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Is group selection a factor modulating the virulence of RNA viruses?

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Summary

RNA viruses consist of populations of extremely high genetic heterogeneity called quasispecies. Based on theoretical considerations, it has been suggested that the unit of selection in such complex genetic populations is not the single viral particle but a set of genetically related particles which form the quasispecies. In the present study we carried out a set of experiments with the vesicular stomatitis virus (VSV) dealing with the evolution of life-history characters under selection acting at two factors either in the same or in opposite directions. The two factors at which selective pressure is applied are the individual and the group. We show evidence that group selection modulates the virulence of VSV populations, in opposition to an unlimited increase in virulence by competitive optimization promoted by individual selection. The results are of relevance for understanding the evolution of parasite virulence.

1. Introduction

The notion that selection may target groups as well as individuals is not new, yet group selection has been and remains a controversial issue. Darwin (1859) noticed that the formation of insect or human societies might have evolved by selection between different groups. Lewontin (1970) agreed with the idea of a hierarchical organization of living systems and proposed that selection acts on the lower levels of the hierarchy. The problem with this view, however, is to define what is meant by the lower level in a given system. Hull (1980) said that some entities commonly regarded as groups are, in reality, treatable as individuals. He also considered that in such groups, in which the components are closely related, group selection could be the main factor determining the evolutionary process. One of the main reasons why group selection has been controversial is because it has been taken as an explanation for the regulation of population size. According to Wynne-Edwards (1986), altruistic individuals will reduce their reproductive rate (i.e. fitness) if such a reduction is beneficial to the whole population.

During the 1970s, some authors (Levins, 1970; Wilson, 1973, 1975; Levin & Kilmer, 1974; Gadgil, 1975; Gilpin, 1975) implicitly or explicitly considered the special case in which group selection and individual selection act in opposite directions, the former being a weaker force than the latter. The general conclusion was that group selection would be of significant strength in natural populations only under some restrictive conditions that could be generated in the laboratory. Wade (1976, 1977) and Wade & McCauley (1980) carried out experiments with the flour beetle, *Tribolium castaneum*, demonstrating that group selection could be an important factor influencing population size in organisms with a population structure promoting rapid genotypic and phenotypic divergence between local demes.

The evolution of virulence has been modelled extensively under different theoretical conditions (Anderson, 1979; Anderson & May, 1979; May & Anderson, 1979, 1983*a*, *b*, 1990; Bremermann & Pickering, 1983; Bremermann & Thieme, 1989; Stewart & Levin, 1984; Levin & Svanborg Edén, 1990; Lenski & May, 1994) and has also been studied extensively using different organisms under different experimental conditions (Chao & Levin, 1981; Scott, 1985; Keymer, 1985; Gill & Mock, 1985; Levin & Lenski, 1985; Bull *et al.*, 1991). However, to our knowledge no experimental design has been proposed to test for the efficacy of a group selection component acting during the evolution of virulence.

Group selection was invoked to explain the reduction in virulence observed in natural populations

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of the myxomatosis virus in Australia (Fenner & Ratcliffe, 1965). In general, group selection has been invoked to justify virulence reduction in parasites (Levin & Pimentel, 1981; Maynard Smith, 1976; Frank, 1992, 1994). A parasite with a high virulence should exterminate the host population, putting an end to replication of other parasites of the same population. Therefore, it is necessary that the individual parasite moderate its virulence to intermediate values such that a sufficient number of copies of a given parasite persist, but not enough to eliminate the host population. The view that parasites evolve towards diminished virulence has been called 'conventional wisdom' by May & Anderson (1983*a*); this view has been criticized by population biologists for its apparent reliance on group selection. At the same time, empirical results also suggest a tendency towards the evolution of reduced virulence (Levin & Svanborg Edén, 1990; May & Anderson, 1990).

An alternative point of view is that individual selection often favours an intermediate level of virulence as a trade-off between parasite transmission and virulence (May & Anderson, 1983*b*; Dwyer *et al.*, 1990). This alternative theory has been called the 'enlightened theory' (Levin & Svanborg Edén, 1990). Individual selection plays an important role in the evolution of virulence during a superinfectious process, that is, when several different strains co-infect the same host resulting in within-host competition. Under such conditions, increased virulence should be favoured (Nowak & May, 1994; Bonhoeffer & Nowak, 1994; van Baalen & Sabelis, 1995). However, because of the necessity for transmission to new hosts, group selection may oppose individual selection, selecting for benign parasites whose hosts live longer and continue to transmit the parasite for a longer period of time.

From another perspective, the quasispecies theory states that, for simple replicon populations with high evolutionary plasticity, such as viruses, selection on the individual virion would be relatively weak. Therefore, the primary target of selection would be the whole quasispecies (Eigen & Schuster, 1979; Eigen & Biebricher, 1988; Nowak, 1992). The reason for this is as follows: the frequency of a given variant in the quasispecies depends not only on its replicating ability (i.e. its fitness) but also on the probability with which it is produced by erroneous replication of other molecules and their frequency in the population; a quasispecies can guide mutations. This does not mean that there is a correlation between the stochastic process of mutation and the selective advantage of the mutant. However, the population can be selected towards higher peaks of the fitness landscapes because the more successful mutants will produce more offspring. Individual RNA genomes may have only fleeting existence, and their evolution is heavily influenced by the mutant spectrum of variants that surround them. De la Torre & Holland (1990) demonstrated that deliberate seeding of very small numbers of highly fit variants into much larger quasispecies populations of lower than average fitness does not always ensure that they will rise to dominance, nor even survive; that is, quasispecies swarms may suppress variants of superior fitness unless they are present above a critical threshold. In relation to the quasispecies concept, Szathmáry & Demeter (1987) suggested that group selection played an important role during the evolution of the first replicons in the prebiotic RNA world.

2. Materials and methods

(i) *Biological materials*

The biological materials and experimental protocols used to obtain the data analysed in the present study have been described previously (Holland *et al.*, 1991; Duarte *et al.*, 1992, 1993, 1994; Clarke *et al.*, 1993). In short, BHK_{21} (baby hamster kidney) cells were grown as monolayers under Dulbecco modified Eagle's minimum essential medium (DMEM) containing 5% newborn bovine calf serum.

All the viral clones employed were derived from the Mudd–Summers strain, Indiana serotype, of the vesicular stomatitis virus (VSV). Two different genetically marked viruses were employed: a wild-type VSV clone, sensitive to a monoclonal antibody (used as reference strain in the competition experiments, see below), and a monoclonal antibody resistant mutant (MARM) clone. The MARM clone selected for this experiment was MARM-*C*, which shows a fitness relative to wild-type of $0{\cdot}90 \pm 0{\cdot}06$. We chose this clone because Muller's ratchet effect (Muller, 1964) is smaller in clones with fitness close to wild-type, as is MARM-*C*, than in high-fitness clones (Novella *et al.*, 1995*a*; Elena *et al.*, 1996). It has been extensively demonstrated (Duarte *et al.*, 1992, 1993, 1994; Clarke *et al.*, 1993) that Muller's ratchet operates under the conditions of the population regimes described below.

Virus was quantified by plaque assays using confluent BHK_{21}cell monolayers under solidified DMEM, providing a structured environment, with 0·7% agarose. Differential quantitation of the MARM clone, compared with total virus (MARM + wild-type), was done by parallel plating of the virus mixtures with and without monoclonal antibody in the agarose overlay. Usually triplicate platings were carried out for each virus plaque number determination. The mouse monoclonal antibody employed was the I_1 (I_1 MAb) produced and characterized by VandePol *et al.* (1986).

(ii) *Experimental populations*

The original MARM-*C* clone was diluted and plated on a monolayer of BHK_{21} cells; then four well-

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isolated plaques were collected (replicates labelled as I, II, III and IV). Each one of these four clones was subjected to three population regimes. The design of these regimes is based on the idea (see Section 1) that group selection is necessarily in opposition to individual selection. Group selection favours intermediate plaques (i.e. intermediate virulence values) while individual selection favours larger plaques (i.e. enhanced virulence). We imposed artificial selection for larger plaques in concert with individual selection and in opposition to group selection, as well as artificial selection for smaller plaques in opposition to individual selection and in the same direction as group selection. The regimes were as follows:

Regime A. Virus from the smallest visible plaque was isolated, diluted, and transferred 20 times on the basis of a daily smallest-plaque-to-smallest-plaque transfer regime. The objective of this regime is to create an artificial selection pressure against large population size, in the opposite direction to individual selection and in the same direction as group selection, thereby selecting populations with reduced virulence. In this case, the expected response is a reduction in virulence.

Regime B. The procedure is the same as that of regime A, except that here the largest plaque is picked and transferred daily. The objective of this regime is to create pressure favouring large groups, acting in the same direction as individual selection and in the opposite direction to group selection, hence selecting populations with increased virulence. The expectation now is an increase in virulence. However, if the magnitude of the group selection factor modulating the virulence of these viral populations is large enough, no significant change would be expected.

Regime C. The procedure is the same as that of previous regimes, but in this case a random plaque is chosen and transferred. Comparing the results of this regime with the original MARM-*C* clone, we have a control for monitoring the action of Muller's ratchet. Also, comparing regime C with regimes A and B, we can determine whether selection on the group character has any effect or not. In this regime we are not acting on (neither boosting nor penalizing) any possible group selection factor.

Infections in all regimes were carried out at 37 °C. In all cases, I_1 MAb was added at transfers 10 and 19 to neutralize possible wild-type revertants. From each replicate and regime, we measured two parameters related to the virulence of each derived strain: mean fitness and the change in growth rate with time.

(iii) *Relative fitness assays*

At the end of each series of transfers the virus was assayed for relative fitness (Holland *et al.*, 1991). Each derived marked clone (resistant to I_1 MAb) was mixed with a known amount of the wild-type clone, and the initial ratio was determined by triplicate plaque assays with and without I_1 MAb in the agarose overlay medium. Each competition mixture was transferred serially up to five times as follows. At each transfer, the resulting virus mixture (MARM-*C* + wild-type) was diluted by a factor of 10^4 (in order to avoid the appearance of defective interfering particles, which can alter the fitness determinations (Horodyski *et al.*, 1983), it is necessary to initiate each new infection with a multiplicity of infection of 0·1 virus per cell or lower) and used to initiate the next competition passage by infection of a fresh cell monolayer. The ratio of MARM-*C* to wild type was determined by plating with and without I_1 MAb in the overlay agarose medium, usually with triplicate plaque assays for each transfer. These determinations gave the proportion MARM (p_t) to wild-type ($1-p_t$) at transfer t. The antilogarithm of the slope of the regression (Hartl & Clark, 1989)

$$\ln\left(\frac{p_t}{1-p_t}\right) = \ln\left(\frac{p_0}{1-p_0}\right) + t \cdot \ln \bar{w}$$

is taken as an estimate of the relative mean fitness of the derived MARM population in relation to the wild-type population ($\bar{w}$).

(iv) *Population growth parameters*

We also allowed for differences among regimes in terms of infectiousness (number of new infections per already infected cell). To do this, we could simply have assigned a different transmission rate (Anderson & May, 1979) to each regime, but there is no way to do this that is not arbitrary. Instead, following the proposal of Dwyer *et al.* (1990), we used the growth data of each replicate within regimes. The virus titre data for a given passage were obtained by counting the total number of observed plaques after each regime passage and taking into account the dilution factor. This counting was done in triplicate, using three independent samples of the same population. From these titre determinations we computed the instantaneous growth rate (μ) for each time interval as $\mu = \mathrm{d} \ln v / \mathrm{d}t$, where v is the decimal logarithm of the titre at each moment.

3. Results

(i) *Estimates of mean fitness*

Table 1 shows the estimates of mean fitness for the four replicates in the three regimes. An ANOVA test (Table 2, second row) showed that the three regimes had a different effect on the resulting fitness, in agreement with the null hypothesis (regime A $<$ regime B $\geqslant$ regime C). A Tukey test (Sokal & Rohlf, 1981) showed that this difference was due to the existence of two groups with different mean fitness:

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Table 1. *Estimates of mean relative fitness* ($\hat{\bar{w}}$) *and the constant of decay in instantaneous growth rate* ($\hat{m}$) *for each replicate and experimental regime*

Regime	Replicate	$\hat{\bar{w}}$	$\hat{m}$	R^2
A	I	0·012 ± 0·004	1·26 ± 0·07	0·953
	II	0·36 ± 0·03	1·4 ± 0·1	0·927
	III	0·000019 ± 0·00006	1·4 ± 0·1	0·898
	IV	0·23 ± 0·04	1·4 ± 0·1	0·889
B	I	0·33 ± 0·02	1·08 ± 0·02	0·994
	II	0·5 ± 0·2	1·04 ± 0·02	0·995
	III	0·7 ± 0·2	1·09 ± 0·04	0·984
	IV	0·46 ± 0·08	1·05 ± 0·02	0·993
C	I	0·57 ± 0·03	0·98 ± 0·02	0·995
	II	0·44 ± 0·03	1·10 ± 0·03	0·984
	III	0·57 ± 0·02	0·98 ± 0·02	0·993
	IV	0·73 ± 0·03	0·94 ± 0·03	0·984

Values are given as mean ± standard error of the mean. The last column shows the coefficients of determination for the non-linear regressions of instantaneous growth rate change on time used to estimate m

one constituted by regime A, with a smaller mean fitness of 0·149, and another group formed by regimes B and C, with a higher mean fitness of 0·531.

The comparison between the fitnesses of the original MARM-*C* clone and the mean fitness of the viruses derived from the control regime C showed that there was a significant reduction in mean fitness ($t_3 = -5{\cdot}500$, one-tailed $P = 0{\cdot}006$). Such a reduction can be explained by the effect of Muller's ratchet due to the continuous plaque-to-plaque passages, as expected.

(ii) *Estimates of growth parameters*

Fig. 1 shows the instantaneous growth rate versus passage number for each experimental regime as well as the regression fitted to the mean titre values. An inverse function of the passage number provided the best fit to the experimental data: $\mu = m/t$. The proportionality constant, m, that characterizes the instantaneous growth rate decay with time for each replicate, as well as the determination coefficients for each individual regression, R^2, are shown in the last two columns of Table 1. An ANOVA test (Table 2, third row) showed that the three regimes had a different effect on the growth parameter, m. As in the previous case, a Tukey test also showed that this difference was due to the existence of two different groups with different growth decay constants. The first group was formed by regime A, with a faster growth decay constant of 1·354, and the second group by regimes B and C, with a slower mean growth decay constant of 0·997.

(iii) *Multivariate analysis*

A Pearson correlation test showed that the two variables, and m, were highly correlated ($r = -0{\cdot}782$, $n = 12$, $P = 0{\cdot}003$), meaning that the greater the decay in growth rate, the smaller the fitness value of the resulting population.

Using the Hotelling's T^2 test in a multivariate analysis of variance, we found that there exists a significant difference between the three experimental regimes (Table 2, first row).

When applying a principal components analysis (Manly, 1994), we found that 99·97% of the observed variability was explained by the major eigenvalue ($\lambda_1 = 9{\cdot}224$) of the variance–covariance matrix of $\bar{w}$ and m^{-1} (the second eigenvalue was lower than 1 and negligible). Finally, summarizing the findings of both variables in only one statistic, we computed the standardized canonical discriminant function (f_1) in the direction of the major eigenvector, which gives the

Table 2. *Multivariate analysis of variance carried out to study the differences among regimes as well as the effect of group selection on the results*

Null model	Analysis	T^2	F	Hypothesis d.f.	Error d.f.	P
Full A < B ⩾ C	Multivariate	9·226	16·146	4	14	0·333
	$\bar{w}$	–	9·625	2	9	0·340
	m	–	35·955	2	9	0·280
Paired A < C	Multivariate	8·978	22·445	2	5	0·499
Paired B ⩾ C	Multivariate	0·555	1·387	2	5	0·667

T^2 is the Hotelling's statistic employed in the multivariate tests. 'Hypothesis d.f.' are the numerator degrees of freedom and 'Error d.f.' are the denominator degrees of freedom. In the case of full model analysis, the univariate tests of significance are also provided. The null hypothesis was that the regimes had different effects and assumed the following effect order among regimes: A < B ⩾ C (see Section 2 for a more precise description of these expectations). P is the probability that the corresponding null hypothesis is true.

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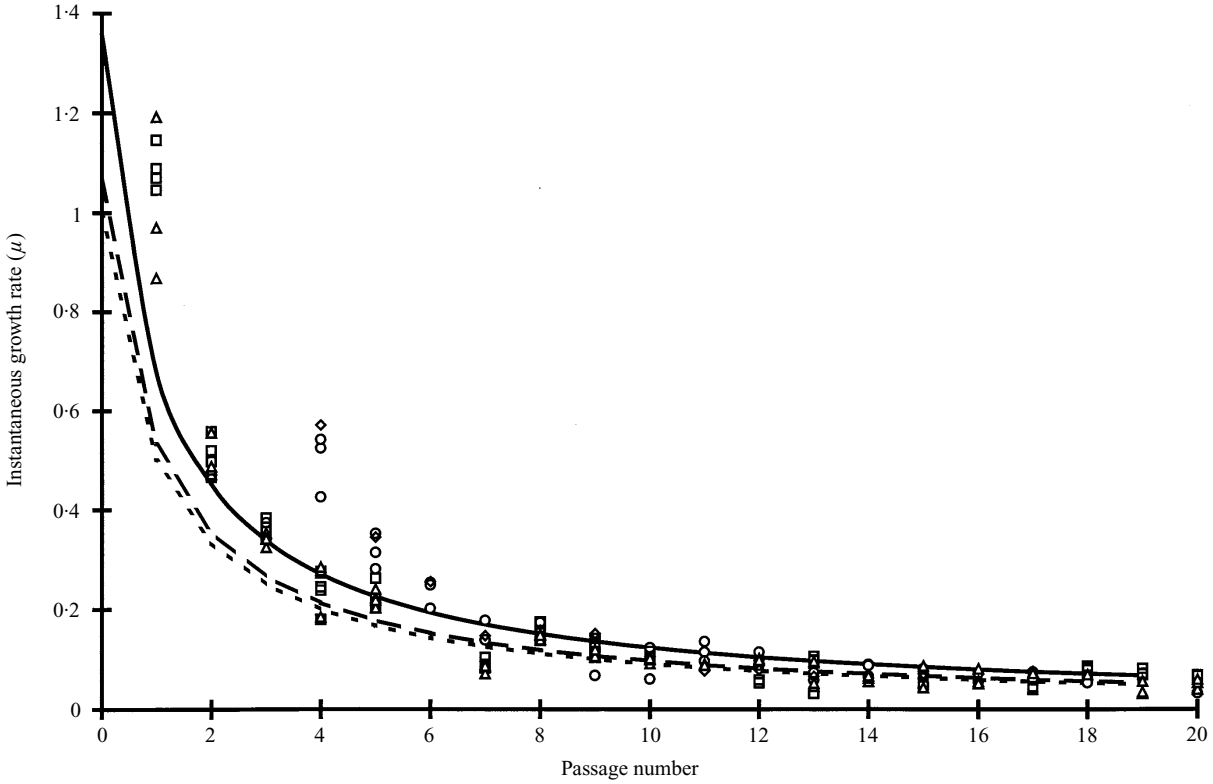

Fig. 1. Change in the instantaneous growth rate (μ) with passage number for each experimental regime. The experimental determinations for each regime are presented as: regime A, circles; regime B, squares; regime C, triangles. The fitted decay curves for each regime are: regime A, continuous; regime B, dashed; regime C, dotted.

following expression: $f_1 = 0{\cdot}884 \cdot \bar{w} + 0{\cdot}369 \cdot m^{-1}$. It should be possible, and interesting, to find a biological meaning for this canonical discriminant function. Anderson & May (1982) suggested that the most important parameter in the study of the evolutionary dynamics of a parasite is its basic reproductive rate (R_0), that is, the number of successful offspring a parasite or parasitic infection is capable of producing in the absence of density-dependent constraints. This is also Fisher's 'net reproductive value' rate (i.e. fitness) for the parasite:

$$\bar{w} \equiv R_0 = \beta K/(\alpha + d + r),$$

where β is the transmission rate, K is the carrying capacity of the system (i.e. the available host population size), α is the virus-induced mortality rate (its virulence), d is the *per capita* host death rate in the absence of viral infection, and r the *per capita* illness recovery. In our particular experimental design, d and r should be assumed equal to zero, or at least, $\alpha \gg d + r$. As Dwyer *et al.* (1990) suggested in their work, and as we have adopted here, the transmission rate, β, is difficult to assign, but the population growth parameters provide a good approximation. Using this approximation, the change in virulence can be obtained as a proportional relationship between the rate of decay in instantaneous growth rate, m, and the mean fitness, $\bar{w}$: $\alpha \propto m/\bar{w}$ (where the proportionality factor is the carrying capacity, also constant in our design). Whether or not this relationship is linear remains uncertain, but as a first approximation and without loss of generality, we will assume the simplest model, i.e. that it is linear. Since f_1 is linearly proportional to the decay in instantaneous growth rate and mean fitness (the latter being more heavily weighted), the f_1 may be interpreted as a measure of virulence of the VSV populations.

Fig. 2 shows the mean virulence (with 95% confidence intervals) for each regime. A first glance at Fig. 2 suggests that there is a difference between regime A and the other two regimes. In the following section these differences will be analysed using paired comparison tests.

(iv) *Changes due to group selection*

In order to gain evidence of an existing group selection factor, we compared by means of paired MANOVA the results of regime C with those of regimes A and B respectively. The results are in concordance with the null hypothesis. The comparison of regimes A and C showed that under regime A, a larger reduction in the traits was obtained than in regime C (Table 2, fourth row). This increased reduction in parameters related to virulence is easily explained by group selection acting in the same direction as Muller's ratchet; that is, the virulence of the populations is reduced. The comparison of regimes B and C showed that there was not a significant difference between these regimes (Table 2, fifth row). These results are what would be

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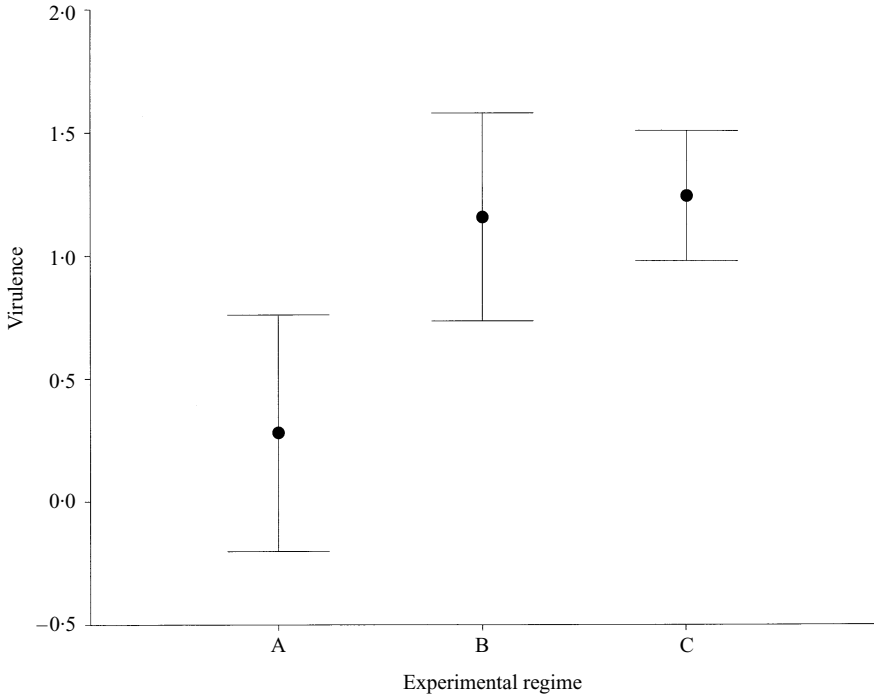

Fig. 2. Mean virulence (with 95% confidence intervals) estimated as the first canonical discriminant function evaluated at group means in each experimental regime.

expected if a strong group selection had been acting during evolution, counteracting any increase in virulence by individual selection.

4. Discussion

On the basis of theoretical (Levins, 1970; Wilson, 1973, 1975; Levin & Kilmer, 1974; Gadgil, 1975; Gilpin, 1975) and experimental (Wade, 1976, 1977; Wade & McCauley, 1980) results it seems clear that in order to detect the existence of any group selection component in the evolution of any population, it is necessary that individual and group selection act in opposite directions. This was the case in our experiment. Under regime A, while individual selection was responsible for an increase in the virulence of each virion, group selection reduced it to an intermediate value. With the artificial selection protocol acting for the group component, we have amplified its effect and shown evidence for its existence; its effect is to reduce fitness more than would be expected if only Muller's ratchet were acting. We observed a larger reduction in fitness under regime A ($\sim 83\%$) than in the control regime C ($\sim 36\%$, due only to the effect of Muller's ratchet) when compared with the original MARM-*C* clone. On the contrary, we would expect regime B to show an equal or higher fitness than control C, because in this case artificial selection was applied in the same direction as individual selection, increasing virulence. The group selection factor acting in the same direction as Muller's ratchet, reducing the fitness, justifies the lack of differences between regime B and the control C.

We also observed that the instantaneous growth rate decayed during the experiment. This is expected due to the action of Muller's ratchet: the virus accumulates more and more mutations producing a decrease in its replicative ability. The interesting point here is the heterogeneity among experimental regimes – the fact that under regime A the decay constant is greater ($\sim 24\%$) than under regimes B or C. We propose as an explanation for this difference that in the case of regime A, besides the action of Muller's ratchet we also powered the effect of group selection, reducing the growth rates.

This similar pattern in the behaviour of fitness and growth rates was reflected in a significant negative correlation between them, showing the existence of a trade-off between different virulence parameters. Several models of virulence evolution have predicted the necessity of these trade-offs between virulence and transmission rate to justify the evolution towards intermediate virulence values in experimental populations without the necessity of any group selection argument (May & Anderson, 1983*b*; Dwyer *et al.*, 1990; Levin & Svanborg Edén, 1990; Frank, 1996). However, Lenski & May (1994) showed that these models do not necessarily contradict the group selection point of view in the long run and, in fact, the group selection models provide a simple mechanistic explanation for the evolution of reduced virulence. Following their arguments, at the beginning of the infection, when the density of uninfected cells is high, selection can favour a highly virulent virus. As the infection progresses, the density of available cells decreases and, as a consequence, the optimal trans-

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mission rate also decreases, allowing a new and less virulent virus to invade the population. The transmission rate does not evolve to zero, but converges on a critical value. This idea of a decrease in both transmission rate and virulence is in concordance with our observations: the greater the decay in growth rate (i.e. smaller growth rate and transmission rate), the smaller the mean fitness of the virus.

Bremermann & Thieme (1989) showed that when new strains with different virulence levels arise through mutation, only a single strain will evolve, and the virulence level of that strain is that which maximizes the mean fitness of the population. In our results, it seems that the maximum fitness is constrained by group selection, which imposes an upper limit.

The quasispecies theory is in agreement with previously expressed ideas about group selection. By its nature, i.e. a 'cloud' or distribution of closely related mutants, the quasispecies fulfils the conditions to be considered the target of group selection: (i) we are dealing with organisms whose population structure promotes fast genetic divergence (Wade, 1976), (ii) the members of the quasispecies are intimately related (Hull, 1980) and, (iii) following from the previous condition, the whole distribution of mutants should be considered as an individual instead of a group (Hull, 1980).

In conclusion, we have observed that, while it is possible to reduce the virulence of a virus, it does not seem possible to increase it, because group selection modulates this increase. This is taken as evidence that group selection may be an important factor affecting viral evolution; its effect is to moderate the virulence of populations towards intermediate values, lending support to the 'conventional wisdom' (May & Anderson, 1983*a*). One could argue that there is other empirical evidence indicating that the fitness of experimental populations of VSV evolves exponentially (Novella *et al*., 1995*b*). It is necessary to point out, however, that there are clear differences between those experiments and the one presented here. Novella *et al*. (1995*b*) worked with large mixed populations in which the main evolutionary force was the competitive interactions between different coexisting variants. In the experiments reported here there is no opportunity for competitive optimization because of the subdivision of populations (single plaques) and, therefore, the opportunity for divergence between groups.

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