these viruses are examined under artificial conditions (such as in laboratory tissue culture) that differ dramatically from their natural ecological settings. When viruses are propagated in laboratory environments where co-infection is common, the evolution of parasitic viruses is often observed. These cheating viruses tend to be shortened forms of the original virus and are generally known as defective-interfering (DI) particles (terminology coined by Huang and Baltimore, 1970). Here, 'defective' refers to their lack of essential genes or parts of the genome that were present in the parental virus. DIs 'interfere' specifically with co-infecting ordinary (helper) viruses by replicating at their expense, usually resulting in reduced titers of the ordinary viruses relative to their reproductive output when DIs are absent. Interestingly, recent evidence shows that DI-helper interactions can be mutualistic, at least in the case of an insect baculovirus that increases the pathogenicity of the DI-helper virus population (Lopez-Ferber *et al*., 2003); future work will determine whether this one reported case is a rare outlier.

In essence, the vast majority of DIs are parasites of parasites, because they rely on one or more proteins synthesized by co-infecting helpers. Use of these intracellular products by DIs allows them to achieve high frequencies in virus populations because their shortened length affords an advantage in terms of rates of genome replication relative to helper viruses. Antagonistic DIs are particularly well-described in vesicular stomatitis virus (VSV), an RNA virus that is transmitted by several insects including mosquitoes, and which infects a wide variety of mammals. VSV has a rich history as a preferred model to study the molecular genetics and experimental evolution of RNA viruses (Holland *et al*., 1991; Turner and Elena, 2000; Rose and Whitt, 2001). But when particles of VSV greatly outnumber their host cells in laboratory tissue-culture experiments, this produces strong selection for DIs (e.g., Li and Pattnaik, 1997; Whelan and Wertz, 1997). Figure 5 depicts one example of the interaction between ordinary (full-length) VSV and an antagonistic DI particle. A thorough description of the varied plant and animal systems where defective (shortened) viruses have been observed extends beyond the space available in this chapter; several recent papers

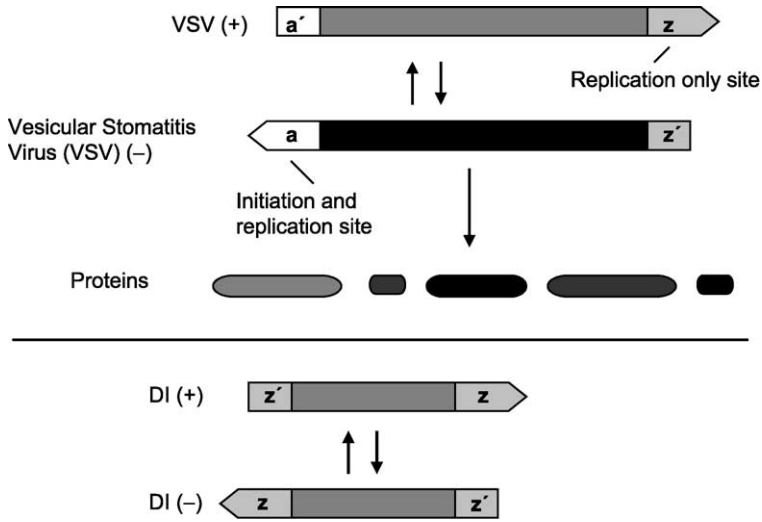

Figure 5 VSV is a single-stranded negative-sense RNA virus, which infects certain insects and mammals. Growth of VSV under high levels of co-infection in the laboratory strongly selects for parasitic defective-interfering (DI) particles. When VSV enters the cell it transcribes viral RNA to make monocistronic mRNAs to produce proteins. However, VSV also replicates by serving as the template for the complementary positive-strand genome, VSV(+), which is used to make more VSV(−). The 3′ terminus of VSV(−) contains an initiation site (**a**) for both transcription and replication, whereas the 3′ terminus of VSV(+) has a site (**z**) for only replication. The complementary sequences of **a** and **z** on the opposite strands are **a**′ and **z**′. DI particles of VSV are typically shorter and lack various protein-coding sequences. In this particular example, the DI particle has a **z** replication site at the 3′ terminus of both its negative and positive strands. Whereas VSV(−) allocates time for transcription, DI(−) does not and is effectively equal to DI(+). Thus, the DI RNA relies on the complete virus to provide proteins, but it has a higher replicative fitness than the VSV genome. Adapted from Turner, 2003.

describe DIs and other defective viruses in these systems (Holland, 1991; Vogt and Jackson, 1999).

Obviously, the long-term persistence of DIs depends on the continued presence of ordinary viruses. But persistence of the helpers is unlikely because DIs typically experience a replication advantage over full-length viruses and may easily out-compete their helpers. Elimination of the helpers results in extinction of the virus population because DIs cannot replicate on their own. Thus, the appearance of DIs in laboratory cultures is often viewed as a nuisance (Holland, 1991). The long-term dynamics of DI-helper virus interactions have received theoretical treatment (Nelson and Perelson, 1995; Frank, 2000) but have rarely been studied in empirical detail (but see e.g., Duhaut and Dimmock, 1998). Unfortunately, the appearance of DIs may be

unavoidable in certain industrial microbiology applications (e.g., virus vaccine production), because the manufacturer of high virus titers (densities) is commercially desirable but can lead to high levels of DIs that reduce titers of ordinary viruses. Beyond the realization that DIs easily occur in laboratory and other artificial environments, there is a pressing need to establish whether DIs are also prevalent in natural populations of viruses. For DIs to flourish in the wild, co-infection must be common enough that viruses lacking essential genes can readily encounter their helpers. Evidence suggests that this condition may be fulfilled in certain systems, because helper-dependent (but non-DI) viruses can be isolated from wild populations of infected animals and plants (see Vogt and Jackson, 1999).

VIII. CONCLUDING REMARKS

As the aforementioned studies demonstrate, recent laboratory experiments with phage ϕ6 are highly valuable for elucidating seldom-studied aspects of phage ecology. Laboratory studies in the evolutionary ecology of viruses can be extremely informative, but even more can be gained by examining the ecology of viruses in the wild. Of course, the latter is extremely challenging due to the complexities and uncontrolled factors inherent to field studies. But worthwhile data may be obtained by combining the study of virus ecology with modern molecular techniques. For example, one can acquire natural samples of viruses, isolate viral DNA or RNA and conduct sequence analyses to examine genetic relatedness within and between samples from given locales (e.g., Paul and Kellog, 2000; Breitbart *et al*., 2002). In addition, valuable data would be biotic factors such as the host genotype or species from which the virus was isolated, as well as abiotic factors such as the local climate or pH at time of isolation. The information can then provide a context for relating the impact of environment on estimates of genetic diversity in the natural samples.

Viruses are so abundant in natural systems that they can influence large-scale ecological processes, such as nutrient cycling and bacterial biodiversity in marine environments (Fuhrman, 1999). The role of parasitic or other interactions between viruses in these ecosystem-level phenomena is unknown. Many viruses are multipartite, featuring genomic segments that are packaged into separate virus particles (Knipe and Howley, 2001). Obviously, frequent co-infection of cells is required to evolve and maintain multipartite genomes, providing further indication that ecological interactions between viruses are not uncommon. For these reasons, it is easy to argue that much greater effort should be placed in examining virus ecology and in determining the influence of virus co-infection on host disease and the evolution of virus traits.

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