

THE ECOLOGY AND GENETICS OF FITNESS IN *CHLAMYDOMONAS*. IX. THE RATE OF ACCUMULATION OF VARIATION OF FITNESS UNDER SELECTION

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Abstract.—Populations of *Chlamydomonas* founded by single cells were cultured in chemostats for 50 days, representing about 125 generations. The mean and variance of division rate was measured daily by withdrawing cells from the effluent and culturing them for 24 h on filtered effluent medium solidified with agar. Mean fitness did not change during the period of culture, and the behavior of neutral markers indicated that no substitutions of novel beneficial mutations occurred. However, the variance of fitness increased markedly at about the same rate in two replicate populations. The standardized rate, or mutational heritability, was $V_m/V_E = 4 - 5 \times 10^{-3}$ per generation. This is substantially greater than most other estimates for characters closely correlated with fitness. Moreover, it seems difficult to reconcile with the absence of any change in mean fitness. We investigated the possibility that frequency-dependent selection was created by spatial heterogeneity within the culture vessel by testing cell populations with different phenotypes from the top, bottom, and surface of the chemostats. However, the differentiation of these populations seemed to be attributable to phenotypic plasticity, with no evidence that their characteristics were heritable. Finally, we report an experiment in which lines were selected for about 100 generations on solid or liquid medium. These lines became specifically adapted to the medium on which they were cultured, showing that liquid and solid media, even when chemically identical, provide different conditions of growth for *Chlamydomonas*. The genetic variance appearing in the cultures was therefore attributed to conditionally neutral mutations that were not expressed in the chemostat. This implies that rates of accumulation of mutational variance measured in the culture environment itself (where this can be done) may greatly underestimate the variation available for a response through selection to environmental change. Moreover, it suggests that chemostat populations may be more dynamic and more diverse than is usually thought.

Key words.—*Chlamydomonas*, conditional neutrality, environmental heterogeneity, fitness, genotype-environment interaction, mutation rate, mutational heritability, selection.

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The long-term response of populations to continued selection depends on the creation of new genetic variation by mutation. This variation accumulates in populations until it reaches an equilibrium determined by the opposed processes of mutation and selection and may constitute a substantial fraction of the total phenotypic variation of quantitative characters, including fitness and fitness components (Lande 1976; Houle et al. 1996). For this reason, the rate at which mutational variance for fitness and fitness components appears in populations is an important population parameter that constrains the rate of response to selection, affects the standing crop of genetic variation; and may affect the evolution of sex, recombination, and mating systems (Charlesworth and Charlesworth 1987; Kondrashov 1988; Houle et al. 1996).

The total phenotypic variance of a character comprises a genetic variance, originally produced in previous generations by mutation, and a recurrent environmental variance. The rate at which new genetic variance arises per generation, V_m , can be appropriately expressed in terms of the standing environmental variance, V_E , as the ratio V_m/V_E . This standardized “mutational heritability” (Lynch 1988) is directly related to the response of the character to selection, and can be used to compare different traits or different organisms. The way in which it is expected to affect levels of genetic variation was originally worked out for stabilizing selection in a randomly mating sexual population, assuming additive allelic

effects, a continuum of alleles at each locus, and a normal distribution of allelic effects (Lande 1976; Turelli 1986). Subsequent models have examined truncation selection (Hill 1982a,b; Zeng and Hill 1986), different life cycles (Lynch and Gabriel 1983), pleiotropy (Lande 1980), and non-Gaussian distributions of allelic effects (Turelli 1986). Although these models differ in their quantitative predictions, all agree that mutation is likely to be an important factor in determining the amount of genetic variance in a population (Lynch 1988). In particular, the expected mutation-selection equilibrium for traits under directional selection, such as fitness, is equal to V_m/s , where s is the average selection coefficient (Barton 1990). The authors of a recent survey of experiments estimating V_m concluded that the available evidence suggested that mutation-selection balance can account for observed levels of genetic variance maintained in populations (Houle et al. 1996).

Although the mutational heritability is commonly used to standardize estimates of V_m for different traits or organisms (Lynch 1988), Houle et al. (1996) have recently demonstrated the value of two other dimensionless measures: the ratio of V_G , the standing genetic variance, to V_m ; and the mutational coefficient of variation, CV_m . Specifically, the value of V_G/V_m is important in assessing the mutation-selection balance hypothesis for the maintenance of genetic variation, and CV_m is correlated to the potential response to selection (Houle et al. 1996).

There have been three types of experiments used to estimate V_m . The first involves analyzing the response to selection of a highly inbred line of a sexual species (Mather and Wigan 1942; Clayton and Robertson 1955). The second involves the examination of inbreeding depression data (Deng and Lynch 1996), for instance, in cyclical parthenogens (Deng and Lynch 1997). The third, and by far the most common, relies on long-term mutation accumulation in isolated lines, using the divergence between inbred lines to obtain estimates of V_m (Durrant and Mather 1954; Mukai 1964; Mukai et al. 1972, 1984; Ohnishi 1977; Houle et al. 1992). Almost all of these experiments have involved highly inbred lines of *Drosophila*, where special chromosomal constructs can be used to prevent recombination and to isolate mutations from selection. Due to the artificiality of this system, its results have recently been called into question (Keightley 1996; Peck and Eyre-Walker 1997). At the same time, several researchers have used demographic means, rather than a balancer chromosome, to limit the selection on mutation-accumulation lines (Lynch 1985; Kibota and Lynch 1996; Keightley and Caballero 1997; Shabalina et al. 1997). The results of these experiments have been mixed: some have produced estimates of V_m roughly in agreement with the older *Drosophila* estimates (Kibota and Lynch 1996; Shabalina et al. 1997), whereas others have resulted in drastically lower values for V_m (Keightley and Caballero 1997).

All of the mutation-accumulation experiments described above share the common aim of minimizing the loss of novel mutational variance through the elimination of deleterious mutations by selection. It is of course unlikely that this situation ever occurs in nature. We have used a different approach by measuring rates of division in clonal populations of a microorganism in mass culture. The populations are not shielded from natural selection (indeed, the large population sizes used in this experiment should make it stringent), and the result is an estimate of V_m associated with the initial generation of genetic variance in a uniform population as the result of novel mutation and simultaneous countervailing selection. This estimate can be compared with comparable estimates of characters related to fitness in *Drosophila* and *Daphnia* and was intended to give a more realistic description of the early stages in the accumulation of mutational load.

Our experimental system consisted of the unicellular chlorophyte *Chlamydomonas reinhardtii* maintained axenically in a chemostat, where the populations are strictly asexual. Because the populations are clonal, V_m and V_E can be estimated from the variance of fitness within a single population and it is unnecessary to maintain a large number of isolated lines. *Chlamydomonas reinhardtii* is haploid, so there are no dominance effects; moreover, because the cultures are also asexual, overdominance cannot contribute to the maintenance of variation. The chemostat provides nearly constant and homogeneous conditions of growth, apparently eliminating the possibility that environmental heterogeneity might sustain variation. Finally, fitness as division rate can be quickly and easily measured at frequent intervals for 100 generations or more. The algal chemostat thus provides a simple and practicable way of studying the appearance of variation in populations.

MATERIALS AND METHODS

Chemostat Apparatus

The chemostats used in these experiments were simple gravity- and pressure-operated devices designed in our laboratory. An Erlenmeyer flask of 1-L capacity was provided with a bung through which three glass tubes passed: (1) a short Pasteur pipette linked to an overhead reservoir by Tygon tubing through a valve, dripping fresh medium into the flask; (2) a long Pasteur pipette supplying sterile air; and (3) a long exhaust tube extending just beyond the air inflow. Air pressure forces culture medium and cells up the exhaust tube, maintaining a constant culture level (approximately 200 ml) just below the opening of the tube. The air itself is blown onto the surface of the water without bubbling to avoid splash contamination of the inlet tube. The components were autoclaved separately and assembled in a laminar flow hood to ensure sterility.

Main Chemostat Experiment

Two replicate chemostats were inoculated with strain CC-2938 (strain designation of the *Chlamydomonas* Genetics Center, Duke University) of *C. reinhardtii* (Sack et al. 1994). The strain was subcloned before beginning the experiment, so that each chemostat was initially inoculated with a single small colony of a few hundred cells. Thus, each chemostat was occupied by the descendants of a single cell. The chemostats were supplied with a minimal Bold's medium (Harris 1989), which was modified by using ammonium nitrate rather than sodium nitrate as the nitrogen source. The chemostats were maintained for 50 days at a dilution rate of 2.5 volumes/day (125 generations of growth) under constant illumination from cool-white fluorescent tubes. The population of each chemostat comprised about $1 - 2 \times 10^8$ cells.

Samples were taken from the chemostat every 24 h through a three-way stopcock fitted to the exhaust tubing. These samples consisted of both cells and spent medium. Volumes of 50 ml of the samples were centrifuged at 2000 rpm for 6 min. All but 5 ml of the supernatant was then poured off: this cell-free medium was then solidified with 1.5% agar and used to make the plates on which the fitness of cells was assayed. The growth conditions for the assay were thus made as similar as possible to the chemostat environment. The pellet was resuspended by vortexing the 5 ml of medium remaining in the centrifuge tube. An aliquot (250 μ l) of this was then pipetted onto the plate and spread by gentle shaking. Our laboratory has found that spreading plates in the normal way, using a platinum-wire loop or a glass "hockey stick," kills up to 70% of the sample, whereas almost all cells commence division after transfer by pipette (S. Leboeuf, unpubl. data). After inoculation, the surface of the plate is covered by a sparse field of isolated cells. The plates were transferred to shelves illuminated by soft-white fluorescent lights and left for 24 h. The number of cells in each of 100 colonies was then scored by examining the plates under a dissecting microscope at 100 \times magnification. The number of divisions per colony was calculated as \log_2 cell number.

Quasi-neutral Markers

Two mutants derived from CC-2938 were also used to detect periodic selection. One was a yellow mutant isolated after mild UV irradiation. The cells of this strain appear normal (green) when grown in the light, but form yellow colonies when cultured in the dark using acetate as a carbon source because they are unable to synthesize chlorophyll in the dark. This mutant was tested against CC-2938 and found to be quasi-neutral, with selection coefficients less than 1%. The second type comprised cells appearing during the experiment that were able to grow on plates supplemented with chlorate. These are defective for nitrate reductase activity (nit⁻; Nichols and Syrett 1978), but can accumulate in media containing ammonium as a nitrogen source. To follow the frequencies of these markers, samples from the pellet were also inoculated onto plates spread with Bold's medium supplemented with either acetate or chlorate. The frequency of the yellow marker was estimated by counting the yellow and green colonies on the acetate plates cultured in the dark. The frequency of chlorate-resistant mutants was estimated by counting the number of live colonies on chlorate plates one week after inoculation and expressing this as a fraction of the total number of colonies (live or dead) on the plate.

Subsampling Experiment

A second experiment was performed to investigate population structure within the chemostat. The protocol was similar to that of the main chemostat experiment, except that the vessels were sampled weekly rather than daily by withdrawing samples directly from them. Samples were taken on each occasion from three locations within the chemostat: the top (cells in suspension), bottom (flocculent clumps of cells), and sides (attached cells). The top and bottom of the vessel was sampled using a pipette. Wall growth was assessed by providing both chemostats with several glass cover slips, one of which was taken on each occasion and washed with distilled water to remove cells. The three cell populations were suspended in filter-sterilized outflow. Fitness was assayed as before by measuring colony growth on solid medium made using this filter-sterilized outflow. Tendency to flocculate was assayed by measuring the size distribution of cells and cell aggregates with a Coulter Multisizer. Adherence was assayed by maintaining cultures in vials containing a cover slip; after three days we counted the number of cells attached to a 25-mm² area of the cover slip.

Liquid and Solid Growth

The relationship between fitness in the experimental conditions (liquid medium) and assay conditions (solid medium) is difficult to investigate using spent chemostat outflow because it will not support sustained growth. We therefore used the results of an experiment where cultures were maintained in Bold's medium, the chemostat inflow. Liquid-medium lines were cultured in 2-L Erlenmeyer flasks bubbled with sterile air. A 250- μ l aliquot was transferred every week, by which time the cultures were at or near maximum density. Solid-medium lines were cultured on agar plates. They were transferred every two weeks by washing off the cells with

sterile medium and inoculating new plates with 250 μ l of this suspension. The base population for this experiment was a line, originally founded from a genetically heterogeneous population, that had been maintained in our laboratory for two years, during which time it passed through 16 sexual cycles and about 250 vegetative generations. This line is known to have possessed genotypic diversity and genetic variance for fitness at the end of this time. Eight liquid-medium lines and eight solid-medium lines were derived from it and maintained in the laboratory for a further nine months, during which they went through about 100–200 vegetative generations. The lines were manipulated so as to express different levels of sexuality, but these treatments had no effect on the mean or variance of fitness, so the eight lines for each medium treatment were analyzed as replicates. Six spores were extracted from each and grown either in liquid medium in culture tubes or in solid medium on agar plates. Fitness in liquid medium was evaluated from complete growth curves obtained from repeated spectrophotometer readings as carrying capacity, estimated by fitting the growth data to the logistic equation with the NLIN procedure of SAS release 6.1. Fitness on solid medium was evaluated by washing off cultures after two weeks' growth and measuring the density of a suspension of standard dilution with a spectrophotometer. In both cases the character assayed was closely related to fitness in the context of the experiment, that is, the growth achieved immediately before transfer. There were two replicate cultures of each spore in both trials, arranged in two independently randomized blocks. The main object of the experiment was to determine whether growth in liquid and on solid media can be selected independently and therefore represent different characters. This hypothesis was tested by the interaction of selection environment with assay environment, using the GLM procedure in SAS release 6.12. This analysis also gave information about the distribution of genotypic variance among and within the selection lines.

RESULTS

Accumulation of Variation

The mean and variance of fitness was estimated from the daily assays. In neither chemostat was there a significant change in mean fitness after 50 days (see Fig. 1). The variance in fitness, however, showed a highly significant increase ($P < 0.0005$) in both chemostats (see Fig. 2). The slopes of the two regressions of variance in fitness versus day of assay were 0.00834 (SE 0.0014) and 0.00956 (SE 0.0017). There was no consistent pattern in the residuals, with second-order regression coefficients insignificant and of opposite sign in the two chemostats (Fig. 3). We conclude that variance increased linearly through time over the period of the experiment. Assuming that the only source of novel variation in fitness was mutational input, these slopes provide estimates of V_m as $0.0083 \times 50/125 = 3.3 \times 10^{-3}$ and $0.0096 \times 50/125 = 3.8 \times 10^{-3}$ per generation for the two replicate chemostats ($V_m = \text{slope} \times \text{day/generations}$).

V_E was estimated as the variance in fitness at the beginning of the experiment from the y-intercept of the fitted linear regression. This is based on the assumption that with a genetically uniform base population, the phenotypic variation

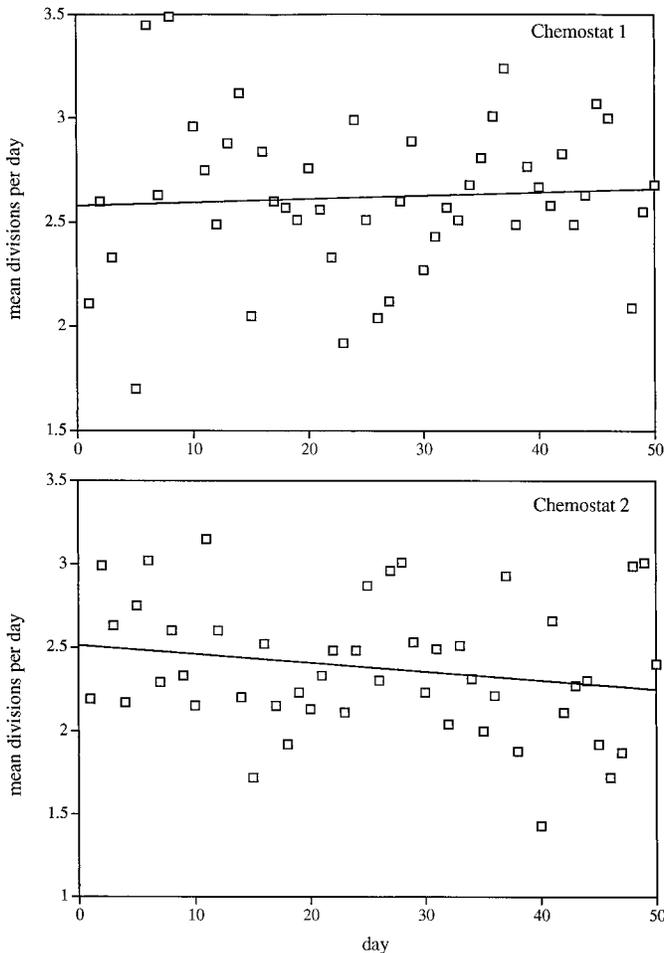


FIG. 1. Mean fitness. The x-axis indicates day of trial; the y-axis mean number of cell divisions in 24 h ("fitness") of 100 colonies on plates made from filter-sterilized chemostat outflow medium. In neither case is the linear regression significantly different from zero at $P = 0.05$.

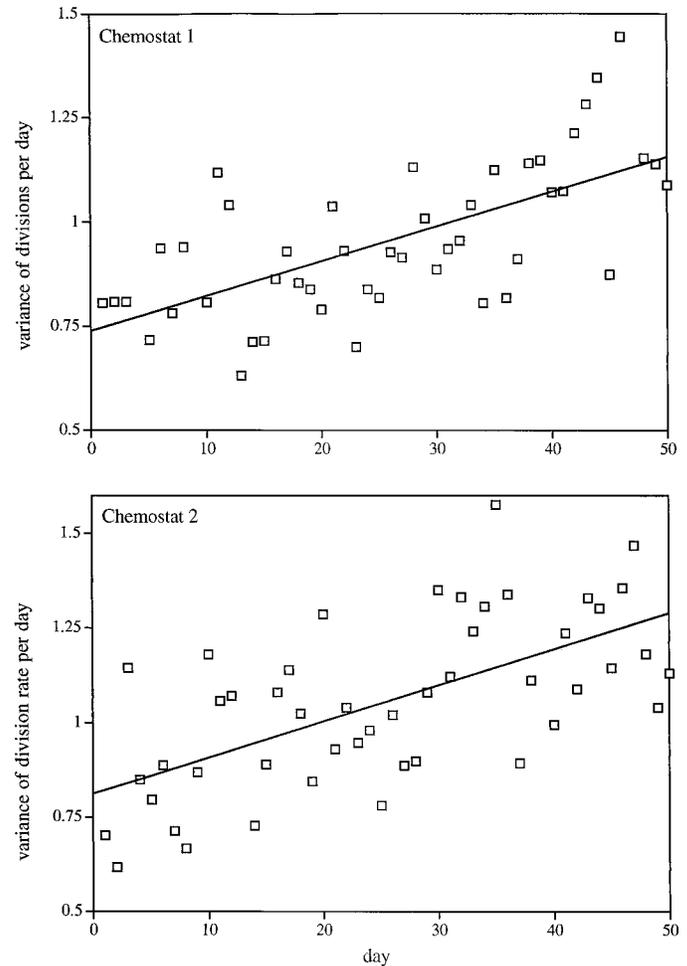


FIG. 2. Variance of fitness. The x-axis indicates day of trial; the y-axis variance of number of cell divisions in 24 h ("fitness") of 100 colonies on plates made from filter-sterilized chemostat outflow medium. The regression equations are: chemostat 1: $y = 0.00834x + 0.739$ ($s_b = 0.0014$; $r^2 = 0.37$) and chemostat 2: $y = 0.00956x + 0.812$ ($s_b = 0.0017$; $r^2 = 0.40$).

is equal to the environmental variation at the start of the experiment. The values obtained for the two chemostats were 0.739 (SE 0.042) and 0.812 (SE 0.051). These estimates of V_m and V_E yield mutational heritabilities of $V_m/V_E = 4.5 \times 10^{-3}$ and 4.7×10^{-3} per generation for the two replicate chemostats. The subsampling experiment yielded two more estimates of $V_m/V_E = 2.6 \times 10^{-3}$ and 3.3×10^{-3} per generation. These are less reliable because sampling was less frequent.

Periodic Selection and Spatial Heterogeneity

The two markers used fluctuated erratically at low frequency with no sign of a periodic selective sweep having taken place (Fig. 4). There was no evidence that the populations sampled from different regions of the chemostat had diverged genetically. Neither the fitness estimates nor the phenotypic scores of flocculation and attachment differed significantly among samples from the top, bottom, or sides of the vessel (data not shown).

Growth in Liquid and Solid Media

The selection \times assay environment interaction evident in the results of this experiment (Table 1) showed that the cultures had responded to selection: Populations that had previously been cultured in liquid medium reached greater densities in liquid than did those that had been cultured on solid medium, whereas those that had been cultured on solid medium grew more on solid medium than did those that had been cultured in liquid. Selectable genetic variation for growth habit therefore existed in the base population or arose during the course of the experiment.

When the assay environments are considered separately, there is substantial genetic variance for fitness within lines. Estimates vary widely among lines, perhaps because relatively few spores were assayed per line, but are positive in 25 of 32 cases and individually significant at $P = 0.01$ in five of 32 cases. When the assay environments are considered together, however, main-effect genetic variance can no longer be detected. The genotypic variance appears instead in the

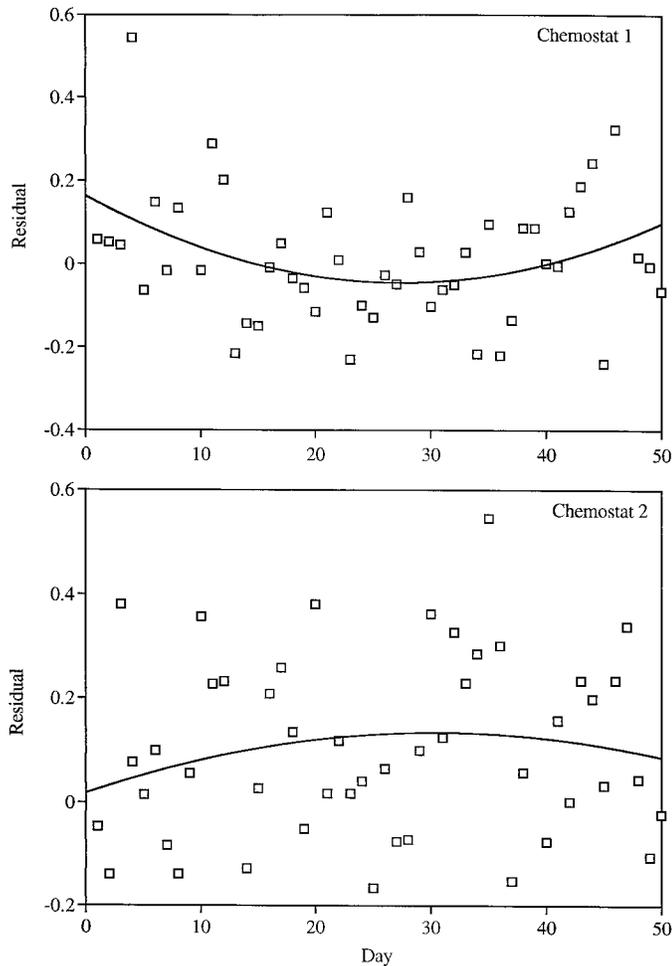


FIG. 3. Residuals of variance of fitness. The x-axis indicates day of trial; the y-axis residuals of the regression of variance on day. The second-order regression equations are: chemostat 1: $y = 0.00028x^2 - 0.015x + 0.164$ ($r^2 = 0.13$) and chemostat 2: $y = -0.00012x^2 + 0.008x + 0.018$ ($r^2 = 0.03$).

form of genotype-environment interaction, both among and within lines (Table 1). Despite their chemical similarity, liquid and solid media seem to represent unexpectedly different environments from the point of view of the algae, with a low genetic correlation of fitness between the two.

DISCUSSION

When a well-adapted, genetically uniform population is allowed to proliferate, genetic variance for fitness will tend to accumulate because of the input of new mutations at a roughly constant rate in each generation. At the same time, deleterious mutations will tend to be removed by selection at a rate equal to the standardized genetic variance of fitness, which will increase over time. The overall rate of increase in variance is the difference between the rate of input through mutation and the rate of elimination through selection, which will fall through time as variance accumulates and selection accelerates. This rate will be initially equal to V_m and will eventually approach zero as mutation-selection equilibrium is approached.

It is never possible to exclude the operation of selection altogether, because there will inevitably be a tendency to transfer more fit individuals within lines or retain lines with greater mean fitness. In the experiments with *Daphnia* reported by Lynch (1985), for example, only eight clones survived of the 50 originally isolated. We did not attempt to exclude selection, but its effect seems to have been limited to preventing an overall reduction in mean fitness. The CC-2938 material has been maintained on minimal medium in our laboratory since its isolation in 1993, and had evidently become well adapted to laboratory conditions of growth. We did not detect any selective sweeps caused by the passage of mutations of major effect, nor did mean fitness increase during the experiment in a manner suggesting the substitution of beneficial mutations of minor effect. This is not especially surprising in an experiment lasting less than 200 generations in an environment containing no radically novel source of stress. Moreover, the increase in variance over the course of the experiment appeared to be linear, with no detectable tendency for the rate of increase in variance to diminish through time. These facts suggest that the two chemostat lineages represent the early stages of the accumulation of mutations, so that the regression slopes are good estimates of V_m , especially because they are based on time series rather than being point estimates.

The values that we obtained for V_m/V_E of fitness, 4.5×10^{-3} and 4.7×10^{-3} per generation, are comparable with those obtained in other studies of mutational heritability (reviewed by Houle et al. 1996). Most of these studies were carried out with *Drosophila melanogaster*, where the characters scored have included abdominal bristle number (Mather and Wigan 1942; Clayton and Robertson 1955; López and López-Fanjul 1993a,b; Mackay et al. 1994), sternopleural bristle number (Mather and Wigan 1942; Durrant and Mather 1954), alcohol dehydrogenase activity (Mukai et al. 1984), life-history traits (Houle et al. 1994), viability (Mukai 1964; Mukai et al. 1972; Ohnishi 1977), and fitness (Houle et al. 1992; V_m only). Other studies have considered body size in mice (Keightley and Hill 1992), pupal weight in *Tribolium* (Enfield and Braskerud 1989), life history traits in *Daphnia* (Lynch 1985; Deng and Lynch 1997), lifetime reproductive output and lifespan in *Caenorhabditis elegans* (Keightley and Caballero 1997), vegetative and reproductive traits in several crop plants (reviewed in Lynch 1988; Houle et al. 1996). Despite the diversity of characters, organisms, and methods in these studies, the estimates obtained for V_m/V_E are remarkably consistent. Almost all fall between 10^{-4} and 5×10^{-2} per generation, and the majority lie around 10^{-3} (Lynch 1988; Houle et al. 1996).

Nevertheless, there are two problematic aspects of our results. The first is the unexpectedly high rate of accumulation of variance, given the unimpeded action of selection in our experiment. The second is the seemingly paradoxical combination of three results: an increase in variance in fitness, a lack of change in mean fitness, and the lack of evidence for selective sweeps. We discuss these problems in turn.

Our estimates of V_m seem to be substantially higher than most of those reported from the most closely comparable studies involving fitness and fitness components. Characters close to fitness seem to have relatively low values of V_m/V_E ,

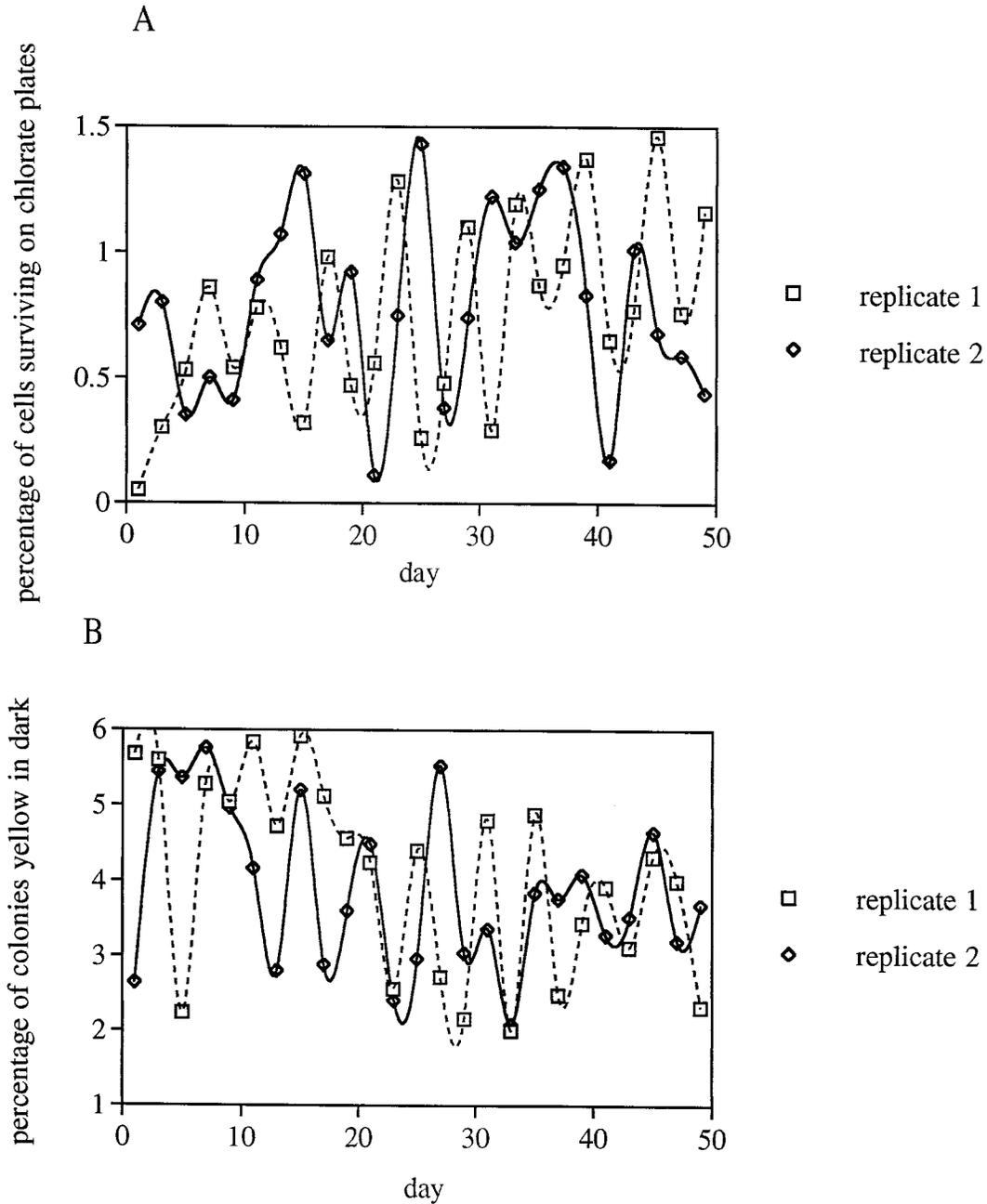


FIG. 4. Behavior of neutral markers. The frequency of two markers was followed. The x-axis indicates day of trial; the y-axis the percentage of cells carrying the neutral marker. (A) Nitrate reductase deficiency: the frequency of nit⁻ cells was indicated by the proportion of cells surviving on chlorate plates. (B) Yellow in dark: the frequency of y mutants was indicated by the proportion of yellow colonies (in contrast to wild-type green) on acetate-supplemented plates in the dark.

perhaps because of the action of selection during the period of mutation accumulation. Estimates of $V_m/V_E = 0.5 - 1 \times 10^{-4}$ per generation for viability in *Drosophila* have been reported by Mukai (1964), Mukai et al. (1972), and Ohnishi (1977). Houle et al. (1992) obtained $V_m = 8.3 \times 10^{-4}$ per generation for fitness in *Drosophila*, and Lynch found $V_m = 1.2 \times 10^{-4}$ and $V_m/V_E = 8.3 \times 10^{-4}$ per generation for fitness in *Daphnia pulex*. Deng and Lynch (1997) estimated V_m/V_E for mean clutch size to be 8.9×10^{-4} and 3.4×10^{-5} in *Daphnia pulicaria* and *Daphnia arenata*, respectively. Lynch

(1988) analyzed data published by Cox et al. (1987) to estimate V_m/V_E for grain yield in barley at $< 1 \times 10^{-4}$ in high-nutrient conditions and 4×10^{-4} in low-nutrient conditions. Our estimates of $V_m = 3.5 \times 10^{-3}$ and $V_m/V_E = 4.6 \times 10^{-3}$ per generation may be comparable with Houle's results (depending upon the magnitude of V_E for fitness in *Drosophila*), but are about four times greater than the estimates reported for *Daphnia pulex* and clutch size in *D. pulicaria* and an order of magnitude greater than estimates for viability in *Drosophila*, mean clutch size in *D. arenata*, and grain yield in

TABLE 1. Analysis of variance for the selection experiment.

Source	df	F denominator	MS	F	P
Selection environment, E_s	1	$L(E_s)$	130,648	3.85	0.07
Assay environment, E_A	1	$L(E_s)$	29,077,219	857	0.0001
Selection \times assay environment, $E_s \times E_A$	1	$L(E_s)$	497,442	14.7	0.0025
Line (selection environment), $L(E_s)$	14	$L(E_s) \times E_A$	33,919	<1	ns
Line (selection environment \times assay environment), $L(E_s) \times E_A$	14	$S(L)$	57,307	5.60	0.0001
Spore (line), $S(L)$	80	$S(L) \times E_A$	9780	<1	ns
Spore (line \times assay environment), $S(L) \times E_A$	80	r	11,372	2.27	0.01
Replicate culture, r	191		5015		
Total	382				

barley. The only study we know of that resulted in a similar estimate of V_m/V_E for fitness is that of Keightley and Caballero (1997) with *C. elegans*, which estimated V_m/V_E to be 1.2×10^{-3} .

The relatively high value for V_m/V_E obtained in our experiment was unexpected, given that selection is expected to bias our estimate of V_m downward. Our system seems to exhibit greater genetic variance or less environmental variance than these other cases.

Our estimate of environmental variance was essentially developmental, the variance among cells at the beginning of the experiment, when mutational variance is assumed to be negligible. It is similar to the within-clutch variance estimated by Lynch (1985) as one component of overall environmental variance. If the within-clutch variance alone is used to calculate V_m/V_E for fitness in Lynch's data, the value obtained is 1.1×10^{-3} , which is somewhat greater than before. Other studies have used the variance among replicate cultures, thereby introducing a component caused by differences in growth conditions into the estimate of V_E . This may be undesirable, because the estimate of V_m/V_E will be affected by the skill and care of the experimenter, with poorly conducted experiments giving lower estimates. However, the variance among individuals in the same homogeneous culture vessel is caused largely by accidents of development that are insensitive to experimental error, and for this reason seems to be a more satisfactory scale for V_m . The surface of a poured agar plate is as nearly homogeneous an environment as can be devised, and the almost complete elimination of variation in growth conditions will lead to low values of V_E and thereby to high values of V_m/V_E .

Even given these qualifications, however, our estimate of V_m/V_E , given the strong action of natural selection in our experimental system, seemed surprisingly high. When combined with the apparent lack of selective sweeps and the lack of decline in the mean fitness, as would be expected under the accumulation of deleterious mutations, the results indeed seem perplexing.

One plausible explanation might be the action of frequency-dependent selection, which would increase the quantity of genetic variation maintained in the population. The evolution of stable polymorphisms in initially uniform, asexually cultured *Escherichia coli* has been described (Helling et al. 1987; Turner et al. 1996). Elena and Lenski (1997) have found that frequency-dependent selection is the most important factor in maintaining within-population genetic variance for fitness in long-term experimental populations of *E. coli*.

We suspected that a similar mechanism was operating, because the chemostat seemed to develop increasing spatial heterogeneity as the experiment progressed. Cells became attached to the walls of the vessel, formed clumps near the bottom of the vessel, and even occasionally formed a transient film on the surface of the medium. This led us to believe that ecological diversification might be taking place. However, a second trial, designed to test this hypothesis, found no difference in fitness, adherence to glass, or propensity to flocculate among cells sampled from the bottom, in suspension, or the surface of the medium. Therefore, the ecological diversification that we observed seems to have been generated through phenotypic plasticity. Although this remains an interesting observation, it does not explain the maintenance of mean fitness in the face of increasing variation.

The results of the assay of solid and liquid growth suggest a different kind of explanation. Liquid and solid media, although chemically very similar, seem to provide very different conditions of growth for *Chlamydomonas*: genotypic variation is dominated by genotype-environment interaction, and selection causes substantial divergence within a few hundred generations. It seems likely that despite our efforts the variation that we measured would not necessarily have been expressed in the chemostat itself. Nevertheless, it was certainly expressed on solid medium. This suggests that the chemostat populations were becoming steadily more diverse through mutations that were neutral, or nearly so, in the chemostat, but caused substantial variance of fitness when expressed in a different environment. Our estimates then refer to a rate of increase of variance associated with conditionally neutral mutations.

It cannot be said that we have estimated *the* rate of increase of variance associated with conditionally neutral mutations, because it is very likely that the amount of variation expressed will depend on the environment in which it is assayed. It is only when the assay is conducted in precisely the same environment as the mutation accumulation that it will provide an unambiguous estimate of V_m as usually interpreted. However, our results suggest two difficulties in the interpretation of such an estimate. The first is that it will usually be impracticable to obtain this estimate, because assay and experimental conditions will usually differ to some degree. It might be argued that the genetic correlation is a declining function of environmental variance (Bell 1992), so that if experiment and assay are conducted in reasonably similar conditions the estimate of V_m will be reliable. It must be borne in mind, though, that this is only a general tendency,

and our experience shows how apparently trivial modifications of the conditions of growth may have profound effects on the expression of fitness. The second difficulty is that estimates of V_m , whether practicable or not, may be of little relevance to evolutionary processes. Natural environments vary continuously in space and time, making any unique estimate of V_m meaningless in the face of substantial genotype-environment interaction. The amount of novel genetic variation available for selection may be much greater than experiments suggest, so that—paradoxically—careful and well-designed experiments may greatly underestimate the rate of response to environmental change.

More fundamentally, our results cast some doubt on the conventional paradigm of the chemostat. The chemostat population is supposed to remain essentially uniform for long periods of time between the appearance and spread of novel beneficial mutations, each of which occurs in the descendent of a previously dominant genotype. Doubt has been cast on this view before, because of the unexpectedly high rates of flux in some experiments (Adams et al. 1985). Our results make it conceivable that a large amount of variation may be contributed by conditionally neutral variants, of which each individually rare. No single genotype may dominate the population, and successful genotypes may descend from one among the myriad of rare conditionally neutral variants, rather than from a previously successful genotype. We need more powerful methods of analyzing populations before this possibility can be properly evaluated, but it may be that chemostats are much more variable and dynamic systems than is commonly imagined.

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LITERATURE CITED

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