

Virus Evolution: Insights from an Experimental Approach

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adaptation, epistasis, error threshold, quasi-species, robustness, trade-offs

Abstract

Viruses represent a serious problem faced by human and veterinary medicine and agronomy. New viruses are constantly emerging while old ones evolve and challenge the latest advances in antiviral pharmaceuticals, thus generating tremendous social alarm, sanitary problems, and economical losses. However, they constitute very powerful tools for experimental evolution. These two faces of virology are tightly related because future antiviral treatments shall be rationally designed by considering evolutionary principles. Evidence indicates that the evolution of viruses is determined mainly by key features such as their small genomes, enormous population sizes, and short generation times, and at least for RNA viruses, large selection coefficients, antagonistic epistasis, and high mutation rates. We summarize recent advances in the field of experimental virus evolution. Increasing our understanding of the roles of selection, mutation, chance, and historical contingency on the ecology and epidemiology of viral infections could determine our ability to combat them.

RdRp: RNA-dependent RNA polymerase

VSV: vesicular stomatitis virus

Fitness: the number of descendants an individual generates per time unit, usually relative to that of a reference genotype

Complementation: interaction between two viral genomes within an infected cell such that the virus can function despite each genome carrying different mutated, nonfunctional genes

INTRODUCTION

Viruses, and in particular those having RNA as genetic material, are the most abundant parasites infecting animals, plants, and bacteria (Domingo & Holland 1997). Despite tremendous economical efforts, the number of eradicated viruses is quite limited and the perspectives for future eradications would most likely be overbalanced by the emergence or reemergence of other viruses (Murphy & Nathanson 1994). The fact that few viruses can be effectively controlled with state-of-the-art pharmacology, as well as the pervasive emergence of new viruses, could be a consequence of both the intrinsic RNA virus ability to evolve and the human-induced alterations in viruses' natural ecosystems. Immune escape strains and strains resistant to antivirals may arise soon after challenged by the immune system or drugs (Althaus & Bonhoeffer 2005, Kalia et al. 2005), and viruses may jump the species barrier from their natural reservoir host to a naïve one (Daszak et al. 2000, Kuiken et al. 2006).

The interest in studying the evolution of RNA viruses is not only motivated by the need to develop new rational antiviral strategies. They also constitute useful tools for experimentally addressing fundamental evolutionary questions while still allowing one to pay attention to the molecular details. After decades of research, some seemingly general properties of RNA viruses have been established. (*a*) As a consequence of the lack of proofreading activity in their RNA-dependent RNA polymerase (RdRp), genomic mutation rates are, on average, in the range 0.13–1.15 (Drake & Holland 1999). (*b*) Genomes are small [e.g., 3569 nt for MS2 to 11,162 nt for vesicular stomatitis virus (VSV)], with many examples of overlapping reading frames and multifunctional proteins. (*c*) Replication rates are fast, reaching tremendous population sizes shortly after infection. (*d*) Variability is a key factor for pathogenicity (e.g., immune escape, antiviral resistant, or host-range mutants). (*e*) Abundant molecular, functional, and structural information makes it relatively easy to map genotypes into phenotypic space. (*f*) The average fitness effect of point mutations are large (~20%, with up to 40% lethals for VSV; Sanjuán et al. 2004a) and the average interaction between deleterious mutations antagonistic, a hallmark of nonrobust genomes (Elena et al. 2006).

In this review, we summarize recent advances in the field of experimental virus evolution, focusing not only on the experiments but also on the underlying evolutionary theories. Particular emphasis is on the dual role of mutation and its interplay with population size. At large population sizes, evolutionary dynamics are driven by competition between beneficial variants. At small population sizes, deleterious mutations have a chance to spread and may drive extinction or the evolution of robustness mechanisms. One such potential mechanism discussed, complementation during multiple infections, allows for the evolution of social behavior, which is briefly discussed at the end of the review.

The Molecular Quasi-species and the Classical Population Genetics Frameworks

Virologists usually, although not always in a precise way, employ the term quasi-species to refer to highly polymorphic viral populations. The quasi-species theory

(Eigen et al. 1989) describes the evolution of an infinite asexual population with short genomes and high error rates. Eigen and coworkers studied mutation-selection dynamics and found that populations reached an equilibrium composed by a polymorphic assemblage of mutant genomes with a rare wild type. Nonetheless, it is worth highlighting that most of the quasi-species formulation is fully equivalent to the classic mutation-selection balance for haploid asexuals developed long ago within the framework of population genetics (Wilke 2005). However, the quasi-species theory has made some specific and relevant predictions (Eigen et al. 1989): (a) There exists an error threshold beyond which selection cannot further maintain the genetic structure of the population, the wild type is lost, and the population randomly drifts in genotypic space; (b) selection does not act on individual genomes but on the cloud of closely related genomes, implying that at high mutation rates a slow-replicating quasi-species can outcompete a faster-replicating one if the first is more robust to deleterious mutational effects (van Nimwegen et al. 1999, Wilke 2001).

Error threshold: critical mutation rate beyond which selection cannot further maintain the information encoded in the genome

THE ADAPTIVE PROCESS

The Dynamics of Adaptation

Similar to other microbes, a common observation in long-term evolution experiments with viruses is fitness trajectory in which gains are initially rapid but tend to decelerate over time (Burch & Chao 1999, Elena et al. 1998, Novella et al. 1995, Wichman et al. 1999) (**Figure 1**). Such dynamics indicate that, after being placed in a new environment, populations are evolving from a region of low fitness toward an adaptive peak or plateau. What determines the rate of adaptation? What kind of molecular changes are associated with fitness increases? These questions are explored below.

In negative-stranded RNA viruses, the genome is complementary to the messenger RNA and hence is not directly translated. In this kind of virus, recombination is rare (Chare et al. 2003), probably owing to the tight binding between viral ribonucleoproteins and genomic RNA, and thus populations behave truly clonally. An important consequence of asexuality is clonal interference. Clones that carry different beneficial mutations compete with one another, thereby interfering with their spread and substitution in the population (Gerrish & Lenski 1998). In general, all but one lineage will be excluded by the one with the most beneficial combination of mutations. Clonal interference becomes increasingly intense for large populations and high mutation rates and thus should be relevant to the evolution of RNA viruses. Its evolutionary consequences include the following: (a) the probability of substitution of a given beneficial mutation declines with increasing population size or mutation rate, but the individual substitutions entail larger fitness gains, (b) the rate of fitness improvement shows diminishing returns with an increasing supply of beneficial mutations caused by large population size or high mutation rates, and (c) many beneficial mutations become transiently common only to be excluded later by interfering mutations, giving rise to a leapfrog event in which the most abundant genotype at a given moment is phylogenetically related to an earlier dominant time than to the immediately preceding one.

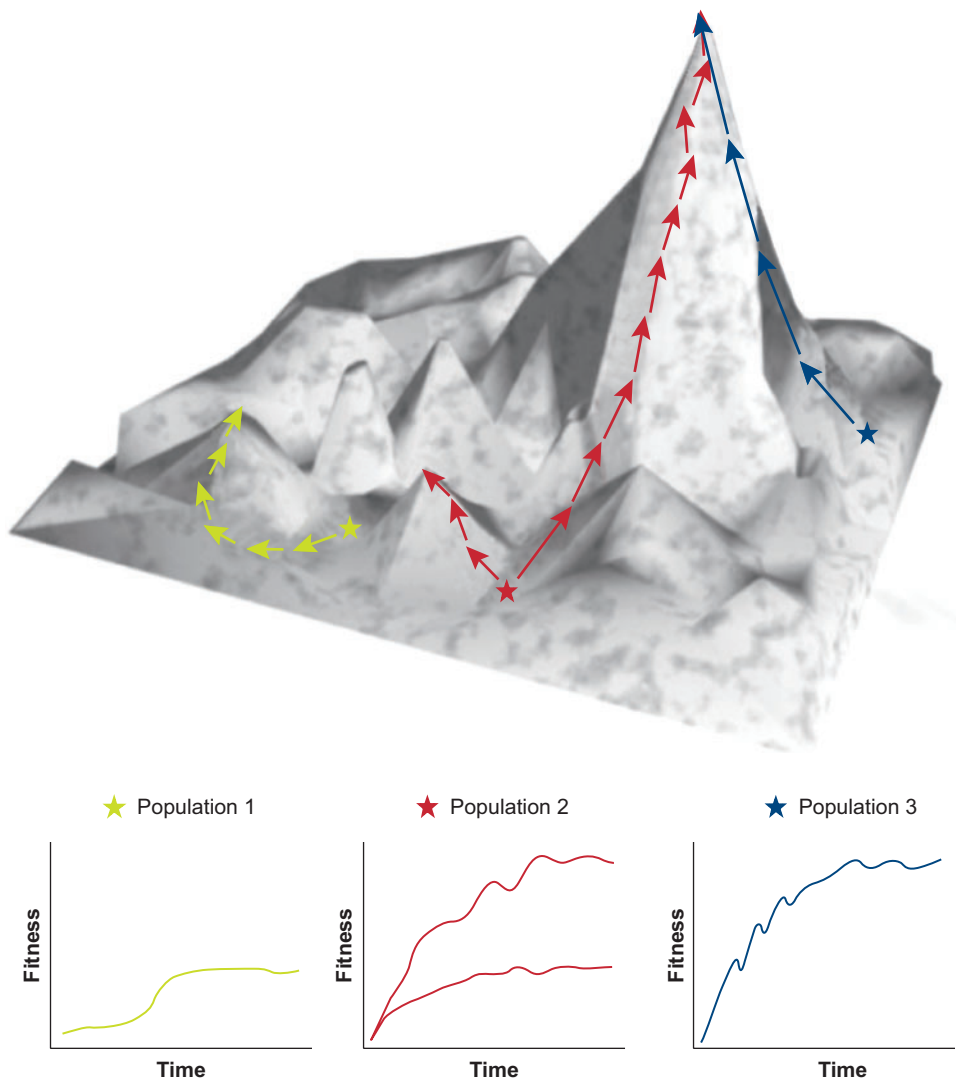


Figure 1

Trajectories of virus fitness evolution. The evolutionary trajectory of three independent populations is represented in a hypothetical rugged fitness landscape by arrows and lines of different colors. Populations starting from different points can reach the same adaptive peak, in a case of convergent evolution (*population 3 and high population 2 dynamics*), and in this particular case, the global optimum. Starting from the same point, replicate populations can diverge into two different trajectories and reach completely different adaptive peaks (*population 2 dynamics*) depending upon the availability of beneficial mutations. A population can be trapped in a local adaptive peak (*population 1 and low population 2 dynamics*).

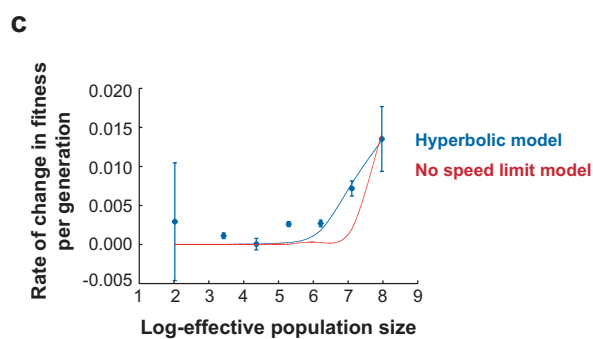
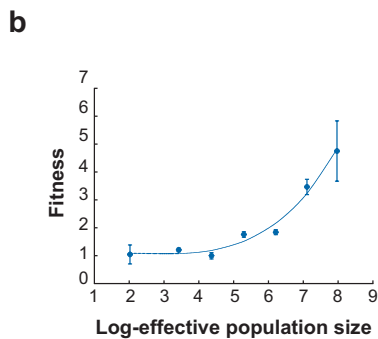
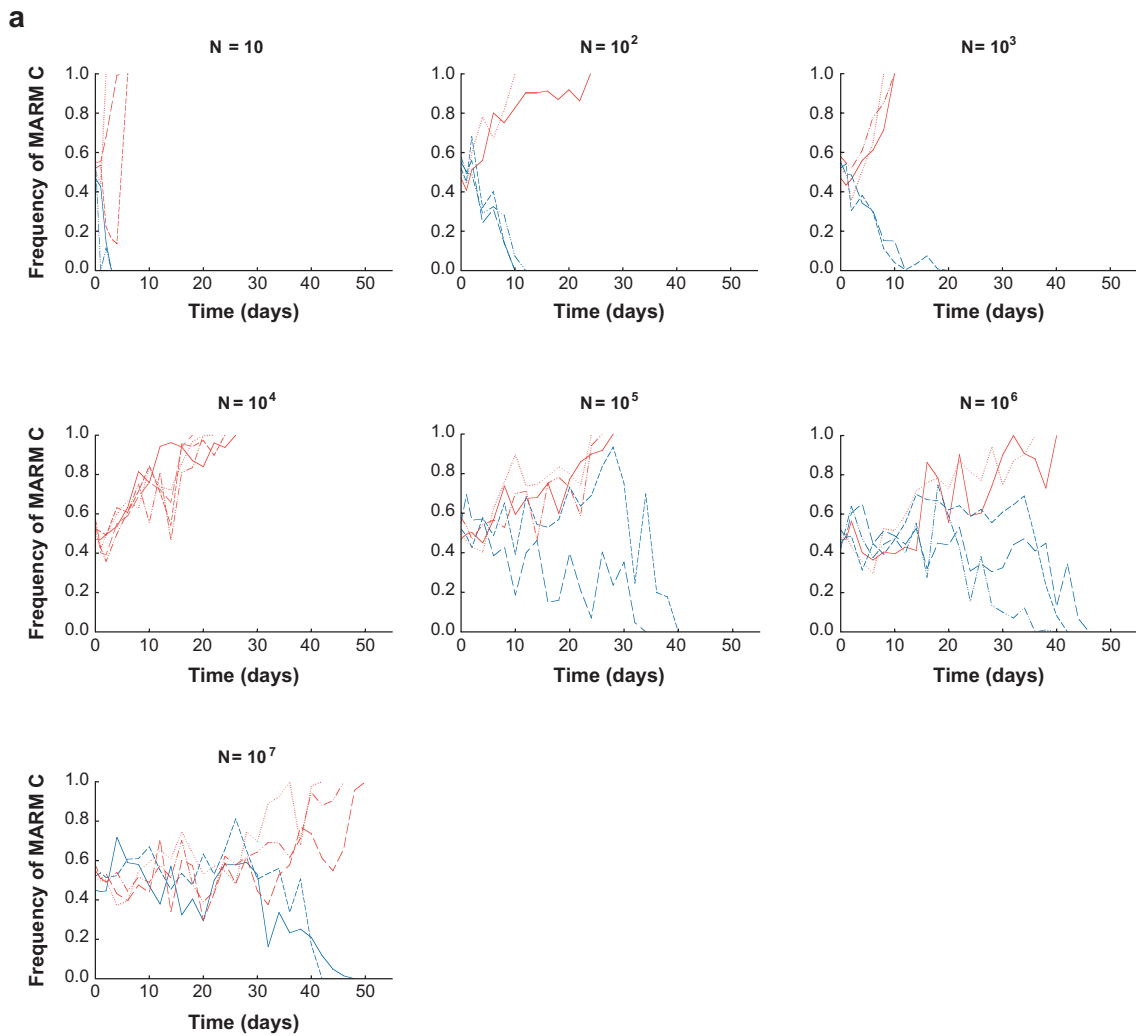
The first two predictions have been confirmed for RNA viruses. Clarke et al. (1994) and Quer et al. (1996) observed that when two equally fit clones of VSV competed, they coexisted for long periods of time until coexistence was suddenly broken and one of them was displaced by the other. Interestingly, clones of both competitors isolated right before the displacement showed an increase in fitness relative to their ancestors, suggesting the existence of an arms race between both competitors, although the reasons for coexistence breaking remained unclear. Later, Miralles et al. (1999, 2000) expanded this study. Two genotypes of VSV carrying distinguishable states of a neutral marker were mixed at equal frequency and allowed to compete at increasing population sizes. Increasing population size has the effect of increasing the number of available beneficial mutations and thus strengthens clonal interference. **Figure 2a** shows the results of the competition dynamics. After one of the two competitors got fixed in the population, the fitness effect of the mutation responsible for the fixation was measured. The magnitude of the fitness effect fixed increased with the intensity of clonal interference, as predicted above (**Figure 2b**). Furthermore, population size had the predicted diminishing-returns effect on the rate of adaptation (**Figure 2c**). With a similar experimental design, Burch & Chao (1999) showed that in $\phi 6$ the beneficial effect of the fixed mutations was proportional to population size (that is, the intensity of clonal interference). Finally, Wichman et al. (2005) showed that clonal interference among co-infecting genotypes of $\phi X174$ was coupled with a sustained molecular evolution after 13,000 generations.

All of these experiments create a picture in which adaptive evolution of RNA viruses occurs throughout the competition of large numbers of variants created continuously by the error-prone replication of their genomes, and only the fittest available genotype will succeed in the population. However, this competition process between multiple genotypes has a diminishing-returns effect on the rate of virus adaptation.

The Molecular Basis of Adaptation

One of the goals of modern evolutionary biology is to learn about the molecular basis of the adaptive process, something that can be easily achieved with the use of RNA viruses as model systems. Perhaps one of the most amazing realizations after sequencing virus lineages evolved in a common environment is the large amount of evolutionary parallelisms and convergences, both at synonymous and nonsynonymous sites (Bull et al. 1997, Cuevas et al. 2002, Novella & Ebendick-Corpus 2004, Wichman et al. 1999). Furthermore, this phenomenon is not exclusive of experimental evolution but also widespread and observed across human immunodeficiency virus type 1 (HIV-1)-infected patients treated with the same antiviral drug (Martínez-Picado et al. 2000), where not only do the same mutations arise, but they do so in a conserved order (Boucher et al. 1992). Although these results could in principle be explained from a neutralist point of view as resulting from mutational bias, it is more likely that parallel and convergent substitutions are adaptive. This pattern would result from organisms facing identical selective pressures, with few alternative adaptive pathways, as expected for viruses' simple and compacted genomes. In addition, the

HIV-1: human immunodeficiency virus type 1



observation that beneficial mutations often become fixed in an ordered way supports clonal interference (Gerrish & Lenski 1998).

Convergence at nonsynonymous sites tells us that RNA itself can be the target of selection. Genomic RNA is involved in many RNA-RNA and RNA-protein interactions, and contains regulatory signals that affect its own replication, transcription, and encapsidation (reviewed in Novella 2003). Furthermore, RNA silencing, at least in plants, acts as an elaborate and adaptive antiviral response (Lecellier & Voinnet 2004), which depends upon sequence complementarities between the target RNA to be sliced and the small interfering RNAs (siRNAs) guiding the nuclease of the RNA-induced silencing complex. Under the selective pressure imposed by RNA silencing, RNA viruses have evolved strategies to avoid it. One such strategy is the evolution of proteins with the ability to suppress the silencing response (Voinnet et al. 1999). An alternative is to get involved in a run-away strategy in which changes in synonymous sites would be constantly selected to minimize homology with siRNAs.

Epistasis and Pleiotropy Generate Trade-Offs on the Rate of Adaptation

Despite the adaptability of viral populations, theoretical considerations suggest that some sort of evolutionary constraint may exist for adaptation as a consequence of environmental, selective, genetic, or functional trade-offs. One source of genetic trade-offs is epistasis. The nature, frequency, and intensity of epistasis are barely known, despite their importance for many evolutionary theories seeking to understand the evolution of genetic systems. Viruses may shed light onto the epistasis problem owing to their easy-to-handle genomes. In particular, site-directed mutagenesis of infectious cDNAs allows creating large numbers of genomes carrying known numbers of mutations and analyzing the fitness effects of each mutation independently as well as in combination. Consequently, in recent years, multiple studies have converged to a common picture in which antagonistic epistasis among pairs of mutations is the rule for RNA genomes (Bonhoeffer et al. 2004, Burch & Chao 2004, Sanjuán et al. 2004b). Other implications of antagonistic epistasis are discussed below in the context of mutational robustness. Here, we focus only on their effect on the rate of viral evolution.

Synergistic epistasis should accelerate the rate of evolution, compared with the case of multiplicative fitness effects, because of the extra fitness differential recovered after the fixation of the two mutations. Conversely, antagonistic epistasis should slow

Antagonistic epistasis:

when the combined effect of mutations is weaker than expected from their individual effects

Mutational robustness:

the constancy of phenotypic expression in the face of mutation

Synergistic epistasis:

when the combined effect of mutations is stronger than expected from their individual effects

←

Figure 2

The effect of clonal interference on the evolutionary dynamics of VSV populations.

(a) Temporal dynamics of the two competitors until one of them gets fixed. Each competitor is represented by a different color. (b) Correlation between the magnitude of the fitness effect fixed and the intensity of clonal interference. (c) Diminishing-returns effect of population size on the rate of viral adaptation. The lines represent the fit to a model with a limit in the rate of an adaptation (hyperbolic) model and the fit to a linear (no speed limit) model. (Panels b and c are taken from Miralles et al. 1999.)

Arboviruses: viruses that use arthropods as vectors of transmission

down adaptation. Sanjuán et al. (2005) tested these predictions by constructing VSV genotypes that carried pairs of deleterious mutations showing different sign of epistasis. In this experimental setting, adaptation consisted mainly of the compensation of the artificially introduced deleterious mutations. As predicted, a negative correlation was observed between the magnitude of fitness improvement and the sign of epistasis: On average, larger fitness increases were associated with pairs of mutations that interacted synergistically, whereas smaller fitness increases were associated with antagonistic epistasis.

In general, differences in cell types and tissues within a given host, differences in host species, or the presence/absence of antiviral responses all represent instances of environmental heterogeneity. Fluctuation between different hosts is an important component of the infectious cycle of arboviruses such as VSV or Easter equine encephalitis virus. Arboviruses are transmitted among vertebrate hosts by insect and tick vectors. Although some can persist by vertical transmission from female arthropods to their offspring, most replicate alternately in vertebrate and vectors during horizontal transmission cycles. Host radiation allows a virus to expand its ecological niche by adapting to one or more novel hosts. With trade-offs, constant environments promote the evolution of specialists, whereas changing environments favor generalists, even if these had suboptimal fitness compared with specialists. Without trade-offs, a single genotype would be expected to prevail in all cases. A simple mechanism for such trade-offs is antagonistic pleiotropy, in which a particular mutation that is beneficial in one environment is harmful in others. Alternatively, the trade-off could be generated if mutations accumulated by drift in genes that are not necessary in some environment but useful in others. Owing to their extremely compacted genomes and the necessity of expressing all genes during the infectious cycle, the latter explanation is unlikely to operate for viruses.

Several studies have confirmed the hypothesis that adaptation to a novel host would decrease competitive ability in the original host and that adaptation is host specific (Crill et al. 2000, Turner & Elena 2000, Weaver et al. 1999). Furthermore, when environments were forced to fluctuate between two novel conditions, two general conclusions were drawn. First, the fitness cost in the ancestral host was as large as it was for the more costly of the two new environments (Turner & Elena 2000). Second, on average, populations evolved in fluctuating conditions improved fitness in each host as much as the populations evolved in each single host, suggesting that fluctuating environments would select for mutations with no pleiotropic effects (Novella et al. 1999, Turner & Elena 2000, Weaver et al. 1999). These results apparently contradict the existence of trade-offs and host specificity. To reconcile these results, it is possible to imagine that there are two classes of beneficial mutations. One class is beneficial only on a particular host and has antagonistic pleiotropic effects on other hosts; mutations in genes that affect interactions with host receptors and other host-specific molecules are good candidates. The other class produces beneficial effects in all hosts; mutations in genes involved in RNA processing and elongation are candidates. Even if mutations with host-specific benefits were more common than the generally beneficial mutations, the latter class would be differentially enriched in viral populations that evolved on alternating host types.

THE EVOLUTION OF HIGH MUTATION RATES IN RNA VIRUSES

The evolutionarily optimal mutation rate of viruses should be determined by the following factors (Sniegowski et al. 2000): (a) Because most mutations are deleterious, a selective pressure exists for reducing mutation rates toward whatever limit is imposed by biochemical restrictions; (b) the mechanisms of replication fidelity could come at a kinetic or energetic cost and thus be selected against (Dawson 1998, Kimura 1967); and (c) raising error rates provides more chances to generate beneficial mutations and to explore adaptive landscapes. On the basis of c , researchers have often argued that elevated mutation rates are maintained in RNA viruses because of the rapid adaptive capacity they bestow (Domingo & Holland 1997, Holland et al. 1982, Pfeiffer & Kirkegaard 2005, Vignuzzi et al. 2006). However, hypotheses about the high adaptability of RNA viruses should take into account the interplay between the three above-mentioned factors.

Biochemical experiments with avian myeloblastosis virus (Kunkel et al. 1986) and VSV (Steinhauer et al. 1992), and structural analyses of HIV-1 reverse transcriptase (RT), have established that RNA virus RdRp lacks 3'-exonuclease activity. This provides a basis for error-prone replication, but it remains to be elucidated whether this lack of activity is a consequence of fundamental biochemical restrictions or the product of natural selection. Variability in mutation rates within and between RNA virus species (Drake & Holland 1999) gives weak support to the latter possibility, as a large portion of this variation is most probably due to inaccurate estimates. However, recent experiments demonstrate that specific genotypic changes modify replication fidelity. For example, serial passage of poliovirus-1 in the presence of increasing concentrations of ribavirin resulted in the fixation of a single nonsynonymous nucleotide substitution in the polymerase gene that conferred a threefold increase in replication fidelity (Pfeiffer & Kirkegaard 2003). Similarly, experiments with HIV-1 RT variants isolated from patients undergoing antiretroviral therapy revealed the existence of substitutions that specifically confer mutator or antimutator phenotypes (Cases-González et al. 2000, Gutierrez-Rivas & Menéndez-Arias 2001). Some of these substitutions have also been assayed in cell culture, suggesting a good correlation between in vitro and in vivo error rates (Furió et al. 2007, Mansky et al. 2003). Finally, the presence of 3'-exonuclease activity in eukaryotic RNA polymerases (Thomas et al. 1998) further suggests that the observed mutation rates may not be due merely to biochemical restrictions.

Mutational Load

Mutations are more often deleterious than beneficial (Sanjuán et al. 2004a). Hence, high mutation rates must be detrimental in the short term. A well-known result for asexual species is that, in the absence of epistasis, the equilibrium mutational load is $L = 1 - \exp(-U_d)$, where L is the mutational load and U_d is the genomic deleterious mutation rate (Kimura & Maruyama 1966). Owing to RNA viruses high error rates, L should be especially elevated for them. Assuming a genomic mutation rate of 1.0 per

RT: reverse transcriptase

L : mutational load

Genomic deleterious mutation rate (U_d): the number of deleterious mutations produced per genome and replication round

Selection coefficient (s):the relative fitness
difference between a mutant
genotype and the wild type

replication event and that approximately 70% of random mutations are deleterious (Sanjuán et al. 2004a), the fitness load due to mutation should be $1 - \exp(-0.7) \approx 0.50$, which means that half the replication capacity would be lost. If the mutation rate was 0.1, L would drop to only 0.07. It is possible that RNA virus mutation rates are actually below 1.0 (Chao et al. 2002, Furió et al. 2005), but otherwise RNA virus populations harbor a considerably high L and, consequently, are under strong selection pressure for reducing mutation rates.

In addition to U_d , the selection coefficient (s) shall be important in determining L in two situations. First, if the population is out of the mutation-selection balance, the larger s is, the faster fitness declines toward the equilibrium (Johnson 1999). Second, for small genomes replicating at high mutation rates, the mean equilibrium fitness depends not only upon the rate of deleterious mutations but also upon their average s (Krakauer & Plotkin 2002, Schuster & Swetina 1988, van Nimwegen et al. 1999, Wilke 2001). In both scenarios, the larger s is, the lower average fitness. This further supports RNA viruses harboring high L .

The Cost of Replication Fidelity

Most polymerases show fidelities well above the substrate specificity predicted from differences in thermodynamic stability between cognate and noncognate base pairs (Showalter & Tsai 2002). However, it was soon realized that the maintenance of replication fidelity must have a replication efficiency cost that would prevent polymerases from evolving the biochemically lowest possible mutation rate (Kimura 1967). Theory has been developed that takes into account the balance between the cost of replication fidelity and L , two opposing forces that should determine a non-null evolutionarily stable mutation rate (Dawson 1998).

Only recently have researchers suggested that this cost may be relevant to the evolution of mutation rates in RNA viruses. Furió et al. (2005) estimated both mutation rate and fitness for VSV mutants carrying single amino acid substitutions in the RdRp gene. Changes leading to lower mutation rates also led to slower growth rates. In good agreement, evolution under rapid growth conditions increased the mutation rate toward the theoretically optimal rate. To shed some light on the biochemical basis of the fidelity cost, data from *in vitro* experiments with HIV-1 RT have been recently analyzed (Furió et al. 2007). A positive correlation between the *in vitro* mutation rate and the catalytic constant for cognate nucleotide incorporation has been observed, suggesting that an increased fidelity could negatively impact the rate of replication. A clue to the mechanism underlying the cost of fidelity in HIV-1 RT came from the observation that, if an incorrect nucleotide is incorporated to the nascent chain, its extension occurs at a much slower rate than for the correct pair (Kunkel 2004). Inefficient misspair extension would hence reduce error rates at the expense of decreasing polymerization speed.

Differences between mutation rates among taxa can be explained by the trade-off between the accuracy and the efficiency of replication because the cost of fidelity should be stronger for species that rely critically on fast replication for survival. This is the case for RNA viruses because their rapid infection cycles allow them to reach

high titers before the onset of the host's defense mechanisms (Coffin 1995). This means that the cost of proofreading functions, in terms of replication rate, may be excessive for RNA viruses. This reasoning may also apply to DNA viruses because their parasitic lifestyle is similar to that of RNA viruses, but the fact is that DNA viruses show substantially lower mutation rates (Drake & Holland 1999). This is an unresolved problem, although it must be noted that genetic information is more compressed in RNA viruses than in DNA viruses because replication and transcription are biochemically equivalent and often catalyzed by a common molecular complex. Increased functional overlapping may impose more functional restrictions and hence more stressed fitness trade-offs.

Lethal mutagenesis: the deterministic extinction of a population due to an excessive mutation rate

Beneficial Mutations and Adaptation

In a changing environment, optimal replication occurs at a nonvanishing error rate to allow the organism to keep up with environmental changes. If deleterious mutations are neglected, the rate of adaptation increases monotonically with mutation rate until clonal interference becomes important (Gerrish & Lenski 1998). However, when deleterious mutations are taken into account, the optimal mutation rate must be high enough to supply adaptive variability but low enough to prevent the accumulation of deleterious mutations (Johnson & Barton 2002, Orr 2000). In asexual species, modifier alleles that increase mutation rate are more likely to be linked to beneficial mutations and hence have a chance to get hitchhiked to fixation. In sexual species, however, linkage is rapidly dissipated by recombination, hitchhiking is weak (Kimura 1967), and thus the advantage of mutator alleles is often not enough to overcome the short-term increase in L . Many RNA viruses, for example HIV-1, show high levels of recombination (Lemey et al. 2006). Hence, the argument that RNA viruses adapt fast owing to their high mutation rates encounters some conceptual flaws.

Mutator genotypes ought to be favored by selection if they often face novel environmental conditions (de Visser 2002), as is the case for rapidly changing environments. Immunity and, in general, variable environments, may favor high mutation rates in RNA viruses. The evolution of viral mutation rates in the presence of an adaptive immune system has been modeled (Kamp et al. 2002), and researchers concluded that the optimal genomic mutation rate per infection cycle should equal the rate at which the immune system adapts to each new viral antigen. However, no experimental work has addressed the influence of immunity in the evolution of RNA virus mutation rates. It is known, though, that the addition of chemical mutagens does not translate into a higher rate of adaptation (Lee et al. 1997). Increasing the mutation rate beyond the already high spontaneous values is counterproductive for RNA virus fitness and adaptation because it favors mutation accumulation and, in some cases, viral extinction through lethal mutagenesis (Anderson et al. 2004).

RNA viruses should be viewed as extant mutators, and thus the adaptive value of their high mutation rates is probably better tested by conducting experiments with increased-fidelity mutants. Given RNA virus' high L , it should a priori be expected that lowering the mutation rate could come about with little loss of adaptive capacity while conferring the benefit of slowing down the accumulation of deleterious

Genetic drift: changes in genotypic frequencies due to random sampling between generations

Effective population size (N_e): the number of viral particles that effectively contribute to the next infectious cycle

mutations. This expectation is supported by the observation that lamivudine-resistant HIV-1 clones with increased RT fidelity do not pay any cost in terms of adaptability (Keulen et al. 1999). However, recent work with poliovirus-1 has challenged this view. A mutant genotype carrying a substitution at the polymerase gene that confers a threefold increase in replication fidelity was less pathogenic in mice than the wild type (Pfeiffer & Kirkegaard 2005, Vignuzzi et al. 2006). An RNA accumulation defect was observed in the high-fidelity genotype (Pfeiffer & Kirkegaard 2005), in agreement with the cost-of-fidelity hypothesis. However, this difference was apparently too slight to explain differences in pathogenesis. Infection of mice revealed that the high-fidelity mutant was less able to spread to the brain tissue, probably owing to its restricted variability and its consequently diminished capacity to invade different local microenvironments.

As a general remark, although immunity and changing environments could facilitate the evolution and maintenance of high mutation rates in RNA viruses, the same should be valid for DNA viruses. A priori, losing the ability to replicate with high fidelity should be evolutionarily easier than gaining it. Therefore, if RNA viruses owed their rapid adaptation to their elevated mutation rates, then there is apparently no reason why DNA viruses should have not evolved high mutation rates as well. Why DNA and RNA viruses, which apparently share similar lifestyles, show different mutation rates, is a major unsolved question.

GENETIC CONTAMINATION

Genetic Drift and Transmission Bottlenecks

Genetic drift can play an evolutionary role comparable with that of natural selection (Kimura 1983). The influence of drift depends upon effective population size (N_e), which in turn is determined by factors such as the reproduction mode, the historical population bottlenecks, the linkage between genes, or natural selection. These factors can reduce genetic variability and hence make N_e substantially lower than census sizes. The relevance of drift also depends on the magnitude of s . Selection should prevail over drift when $N_e s > 1$, whereas drift should prevail otherwise (Ohta 1992). Under drift, deleterious mutations behave as nearly neutral and hence accumulate in the population. This can lead to fitness declines and potentially jeopardize the survival of small populations. A mechanism for mutation accumulation is Muller's ratchet: In finite populations, mutation-free individuals will become rare at low population sizes, hence making it plausible that they get lost by drift (Haigh 1978). In the absence of compensatory and back mutations or recombination, the loss is irreversible and the ratchet clicks. If mutation accumulation is sustained, Muller's ratchet can result in extinction by mutational meltdown (Lynch et al. 1993).

Considering the fact that RNA viruses show large s and rapidly reach population sizes of several billion particles (Coffin 1995), it should be concluded that selection is the main factor determining their evolution and that drift plays only a minor role. However, data indicate that, in natural populations, N_e in RNA viruses is several orders of magnitude lower than particle counts (Brown 1997, García-Arenal et al.

2001). One reason is that viral populations experience strong bottlenecks upon transmission, especially between individual hosts (Edwards et al. 2006), but probably also between organ compartments within individuals (Itescu et al. 1994). For example, bottlenecks take place during plant virus systemic movement between leaves of the same plant (Hall et al. 2001, Li & Roossinck 2004), being the number of viral particles propagated on the order of tens for tobacco mosaic virus (Sacristán et al. 2003) or as low as 4 for wheat streak mosaic virus (Hall et al. 2001). Similarly, strong bottlenecks upon aphid-mediated plant-to-plant transmission have been reported (Ali et al. 2006). Furthermore, in HIV-1, N_e upon homosexual transmission has been estimated by coalescence methods to be as low as 1.6–2.0 particles (Edwards et al. 2006).

The evolutionary consequences of these transmission bottlenecks have been studied extensively in cell cultures by performing serial transfers of randomly chosen lytic plaques. The sampling of individual infection units at each passage dramatically reduces N_e to little more than one individual, which maximizes genetic drift and onsets Muller's ratchet. Sustained plaque-to-plaque passages of a variety of RNA viruses, including $\phi 6$ (Chao 1990), MS2 (de la Peña et al. 2000), VSV (Duarte et al. 1992), foot-and-mouth disease virus (FMDV) (Escarmís et al. 1996), and HIV-1 (Yuste et al. 1999), typically resulted in significant fitness losses, ranging from 20%–90% relative to the ancestor after approximately 20–30 passages, and in a few cases achieved viral extinction. Several mutations were fixed in these low-fitness populations, albeit less than expected. For example, in FMVD, an average of two to three mutations was found in lines that had experienced 35% fitness losses. These results are not striking given the elevated s showed by RNA viruses. One or few changes are sufficient to produce dramatic fitness losses, and lines with more mutations went extinct.

The main difference between plaque-to-plaque experiments and natural infections is that in the latter, the number of generations between bottleneck events is much larger, thus providing more chances for beneficial or compensatory mutations to get fixed. Together with the fact that RNA viruses can recover from fixation of deleterious mutations even at small population sizes (Burch & Chao 1999), this difference should explain why natural RNA virus populations are not extinguished or even endangered by Muller's ratchet.

Lethal Mutagenesis versus Error Catastrophe

Genetic contamination can also take place at large population sizes. Population genetics predicts that in an arbitrarily large population, the frequency of genotypes carrying deleterious mutations at the mutation-selection balance is U_d/s and the frequency of the mutation-free genotype is $\exp(-U_d/s)$ (Kimura & Maruyama 1966). Therefore, mutagenesis will make the mutation-free class rarer and average fitness will rapidly decrease. However, this does not provide enough information about the probability of extinction by lethal mutagenesis. To address this point, it is necessary to express fitness in an absolute instead of relative scale (Bull et al. 2007). If the absolute number of progeny per individual is lower than one, population size will deterministically decline, leading to lethal mutagenesis. Once the population size is low enough to make

FMDV: foot-and-mouth disease virus

drift important relative to selection, extinction would be boosted by the stochastic mutation-accumulation processes discussed in the previous section.

A concept related to mutation accumulation in large populations is the error catastrophe, a prediction of the quasi-species theory (Eigen et al. 1989). Beyond an error threshold, the mutation-selection balance is expected to be lost and the population becomes a pool of randomly drifting genotypes. In general, the existence of an error threshold depends critically upon the assumed fitness landscape, which in the original formulation consisted of a single fit wild-type sequence and all other genotypes with a constant, non-null, fitness, regardless of the number of mutations they carried. This is an unrealistic model because lethal mutations are highly frequent in RNA viruses (Sanjuán et al. 2004a) and average fitness generally declines with increasing numbers of mutations. Thus, despite error thresholds that arise in some generalizations of the quasi-species model (e.g., Tarazona 1992), it takes place only under some special set of conditions (Summers & Litwin 2006, Wagner & Krall 1993, Wiehe 1997).

The consequences of artificially increasing error rates in RNA viruses have been explored in several cell culture experiments. Chemical mutagens have been used in a variety of RNA viruses, including VSV (Lee et al. 1997), HIV-1 (Loeb et al. 1999), poliovirus-1 (Crotty et al. 2001), FMDV (Sierra et al. 2000), and lymphocytic choriomeningitis virus (Grande-Pérez et al. 2002), among others. All these studies have proven that mutagens are detrimental to viral fitness and, in some cases, extinction was observed. For example, in HIV-1, the addition of the base analog 5-hydroxydeoxycytidine resulted in a loss of infectivity after 9–24 serial passages. Similarly, in FMDV, 5-fluorouracil or 5-azacytidine caused occasional extinction after 11–21 passages. In many cases, extinction was accompanied by an increase in the average number of mutations per genome. Virologists have often relied on the notion of error threshold to explain these experimental observations. The very idea behind this interpretation is that the error catastrophe is a form of lethal mutagenesis. However, the two concepts are not equivalent (**Table 1**) and, ironically, the

Table 1 Differences between lethal mutagenesis and error threshold models

| | Lethal mutagenesis | Error catastrophe |
|--------------------------------|---|---|
| Nature of the process | Mutation accumulation and population extinction | Change in the genetic composition of the population |
| Name of the threshold | Extinction threshold | Error threshold |
| Key parameters | U_d , fecundity of the wild type, and s | U_d and s |
| Demography | Population size declines | No changes in population size are required |
| Fate of the wild type | Not necessarily extinguished | Extinguished |
| Dependence on mutation rate | Gradual; extinction is more likely and occurs faster at higher mutation rates | Phase transition; beyond the error threshold, further increases in mutation rate have no effect |
| Effect of mutations on fitness | The fitness of mutant genotypes decays with mutation number | The fitness of mutant genotypes does not decay with mutation number |
| Mutational pattern | Only that induced by the drug; no specific changes are required | The consensus sequence randomly drifts through time |

crucial assumption leading to the prediction of an error catastrophe (that is, a lower bound to fitness) actually retards extinction (Bull et al. 2007). Making the distinction is important because error catastrophe has been proposed as a candidate therapeutic strategy (Anderson et al. 2004, Domingo et al. 2001).

MOI: multiplicity of infection

MUTATIONAL ROBUSTNESS

Mechanisms of Robustness

Both s and the fraction of mutations that are neutral are useful measures of mutational robustness. For any given protein, the latter fraction can be accurately predicted from thermodynamic parameters (Bloom et al. 2005). Thermostability is a form of environmental robustness and, on the ground of theoretical arguments and RNA-folding simulations, it has been proposed that environmental and mutational robustness ought to be correlated (Ancel & Fontana 2000). Although mutational robustness has now a well-founded biophysical ground, it may sound intuitive that the transition from a nonrobust to a robust state should be complex, involving many genetic changes and adjustments. However, using β -lactamase as a model, it has been shown that a single amino acid substitution is enough to increase thermostability and render a more robust protein (Bloom et al. 2005). Additionally, the virological literature provides many examples of thermosensitive phenotypes associated with one or few nucleotide substitutions (Dupraz & Spahr 1993, Marriott et al. 1999). Therefore, current data and theory support the notion that robustness does not constitute a difficult evolutionary transition.

Misfolded proteins, in addition to being nonfunctional, aggregate and are often toxic for the cell, imposing metabolic burdens (Goldberg 2003). Molecular chaperones, originally identified as heat-shock proteins, assist folding and tag unfolded proteins for degradation (Feldman & Frydman 2000). Chaperones ought to have an important role in buffering not only environmental noise, but also mutational effects (Fares et al. 2002). Although the fact that chaperones are overexpressed in response to viral infections may suggest that chaperones are antiviral factors, animal viruses and bacteriophages depend upon chaperones to complete many steps of their infection cycle, including endocytosis, capsid disassembling, early replication, enhancement of transcription and translation, and virion assembly (Mayer 2005). Interestingly, thermosensitive mutants of tobacco mosaic virus trigger a much stronger expression of heat-shock protein 70 than the wild type, suggesting that the host's chaperones also assist the folding of viral proteins (Jockusch et al. 2001). Therefore, it is possible that chaperones may provide a mechanism for buffering mutational effects in RNA viruses. However, this hypothesis remains to be tested.

An additional putative mechanism for mutational buffering could be genetic complementation during co-infections. It is well known that defective viral particles lacking a portion of the genome can be stably maintained in populations at high multiplicity of infection (MOI), that is, the average number of viral particles infecting a host cell, impacting the evolution of the full virus (Bangham & Kirkwood 1993). Tobacco aspermy virus genotypes carrying single lethal substitutions have also

been reported to be at higher frequencies than expected, presumably maintained by complementation (Moreno et al. 1997). It has also been hypothesized that deleterious mutants of FMDV could be stably maintained by complementation at frequencies higher than the mutation-selection balance expectation (Wilke & Novella 2003). Similarly, high levels of co-infection lessen the effectiveness of selection at purging deleterious mutations (Froissart et al. 2004).

The Evolution of Robustness

Insofar as robustness has a heritable basis, shows variability among individuals, and affects the probability of survival, it is a potential target for selection and evolutionary optimization. However, for mutational robustness to provide a selective advantage, the genes involved in expression of the phenotype have to be mutated. Therefore, the selective advantage of robustness can be, at most, equal to the mutation rate, which is typically small (Wagner 2005). The difficulty is somehow lessened in RNA viruses because the per site mutation rate is orders of magnitude higher than for their cellular hosts. Also, this putative advantage would also be fuelled by the strong deleterious coefficients characteristic of RNA viruses.

Even though robustness could potentially evolve in RNA viruses, the fact is that they remain highly sensitive to mutation compared to more complex microorganisms (Elena et al. 2006). In general, a lack of robustness is expected in small, compact genomes that have no redundancy, no repair systems, and exhibit strong pleiotropy (Krakauer & Plotkin 2002). These systems usually exist as very large populations, thus making selection very efficient at purging deleterious mutations ($N_e s \gg 1$) and promoting the preservation of the unmutated genotype at high frequencies. Individual hypersensitivity seems to be the predominant survival strategy for RNA viruses, but, under some circumstances, the evolution of mutational robustness may be favored. These conditions were identified using digital organisms (Wilke et al. 2001). Digital organisms that had been replicating at low population sizes and high genomic mutation rates evolved increased mutational robustness but paid the cost of reduced replication rates. Using Wright's adaptive landscape metaphor, these organisms had evolved toward a flat peak, as opposed to organisms that had been replicating at high population sizes and low mutation rates (**Figure 3**). The latter were faster replicators, but were more sensitive to mutation and hence were located in a higher peak (**Figure 3**). The flatter population was readily outcompeted by the fitter at low mutation rates, but it benefited from a selective advantage at high mutation rates. Similarly, flat populations should be good competitors in small populations, where genetic drift favors mutation accumulation (Krakauer & Plotkin 2002, Schuster & Swetina 1988, van Nimwegen et al. 1999, Wilke 2001). Quasi-species theory provides a suitable theoretical framework for these results because neutral and back mutations are not ignored and, as a consequence, the average fitness of the population at the mutation-selection balance depends upon the geometry of the fitness landscape, that is, upon s (Schuster & Swetina 1988, van Nimwegen et al. 1999).

Experiments proving the evolution of mutational robustness in RNA viruses are scarce. Some clues first came from work with $\phi 6$ (Burch & Chao 2000) showing that

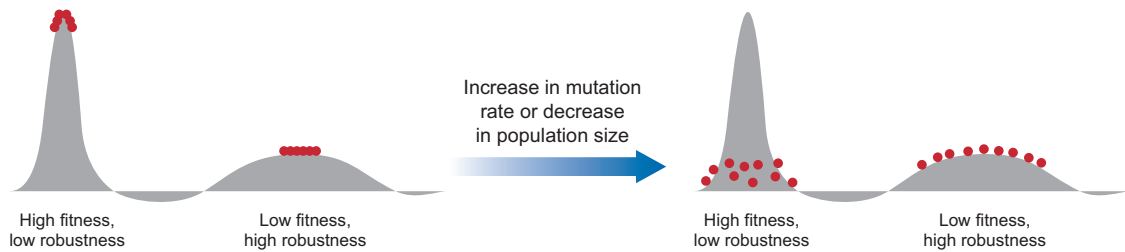


Figure 3

Schematic representation of a landscape characterized by a peak of high fitness but low robustness and another one of low fitness but high neutrality (that is, robustness). As mutation rate increases or population size decreases, populations at the high peak suffer disproportionately larger fitness declines than those inhabiting the flatter surface.

the evolution of different genotypes depended on the topology of the neighboring adaptive landscape. Interestingly, one genotype repeatedly evolved decreased fitness despite passages done at high population sizes, whereas another genotype repeatedly increased in fitness owing to the availability of beneficial mutations in its mutational neighborhood. More recent work with $\phi 6$ has provided indirect evidence for the adaptive evolution of robustness (Montville et al. 2005). If robustness had a selective value, selection for robustness might be relaxed under co-infection regimes owing to genetic complementation. This prediction was tested by subjecting lineages that had previously evolved under high versus low MOI to mutation accumulation through plaque-to-plaque passages. In good agreement with the hypothesis, fitness declined faster in lineages that had evolved under high MOI.

A more direct approach was undertaken with viroids, plant pathogens constituted by small, noncoding, RNA molecules (Codoñer et al. 2006). Two viroid species belonging to different families and characterized by different secondary structures were competed in common host plants. One of the viroids was characterized by fast population growth and genetic homogeneity, whereas the other showed slow population growth and a high degree of variation. As expected, under standard nonmutagenic infection conditions, the faster replicator outcompeted the slower one. However, this advantage vanished when mutation rate was artificially increased, and the slower but more robust replicator was not outcompeted anymore.

SOCIAL CONFLICT AND COOPERATION IN THE VIRAL WORLD

Usually when talking about sociality, we tend to imagine the intricate relationships established between higher organisms (from social insects to the human society), but we hardly imagine viruses to be good candidates for testing theoretical predictions about cooperation, altruism, selfishness, or cheating. This appreciation is not correct because social interactions among viruses are quite common in nature, as, for example, synergistic symptoms among co-infecting plant virus (Malpica et al. 2006),

predator-prey-like coevolutionary dynamics between some virus and their defective interfering particles (Bangham & Kirkwood 1993, DePolo et al. 1987), or the existence of coviruses, that is, genome segments encapsidated into separate particles, which are required for a successful infection (Nee 2000).

Viral sociality has also been studied in the laboratory. In a series of clever experiments, Turner & Chao (1999) explored the evolution of competitive interactions among $\phi 6$ at different MOIs. At high MOI, many viruses infected the same cell; at low MOI, only one virus infected each cell. Hence, they argued that different selective constraints were acting on each type of experiment. They found that the fitness of the virus evolved at high MOI relative to their ancestors generates a payoff matrix similar to the prisoner's dilemma strategy (that is, the cost of defecting is smaller than its advantage). In this strategy, selfishness evolves. How can cooperation and defection be defined in a viral system? Simply by manufacturing (cooperation) or sequestering (selfishness) diffusible intracellular products; this is a different side of the complementation phenomena discussed in the previous section. In single infections, either competition was absent or selection favored the evolution of cooperation among closely related individuals (resulting from the replication of the initially infecting phage). At high MOI, however, strong intracellular competition occurred between less-related individuals (originated from different infected hosts). As Turner & Chao postulated, the evolution of a prisoner's dilemma strategy can be attributed to the absence of clonal structure at high MOI, leading to the evolution of selfishness. Indeed, later on Turner & Chao (2003) showed that clonal selection allowed viruses to escape from the prisoner's dilemma. The ancestral $\phi 6$ clone was propagated under strict clonal conditions, creating the opportunity for cooperation to evolve. When the evolved cooperators were mixed with their selfish counterparts, both strategies coexisted in mixed polymorphism (Turner & Chao 2003). The transition from the prisoner's dilemma can occur either by selection for cheating and the associated cost or by selection for decreased sensitivity to cheaters. The Turner & Chao (2003) results supported the latter possibility. One plausible explanation for the evolution of selfish viruses relies on the fact that RNA virus genomes serve both as a template for replication and transcription. A virus with increased transcription activity, that is, contributing more proteins to the pool, would in fact act as a cooperator. By contrast, a virus that changes its schedule and spends more time on replication would act as a cheater and would encapsidate using the cooperator's coat proteins.

Sachs & Bull (2005) observed the evolution of cooperation between $\phi 1$ and IKE. Both phages produce nonlytic infections in *Escherichia coli* and they were engineered to contain distinct antibiotic resistance markers. When both antibiotics are present in the media, only co-infected cells can survive and produce viral progeny. Thus, it is to the benefit of both viruses to remain together, despite the fact that cellular resources consumed by one virus are detrimental to the replication opportunities of the other. The evolutionary solution to this conflict was that both phages copackaged their genomes into $\phi 1$ protein coats, ensuring cotransmission. In parallel, the IKE genome got smaller by deleting its own coat protein gene.

SUMMARY POINTS

1. Small genome size, high mutation rates, large selection coefficients, short generation times, and large population sizes are the key parameters for understanding RNA virus evolution.
2. Despite the remarkable evolutionary potential of RNA viruses, it is important to stress that factors such as clonal interference among coexisting beneficial mutations and negative epistasis among beneficial mutations and their pleiotropic effects can create trade-offs, limiting their evolution.
3. RNA viruses' high mutation rate may or may not have evolved as a strategy for accelerating adaptation. It is possible that the mechanisms of replication fidelity impose a fitness burden to systems that, as RNA viruses, rely critically on fast replication.
4. When the purifying selection is relaxed, for instance, during bottleneck transmission events, or when the mutation rate is artificially increased, deleterious mutations accumulate in viral populations, potentially triggering their extinction.
5. The general viral strategy to cope with deleterious mutational effects is individual hypersensitivity, which makes selection efficient at preserving the unmutated genotype. However, alternative strategies such as increased mutational neutrality, genetic complementation during co-infection, genome segmentation, or the use of cellular buffering mechanisms are also likely.
6. RNA viruses evolve social behaviors, including cooperative synergistic interspecies interactions or cheating.

FUTURE ISSUES

1. Researchers must adopt a unified theoretical framework for viral evolution from quasi-species and classical population genetics theories. The two are fundamentally equivalent but differ at some sets of assumptions, which give rise to sometimes different predictions.
2. Is there an error threshold beyond which genetic information is lost? And if so, is it equivalent to lethal mutagenesis? Can it be the basis for a new approach to antiviral therapies?
3. Is RNA viruses' high mutation rate beneficial per se or an unavoidable consequence of selection for fast replication?
4. From an evolutionary perspective, why do DNA viruses maintain mutation levels much lower than those of RNA viruses despite sharing the same lifestyle?

5. Can RNA viruses evolve mechanisms that buffer the deleterious effects of mutations and how may such mutations affect virus evolvability?

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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