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Additional Information

RNA Virus Genetic Robustness: Possible Causes and Some Consequences

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Abstract

In general terms, robustness is the capacity of biological systems to function in spite of genetic or environmental perturbations. The small and compacted genomes and high mutation rates of RNA viruses, as well as the ever-changing environments wherein they replicate, create the conditions for robustness to be advantageous. In this review, I will enumerate possible mechanisms by which viral populations may acquire robustness, distinguishing between mechanisms that are inherent to virus replication and population dynamics and those that result from the interaction with host factors. Then, I will move to review some evidences that RNA virus populations are robust indeed. Finally, I will comment on the implications of robustness for virus evolvability, the emergence of new viruses and the efficiency of lethal mutagenesis as an antiviral strategy.

Highlights

- Experimental evidences suggest that viruses may have evolved robustness mechanisms.
- Virus populations are robust at the cost of individuals being fragile.
- Genetic robustness evolves linked to environmental robustness.
- Genetic robustness affects virus evolvability.
- Robustness does not play a substantial role in lethal mutagenesis.

RNA viruses are the most successful parasites on Earth, infecting hosts from all biological kingdoms, including other parasites. This success results from their evolutionary plasticity (*i.e.*, evolvability): a combination of short generation times, huge population sizes and high mutation rates [1,2,3]. Alas, these properties come along with some costs. First, fast replication requires that genomes must be kept small, with overlapping reading frames and encoding multifunctional proteins [4,5]. Second, high mutation rates limit the length of the genome that can be transmitted without incurring in too many errors [6]. High mutation rates may be favored in stressful situations where the input of beneficial mutations allows for escape and survival (*e.g.*, changing cell types, tissues and hosts or the presence of antiviral responses or drugs). However, in all situations deleterious and lethal mutations represent the larger fraction of all possible mutations [7], thus jeopardizing viral fitness [8,9]. How do RNA viruses maintain their functionality in this scenario? Are they robust to the accumulation of deleterious mutations? In this review I try to answer these questions and look beyond to the consequences of RNA virus robustness.

What is robustness and how can it be measured?

In a hallmark article, De Visser et al. [10**] reviewed the notion of robustness and explored its causes and consequences. *Robustness is the preservation of the phenotype in the face of perturbations.* The robustness of phenotypes appears at various levels of organization: from gene expression, protein folding, metabolic flux, physiological homeostasis, and development, to fitness. From an evolutionary standpoint, fitness is the most relevant level. Phenotypes can be robust either against mutations or environmental perturbations.

Three reasons may account for the evolution of genetic robustness (GR). First, as long as it is heritable, shows variability among individuals and affects fitness, GR can be a target for selection [11]. The more frequent mutations are, the more efficient selection will be at promoting the evolution of GR. Second, GR is a side effect of stabilizing selection acting on different traits [12]. Third, given that environmental fluctuations often have strong impact on fitness, selection would favor mechanisms of environmental robustness (ER), emerging GR as a correlated response (plastogenetic congruence) [13,14]. This is particularly appealing in the case of RNA viruses because

they must cope not only with deleterious mutations but also with dramatic and fast fluctuations in their environments.

Keeping in mind the definition of GR, a way of estimating it is to evaluate the effect of large collections of individual point mutations on viral fitness. If a point mutation i reduces the fitness of a genotype with respect to that of the wild-type in an amount s_i , then the average effect \bar{s} across the collection of point mutations can be seen as a measure of mutational sensitivity and, henceforth, as an inverse of GR. In other words, if the average effect of mutations on a virus is small, we conclude it is robust. By contrast, if the average effect is large, we conclude the virus is brittle.

Potential mechanisms for viral GR

In a previous review, we elaborated on possible mechanisms by which RNA viruses may attain GR [15^{**}]. We distinguished two classes of mechanisms. Mechanisms of intrinsic GR are the consequence of RNA-genome architecture, replication peculiarities and population dynamics. Intrinsic GR mechanisms operate efficiently at the population level. By contrast, extrinsic GR results from the exploitation of cellular buffering mechanisms by viruses.

This review has been written from the perspective of evolutionary biology. I refer readers interested in molecular details to the excellent review by Barr and Fearn [16^{**}] on the several mechanisms by which RNA viruses maintain genome integrity.

Intrinsic mechanisms

RNA virus genomes are very sensitive to the effect of mutations [7,15^{**}], with most mutations being either lethal or strongly deleterious, and with \bar{s} well above 10% [7]. Furthermore, RNA viruses also show a second hallmark of mutational hypersensitivity, namely, a dominance of antagonistic epistasis among pairs of deleterious mutations [15^{**},17]. Paradoxically, individual hypersensitivity to mutations generates GR at the population level [18]. The efficiency by which natural selection purges deleterious mutations from a population depends on the product $N_e\bar{s}$, where N_e is the effective population size. RNA viruses reach enormous N_e even within infected hosts; hence the above product tends to be large, making selection remarkably efficient removing

mutants and preserving only non-mutated genomes [18]. In good agreement with this *individual hypersensitivity* strategy, recent evidences from ultra-deep sequencing of viral populations suggests that much of the variation is rapidly purged from populations and that the wild-type sequence remains numerically dominant, while adaptation to new conditions depends on the fixation of few beneficial alleles [19,20].

Opposite to the individual hypersensitivity strategy is the idea of the *survival of the flattest* (SF) [21,22] (Figure 1A). When neutral and back mutations are considered, the population average equilibrium fitness depends on the geometry of the fitness landscape, which can be described by \bar{s} [11]. In this scenario, a new selective pressure comes into play at high mutation rates, pushing populations towards regions of the landscape with high density of neutral mutations –a *neutral network* (NN) (Figure 1B)- [11,21,22]. As a consequence, the whole population evolves increased GR.

A third mechanism of GR is high ploidy [15,23]. Viruses are n -ploid organisms, as n is variable during infection. At initial stages, multiplicity of infection (MOI) is low and viruses are effectively haploid. However, as infection progress, high MOIs ensure frequent co-infections and increasing ploidy. An immediate consequence of polyploidy is genetic complementation. Strong complementation slightly reduces the average population fitness by weakening the efficiency of purifying selection but significantly enhances population diversity and GR, especially if epistasis among deleterious mutations is antagonistic [23].

Different modes of genome replication may also affect GR [24]. By always using the same molecule as template, the stamping-machine strategy produces offspring with a minimal number of mutations, whereas the geometric replication strategy, by using progeny genomes as templates, generates offspring with a number of mutations that increases geometrically. Furthermore, it has been shown that, in combination with selection, the stamping-machine accumulates less mild-effect mutations than geometric replication [24]. Indeed, the difference between both replication schemes in terms of minimizing deleterious mutational load is enhanced if mutations show antagonistic epistasis [24].

A last mechanism of intrinsic GR is viral sex, resulting from recombination between homologous molecules or in segregation of segments in a multipartite genome. Sex recreates mutation-free genotypes and helps to keep the average population fitness high. Both forms of sex are common among RNA viruses [15,25*].

Extrinsic mechanisms

It is well known that viral infections induce the cellular stress response [26]. However, is it possible that viruses coopt chaperones to buffer mutational effects? The answer is yes. It has been shown that most viruses need cellular chaperones during their life cycle to solve their own protein-folding problems [27], to assist during RNA replication [28] and to interfere with cellular processes such as signal transduction [29].

Evidences of GR in RNA viruses

The first evidence that RNA viruses have evolved some sort of GR comes from *in silico* studies analyzing the stability of RNA folding. In a pioneering study, Wagner and Stadler [30] compared the GR of highly conserved RNA secondary structure elements with that of non-conserved elements for three viruses (*Denge virus*, *Hepatitis C virus* (HCV) and *Human immunodeficiency virus* type 1). They hypothesized that conserved elements, given their functional importance must be more robust than non-conserved elements. This hypothesis was supported by the data, thus concluding that the sequences and structures of important conserved domains had evolved to minimize the impact of mutations. Recently, the observation for HCV has been confirmed using a much larger dataset [31].

In a set of computational studies, Sanjuán *et al.* [32,33] explored the GR of all viroid species. Viroids have been classified into two families according to biological properties and sequence similarity [34]. Interestingly, members of the *Avsunviroidae* fold into highly branched structures, whilst those from the *Pospiviroidae* fold into very compact rodlikes. Given that a branched structure seems more fragile than a rodlike, it can be hypothesized that the pospiviroids may show characteristics of GR whereas the avsunviroids may not. Results confirmed this expectation: \bar{s} was much larger for the avsunviroids than for the pospiviroids [32] and epistasis was, on average, antagonistic for the former but synergistic for the later [33].

Montville *et al.* provided the first empirical evidence of evolved GR in an RNA virus [35*]. These authors hypothesized that $\phi 6$ populations evolving at high MOI will experience intense complementation and thus, selection for alternative GR mechanisms will be weak. By contrast, populations evolved at low MOI will evolve alternative GR mechanisms. After 300 generations of experimental evolution, clones from each of three independent evolution lineages per treatment were isolated and subjected to 100 generations of mutation-accumulation (MA) by genetic drift at low MOI [35*]. If the initial hypothesis was true, then viruses evolved at high MOI will show no GR and will experience larger fitness declines than those evolved at low MOI. The results significantly matched this expectation, thus confirming that GR could evolve in $\phi 6$ after just 300 generations.

Confirmation of the SF effect as a mechanism of GR came from two different experiments. Codoñer *et al.* [36] selected two different viroids that infected a common host. These two viroids largely differ in their replication rates and in the extent of genetic variability they generated within host. *Chrysanthemum chlorotic mottle viroid* (CChMVd) generated lots of variants after being inoculated but accumulated to very low titers. *Chrysanthemum stunt viroid* (CSVd) accumulated to very high titers but showed little genetic variation. The authors hypothesized that CChMVd may represent a case of a flat organism replicating in a NN whereas CSVd may not so. To test this hypothesis both viroids were co-inoculated into the same plants and allowed to compete. As expected, CSVd quickly outcompeted CChMVd owed to its faster replication rate (Figure 1A). However, when mutation rate was artificially increased by UVC radiation, the situation was reversed and CChMVd persisted in the mixed population (Figure 1A).

Sanjuán *et al.* [37] provided a second confirmation of the SF effect. Two *Vesicular stomatitis virus* (VSV) populations that differed in evolutionary history were chosen. Population A was formed by individuals that on average had lower fitness than those from population B but that were more diverse in fitness (Figure 1A). The authors hypothesized that population A was the flattest while population B was the fittest. As in the viroids case, these two populations were allowed to compete in standard conditions and at increasing mutation rates (by adding either 5-FU or 5-AzC). The results showed

that while population B outcompeted population A at standard conditions, B was able of reverse its fortune as the concentration of mutagens increased.

As I mentioned at the beginning of this review, it has been proposed that plastogenetic congruence may drive the evolution of GR. To test this hypothesis Domingo-Calap *et al.* [38*] evolved independent populations of Q β under periodic temperature pulses to select for thermotolerant viruses. Thermotolerant and control viruses were then subjected to MA by treating populations with HNO₂ at each experimental passage. If selection for ER has a positive effect on GR, then thermotolerant viruses may suffer a smaller reduction in fitness than the control viruses during the MA phase. The results confirmed this expectation, thus supporting the view that GR evolves as a correlated response to selection for ER.

Consequences of GR

Does GR promote evolvability?

There is not easy answer to this question since opposing results have been reported.

McBride *et al.* [39] used some of the ϕ 6 robust and brittle clones generated in [35*] to test whether they differed in their ability to adapt to a new thermal niche. All clones were originally evolved at 25 °C and had very low viability above 45 °C. The selected clones were evolved for 50 generations under periodic pulses at 45 °C. At the end of this evolution phase, the fitness of all evolved lines was tested at 45 °C. As expected, the robust clones had achieved higher fitness than the brittle ones.

The existence of NN has a strong implication for the antigenic evolution of *Influenza A virus* H3N2 [40,41*]. The observed patterns of epochal antigenic evolution of H3N2, alternating periods of phenotypic stasis punctuated by sudden changes in the antigenic phenotype [40] can easily be explained in terms of NN [41*] (Figure 1B). At the onset of an epochal evolution cycle, a H3N2 population is distributed over the NN of an antigenic cluster (Figure 1B). Neutral mutations accumulate, allowing the virus to explore and reach distant regions of the NN. At some point, a mutation in the edge of the network will create an individual that belongs to a new NN that corresponds to a different antigenic cluster (Figure 1B). This antigenic innovation corresponds to a peak

in infections. The new antigenic variant now starts exploring the new NN, and the process repeats itself.

Turner *et al.* [42] have tested whether generalist (*i.e.*, environmentally robust) viruses were more evolvable than specialist (*i.e.*, environmentally brittle) ones. To do so, they used VSV populations that were previously evolved as generalists or as specialists. The fitness of all these populations was tested on four novel hosts. The prediction was that the ER generalists would show higher mean fitness and less variance in mean fitness across the novel hosts than the brittle specialists. These predictions were fulfilled, thus linking robustness to the likelihood of viral emergence.

Contrasting to the above results, Cuevas *et al.* [43] have shown that brittle VSV populations were more evolvable than genetically robust ones when facing a new host cell type. Brittle populations reached higher infectivity and fitness in the new cell line than the robust ones, while both paid the same fitness cost in the ancestral host cell type and accumulated similar number of mutations.

Two arguments can be brought forward to explain this discrepancy. First, the relationship between robustness and evolvability may be time-dependent [44,45]. At the short term GR will buffer the effect of any potential beneficial mutation, thus hampering adaptation. Only at the long-term will GR bolster evolvability by allowing populations to drift in the NN until reaching distant parts and facilitate the switch to different NN. Second, to confer GR and evolvability, the size of the NN needs to cover most of the genotypic space; otherwise only small regions would be explored [46].

New data need to be obtained to solve this controversy.

Does GR diminish lethal mutagenesis?

Lethal mutagenesis (LM), that is viral extinction mediated by enhanced mutation rates, has been proposed as a potential therapeutic strategy [47*]. A critical issue regarding LM as an antiviral strategy is whether virus mutants resistant to the mutagens can be selected. Obviously, GR may be relevant for the emergence of such mutants. The scarce data available do not provide a definitive answer to the above question.

To tackle the problem of whether passages in presence of mutagens may select for genetically robust genomes that will jeopardize the efficiency of LM, Martín *et al.* [48] evolved populations of LCMV in absence and in presence of a sub-lethal concentration of 5-FU. Populations and clones isolated at different time points during the evolution experiment were then tested for their resistance to a concentration of 5-FU large enough as to induce LM. No differences in the outcome of the experiment were observed between viruses evolved in presence or absence of 5-FU. This observation led to the conclusion that evolution in presence of sub-lethal concentrations of mutagen did not select for GR.

Recently, Graci *et al.* [49] have provided evidences that GR determines the success of LM. These authors have shown that the enteroviruses *Coxsackie virus* B3 (CVB3) and *Polio virus* (PV) differ in their degree of GR according to a series of evidences, the former being less robust than the latter. In agreement with the hypothesis that GR will diminish the efficiency of LM, the results showed that CVB3 was more sensitive to ribavirin than was PV.

However, a theoretical analysis of the effect of GR on the likelihood of extinction by LM [50] has shown that the effect of GR on LM shall be minor. Viruses will obtain a benefit from GR only if the increase in mutation rate by the mutagen is small. When the mutation rate goes beyond a critical value that depends on the ratio between the logarithm of the virus reproductive capacity and the fraction of all deleterious mutations, the virus will not have time enough as to expand its NN. The study concludes that GR does not impose a strong burden to LM therapy.

Concluding remarks

Far from being passive victims of their error-prone replication, RNA viruses cope with the deleterious effects of mutations. Growing evidences suggest that viruses have evolved mechanisms to increase their GR at the population level at the cost of being very fragile at the individual level. The role of GR in the emergence of new viruses or in the durability of antiviral therapies needs to be further explored.

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Figure 1. (A) Schematic representation of the survival of the flattest effect. In this two-dimensional representation of fitness landscapes, fitness corresponds to the height on the peaks, whereas the horizontal axis represents different genotypes. Fast replicating but brittle populations inhabit the left peak, whereas slow replicating yet robust populations inhabit the right peak. At low mutation rates (upper panel), populations remain located at their peaks and the fittest (brittle) outcompetes the flattest (robust). At high mutation rates (lower panel), mutations move genotypes away from their original position on the peaks. The fittest viral population experiences large changes in fitness, as genotypes slide down from the narrow fitness peak. Because the flattest population inhabits a neutral portion of the landscape, its fitness is buffered against mutational change. (Modified from ref. [37]. Reprinted with permission from the authors.) (B) *Influenza A virus* H3N2 antigenic evolution in a neutral network model. Phenotypes are genetically robust, allowing genotypes to drift through genotypic neutral space until reaching the edge of a new neutral network (that corresponds to a different antigenic variant) before instant shifts in phenotype. (Modified from Ref. [41]. Reprinted with permission from AAAS.)

