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The accumulation of variance in fitness in clonal populations of *Chlamydomonas reinhardtii* in normal and stressful environments.

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Master of Science.

Table of Contents

Abstract

The work presented here investigates two basic properties of mutation rates in the unicellular chlorophyte *Chlamydomonas reinhardtii*. The first chapter is devoted to an investigation of the mutational heritability (V_M) of fitness in asexually propagated populations. This is the rate at which novel variation for fitness accumulates in a population. In two trials, values of V_M = 4.5 and 4.7 x 10^{-3} of the environmental variance (V_E) were obtained. These values were at least an order of magnitude greater than estimates from other organisms of V_M/V_E for fitness or for quasineutral variation. The possibility that this was due to disruptive selection for types specialized for different parts of the culturing environment was investigated, and rejected. Other possible explanations, and future avenues for research, are discussed.

The second chapter extends the investigation from normal culturing conditions into stressful ones. Specifically, it considers the hypothesis that *C. reinhardtii* might increase its mutation rate as a general response to environmental stress. Stressed lines were found to display reduced mean fitness and an increased variance of fitness after being returned to normal culturing conditions. This was interpreted as evidence for increased mutation rates in treated lines relative to controls. Possible mechanisms underlying this phenomenon are discussed, along with suggestions for further research.

Résumé

Le travail presenté ici explore deux proprietés du taux de mutation chez le chlorophyte unicellulaire *Chlamydomonas reinhardtii*. Le premier chapitre est devoué à une investigation de l'héritabilité mutationelle (V_M) de la valeur sélective dans les populations asexuées. V_M est le taux auquel la variation de la valeur sélective augmente dans une population. Dans deux essais, des valeurs de V_M = 4.5 et 4.7 x 10^{-3} de la variation environnementale (V_E) ont été obtenus. Ces valeurs étaient au moins un ordre de grandeur plus élevés que des estimations obtenues chez d'autres organismes pour V_M/V_E de la valeur sélective ou pour la variation quasi-neutre. On a investigué, puis rejeté, la possibilité que la différence était causée par une sélection diversifiante pour des types spécialisés pour differentes portions du milieu de culture. D'autres explications possibles et des possibilités de recherches futures sont discutées.

Le deuxième chapitre étend l'investigation à une comparaison des milieux de cultures normales à des milieux stressants. Plus particulièrement, on considère l'hypothèse que *C. reinhardtii* augmente son taux de mutation comme réponse générale au stress. Des lignées stressées avaient une valeur sélective moyenne diminuée et une variance de valeur sélective augmentée. Ces résultats furent interprètés comme évidence d'une augmentation du taux de mutation dans les lignées stressées par rapport aux lignées contrôles. Des mécanismes possibles pour expliquer ce phénomène sont discutés, avec des suggestions de recherche future.

Preface

In accordance with the regulations of the Faculty of Graduate Studies, the following text is here reproduced in full.

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The results presented here have not yet been published, but are in preparation for publication. A general introduction and conclusion have been added so as to unite the chapters into a cohesive thesis. All of the text in this thesis is my own.

Acknowledgements

I must first of all mention my supervisor, Graham Bell. Without him, it would not have been possible for me to complete this. His aid was invaluable at every stage of this project, from suggestions for experiments, to advice on design and interpretation of data, to editorial comments on earlier versions of this thesis. His enthusiasm and creativity on many occasions revived me from the stupor enduced by the dull routine of laboratory drudgery. I owe him many thanks for his encouragement, and his tolerance of my somewhat irregular work habits.

I also would like to express my gratitude to all of the other people who inhabited our lab during some or all of my stay, for providing a friendly and stimulating atmosphere in which to work. They included: Jamie Bacher, Stephan Baral, Torsten Bernhardt, Yannick Ducharme, Rees Kassen, Jessica Rabe, Ian Rae, Monique Richard (to whom I owe special thanks for help in the translation of the abstract), Kathy Tallon, Gabe Yedid, and Cliff Zeyl.

Last, and certainly not least, I thank my family, friends and Alex, for supporting me through this work and making it worthwhile.

Mutability

We are as clouds that veil the midnight moon:
  How restlessly they speed, and gleam, and quiver,
Streaking the darkness radiantly! - yet soon
  Night closes round, and they are lost for ever:

Or like forgotten lyres, whose dissonant strings
  Give various response to each varying blast,
To whose frail frame no second motion brings
  One mood or modulation like the last.

We rest. - A dream has power to poison sleep;
  We rise. - One wandering thought pollutes the day;
We feel, conceive or reason, laugh or weep;
  Embrace fond woe, or cast our cares away:

It is the same! - For, be it joy or sorrow,
  The path of its departure still is free:
Man's yesterday may ne'er be like his morrow;
Nought may endure but Mutability.

Percy Bysshe Shelley

General Introduction

Heritable genetic variation is the basis of all evolutionary change; all novel variation is produced by mutation. Natural selection sorts this variation by favouring those variants best adapted to prevailing environmental conditions.

Measures of the mutation rate per generation are needed to determine whether mutation alone is sufficient to account for observed levels of quantitative variation (Lande 1976), or whether selective mechanisms such as heterozygote advantage or environmental heterogeneity must be invoked.

To date, many studies have been carried out examining the rate of mutation for quantitative characters. However, these have been concentrated upon a few model organisms. The vast majority have been carried out upon one, *Drosophila melanogaster.* It is well-established that mutation rates vary between individuals of a species, and between species as well (Smith 1992). Therefore, it is of interest to broaden the range of species that have had their mutation rate measured. Moreover, only one study (Houle et al. 1992) has considered the genomic mutation rate for fitness.

Therefore, I have examined the rate of mutation for fitness in the unicellular chlorophyte, *Chlamydomonas reinhardtii.* Whereas most studies to date have been performed upon obligately sexual, multicellular organisms, this study considers a facultatively sexual unicell. Therefore, not only am I examining yet another organism, but it is a rather different sort of organism than has hitherto been considered.

When a population is well-adapted to its environment, almost all mutations are deleterious. Since error is an inevitable characteristic of any

self-replicating system (Bell 1997), mutations will occur, and will decrease the mean fitness of a population. The extent of this reduction is a component of the "genetic load" (Crow 1970). This is the traditional perspective, where mutation is regarded as a unavoidable evil, which selection keeps in check.

The complement of this is mutation's nature as the source of potentially adaptive novelty. This can become especially important when the population is faced with a change in the environment. Therefore, one encounters remarks like the following: "The power of mutation to maintain genetic variation against the force of stabilizing selection..." (Lande 1976, p. 232). Here the emphasis is placed on the potentially beneficial variation, which must be maintained at all costs against the inexorable force of selection.

These differences in emphasis reflect the differences in the significance of mutation in stable and changing environments. When a population is well-adapted to its environment, mutations are virtually always harmful; yet when the environment is changing and a population is stressed, mutations are the only source of adaptive novelty. Therefore, one would expect that organisms in fluctuating environments have higher mutation rates than those in stable environments (Levins 1967, LeClerc et al. 1996).

From these considerations, one is logically led to the question of whether organisms can modify their mutation rates to suit the prevalent environmental conditions. This is not a hypothesis of "directed mutation", but rather one of an increase in the rate of spontaneous, undirected mutations in conditions when they are more likely to be favourable.

The second chapter of this thesis is devoted to an experimental examination of this question. Cultures of *Chlamydomonas reinhardtii* were exposed to a variety of stresses. Several generations after being returned to normal conditions, stressed and control populations were assayed for their

mean and variance of fitness. An increased mutation rate would be manifested as a decrease in the mean, and an increase in the variance, of fitness.

Therefore, taken as a whole, this thesis is an examination of, first, the rate of mutational input to variation in fitness in regular culturing conditions, and second, whether this rate is changed by exposure to stressful conditions.

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Chapter One

The Accumulation of Variance for Fitness in a Clonal Population

Introduction

Heritable genetic variation is the basis of all evolutionary change; all novel variation is produced by mutation. Natural selection acts upon this variation by favouring those types best adapted to prevailing environmental conditions. In sexual populations, lineages can incorporate new variation by the exchange of genetic material. However, in asexual populations each lineage evolves independently of all others, and new variation can only be incorporated by mutation. Therefore the study of the rate of the incorporation of such variation into a population provides us with information vital to our understanding of its evolutionary potential.

The amount of genetic variation present in a population sets the limit to that population's ability to respond to natural selection. Fisher's "fundamental theorem" of natural selection (1930) states that a population's rate of response to selection is directly proportional to its genetic variance of fitness. Therefore, Crow (1970) has called a measure of such variation the *index of opportunity for selection.* Two important questions can be asked about such variation: how much of it is maintained in populations? And at what rate does it arise? While the former question has been addressed by many investigators (Falconer and Mackay 1996), the latter has received little attention.

Mutation is unrelenting, and selection continually acts to eliminate genetic variation, maintaining the adaptedness of a population. Selection on fitness is, of course, directional, but selection on the underlying quantitative

traits will be stabilizing (Falconer 1957). Fisher (1930) established theoretically that stabilizing selection should deplete genetic variation for a quantitative character under selection. It has been empirically demonstrated, both in the laboratory (Falconer 1957, Bos and Scharloo 1973a, 1973b) and in natural populations (Grant et al. 1976, Bell 1978), that mutation increases the variation in quantitative traits, and that selection reduces it .

The conflicting influence of these two forces will lead to an equilibrium value of genetic variation, the mutation-selection equilibrium. Historically, investigators were first interested in establishing whether a substantial amount of genetic variation for quantitative traits existed in natural populations. Many studies have shown that there indeed exists a considerable amount of heritable variation for such characters (Falconer and Mackay 1996, Roff and Mousseau 1987). Interest has since then been directed at investigating how such variation is maintained.

The problem was that observed levels of variation appeared to be too great to be accounted for by mutation alone (Lande 1976). Therefore, for many years, selective mechanisms such as heterozygote advantage and environmental variability were considered to be the chief maintainers of genetic variation in quantitative traits. However, very few loci are known to exhibit heterozygote advantage (Lewontin 1974), and even populations maintained in stable, uniform environments exhibit considerable genetic variability in quantitative traits (Lopez-Fanjul and Hill 1973).

It was Lande (1976) who suggested that mutation alone could maintain variation in quantitative traits in the face of selection. In Lande's model, the critical parameter was V_M/V_E. V_M/V_E expresses the rate of mutational input to phenotypic variance per generation (V_M) as a proportion of the environmental variation (V_E) for that character. Lynch (1988) has called this

the "mutational heritability". Lynch and Gabriel (1983) have established that a value of V_M/V_E of 10^{-3} is sufficient for a new clonal lineage to reach equilibrium levels of genetic variance within 1000 generations. The environmental variance in the denominator serves to normalize estimates of V_M for different traits, and also reflects the maximum force of selection that can act upon the character of interest (Lande 1976).

The width of a fitness function (V_S) is a measure of the intensity of natural selection; the narrower the function, the more intense the selection. It must be at least as broad as the environmental variance, since this represents the range of phenotypes that can be produced by identical genotypes. Empirical studies of stabilizing selection in natural populations have found that V_S usually ranges between 100 V_E and 10 V_E (Falconer and Mackay 1996).

The value of V_M, on its own, is also of paramount importance in Lande's neutral model, as the predicted equilibrium of additive genetic variance is $2NV_M$, where N is the population size (Lande 1976). Lande's original model of stabilizing selection considered a randomly mating sexual population, and assumed only additive allelic effects, a continuum of alleles at each locus, and a normal distribution of allelic effects (Turelli 1986). Subsequent models have examined truncation selection (Hill 1982 a, b, Zeng and Hill 1986), different life cycles (Lynch and Gabriel 1983), pleiotropy (Lande 1980) and non-Gaussian distributions (Turelli 1986). While these models differ in their quantitative predictions, all agree that mutation is an important factor in determining the level of genetic variance in a population (Lynch 1988).

In some formulations (e.g. Turelli 1984), the genomic mutation rate is a more relevant parameter for predicting equilibrium levels of genetic variance than V_M in models of stabilizing selection. However, Lynch (1988)

points out that V_M will still be appropriate for some parameter values. As well, V_M is the appropriate measure when selection takes the form of truncation selection (Hill 1982a, b). Most importantly, the expected mutation-selection equilibrium for traits under directional selection, such as fitness, is equal to V_M/s, where s is the intensity of selection (Barton 1990).

Experiments intended to estimate V_M have traditionally followed one of two protocols (more detailed methods for each are given in Lynch 1988). In the first, the response to selection of a highly inbred line of a sexual species is analysed (Mather and Wigan 1942, Clayton and Robertson 1955). The second relies on long-term mutation-accumulation in lines of *Drosophila* with special chromosomal constructs (Durrant and Mather 1954, Mukai 1964, Mukai et al, 1972, 1984). In these experiments, the divergence between inbred lines is measured in order to obtain estimates of V_M.

To date, experiments have been carried out on a variety of plants and animals, and have considered a variety of quantitative traits. Not surprisingly, the most popular organism to be studied has been *Drosophila melanogaster.* Characters examined in *Drosophila* have included abdominal bristle number (Mather and Wigan 1942, Clayton and Robertson 1955), 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), and fitness (Houle et al. 1992).

Other studies have considered body size in mice (Keightley and Hill 1992), pupa weight in *Tribolium* (Enfield and Braskerud 1989), life history traits in *Daphnia* (Lynch 1985), vegetative and reproductive traits in several crop plants (reviewed in Lynch 1988), and cell size in *Chlamydomonas reinhardtii* (Bell, 1996).

Despite the diversity of characters, organisms, and methods in these studies, the estimates obtained for V_M/V_E are remarkably consistent. With the exception of the viability estimates from *Drosophila*, all have fallen between 10^{-4} and 5×10^{-2}. The majority lie at about 10^{-3}.

The experiments presented here provide estimates of V_M/V_E for fitness in *Chlamydomonas reinhardtii*. Despite the copious literature on mutational heritabilities, this study differs from previous work in several respects. First, only Lynch (1985) and Bell (1996) have examined asexually-reproducing organisms. There is some reason to believe that mutation rates might be different in asexual versus sexual species, since asexual lineages cannot incorporate variation from other lineages (Lynch and Gabriel 1983). Furthermore, only Bell (1996) has examined a unicellular microbe.

As well, this is only the second time that V_M has been estimated for fitness [the other being Houle (1992)]. That study had no estimate for the environmental variance for fitness, making this the first study to estimate the compound parameter V_M/V_E. Finally, the methodology of this study is novel. With clonally reproducing organisms, one can avoid the labour-intensive maintenance of several replicate inbred lines necessary when one is examining sexual species. Instead, V_M and V_E can be estimated from variances in fitness within a single population (Bell 1996).

Experiment One

Introduction

The experiment presented here differs from most previous work on the accumulation of variance in two ways. First, I looked at the increase in variance for fitness, rather than for a single quantitative trait. Second, I considered the rate of increase of variance in the presence of natural selection, whereas previous studies have attempted to eliminate selection upon the traits of interest. Such a study is of interest because it is fitness, rather than any given trait, upon which natural selection acts, and because variation must accumulate in natural populations in the face of selection.

Researchers have to date usually estimated V_M/V_E by the method of investigating the divergence in the mean of a character in replicate lines (e.g. Mukai et al. 1972). Highly inbred stocks are used to create founders as homozygous as possible. Then many replicate lines are maintained descending from these founders by full-sib matings. The idea is to reduce to a minimum the force of selection, and to allow mutations and random drift to drive the replicate lines apart in their values for the trait of interest. The increase in variance per generation among replicate lines is the estimate of V_M. V_E is estimated from the variance within replicate lines. While effective, and indeed necessary for the examination of sexual species, such methods have the disadvantage of being extremely labour-intensive.

The experiment described here is based on a simpler protocol, involving the use of an asexually-reproducing population. Here one does not need to painstakingly maintain many replicate lines, since each clonal lineage within a single population is effectively isolated from all others. Therefore, one can estimate V_M from the increase in variance in a single population through

time. As well, V_E can be estimated from the variability in fitness in the immediate descendants of a single individual.

The unicellular chlorophyte, *Chlamydomonas reinhardtii*, provided a convenient model system. Fitness could be unequivocally scored as the rate of cell division in a standard growth medium (Bell 1990). As well, the short generation time (4-12 hours) allows experimental trials of hundreds of generations to be carried out in a relatively short period of time.

In addition, since *C. reinhardtii* is a facultatively sexual species, populations can be propagated asexually starting from a single founder. In this way, one can start with a genetically uniform population. Therefore, V_E can be estimated as the variance in fitness, in the chosen environment, of the immediate descendants of the founding clone. If one then follows the rate of increase of variance in fitness through time, the slope of this line will give one an estimate of V_M within the population.

It might be argued that by making no effort to protect the cells from natural selection, I am bound to underestimate V_M. This is indeed true, and is true of all mutation-accumulation experiments. Strongly deleterious mutations will not survive to be assayed in any experimental protocol devised to date. Therefore, the mutational heritability estimated is perhaps best thought of as that for "quasineutral" mutations, and not for all mutations.

Moreover, since *C. reinhardtii* is haploid, and can be cultured clonally, and since the chemostat represents a uniform, stable environment, one can isolate the effects of mutation from other postulated influences on the maintenance of genetic variation in a population. There can be no dominance effects, since the cells are haploid, and one cannot invoke environmental heterogeneity, either temporal or spatial, for the maintenance of variance, since the culturing environment is uniform.

Materials and Methods

Chemostat apparatus.

The chemostats used in these experiments were simple, gravity- and pressure-operated devices designed in our laboratory (figure 1). Each chemostat had three main components. The first was the medium supply vessel. This was a 15 litre "Lowboy" carboy. It was connected to the growth vessel by Tygon tubing. The flow of medium into the growth vessel was powered by gravity, as the supply vessel was placed on a shelf above the growth vessel. The flow was regulated by a needle valve.

The growth vessel was a 500 ml Erlenmeyer flask. The top of the flask was sealed by a rubber stopper, in which three holes had been drilled. The first hole allowed the entry of fresh medium from the supply vessel. The supply tubing was attached to the wide end of a pasteur pipette, which passed through the hole in the stopper and allowed fresh medium to drip into the culture.

Through the second hole, the growth vessel was supplied with filter-sterilized, pressurized air from a lab counter-top valve. The air was sterilized by passing it through a 0.2 micron syringe filter. The air supply tubing was attached to a pasteur pipette, which passed through the hole in the rubber stopper. The tip of the pipette was placed so as to release the air just above the surface of the medium. This agitated the surface lightly, allowing for the aeration of the culture. The culture was not "bubbled" by placing the tip of the pipette below the surface of the medium, because in trial runs this had

figure 1: Photographs of chemostat apparatus. a. Entire apparatus. b. Culturing vessel.

b

a

been found to produce splashes of culture that could reach the tip of the medium inflow pipette, thus permitting contamination of the medium supply.

Besides the aeration of the culture, the inflow of pressurized air served a second function. In the air-tight culture vessel, it generated the pressure necessary to allow the removal of spent medium, thus maintaining a stable volume of medium in the vessel.

The third hole in the stopper at the top of the culturing vessel allowed for the removal of spent medium. A hollow glass tube, whose bottom opened just above the surface of the medium, passed through the hole, and was connected to tygon tubing. When the level of medium in the growth vessel reached the base of the glass tube, the pressurized air created a suction sufficient to pull the excess medium up the tube and out of the vessel. The tubing passed down to the waste carboy, a 20 litre carboy. It was placed on the floor, below the culture vessel. Halfway along the tubing connecting the culture and waste vessels was placed a three-way stopcock, to allow the collection of samples.

All of the materials used in the chemostat apparatus were autoclavable. The components were autoclaved separately, and assembled in a laminar flow hood, to ensure sterility.

Algal Strains.

For both experiments, the strain CC-2938 (strain designation of the *Chlamydomonas* Genetics Center, Duke University) of *Chlamydomonas reinhardtii* (Sack et al. 1994) was used. This strain was collected by members of our laboratory from a corn field in Farnham, Quebec in the summer of 1993. It has been maintained in the lab since this time. In order to follow periodic selection (see below), a yellow mutant of CC-2938 was inoculated in the

chemostats at a low frequency. This strain was derived from CC-2938 by UV-irradiation. The cells of this strain appear normal when grown in the light. However, when cultured in the dark, they fail to produce chlorophyll, and thus form yellow colonies. These colonies can be easily differentiated from those of CC-2938, which are green in both light and dark. The yellow strain was considered to be selectively neutral in the chemostat environment, as its frequency did not change significantly after a 10-day trial run, in which the green and yellow strains had each been inoculated as 50% of the original culture.

Culture media.

All media used in these experiments were modifications of a standard Bold's medium (Harris 1989). The chemostats were supplied with a modified minimal Bold's, with NH_4NO_3, rather than $NaNO_3$, as the nitrogen source. The fitness of cells sampled from the chemostat was assayed on plates made from the spent, filtered chemostat medium. The reason for using this medium, rather than fresh Bold's, was to assay fitness in an environment closely resembling that of the chemostat. In a chemostat at equilibrium, the levels of nutrients present are far lower than in fresh medium (Dykhuizen and Hartl 1983).

Periodic Selection.

Periodic selection is the name given to a phenomenon commonly observed in chemostat cultures (Atwood et al. 1951, Dykhuizen and Hartl 1983). When a favourable mutation occurs in the population, types bearing this mutation will sweep through the population until it is fixed. Since such a phenomenon will obviously decrease the genetic variability of the population

and therefore bias estimates of V_M, it is important in these experiments that the occurrence of periodic selection be monitored.

The standard method for monitoring periodic selection is to follow the frequency of a neutral marker in the population. Such a marker, beginning at a low frequency, will tend to accumulate through time due to mutations. However, since cells bearing it are present at such a low frequency, when a favourable mutant does arise, it is virtually certain to do so among the population of cells not bearing the marker. Therefore, as the novel type sweeps through the population, the frequency of the marker will decrease as cells bearing it are selected out of the population. However, once the new type is fixed, these cells will also begin to accumulate mutations for the marker. This pattern of accumulation followed by rapid disappearance leads to a characteristic "zig-zag" pattern for the frequency of neutral markers. In this experiment, the frequencies of two markers, y (yellow) and nit^- (nitrogen reductase deficiency) were followed in order to detect periodic selection, in case it occurred.

The frequency of yellow mutants was checked by inoculating samples onto agar plates made from Bold's medium supplemented by 1.2 g/l sodium acetate. The frequency of nitrogen reductase deficient (nit^-) mutants was followed by plating samples on "chlorate plates" (Nichols and Syrett 1978). These plates are made from a modified Bold's medium: they have five times the normal concentration of $NaNO_3$, as well as being supplemented with sodium chlorate, urea and sodium acetate.

The chemostat medium provided cells with a reduced nitrogen source (NH_4NO_3). In addition, ammonia inhibits the activity of nitrate reductase, the enzyme normally used to reduce nitrate to nitrite(Harris 1989). Cells carrying loss-of-function mutations for nitrate reductase would therefore be selectively

neutral in the chemostat environment, and should tend to accumulate through time. Chlorate plates allow one to detect nit$^-$ mutants by taking advantage of the lack of substrate specificity of nitrate reductase. The enzyme will accept sodium chlorate instead of nitrate as a substrate. It reduces sodium chlorate to sodium chlorite, which is toxic for *Chlamydomonas* cells. Therefore, any cells with a functioning nitrate reductase enzyme will quickly accumulate toxic chlorite when spread on chlorate plates, and will soon die. However, nit$^-$ mutants will continue to grow, as long as another nitrogen source, in this case urea, is provided.

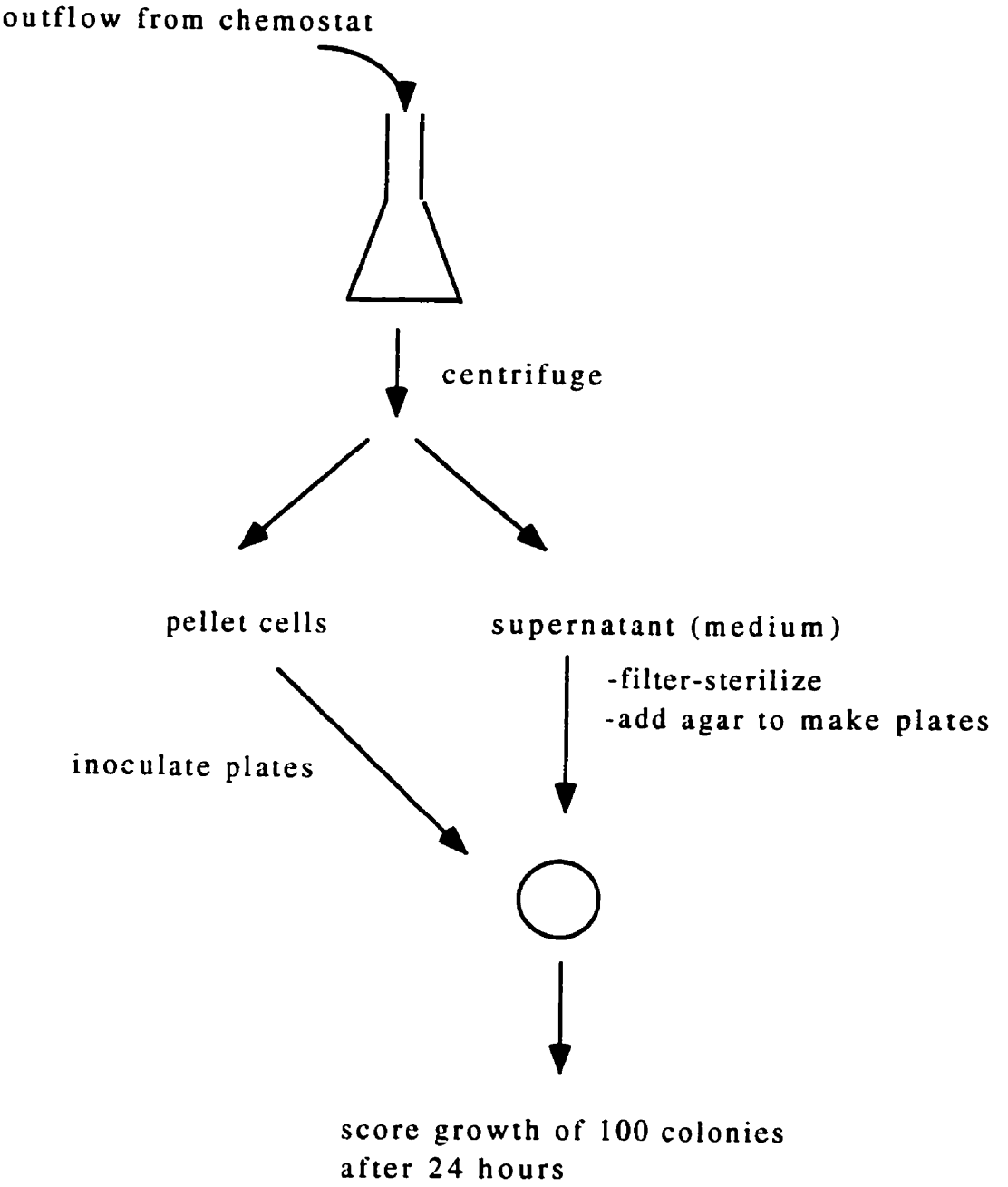
Experimental protocol.

Two replicate chemostats containing the strain CC-2938 of *Chlamydomonas reinhardtii* (Sack et al. 1994) were maintained for 50 days at a dilution rate of about 2.5 volumes/day (100-150 generations of growth). Two neutral markers were followed as indicators of periodic selection: ability to grow on plates supplemented with chlorate (indicative of nitrate reductase deficiency) and yellow growth in the dark (a yellow strain derived from CC-2938 was included as 5% of the initial inoculum).

Samples were taken from the chemostat daily. They were gathered by attaching a sterile flask to the three-way valve on the outflow tubing. The flow of waste from the growth vessel was then temporarily redirected into the sample flask. These samples consisted of both cells and spent medium (figure 2). 50 ml of the samples were centrifuged at 2000 rpm for 6 minutes. All but 5 ml of the supernatant was then poured off: this cell-free medium was then used to make the agar plates on which the fitness of cells would be assayed. It was first filter-sterilized by passing it through a 0.2 micron syringe filter. To make an agar plate, 17 ml of supernatant was added to 3 ml double-distilled

figure 2: Schematic diagram of sampling protocol for fitness assay in first mutation-accumulation experiment.

sampling protocol



water to which 0.06 g of agar had been added. This mixture was heated in a test tube over a bunsen burner until the agar melted. It was then allowed to cool until it reached a temperature of 40 degrees Celsius. At this point, the sample of cells was inoculated into the tube. This sample was taken from the pellet, which had been resuspended by vortexing in the 5 ml of medium remaining in the centrifuge tube. 30 microlitres of the sample was inoculated into the test tube containing the cooling medium; the tube was then vortexed and the contents poured into an empty, sterile petri dish. The plates were allowed to cool for 30 minutes before being transferred to shelves illuminated by soft-white fluorescent lights. They were left on the shelf for 24 hours, exposed to continuous light. After this, the number of cells in 100 colonies was scored by examining the plates under a microscope.

To follow the frequencies of the neutral markers, samples from the pellet were also inoculated onto Bold's plus acetate and chlorate plates. These plates were placed on dark and light growth shelves, respectively. The frequency of the yellow marker was followed by counting the relative number of yellow and green colonies on the Bold's plus acetate plates. The frequency of nit^- mutants was followed by counting the number of live colonies on chlorate plates one week after inoculation. Growing colonies were large and green, whereas dead colonies were small and white.

Heritability trials.

One way of examining the extent to which differences in fitness among the cells had a genetic basis was to measure the heritability of the fitness scores. At the conclusion of 50 days, the chemostats were sampled a final time. When the fitness of 100 colonies was scored on plates made from chemostat outflow medium, the position of three colonies of each of 2, 4, 8 and 16 cells

were marked on the bottom of the plate with a felt marker. The descendants of these cells were to be assayed to determine the heritability of these fitness measures. In other words, would the descendants of a colony that had only 2 cells after 24 hours have a lower mean fitness than those of a colony that already had 16 cells after the same length of time?

After allowing the colonies a few days to grow, they were picked off the plates with a loop, and inoculated into NH_4NO_3 Bold's medium in the wells of a 16-well plate. After two days, the contents of each well were inoculated onto an agar plate made from the spent chemostat medium. As is the previous fitness assays, the number of cells per colony was scored for 100 colonies.

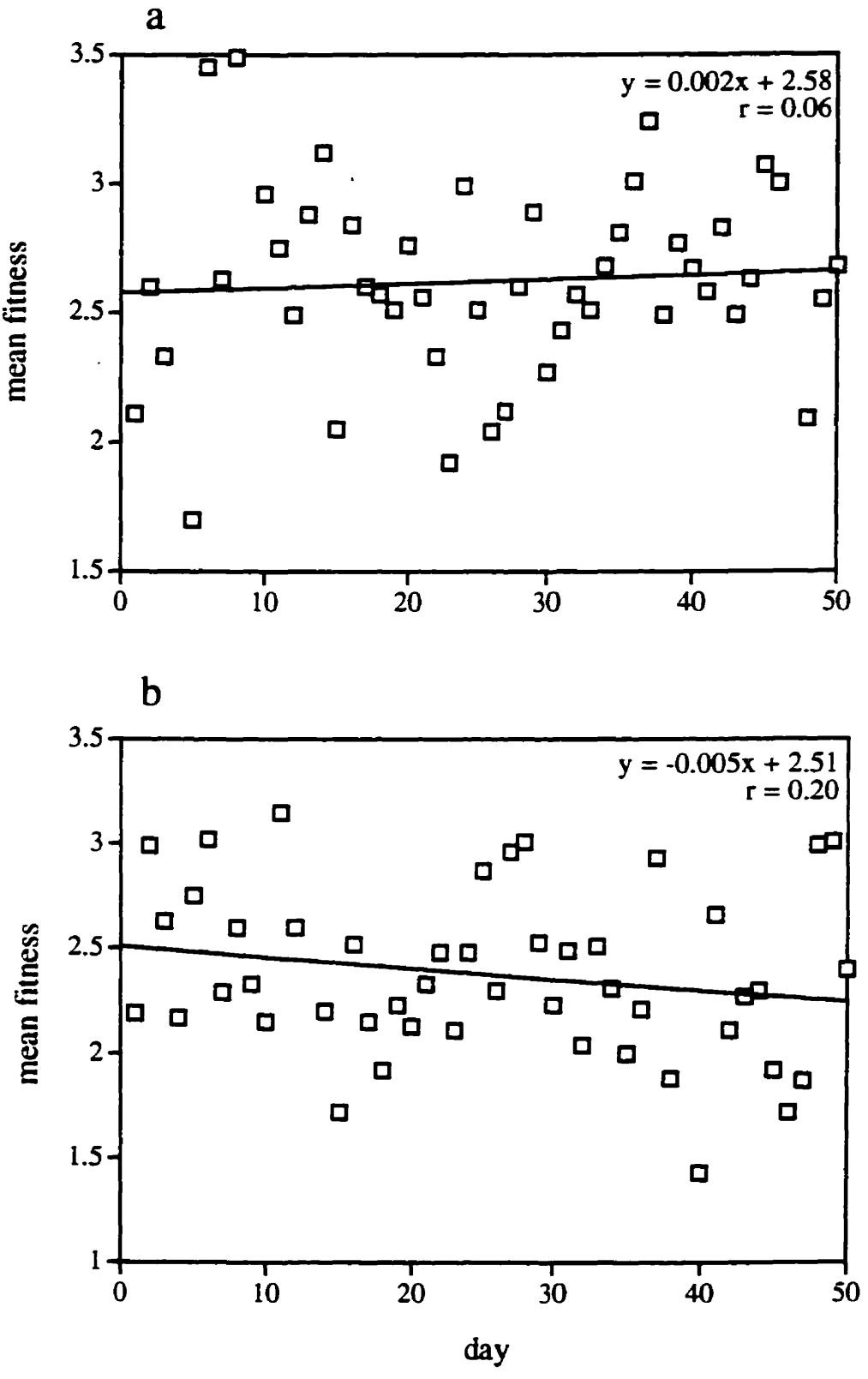
Because the first such assay produced surprising results (see below), the assay was repeated. The surface of the plates on which fitness was assayed in the first trial were washed with NH_4NO_3 Bold's medium to remove the attached cells. These cells were then inoculated into liquid culture, grown up for two days, and then inoculated onto new plates. These plates were made from NH_4NO_3 Bold's medium, rather than the spent chemostat medium, as all of the latter had been used up in the first assay. The fitness of 100 colonies was scored on these plates.

Results

Mutation accumulation.

From the 100 observations per plate of number of cell divisions, the mean and variance in fitness of the cultures could be estimated. These values were collected every day, for the duration of the experiment. In neither chemostat was there a significant change in mean fitness after 50 days (figure

figure 3: Mean fitness in the first mutation-accumulation experiment. The x-axis indicates day of trial; the y-axis mean number of cell divisions in 24 hours ("fitness") of 100 colonies on plates made from filter-sterilized chemostat outflow medium. **a.** First replicate chemostat. **b.** Second replicate chemostat.


a
y = 0.002x + 2.58
r = 0.06
mean fitness
3.5
3
2.5
2
1.5
0
10
20
30
40
50
b
y = -0.005x + 2.51
r = 0.20
mean fitness
3.5
3
2.5
2
1.5
1
0
10
20
30
40
50
day

3). However, in both, the variance in fitness showed a strongly significant increase ($p < 0.0005$). This statement should be qualified by the admission that parametric regression analysis is not strictly correct for this type of data, because of autocorrelation among estimates from different days. Nonetheless, the data do indicate a linear trend among estimates of the variance in fitness, while there is none among estimates of the mean. What is of interest in calculating V_M is the value of the slope, and not the significance of the regression.

The slopes of the two regressions of variance in fitness versus day of trial (figure 4) were 0.0096 and 0.0083. Under the assumption that the only source of novel variation in fitness is *de novo* mutational input, these slopes provide estimates of V_M. Since the mean growth rate throughout the trial was about 2.5 divisions per day (figure 3), there were estimated to be about 2.5 x 50 = 125 generations of accumulation. Therefore, V_M, expressed per generation, was 3.3×10^{-3} and 3.8×10^{-3} for the two chemostats, respectively.

V_E was estimated as the variance in fitness at the start of the experiment. The values obtained for the two chemostats were similar: the Y-intercepts for the least-squares regression lines were 0.74 and 0.81. These values for V_M and V_E give V_M/V_E of 4.5×10^{-3} and 4.7×10^{-3} for the two replicate chemostats.

The two markers used did not show any sign of periodic selection having taken place. The frequencies of the nit$^-$ and yellow phenotypes, though erratic (figure 5), do not follow the zig-zag pattern characteristic of periodic selection (Atwood et al. 1951, Dykhuizen and Hartl 1983). Instead, there is a slight tendency for the frequency of nit$^-$ to increase and for that of the yellow type to decrease.

figure 4: Variance of fitness in the first mutation-accumulation experiment. The x-axis indicates day of trial; the y-axis variance of number of cell divisions in 24 hours ("fitness") of 100 colonies on plates made from filter-sterilized chemostat outflow medium. **a**. First replicate chemostat. **b**. Second replicate chemostat.

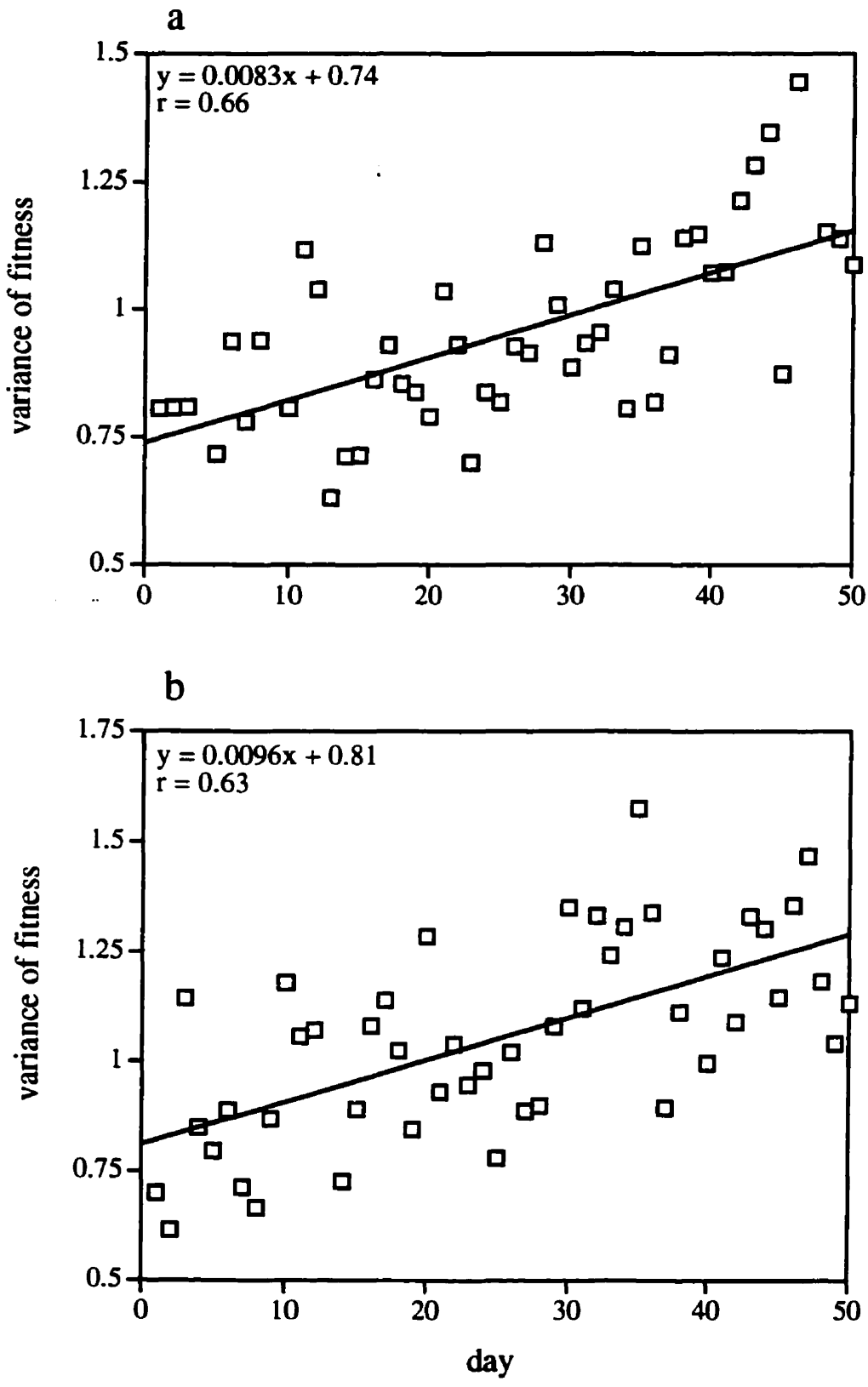
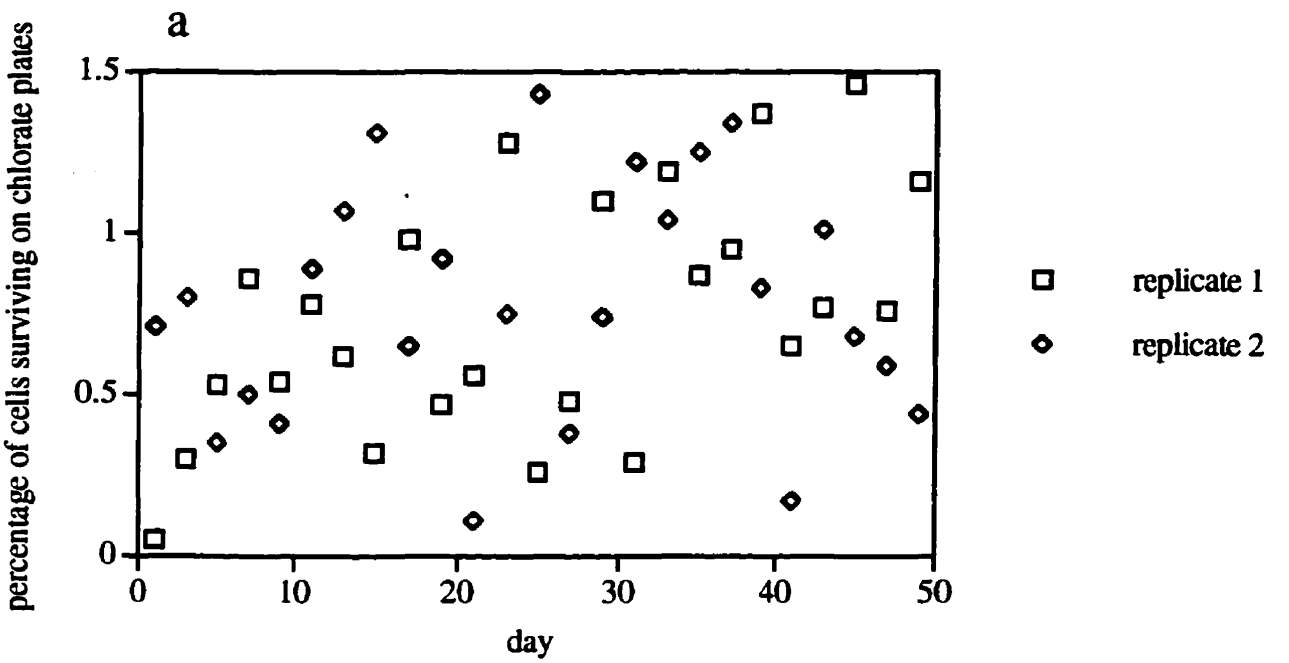

a
y = 0.0083x + 0.74
r = 0.66
variance of fitness
1.5
1.25
1
0.75
0.5
0
10
20
30
40
50
b
y = 0.0096x + 0.81
r = 0.63
variance of fitness
1.75
1.5
1.25
1
0.75
0.5
0
10
20
30
40
50
day

figure 5: Monitoring of periodic selection in the first mutation-accumulation experiment. **a**. Frequency of nitrate-reductase deficient cells. The x-axis indicates day of trial; the y-axis the percentage of cells spread on a chlorate plate which had survived and formed growing colonies one week after inoculation. **b**. Frequency of yellow-in-dark cells. The x-axis indicates day of trial; the y-axis the percentage of cells spread on a Bold's plus acetate plate which formed yellow colonies when kept in the dark.


a
percentage of cells surviving on chlorate plates
1.5
1
0.5
0
0
10
20
30
40
50
day
replicate 1
replicate 2

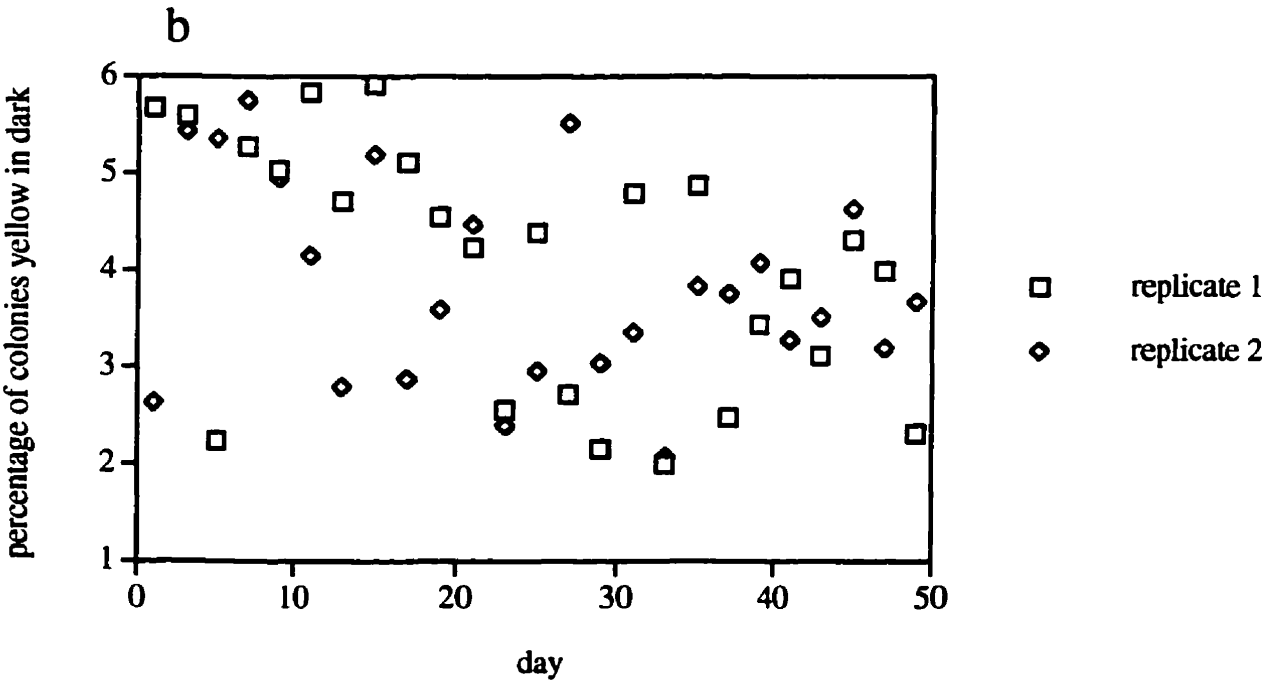
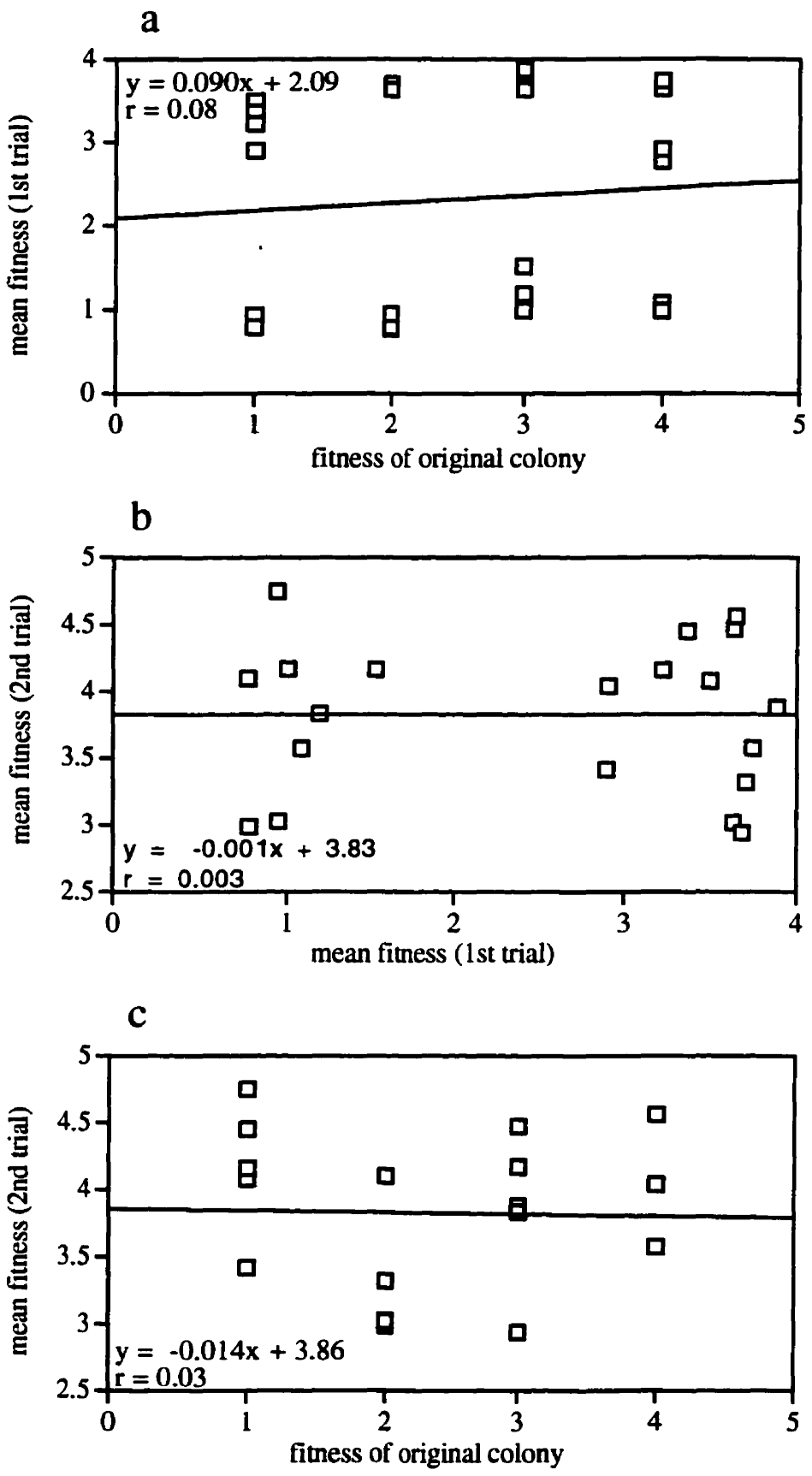

b
percentage of colonies yellow in dark
6
5
4
3
2
1
0
10
20
30
40
50
day
replicate 1
replicate 2

figure 6: Heritability trials. **a**. First trial vs. founder. The x-axis indicates the number of cell divisions in 24 hours of the original colony. The y-axis indicates the mean number of cell divisions in 24 hours in colonies produced by the descendants. **b**. Second trial vs. first. The x-axis indicates the mean number of cell divisions in 24 hours of the lineage in the first trial. The y-axis indicates the mean number of cell divisions in 24 hours of the descendants of these cells in the second trial. **c**. Second trial vs. founder. The x-axis indicates the number of cell divisions in 24 hours of the original colony. The y-axis indicates the mean number of cell divisions in 24 hours of the descendants in the second trial.


a
y = 0.090x + 2.09
r = 0.08
mean fitness (1st trial)
fitness of original colony
b
y = -0.001x + 3.83
r = 0.003
mean fitness (2nd trial)
mean fitness (1st trial)
c
y = -0.014x + 3.86
r = 0.03
mean fitness (2nd trial)
fitness of original colony

Heritability Trials.

The first heritability trial demonstrated no correlation between fitness of the founder colony and mean fitness of the descendants (figure 6a). This unexpected result was made still more perplexing by the obviously bimodal distribution of fitness among lines.

When the descendants of the plates from the first heritability trial were assayed a second time, again no correlation was found, either with mean fitness from the first assay (figure 6b), or with fitness of the founder colony (figure 6c). However, the fitness scores among plates were approximately normally, rather than bimodally, distributed in this assay.

It should be noted that although the heritability trials were inconclusive in this case, other, more extensive trials performed in our laboratory have demonstrated that the measure of fitness used in these experiments (number of cell divisions in 24 hours) does have a substantial heritability (R. Timms, unpublished data).

Discussion

The values obtained for V_M/V_E for fitness, 4.5×10^{-3} and 4.7×10^{-3}, fall within the range of previously observed values (Lynch 1988). Indeed, most studies have produced results of the same order of magnitude.

Therefore, at first glance the estimates obtained here are in agreement with those obtained in earlier studies. However, there are reasons to suspect that these values are rather high.

When an aspect of fitness such as viability has been examined, the values found for V_M/V_E were generally at least an order of magnitude lower

(Mukai et al. 1972, Lynch 1988). With no estimate of V_E, Houle et al. (1992), estimated V_M for fitness in *Drosophila* to be 8×10^{-4}. As well, studies of other characters that considered only "quasineutral" mutational variance, and excluded highly deleterious mutations, also obtained estimates for V_M/V_E on the order of 10^{-4} (Lopez and Lopez-Fanjul 1993). Since the cultures in this experiment were exposed to selection, only "quasineutral" variation should be detected.

There are several possible explanations for this discrepancy. They will be enumerated below, and the plausibility of each will be discussed. As well, experimental tests of each explanation will be discussed.

One explanation in particular seemed particularly promising, and was examined in a second mutation-accumulation experiment.

Experiment Two

Introduction

Chemostats are designed to provide a stable, uniform culturing environment (Dykhuizen and Hartl 1983). This was one of the reasons why a chemostat was chosen as the culturing environment for the mutation-accumulation experiment. If the environment is stable and homogeneous, then one can ignore selective arguments for the maintenance of genetic diversity through temporal or spatial environmental heterogeneity. Instead, the effects of mutation can be isolated.

However, in the course of the first experiment it became clear that the chemostat could in fact be viewed as a spatially complex environment. Cells were observed to grow attached to the walls of the vessel, and sometimes cells would flocculate out near the bottom of the vessel, or even form a transient film on the surface of the medium. These observations, along with the high estimate of V_M/V_E obtained in the first experiment and the bimodal distribution of fitness scores in the first heritability trial, suggested that ecological diversification might be taking place among the cells in the chemostat.

Diversification of an initially uniform *E. coli* population in a chemostat has been described by Helling et al. (1987). They found that a novel "small-colony" type arose in glucose-limited chemostats, but that it did not take over the population in the classic "periodic selection" manner. Instead, it remained at an intermediate frequency. A stable polymorphism was established between the original "large-colony" type and the small-colony mutants. The small-colony types were able to process glucose more rapidly than the large-colony types, but the large-colony types were able to survive as scavengers of

substances excreted by the small-colony types. Thus, social interactions increased the amount of genetic diversity maintained in a chemostat environment.

It was hypothesized that a similar phenomenon was taking place here. To successfully propagate itself in a chemostat, a cell must ensure that its rate of cell division is at least equal to the rate at which it gets washed out of the growth vessel. To increase one's fitness in such an environment, there are two basic strategies that may be adopted. The first is to optimize the rate of uptake of nutrients. The obvious way to do this is to remain in suspension in the liquid, constantly drifting about and coming into contact with fresh medium. The second option is to reduce the rate at which one is washed out of the vessel. From this perspective, remaining in suspension is the worst possible strategy. Much better is it to remain attached to the walls of the culture vessel, or to flocculate out at the bottom (since medium is removed from the top). Two or more types, each specialized for one such niche, might stably coexist in a chemostat. It was hypothesized that the slow-growing lines observed in the heritability trial were representatives of the attached type, while the faster-growing lines were from the suspension type.

A second experiment was carried out to test the hypothesis that the large value for V_M/V_E obtained in the first experiment was the result of ecological diversification among the descendants of the colony first inoculated into the chemostat.

Materials and Methods

The chemostats, strains and culturing media used were the same as those described above for the first experiment. The two chemostats were maintained and sampled in a similar manner to those from the first experiment. Only the following modifications were made in the protocols.

First, samples were collected only weekly, rather than daily. Second, when samples were collected, three additional assays were performed in addition to those carried out in the first experiment. These assays were designed to test for adaptation of subpopulations of the cells to different ecological niches in the vessel.

Though the medium was collected, as before, by attaching a flask to the three-way valve on the outflow tubing, samples of cells were collected directly from the culturing vessel. The vessel was detached from the rest of the chemostat apparatus and taken to a laminar flow hood, where the samples were removed.

Three sets of samples were taken from the vessel. These were meant to represent the three potential niches in the chemostat: top (suspension), bottom (flocculation) and wall (attached). Samples of liquid medium, including cells, were taken from near the surface of the medium and from near the bottom. As well, the chemostats had several glass cover slips placed in the culturing vessels. These were meant to be additional pieces of "wall" environment, to which cells might attach. Each time a sample was taken from the chemostat, one of these cover slips was removed. Cells were removed from the glass by gently washing the surface of it with distilled water, and then by suspending the slip in a vial containing filter-sterilized medium from the chemostat.

The following assays were carried out on the cells sampled from all three environments. Their fitness was assayed in the same manner as in the first experiment: number of cell divisions in 24 hours was scored for 100 colonies on agar plates made from filter-sterilized chemostat outflow medium. Periodic selection was followed by plating on Bold's plus acetate and on chlorate plates. Tendency to flocculate was assayed by passing samples of cultures through a Coulter Counter and measuring the mean size of cells. If cells tend to clump together and flocculate, then the mean size of the "cells" passing through should be greater than for cells without this tendency. Adherence was assayed by placing samples of cultures in vials containing a cover slip. After three days, the number of cells per unit area on the cover slip was measured by counting them under a microscope. A 5mm by 5mm square was marked off on each cover slip, and the number of cells that had attached to this surface were scored.

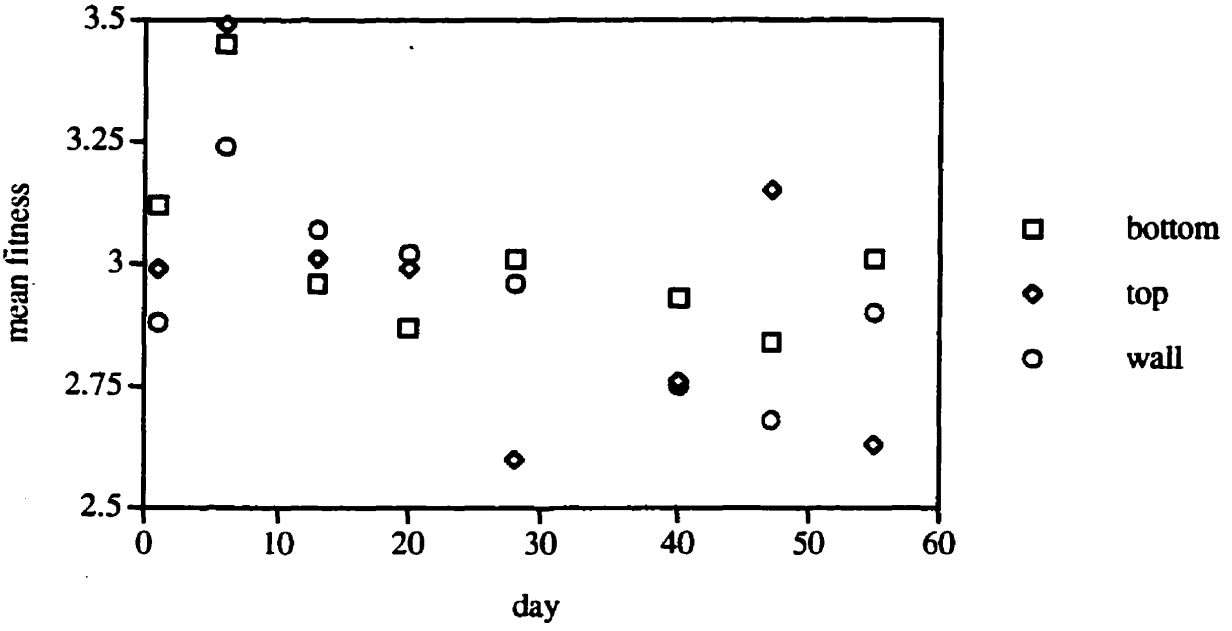
Results

No evidence was found for divergence among the populations inhabiting different niches in the chemostats. The fitness scores (figure 7), as well as the phenotypic assays (figures 8 and 9), showed no significant difference between top, bottom and surface-sampled populations.

Further estimates of V_M/V_E were obtained from this experiment (figure 10), though the values are probably not as reliable as those from the first experiment, due to the reduced frequency of sampling. This is reflected in the lower r^2 values (compare figures 4 and 10). The values for V_M/V_E obtained for the two chemostats were 2.6×10^{-3} and 3.3×10^{-3}, respectively. Once again,

figure 7: Mean fitness in the second mutation-accumulation experiment. The x-axis indicates day of trial; the y-axis mean number of cell divisions in 24 hours ("fitness") of 100 colonies on plates made from filter-sterilized chemostat outflow medium. **a**. First replicate chemostat. **b**. Second replicate chemostat.

a


3.5
3.25
3
2.75
2.5
mean fitness
0
10
20
30
40
50
60
day
bottom
top
wall

b

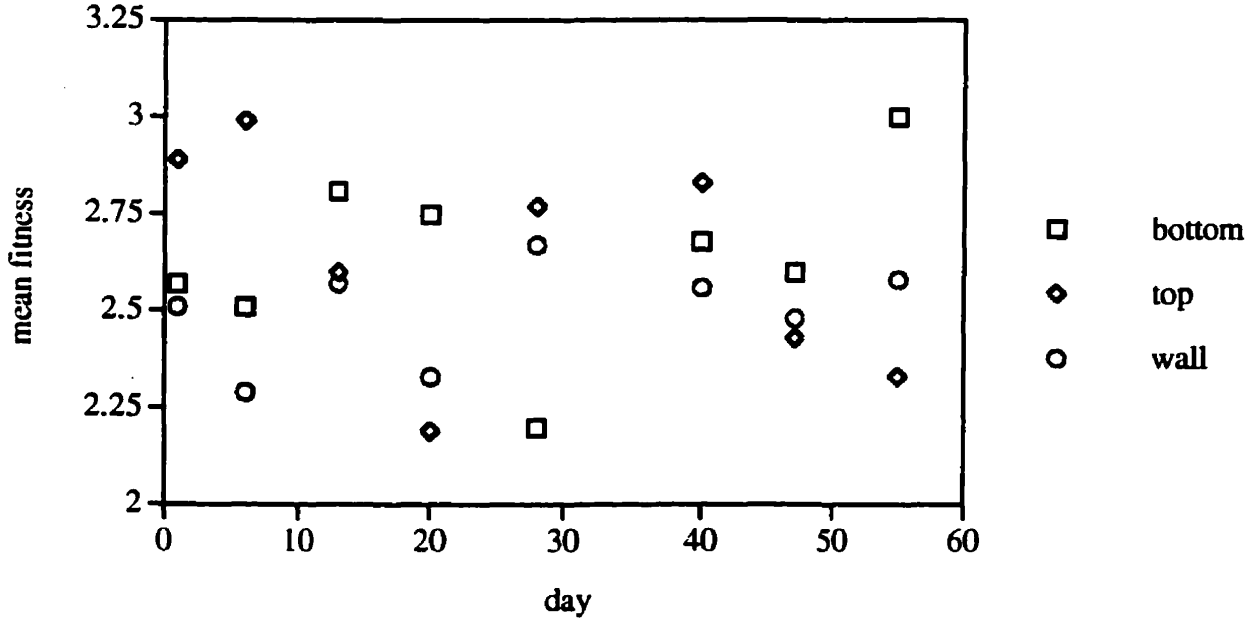
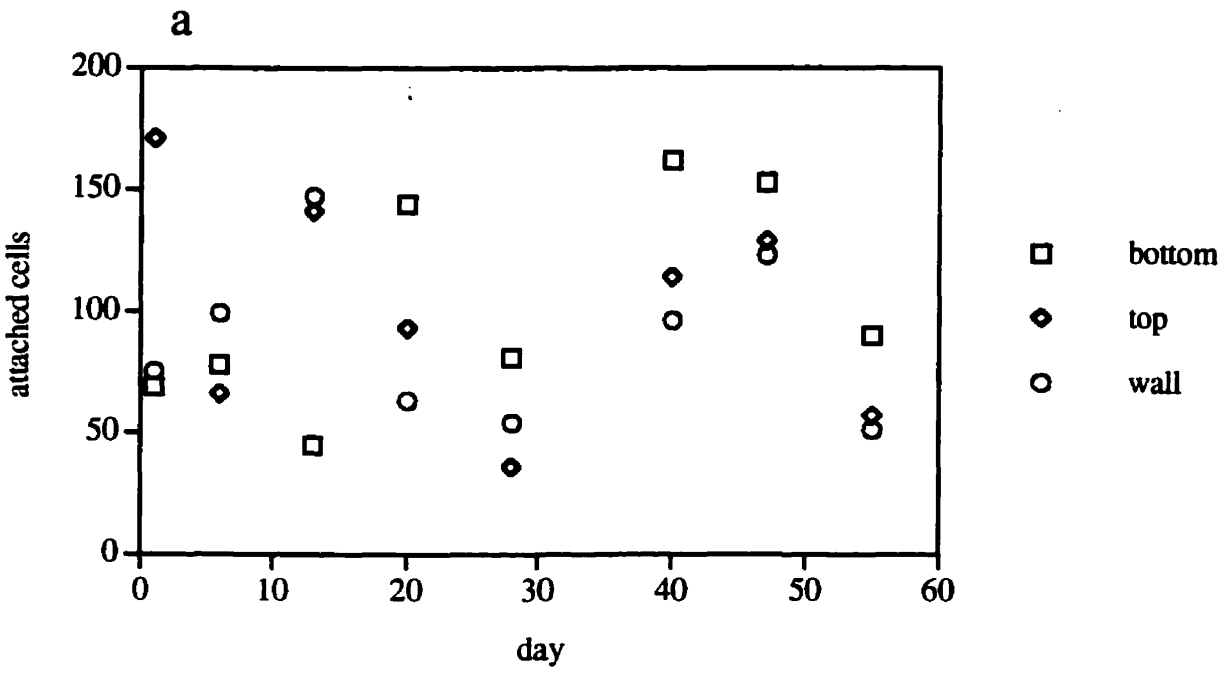

3.25
3
2.75
2.5
2.25
2
mean fitness
0
10
20
30
40
50
60
day
bottom
top
wall

figure 8: Adherence assay. The x-axis indicates day of trial; the y-axis the number of cells found attached to a glass coverslip in a 0.5 mm by 0.5 mm square three days after inoculation of a vial in which the slip was immersed. **a**. First replicate chemostat. **b**. Second replicate chemostat. The legend indicates the region of the chemostat from which samples were taken.


a
200
150
100
50
0
attached cells
0
10
20
30
40
50
60
day
bottom
top
wall

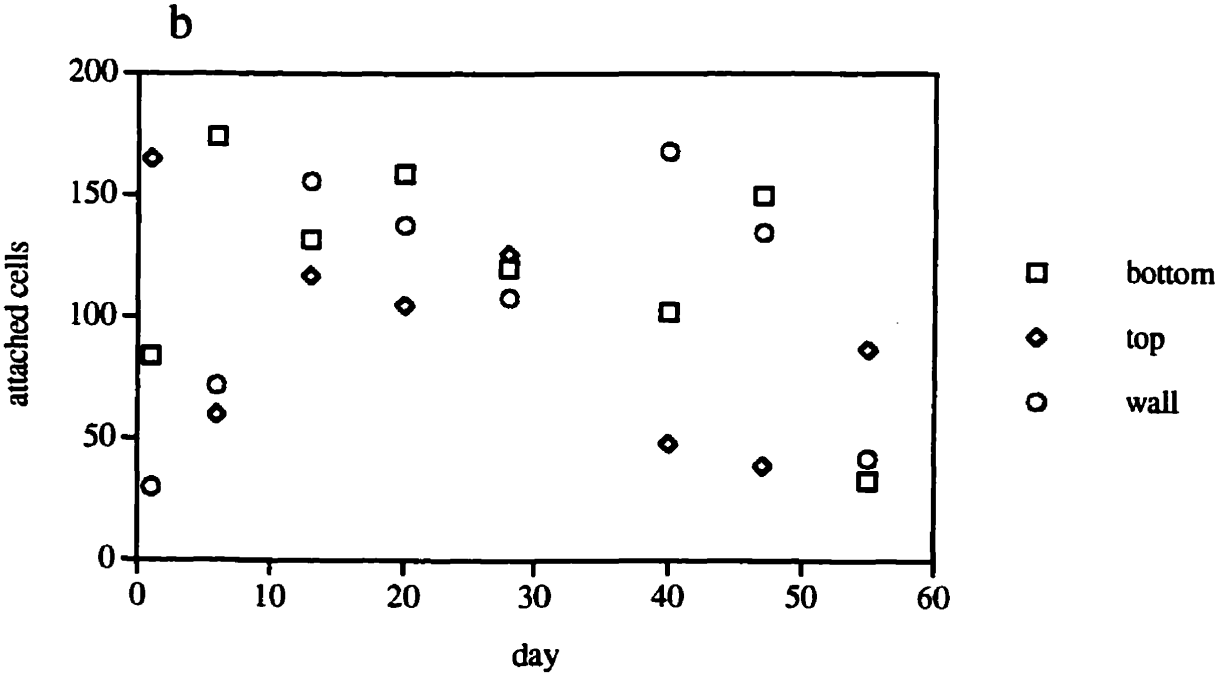
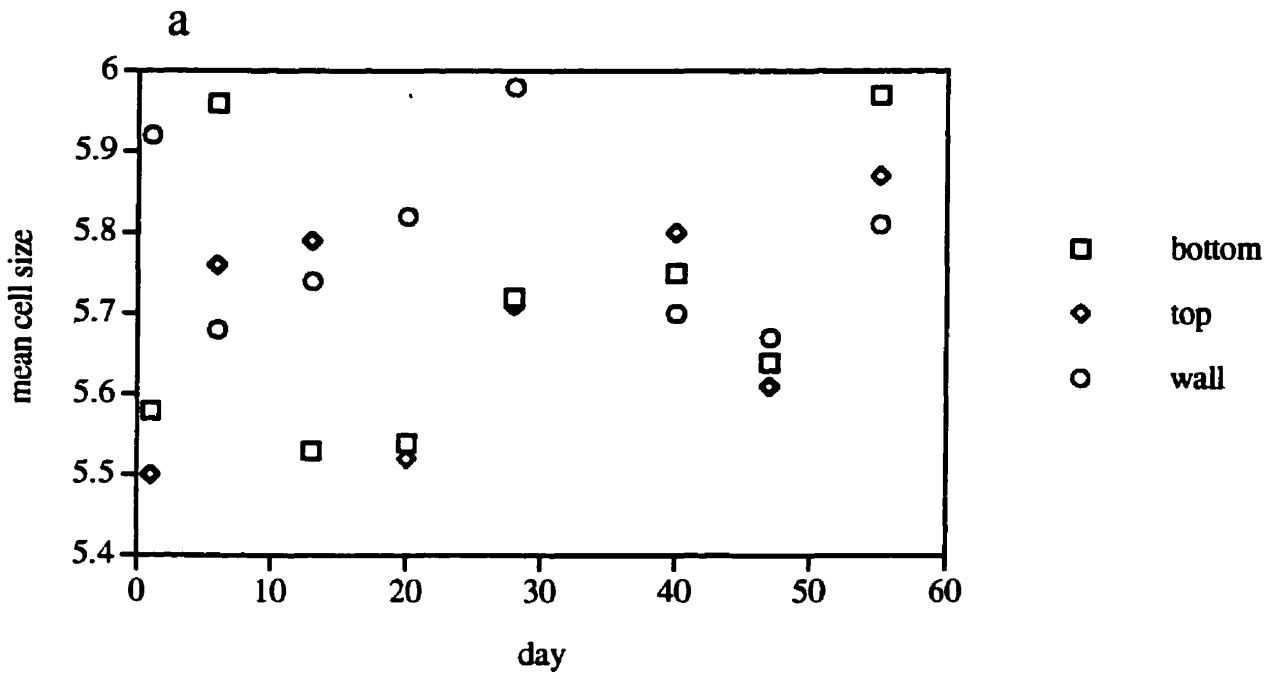

b
200
150
100
50
0
attached cells
0
10
20
30
40
50
60
day
bottom
top
wall

figure 9: Flocculation assay. The x-axis indicates day of trial; the y-axis the mean size of cells or clumps of cells passing through a multisizer. **a**. First replicate chemostat. **b**. Second replicate chemostat. The legend indicates the region of the chemostat from which samples were taken.


a
mean cell size
6
5.9
5.8
5.7
5.6
5.5
5.4
0
10
20
30
40
50
60
day
bottom
top
wall

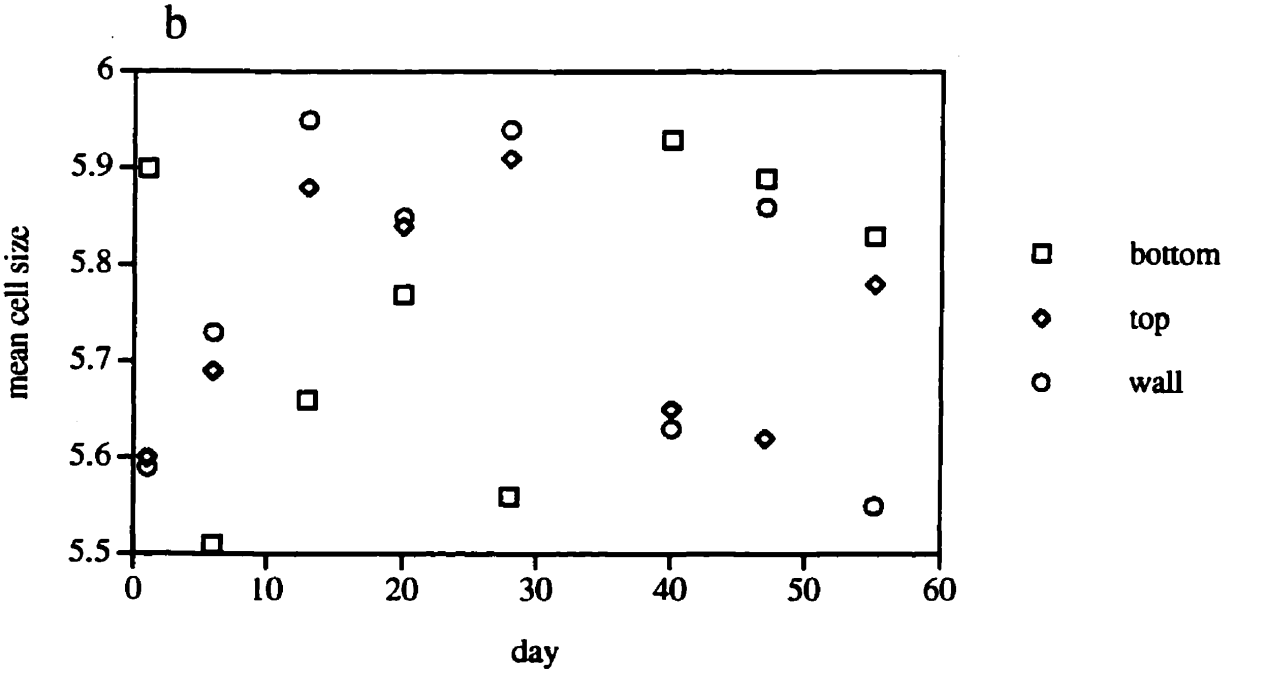
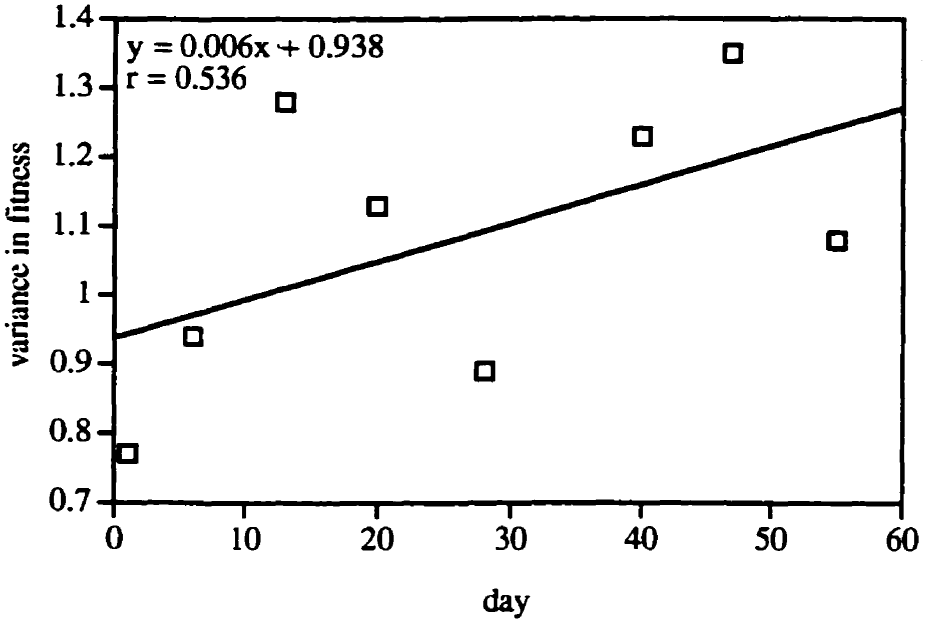
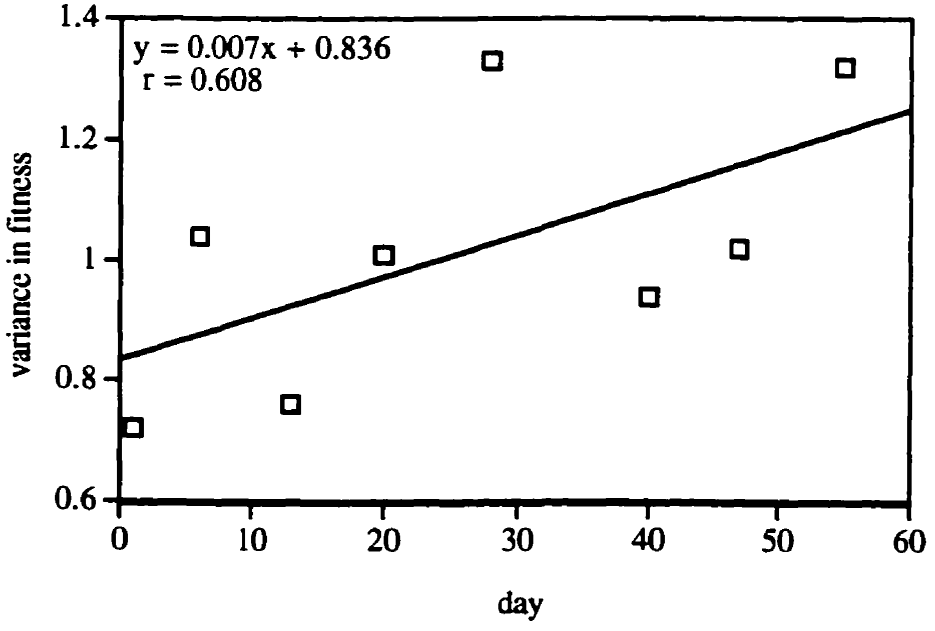

b
mean cell size
6
5.9
5.8
5.7
5.6
5.5
0
10
20
30
40
50
60
day
bottom
top
wall

figure 10: Variance of fitness in the second mutation-accumulation experiment. The x-axis indicates day of trial; the y-axis variance of number of cell divisions in 24 hours ("fitness") of 100 colonies on plates made from filter-sterilized chemostat outflow medium. **a**. First replicate chemostat. **b**. Second replicate chemostat.

a


y = 0.006x + 0.938
r = 0.536
variance in fitness
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0
10
20
30
40
50
60
day

b


y = 0.007x + 0.836
r = 0.608
variance in fitness
1.4
1.2
1
0.8
0.6
0
10
20
30
40
50
60
day

periodic selection did not seem to be occurring, although it would be harder to detect because of the infrequency of sampling (figure 11).

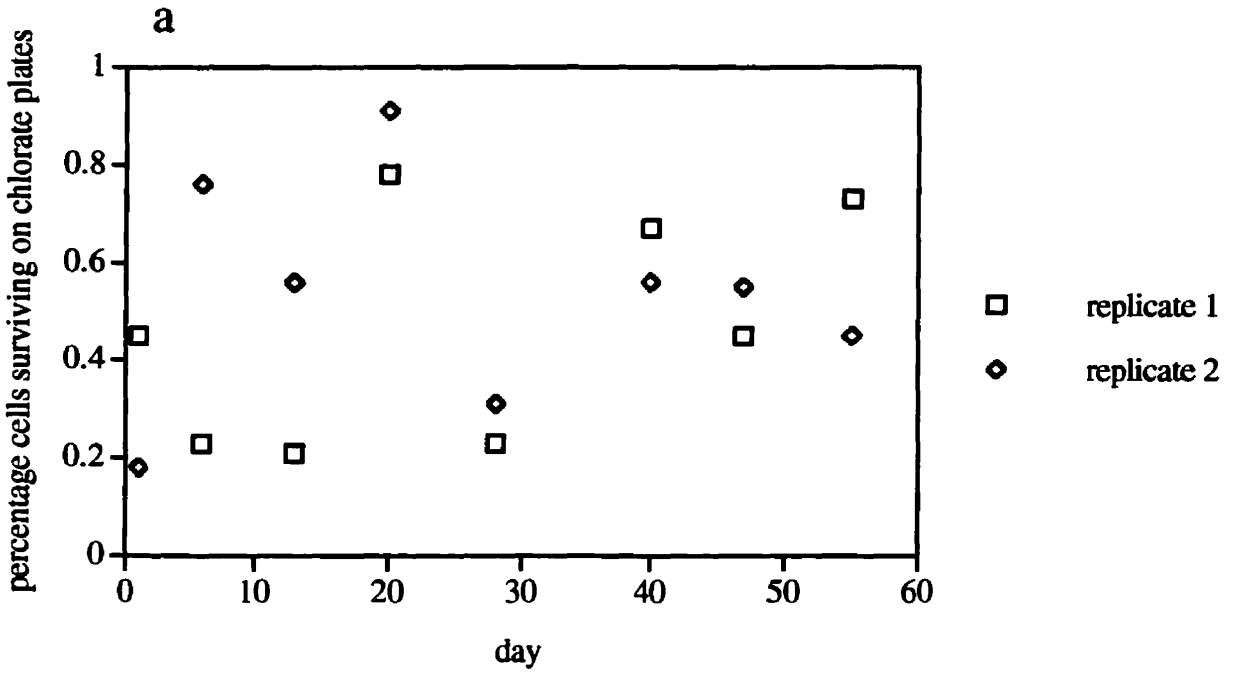
Discussion

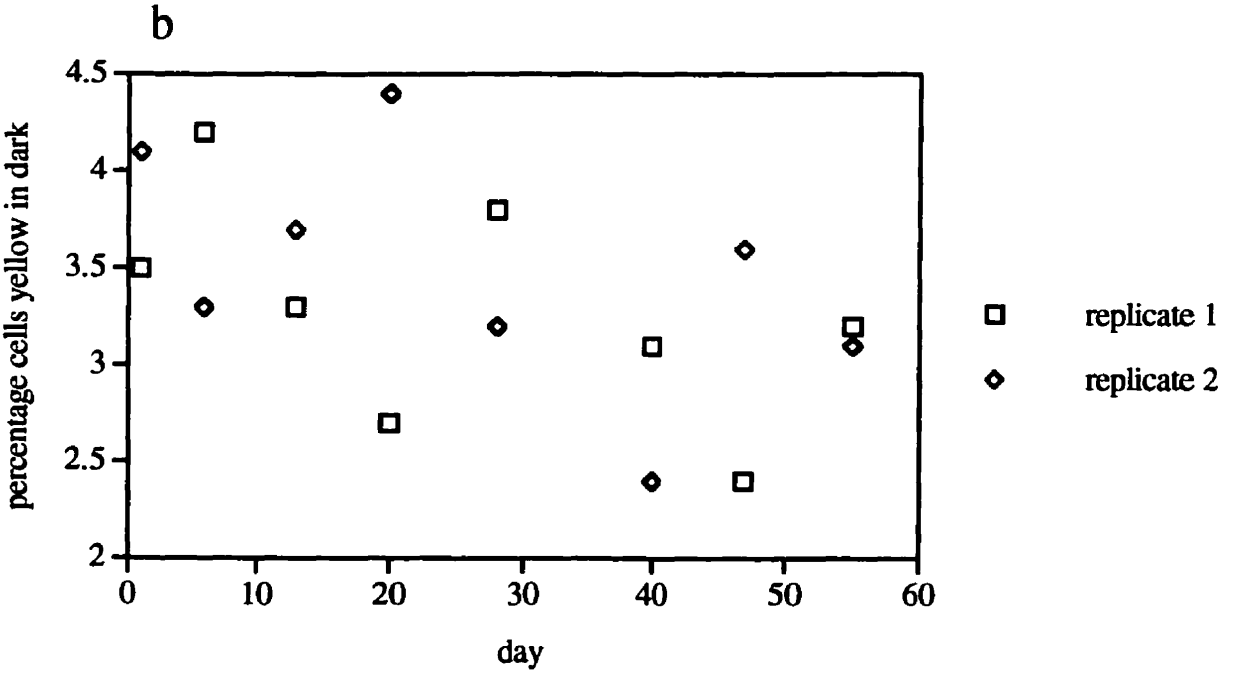
The estimates of mutational heritability obtained from the first mutation-accumulation experiment were higher than those previously obtained for fitness or components of fitness. Therefore, the hypothesis that this was due to ecological diversification among the inhabitants of the chemostat was proposed. The results obtained here provide no support for this hypothesis.

Cells sampled from different regions of the chemostat did not differ significantly in fitness or in terms of the phenotypes that would result from adaptation to a particular niche. Therefore, the cells occupying different regions do not seem to be members of distinct populations of cells. Even if they are, the identity of all populations in fitness suggests that this does not account for the high levels of variance in fitness observed in the first experiment.

It seems more likely that all of the cells in the chemostat are part of a single population, and that cells can migrate back and forth among the different environments. Such a phenomenon has been previously observed in populations of *Chlamydomonas reinhardtii.* maintained in chemostats (Olsen et al. 1983) and in batch culture (Straley and Bruce 1979).

figure 11: Monitoring of periodic selection in the second mutation-accumulation experiment. **a**. Frequency of nitrate-reductase deficient cells. The x-axis indicates day of trial; the y-axis the percentage of cells spread on a chlorate plate which had survived and formed growing colonies one week after inoculation. **b**. Frequency of yellow-in-dark cells. The x-axis indicates day of trial; the y-axis the percentage of cells spread on a Bold's plus acetate plate which formed yellow colonies when kept in the dark.


a
percentage cells surviving on chlorate plates
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
day
replicate 1
replicate 2


b
percentage cells yellow in dark
4.5
4
3.5
3
2.5
2
0
10
20
30
40
50
60
day
replicate 1
replicate 2

General discussion

Both mutation-accumulation experiments resulted in mutational heritabilities of the order of 10^{-3}. While these are in accordance with observations of mutational heritabilities for other characters in other organisms (Lynch 1988), they are at least an order of magnitude higher than the values obtained from other experiments where fitness, components of fitness, or quasineutral mutations were studied (Mukai et al. 1972, Lynch 1988, Houle et al. 1992, Lopez and Lopez-Fanjul 1993). The first explanation proposed to account for this discrepancy, that ecological diversification was occurring among the cells in the chemostat, was examined in a second experiment. However, no support was found for this hypothesis. Some other possible mechanisms are described below, along with suggestions for further research.

Sexual vs. Asexual Species.

Lynch and Gabriel (1983) suggested that mutation rates could be higher in asexual than in sexual species, to make up for the lack of recombinational input to variability in quantitative traits. It is possible that species which only occasionally undergo a sexual episode, such as *Chlamydomonas reinhardtii*, might also have elevated rates of mutation for quantitative traits, for the same reason. The particular strain used in these experiments has been maintained vegetatively in the laboratory for several years, equal to thousands of generations of cell division.

However, Lynch's own study (1985) of parthenogenetic *Daphnia pulex* did not demonstrate the expected increase. He obtained a mean value of $V_M/V_E = 1.7 \times 10^{-3}$ for a suite of life history characters. Yet as long as Lynch's (1985) study is the only other one examining an asexual organism, the matter cannot

be considered to be settled. As well, no study before the one reported here has considered fitness in a clonally-reproducing population.

Microbes in variable environments.

It has recently been demonstrated by LeClerc et al. (1996) that some populations of pathogenic bacteria have elevated mutation rates, with mutator alleles being maintained in natural populations at a frequency of 1 to 5 percent. This phenomenon might be more widespread among unicellular organisms inhabiting variable environments. Though little is known about the ecology of *Chlamydomonas reinhardtii*, it may also experience selection in the wild for elevated mutation rates.

One difficulty with this argument is that the strain of *C. reinhardtii* used in this experiment, CC-2938, had been maintained in a stable laboratory environment for over two years prior to the commencement of the experiment. Stable conditions should select against mutator alleles (Leigh jr. 1973).

Increase in V_E in course of experiment.

One assumption of the experimental design was that the environmental variance (V_E) remained constant for the course of the experiment. I defined V_E as the variance in fitness (measured as number of cell divisions in 24 hours) of the descendants of a single cell, without any mutation. This measure thus incorporates variance due to developmental accidents as well as microenvironmental variability in the agar plates.

While it is in practice impossible to ensure that no mutations occur, a good estimate of this value can be obtained by plating a sample very soon after

the inoculation of a flask with a single colony. Thus, the variance obtained from the first few samples of the experiment should give a good estimate of V_E.

This value was assumed to remain constant because the method of sampling and preparing plates was the same throughout the course of the experiment. As well, the medium used to prepare the plates should have been nearly constant, as the composition of the medium in a chemostat is known to remain stable after it has reached equilibrium (Novick and Szilard 1950b).

Therefore, the environment of the assay plates was probably constant throughout the experiment. However, V_E is determined both by the environment and by the response of the descendants of single cells to that environment. If the variability of the descendants of a cell, or the *versatility of a lineage*, increased through the course of the experiment, then the value of V_E would have increased. This undetected increase could inflate the estimate of V_M.

Selection for versatility could be a result of the multiple niches available in the culture vessel. While the population might not differentiate into distinct wall and suspension types, the population as a whole might be selected so that the ability of a lineage to produce cells adapted to either environment would be increased.

Though the data gathered in the experiments were not strictly appropriate for the testing of this hypothesis, a rough attempt can be made by using the data from the heritability trials from the first experiment. These trials can give us estimates of V_E at the end of the mutation-accumulation experiment, which can then be compared with the V_E estimates from the start. The data from the first heritability trial, which assayed fitness in filter-sterilized chemostat outflow medium, is better for this purpose than the second one, which used Bold's.

In the first heritability trial, the mean within-plate variance (V_E) was 0.85. This is larger than the estimates of V_E at the beginning of the experiment (0.74 and 0.81), though with a sample size of only two the difference is not statistically significant. The data from the first heritability trial are likely to produce underestimations of V_E, since the mean fitness of some of the plates was quite low.

If one instead considers the coefficient of variation (CV), the standard deviation divided by the mean, then the effect of the difference in means should be negated. The mean CV from the first heritability trial was 0.54. Using the Y-intercepts of the regressions of mean and variance on day of trial (figures 3 and 4), one can calculate the initial CV for the two replicate chemostats as 0.33 and 0.36. Once again, the difference is not significant, because of the small sample size, but it is suggestive.

General conclusions.

The experiments presented here provide the best estimates I know of for the mutational heritability of quasineutral mutations influencing fitness. Since it is such mutations that are the most likely source of adaptive mutations (Lynch 1988, Lande 1995), such an estimate is a valuable source of information about the adaptive potential of the organism studied. As well, since genomic mutation rates seem to be similar among microbes with genome sizes varying by orders of magnitude (Drake 1991), this result might be extendable to a variety of other unicellular eukaryotes.

The mutational heritability estimated from these experiments is an order of magnitude greater than that obtained in comparable experiments in *Drosophila*. One possible explanation for this, disruptive selection for

adaptation to different ecological niches, was examined and rejected. Two other possibilities seem promising and should be further investigated.

The first has to do with the nature of the organism studied. *Chlamydomonas reinhardtii*, a unicellular alga that can reproduce either sexually or asexually, is a rather different sort of creature than *Drosophila melanogaster*, a multicellular, obligately sexual metazoan. Experiments examining mutational heritabilities should be extended to other asexual organisms and to other microbes. Such studies would allow one to conclude whether the elevated values obtained here were due to idiosyncrasies of the experimental set-up, or due to the nature of the organism itself.

The other possibility is a selective explanation. Though the populations inhabiting the chemostats did not seem to genetically differentiate into specialized types, their versatility may have increased. The data gathered in the heritability trials provide some support for this hypothesis. It could be tested by further experiments similar in design to those reported here, in which the "environmental variance" was monitored through the course of the experiment by the regular isolation of single cells and the estimation of the variability in fitness of their descendants.

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Chapter Two

The Effect of Environmental Stresses on the Mutational Input to Variance in Fitness

Introduction

Mutation is the ultimate source of all genetic variation. Genetic variation in populations is necessary if they are to respond to selection. Fisher (1930) proposed that the rate of a population's response to selection would be equal to its scaled variance for the trait under selection. The greater the mutation rate, the greater this scaled variance will be. However, in a constant environment to which a population is well adapted, virtually all mutations will be deleterious. One sees in these two statements the basis of the conflicting forces that influence the evolution of mutation rates.

The experiments presented here examine the possibility that organisms (in particular, eukaryotic microbes) may increase their mutation rate as a general response to environmental stress. An environmental stress is here defined as a change in the environment that results in a decrease in organismal fitness.

Before describing the experiments performed to test this idea, I will describe the experimental and theoretical background that makes it seem like a plausible hypothesis. The reasoning leading to this conclusion has four parts. First, mutation rate is a character with a genetic basis, and as such, can be acted upon by selection. Second, an increased mutation rate would be selectively favourable in clonal populations exposed to stress. Third, cellular signaling pathways exist for the detection of stress and the eliciting of

responses to stress. Fourth, some potentially mutagenic genetic elements (i.e. mutator alleles and transposons) can show increased rates of activity in response to stress.

This argument makes it seem, at the very least, reasonable that an increase in the mutation rate may be a generalized response to stress. However, it remains to be demonstrated that such a response can be produced by a variety of stresses, or that the mutations produced by such a response influence the organism's fitness. It is the aim of the experiments described below to address these two questions.

Mutation rate is a character.

The rate of mutation is a character, and as such can be acted upon by selection, provided that there exists heritable genetic variation for it. It has long been known (Muller 1928, Demerec 1937, Ives 1950) that variation in mutation rate exists in natural populations. In particular, alleles of some genes that result in elevated mutation rates, known as mutator alleles, have been identified in many species (Demerec 1937, Neel 1942, Ives 1950, von Borstel et al. 1973, Smith 1992). These can be simple loss-of-function alleles at loci involved in DNA replication, repair, or (in sexual species) recombination (Smith 1992). It has recently been demonstrated (LeClerc et al. 1996) that such alleles are present at relatively high frequencies (1-5 %) in natural populations of some pathogenic bacteria. Interestingly, antimutator alleles, which reduce the mutation rate, have also been identified (von Borstel et al. 1973, Smith 1992), suggesting that most organisms do not have the lowest possible spontaneous mutation rate.

In some situations, selection may favour the spread of mutators, since they increase the likelihood that a carrier will experience a beneficial

mutation. Chao and Cox (1983) examined the dynamics of the *E. coli mutT* mutator in chemostat cultures. They found that, when present as a sufficiently high proportion of the base population, a strain carrying *mutT* out competed an isogenic line (*mut*$^+$). Moreover, the replacement of the *mut*$^+$ strain by the *mutT* strain only occurred after a lag of about 60 generations, and did not occur if the initial ratio of *mutT*/*mut*$^+$ strain was less than 7×10^{-5}. These two additional observations strongly suggest that the replacement of the *mut*$^+$ line by the *mutT* was due to the incorporation of novel, beneficial mutations in the *mutT* population, and not to any intrinsic competitive superiority.

However, Leigh jr. (1973) has concluded that in sexual populations in a stable or slowly changing environment, selection will never favour alleles that increase the mutation rate. Recombination and the independent assortment of chromosomes will separate the mutator allele from the favourable mutations it produces.

Even in asexual populations, one would expect the frequency of mutator alleles to be generally low. A mutator may increase in frequency as it hitchhikes through a population on a favourable mutation it produces, but it should then decrease in frequency as revertants to wildtype will be favoured because they produce fewer deleterious mutations (Bell 1997).

In the experiments of Chao and Cox (1983), the *mutT* strain was not favoured if present at a low frequency, suggesting that in such conditions a novel mutator allele could not invade the population.

In stable environments, the mutation rate will evolve to an equilibrium determined by the tradeoff between the costs of allowing deleterious mutations and of maintaining an efficient DNA replication and repair system(Leigh jr. 1973).

In stressful conditions, an increase in the mutation rate could be favoured.

While a population is in an environment to which it is well-adapted, selection will favour the lowest possible mutation rate. However, if this same population is exposed to a novel environment in which its fitness is drastically reduced, a higher mutation rate could be selected.

Should the fluctuation of the environment from favourable to unfavourable be frequent enough, an increased mutation rate could be favoured (Levins 1967). Simple mutator alleles could reach a relatively high frequency in the population. This seems to be what is happening in the populations of *E. coli* and *Salmonella* examined by LeClerc et al. (1996).

However, another possibility exists. A mutagenic genetic element could be regulated in such a way that it was inactive when conditions were favourable, but activated once the carrier was exposed to stressful conditions.

For this to be feasible, organisms must have some way of detecting environmental stress. Examples of such detection systems are not unknown. The heat shock response is found in organisms ranging from bacteria to mammals. A molecular pathway for signaling starvation has been discovered in *E. coli* (Hengge-Aronis 1993, Huisman and Kolter 1994). The sigma^S protein, part of this pathway, has also been implicated in the regulation of osmoprotection and general stress resistance (Hengge-Aronis 1993).

One way in which an organism may actively increase its mutation rate in response to the stress is by the upregulation of mutator loci. LeClerc et al. (1996) have already found some evidence for the possible regulation of a mutator allele in *E. coli*. One strain they examined had *rpoS*, the gene which encodes sigma^S, positioned close to *mutS*. If *rpoS* could regulate *mutS*, then mutator activity could be turned on only when needed most.

Transposons can be activated by environmental stress.

Mobile genetic elements (transposons) are widespread in eukaryotic genomes. They are thus obvious candidates for endogenous mutagenic agents. Indeed, when examined at the molecular level, the majority of morphologically-detectable mutations in *Drosophila melanogaster* have been traced to the activity of transposable elements (Sankaranarayanan 1988). It has also been demonstrated that transposon-bearing strains of *E. coli* can spread in chemostat culture in a manner analogous to that in which mutator strains do (Chao et al. 1983). Chao and McBroom (1985) went on to show that strains bearing a novel transposon insertion observed by Chao et al. (1983) were competitively superior to isogenic strains, proving that this result was due to favourable mutations produced by transposition.

However, most mobile elements are not likely to be transpositionally active, either because of self- or host-regulation. Charlesworth and Langley (1986) have provided the population-genetics argument demonstrating the selective feasibility of both types of regulation. In each case, it is the induction of lethal or sterile mutations (dominant in diploids) by transposition that provides the selective pressure favouring regulation. Experimental work has shown that both *Drosophila* P-element DNA transposons (Anxolabehere et al. 1988, Engels 1989) and I factor RNA LINE-like transposable elements (Udomkit et al. 1996) self-regulate their transposition rates.

Therefore, while transposable elements have the potential to produce many mutations, most transposons are usually transpositionally dormant. This raises the possibility that previously-dormant transposable elements may be activated in times of stress, through the loosening of either host- or self-regulation. The initial discovery of transposable elements was due to the

activation of previously silent elements by genomic stress: the rupture of a chromosome end (McClintock 1984). McClintock herself saw transposable elements as providing the genome with a way of responding to "unanticipated challenges." (McClintock 1984, p. 792) Several authors have explicitly proposed that transposable elements may be activated by environmental as well as genetic stresses (Echols 1981, Wills 1984, McDonald 1987, Wessler 1996).

The experimental evidence is not unequivocal, but generally supports this hypothesis. Edlin et al. (1986) found that transpositional activity of Tn10, a bacterial transposon found in *E. coli*, was not induced by chemical, nutritional or thermal stresses to the host. However, Datta et al. (1983) observed an increase in Tn9 transposition in *E. coli* cells exposed to several chemicals, including acetate and DMSO. They also report unpublished data gathered by two of the authors demonstrating differences in Tn9 transposition in response to variation in temperature.

Among eukaryotes, the best evidence comes from *Drosophila*. Heat-shock and gamma radiation increase transcript levels of *Drosophila copia* elements (Strand and McDonald 1985, McDonald et al. 1988). Ratner et al. (1992) found that the transposition rate of Drosophila *copia*-like Dm-412 elements increased by two orders of magnitude in the next generation following heat shock treatment. Plant retrotransposons are known to be transcriptionally activated by microbial infections (Pouteau et al. 1994) and cell culture (Hirochika 1993). Johns et al. (1985) have suggested that infection of maize by barley stripe mosaic virus may activate more than one class of transposons. However, Menees and Sandmeyer (1996) report that cellular stress caused by heat or alcohol actually inhibited the transposition of the retrotransposon Ty3 in *Saccharomyces cerevisiae*.

In addition to these empirical results, the activation of transposons by stressful conditions is also theoretically plausible. Inactive transposons are likely quiescent because of selection among hosts (Charlesworth and Langley 1986). This sort of selection will occur when hosts are well-adapted to their environment. However, if host fitness is low, e.g. because of exposure to a stress, such selection may be much weaker. On the contrary, in order to make the best of a bad situation, selection might even favour the release of transposons from regulated inactivation.

This is the theoretical and empirical background that makes the hypothesis of stress-triggered increases in mutation rates seem plausible. Before proceeding to a description of the experiments I performed, I will give a brief overview of previous research on environmental influences on mutation rate.

Changes in the environment influence mutation rates.

It is well-established that a variety of changes in environmental conditions, including many not normally thought of as mutagenic, can influence mutation rates (Smith 1992). Besides the many chemicals and forms of radiation known to be directly mutagenic, some of the factors implicated in increases in mutation rates include temperature, nutrient deprivation, pH and viruses (Smith 1992).

Temperature.

In the late 1920s and early 1930s, a number of experiments were carried out examining the influence of temperature on mutation rate in *Drosophila* [summarized in Timofeeff-Ressovsky (1934) and Plough and Ives (1935)]. Some of the studies indicated an increase in the frequency of certain mutations

under conditions of elevated temperature. These experiments fell into two classes. The first considered the rate of mutation at different temperatures within the normal range of physiological tolerance of the species. Muller (1928) reported that the frequency of lethal mutations in *Drosophila* cultures maintained at 27 degrees Celcius was three times higher than in cultures maintained at 19 degrees Celcius. Timofeeff-Ressovsky (1934) confirmed these results. Many years later, analogous experiments with *E. coli* found that mutation rates increased 6-fold as temperature was increased from 20 to 42 degrees Celsius (Savva 1982).

Another series of experiments looked at the effects of a pulsed temperature shock. The flies were briefly exposed to temperatures of 35-37 degrees Celcius. Here the results were less unequivocal. While some authors (Grossman and Smith 1933, Plough and Ives 1935) reported slight increases in the frequency of gross morphological mutations following such treatment, the majority of such experiments [reviewed in Timofeeff-Ressovsky (1934)] produced negative results.

Nutrient Deprivation, pH and viruses.

Herskowitz (1963) found that maternal malnutrition increased the frequency of gross cytological mutations observed in offspring produced with X-irradiated sperm. However, it is not clear whether this was just one symptom of a general pathological decline, or indeed whether the increased mutation rate could be observed independently of the irradiation of the sperm. Some *E.coli* strains have shown elevated mutation rates under starvation conditions

(Rebeck and Samson 1991). However, other strains have shown elevated mutation rates in rich medium, as compared to minimal medium (Smith 1992).

Savva (1982) found that *E. coli* mutation rates were 2 times greater at pH 6 than pH 7.

Some researchers have also concluded that viruses are mutagenic agents in *Drosophila* (e.g. Baumiller 1967). Golubovsky and Plus (1982) found an increase in the frequency of lethals in stock infected with C Picornavirus. Gershenson (1986) concluded that the phenomenon was more general, and did not require infectiousness. Several RNA and DNA viruses that were non-infectious for *Drosophila melanogaster*, produced significantly elevated rates of lethal mutations in the offspring of individuals that were injected with them.

Adaptive mutation.

Alongside this work, another group of experimenters were examining the influence of stressful conditions on the mutation rate in microbes. These experiments, of which the canonical example is that of Luria and Delbruck (1943), were aimed at detecting whether mutations favouring survival in a particular stressful environment would occur at a greater frequency in that environment than in others. These experiments were therefore only concerned with the production of appropriate mutation, and were only assaying the rate of one mutant phenotype, such as virus resistance. Therefore, though the conclusion reached was that there was no increase in appropriately-directed mutations, the negative results obtained by these workers do not preclude the possibility of a general increase in mutation rate in response to stress.

The accepted wisdom that mutations are not appropriately directed has recently been challenged. In the late 1980s, a controversy arose over the nature of "directed mutations" (Cairns et al. 1988, Charlesworth et al. 1988, Lenski et al. 1989, Hall 1990). Though these experiments are not directly relevant to the work discussed here, it is worth noting that again the mutation rates of only a few loci were examined. Therefore the generally negative results found for the influence of starvation upon "general mutation rate" are not definitive. For instance, Hall (1990) ruled out the possibility that the stressful conditions (tryptophan starvation) were generally mutagenic by showing no increase in the rate of mutation to valine resistance. Though he reports that "several" loci can produce mutations with this effect, they still must represent only a negligible proportion of the genome.

It must be emphasized that in reviewing this literature, the point is not to argue whether mutation rates vary in different environments. Some environmental stresses (UV radiation, EMS) are clearly mutagenic. However, in such cases, a mechanistic explanation for the direct influence of the mutagen upon DNA is known. Rather, the question is whether there is a generalized organismal response to stress that increases the rate of mutation. Whatever mechanism underlies such an effect, the influence of the environmental factor on the DNA will be indirect. The distinction between these two types of mutagenesis (direct and indirect) is related to Wills' (1984) division of mutagenic agents into "external, autonomous" and "internal, cooperative" agents. If such a phenomenon exists, then it will have a profound effect on the way we view the relationship between organisms and their environment. To the best of my knowledge, this phenomenon has not

previously been reported. The experiments described below investigate this possibility.

The experiments.

Most experimental investigations of mutation rates consider the mutation rate at only one or a few loci (Smith 1992). From such experiments, extrapolations are made to the genomic mutation rate (Drake 1991). However, such a method did not seem appropriate for the present study. Mutation rates are known to vary greatly between loci in the same species (deMarini et al. 1989, Smith 1992). As well, mutations at single loci of large effect are not necessarily the right ones to consider when looking for an adaptive response to stress. Rather, one should consider the genomic mutation rate for fitness. This is best measured phenotypically, as fitness itself.

To do this I decided to carry out the experiments on *Chlamydomonas reinhardtii*, a unicellular chlorophyte. Since *C. reinhardtii* can be propagated vegetatively, fitness can be easily and unequivocally assayed as rate of cell division. As well, since one can start the experiment with a single cell, all variation observed in the experiment must be due to *de novo* mutations.

I exposed replicate cultures to a variety of sublethal, pulsed stresses. If the stresses were too harsh, or applied for too long, they would select for a single type best-adapted for them, thus depleting genetic variation.

About 10 generations after being removed from the stresses, cultures were assayed for mean and variance of fitness. The delay allowed physiological effects of the stresses to be ruled out as explanations of the results. It was hypothesized that an increased mutation rate in stressed cultures would be manifested as a decrease in the mean fitness, and an increase in the variance of it. The mean should decrease because most

mutations will be deleterious in an environment to which the organism is well adapted.

Materials and Methods.

Strains and culture media.

Two strains of *Chlamydomonas reinhardtii* were used in these experiments: CC-1952 and CC-2938. CC-1952 was collected from Minnesota in the 1980s (Harris 1989). CC-2938 was collected by members of our lab from a corn field in Farnham, Quebec in the summer of 1993 (Sack et al. 1994). Both strains have been cultured in our lab for at least three years on Bold's medium (Harris 1989). They are therefore well-adapted to standard laboratory conditions and media.

Culture media were either Bold's medium (Harris 1989), or in the case of the stressful media, variations upon it. See table 1 for the modifications to the standard recipe made to produce the stressful media.

Methods. Demonstrating stressfulness of proposed stresses.

Since we were defining "stress" as an environment which results in a reduction in fitness, the first task was to demonstrate that the proposed stresses were in fact stressful for the strains in question. Therefore, the following assay was carried out.

Single colonies of each CC-1952 and CC-2938 were isolated from storage plates made of Bold's medium plus 2 grams per litre yeast extract (Harris 1989). These colonies were inoculated into flasks containing minimal Bold's medium. After four days, corresponding to the middle of the exponential phase of

Table 1: List of stresses to which cells were exposed.

The Stresses

1. cold	5 degrees Celsius for 24h
2. heat	40 degrees Celsius for 24h
3. UV	irradiated with 3000 $\mu J/cm^2$ UV light
4. pH	in media with pH = 6.0 for 24h
5. osmotic	0.125 g/L NaCl (5x greater than in Bold's)
6. starvation	distilled water for 24h
7. cyclohexamide	in media with 5 µg/L cyclohexamide

growth, a small quantity (25 microlitres) of the medium was inoculated onto plates representing each of the stressful environments, as well as control plates. Each treatment, including controls, was replicated three times. To ensure an even distribution of cells on the plates, the inoculum was diluted in 0.75 ml double-distilled water (dd H_2O), which was spread by gently agitating the plates by hand. Plates were then left open inside a laminar flow hood for approximately 45 minutes, in order to let the excess liquid evaporate.

Plates were then placed on growth shelves exposed to continuous light for 24 hours. At this time, 1 ml of a dilute Lugol's solution (Harris 1989) was added to all plates to fix the cells and stop growth. The number of cells in each of 100 colonies were scored on each plate by examining them under a microscope.

Plate assay.

Two assays were planned to test the effects of stress on the mutation rate affecting fitness (figure 12). These assays would measure the fitness of strains in two environments: solid and liquid media. The former assay considered the fitness of cultures on agar plates made from Bold's medium.

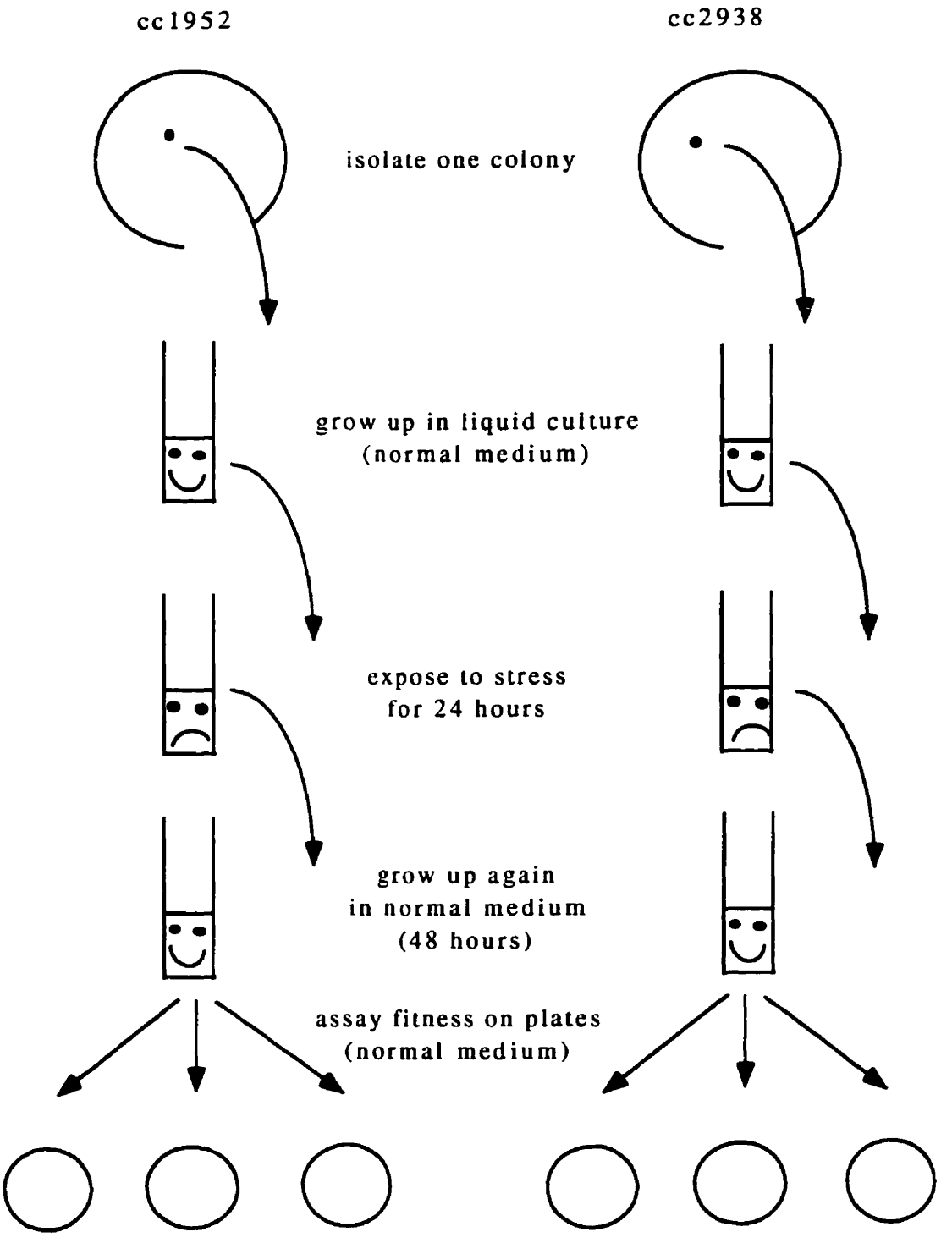
As in the assay for the stressfulness of the environments, single colonies of CC-1952 and CC-2938 were isolated to begin the experiment. The purpose of this was to start the experiment with as genetically homogeneous a population as possible. These colonies were then inoculated into flasks containing minimal Bold's medium and were allowed to grow for four days. One ml from each flask was then transferred into each of two test tubes containing 20 ml of the treatment media.

Twenty-four hours later, the cells were removed from the stressful conditions. Because some of the treatments involved chemical modifications of

figure 12: Schematic diagram of methods for plate assays in stress experiment.

Experimental Outline

two genotypes:



the media, the cultures could not be directly transferred into fresh media. Inevitably, some of the "stressful" medium would be transferred with the cells. Therefore, the cultures were washed by being centrifuged and then resuspended in minimal Bold's medium. All cultures, including controls, were washed in this manner.

The cultures were then transferred into test tubes containing 20 ml minimal Bold's, and allowed to grow for two days.

The cultures were then transferred onto agar plates made of minimal Bold's medium. Five plates were inoculated from each tube. The inoculum consisted of 25 microlitres of the tube culture diluted in 0.8 ml dd H_2O. Plates were gently agitated, and then left under the flow hood for 45 minutes to dry after inoculation. They were then placed on growth shelves, under conditions of continuous light. After 24 hours' growth, plates were removed and fixed by the addition of 1 ml Lugol's solution. 100 colonies were then scored on each plate.

This assay was carried out in September 1996. A second assay, in which every effort was made to duplicate the protocol of the first, was carried out in January 1997. In the results and discussion, these two assays will be referred to as replicates 1 and 2 of the plate assay.

Tube assay.

The initial stages of the tube assay were those described above for the plate assays. In the first plate assay, when the growth assay plates were inoculated from the tube cultures, two extra plates were inoculated from each tube, besides the five needed for the plate assay. These plates were not fixed after 24 hours, but rather were allowed to grow until colonies were visible to the naked eye.

At this point, 10 colonies were isolated from each plate. Thus, for each replicate tube that was originally exposed to the stress, a total of 20 colonies were isolated. To allow them to adjust to liquid, as opposed to plate, culture, each colony was added to 200 microlitres of Bold's medium in one well of a 96-well tissue culture plate. These plates were left on growth shelves for 24 hours.

These cultures were then inoculated into test tubes containing 20 ml of minimal Bold's medium, the well corresponding to each colony being used to inoculate a separate tube. These tubes were then placed on growth shelves, exposed to continuous light. Growth was assayed by measuring the transmittance of cultures at 665 nm in a spectrophotometer (Bell 1991). The cultures were assayed every second day until transmittance ceased to decline, indicating that the carrying capacity had been reached. The only exception to this occurred near the beginning of the experiment, when a power failure on the McGill University campus deprived the Stewart building, where these experiments were being carried out, of power for approximately 48 hours. During this time, the lights on the growth shelves were off, and cultures only received indirect natural light. During the night, the cultures were not illuminated. As well, during this time, measurement of growth in the spectrophotometer was impossible. The power failure occurred one day after the inoculation of tubes containing cultures of CC-2938, and two days after the inoculation of CC-1952.

Analysis. Plate assays.

As described above, the number of cells in each of 100 colonies were scored on each plate. Colonies tended to fall into discrete size categories of 2, 4, 8, 16, 32 or 64 cells, representing 1, 2, 3, 4, 5 or 6 cell divisions, respectively.

Therefore, from the 100 colonies scored on each plate, one could obtain an estimate of the mean and variance of the number of cell divisions completed in 24 hours by the cells used to inoculate the plate. These were the basic values used in the subsequent analysis.

As there were five plates for each tube, and two tubes for each treatment per genotype, a nested analysis of variance was an appropriate method for analysing the data (Sokal and Rohlf 1995). Comparisons were made between treatments for the following parameters: mean fitness per plate, variance in fitness per plate, and the scaled variance in fitness (variance divided by the square of the mean fitness) per plate. The reasoning behind the use of the last parameter will be given in the results section.

The error variance used was the variance among plates within tubes. This was appropriate for the comparison of variance and of the scaled variance, as only one estimate of each could be obtained per plate. In the case of the means, this was a conservative test, as the degrees of freedom available would have been much greater had each colony been the unit measure, rather than the plate as a whole.

Tube assays.

The original intention with the tube assay had been to describe complete growth curves for each culture, and estimate the parameters r and K. These would then be the measures of fitness used in the subsequent analysis. However, the power failure and subsequent lack of illumination that the laboratory experienced near the beginning of the assay produced irregularities in the growth of many cultures. In many cases, there was a lag of up to two weeks before growth was detectable in a tube. As well, a difference in growth was noticed in tubes depending upon the colour and

placement on a shelf of the racks they were resting in. These effects were most pronounced early in the experiment, that is, shortly after the power failure.

As a result of these complications, the fitting of a logistical curve to the transmittance data proved problematic. For this reason, a preliminary analysis was carried out on two sets of data: transmittance at day 10, and minimum transmittance (equivalent to maximum growth or, approximately, K). It was felt that these parameters, particularly minimum transmittance, were the most likely to show a significant treatment effect, because of the above-described perturbations in the early growth of the cultures.

These parameters were analysed by a single-factor (treatment) analysis of variance. Because of the observations of variability in growth by rack, the data were reanalysed, to see if such effects were significant. Then the analysis was repeated as a two-factor ANOVA, with treatment and rack as the effects.

Since, as it happened, no significant effects of treatment were found in the analysis of these parameters, further analysis was abandoned as futile.

Results

Stressfulness Assay.

The stresses applied seemed to produce three qualitatively different fitness distributions among treated cells . The cold, UV and cyclohexamide stresses resulted in virtually no cell divisions by the stressed cultures during the 24 hours for which they were assayed. The pH and osmotic stresses resulted in a general, but slight, decrease in mean fitness compared to the controls. The starvation-stressed lines produced the most unusual fitness distributions. Whereas many cells only divided once or twice, many others

divided five or six times in 24 hours. In those colonies where many cell divisions had taken place, the cells were very small, as though cell division had taken place without normal intervening growth.

In summary, all treatments resulted in a decrease in mean fitness (figure 13). These differences were not always significant, but this is probably due to the small sample size (n=3) used in the assay. If the same difference between the mean fitness of stressed and control lines was maintained for sample sizes comparable to those used in the later experiments, then these differences would be statistically significant.

A heat stress proved impossible to apply in this assay because the desired temperature (40 degrees Celcius) was hot enough to melt petri plates. However, because it is well established that high temperature are stressful for *C. reinhardtii* (Harris 1989), the decision was taken to use the heat stress in the main experiments, where the stresses would be applied to cultures in Pyrex tubes.

Plate assay. Mean fitness.

In most cases, the fitness of stressed lines was reduced relative to that of control lines. In the first replicate, eight of fourteen stressed lines showed a significant decrease in mean fitness relative to the controls (figure 14a). For CC-2938, three of seven treatments (pH, NaCl, and cyclohexamide) resulted in a statistically significant decrease in mean fitness. With CC-1952, five of seven treatments resulted in strongly significant (at least $p < 0.01$) decreases in mean fitness. The results of the second replicate were even stronger. Both genotypes showed strongly significant ($p < 0.005$) decreases in mean fitness for all stresses (figure 14b).

figure 13: Assay of stressfulness. The y-axis indicates mean number of cell divisions in 24 hours of 100 colonies on treatment plates; the x-axis indicates the treatment (see table 1). The legend indicates the strain of *C. reinhardtii* assayed.

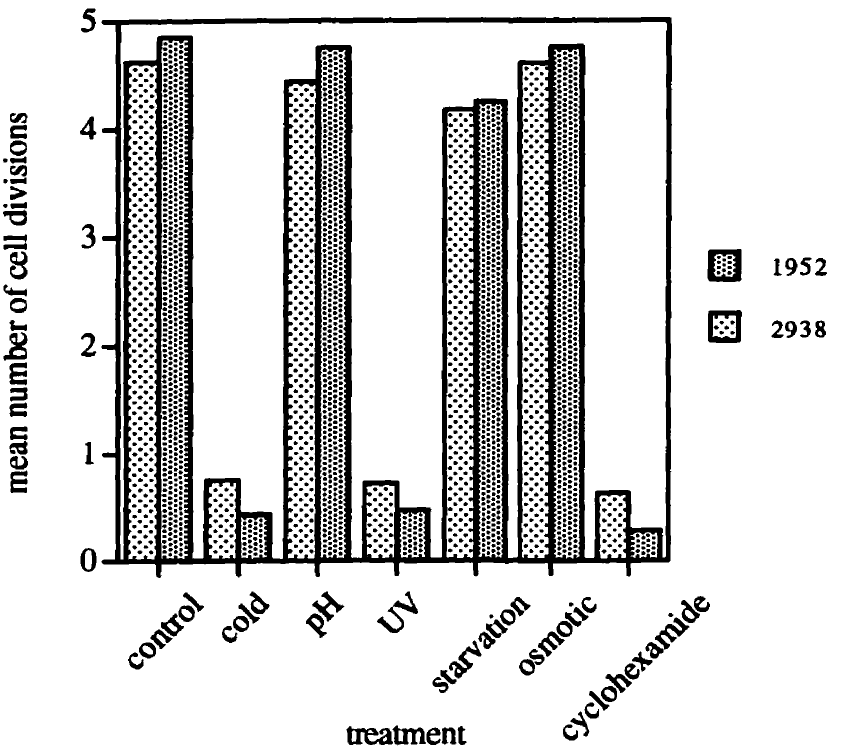
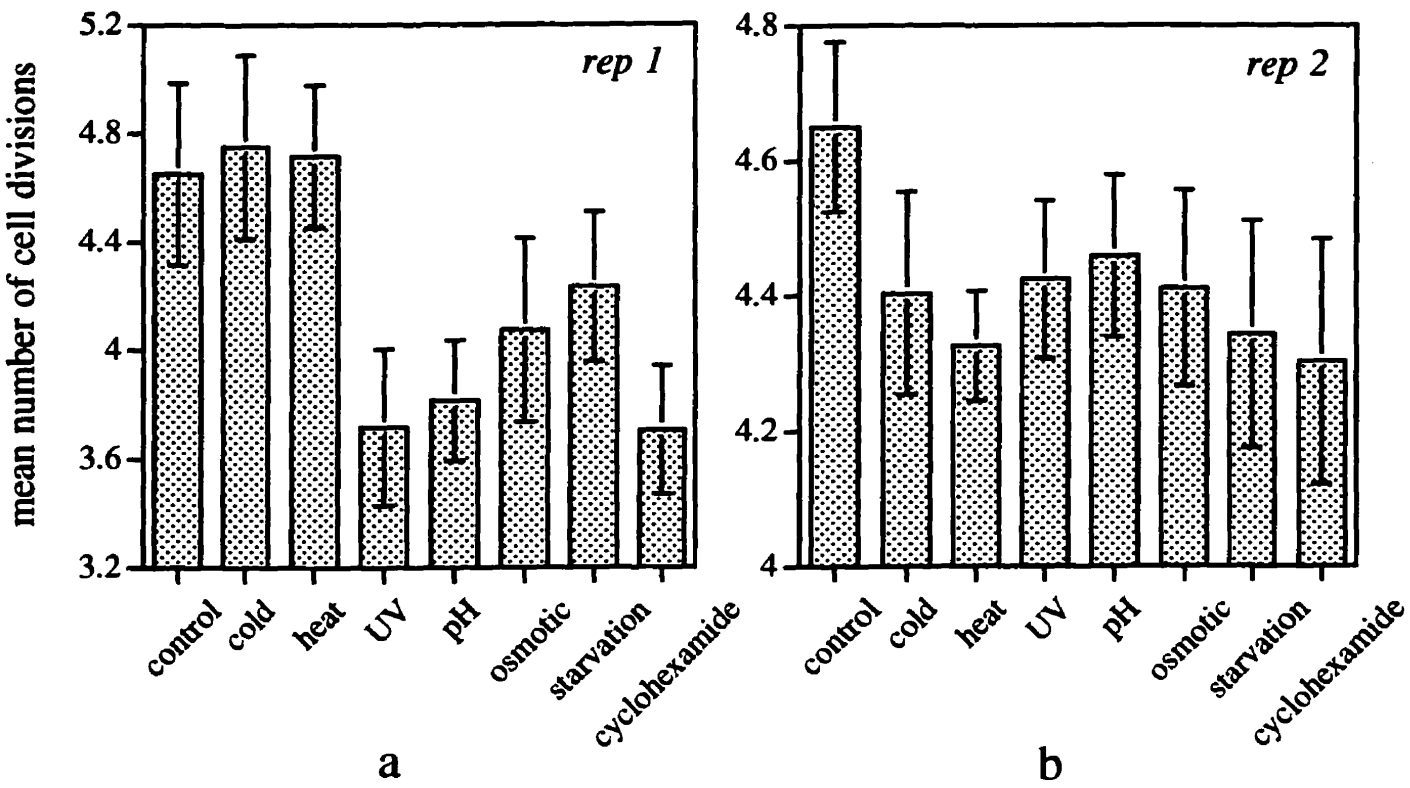
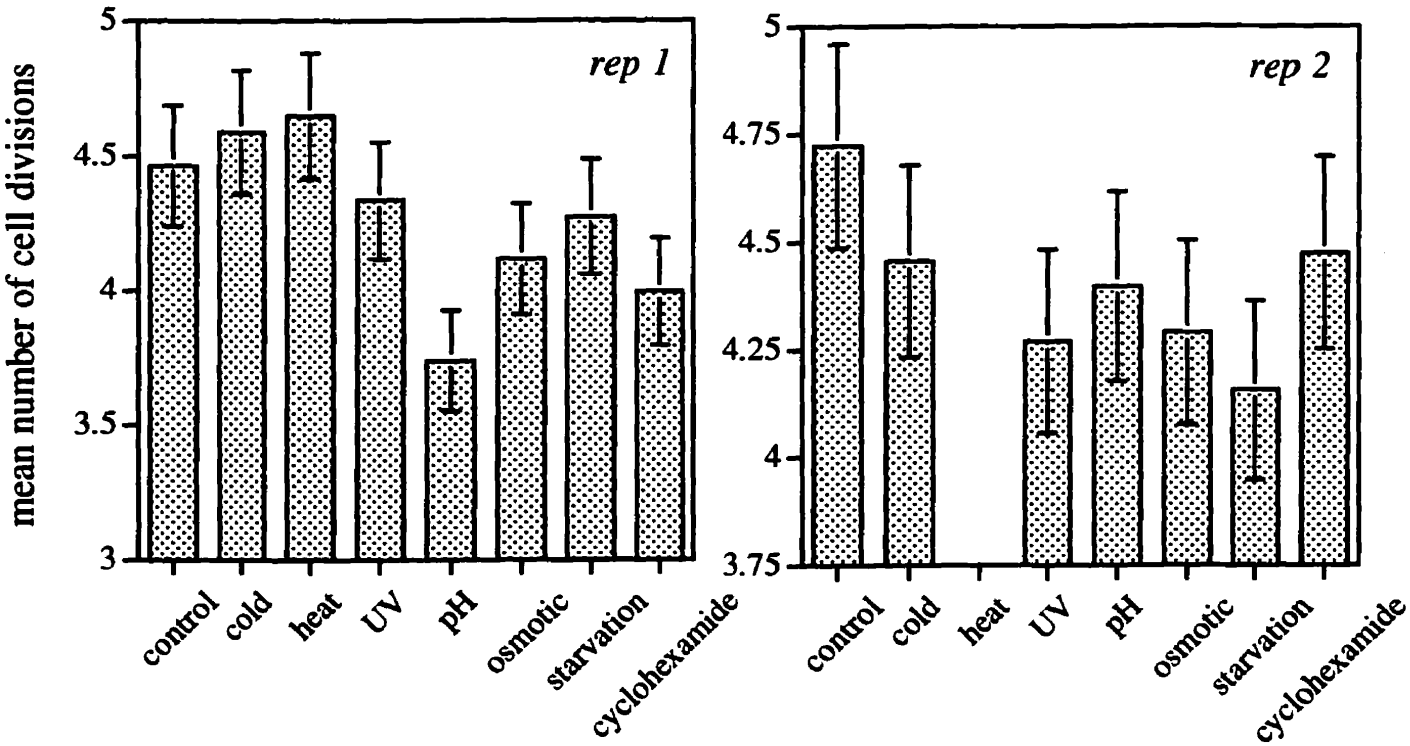

mean number of cell divisions
5
4
3
2
1
0
control
cold
pH
UV
starvation
osmotic
cyclohexamide
treatment
1952
2938

figure 14: Mean fitness in plate assays. The y-axis indicates mean number of cell divisions in 24 hours of 100 colonies; the x-axis indicates the treatment (see table 1). **a**. Results from first plate assay ("replicate 1") for CC-1952 and CC-2938. **b**. Results from second plate assay ("replicate 2") for CC-1952 and CC-2938.


mean number of cell divisions
rep 1
rep 2
5.2
4.8
4.4
4
3.6
3.2
4.8
4.6
4.4
4.2
4
control
cold
heat
UV
pH
osmotic
starvation
cyclohexamide

a b


mean number of cell divisions
rep 1
rep 2
5
4.5
4
3.5
3
5
4.75
4.5
4.25
4
3.75
control
cold
heat
UV
pH
osmotic
starvation
cyclohexamide

Variance in fitness.

In general, stressed lines had a greater variance in fitness than control lines. In the first replicate, with CC-2938, five out of seven treatments resulted in significant increases in variance in fitness. In CC-1952, only one treatment (NaCl) resulted in a significant increase in variance in fitness, though the starvation treatment was also on the borderline of significance ($P = 0.05$). In summary, seven out of fourteen treatments led to significant increases in variance in fitness (figure 15a), as predicted, while the rest were not significantly different from the controls.

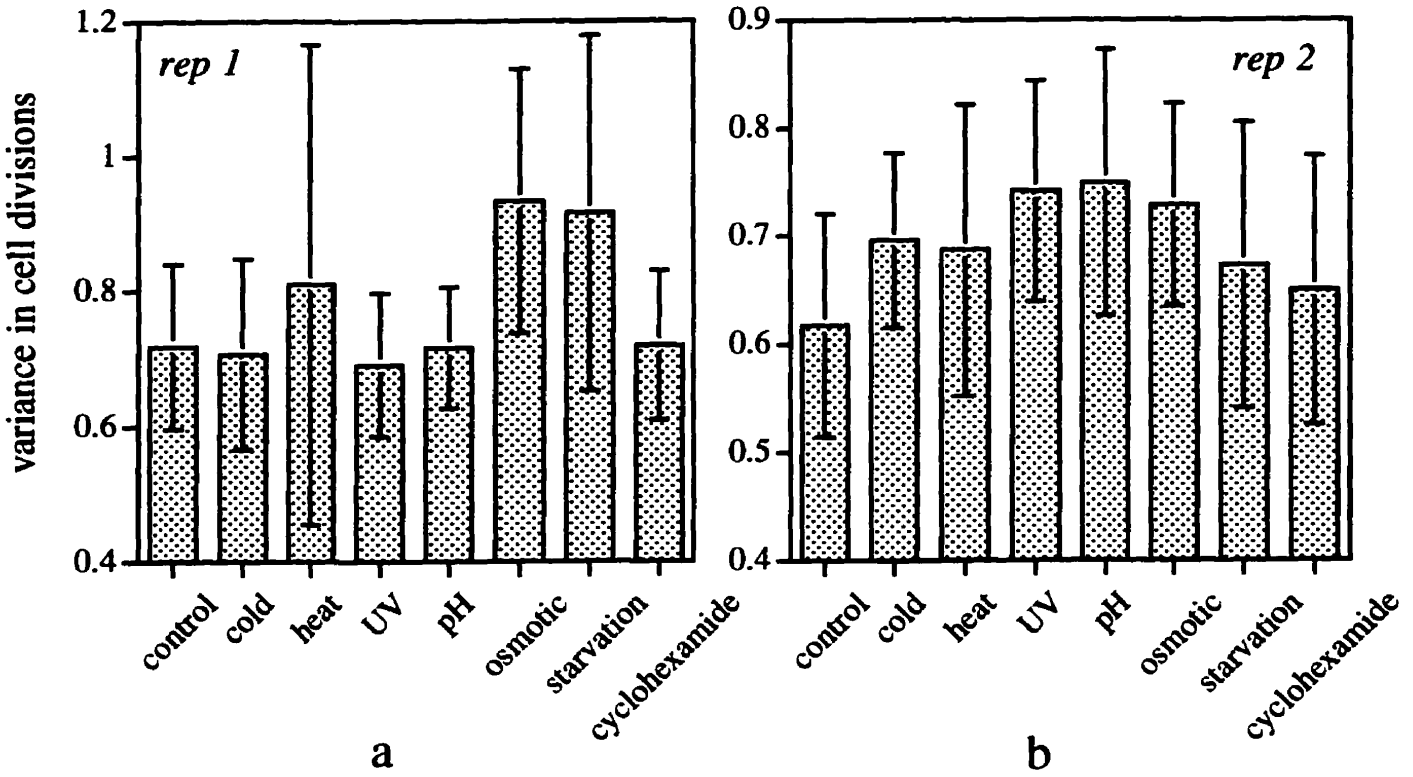
However, in the second replicate, only four of 13 treatments (heat treatment of CC-2938 was lost because one of the replicate cultures died) produced significant increases in the variance in fitness (figure 15b).

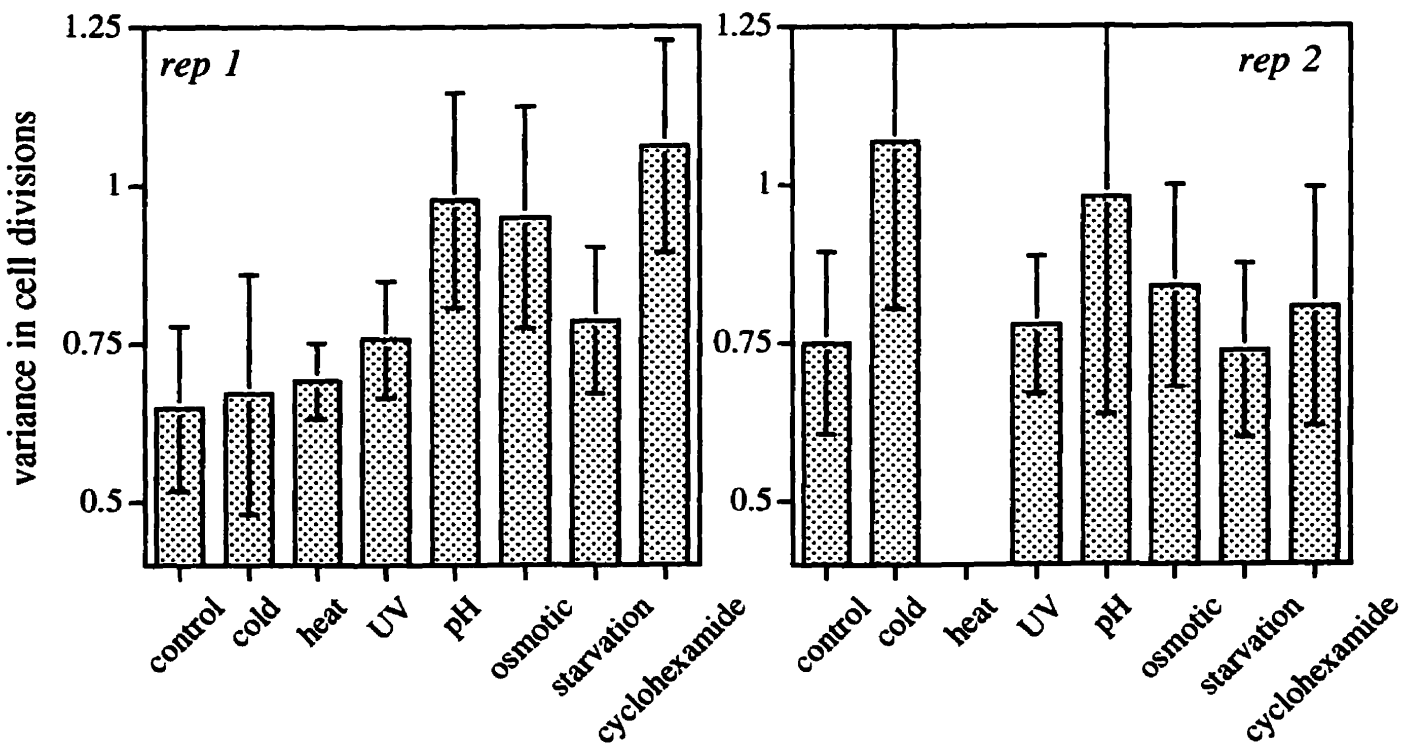
Scaled variance.

While these results were suggestive, the use of variance as the measure of variability did not seem entirely satisfactory. Since variance tends to increase as the mean does, it is only a proper comparison of the variability of two populations if these populations have the same mean for the parameter of interest. Otherwise the population with the greater mean would be expected to have the greater variance. In the case of the data presented above, this makes the use of variance an extremely conservative test of differences in variability between control and stressed lines. Since the control lines have in general greater means, they should have greater variances, even if the underlying variability is the same. Thus the null hypothesis of equal variances is conservative.

One solution to this difficulty is to use a scaled measure of variability. The obvious choice is the "opportunity for selection" (Crow 1970). This is the

figure 15: Variance in fitness in plate assays. The y-axis indicates the variance of the number of cell divisions in 24 hours of 100 colonies; the x-axis indicates the treatment (see table 1). **a**. Results from first plate assay ("replicate 1") for CC-1952 and CC-2938. **b**. Results from second plate assay ("replicate 2") for CC-1952 and CC-2938.


rep 1
rep 2
variance in cell divisions
1.2
1
0.8
0.6
0.4
0.9
0.7
0.5
control
cold
heat
UV
pH
osmotic
starvation
cyclohexamide
a
b


rep 1
rep 2
variance in cell divisions
1.25
1
0.75
0.5
control
cold
heat
UV
pH
osmotic
starvation
cyclohexamide

variance in fitness divided by the squared mean fitness. This value comes from Fisher's "fundamental theorem of natural selection," (Fisher 1930) which states that the rate of response to selection is equal to the variance in fitness divided by the squared mean; in other words, is equal to the "opportunity for selection".

In the first replicate, for both CC-1952 and CC-2938, all treatments except for cold and heat showed significant ($p < 0.05$) increases in the opportunity for selection when compared to the appropriate controls (figure 16a). Thus ten out of fourteen treatments led to significant increases in the scaled variance in fitness.

In the second replicate, a significant increase in the scaled variance was observed in 12 out of 13 treatments (figure 16b). Only the cyclohexamide treatment of CC-2938 was not significant, though it was nearly so ($p = 0.12$).

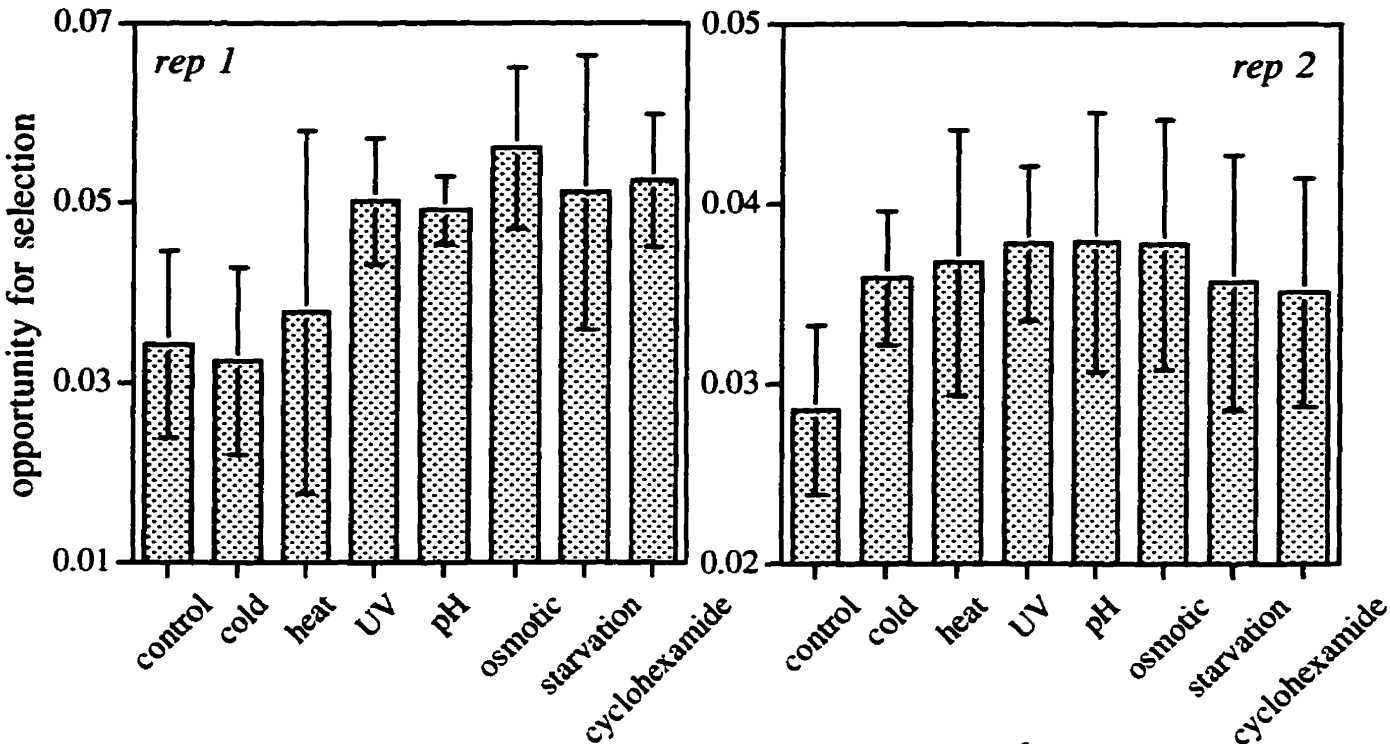
Tube assay.

In the tube assay, none of the treatment lines differed significantly from the controls in either mean or variance of fitness. However, significant rack effects were observed. Specifically, tubes in white racks had significantly greater "K" values than tubes in coloured racks (for CC-1952, $p < 0.005$; for CC-2938, $p < 0.04$). Even when corrected for rack differences, there were still no significant differences between treatments and controls.

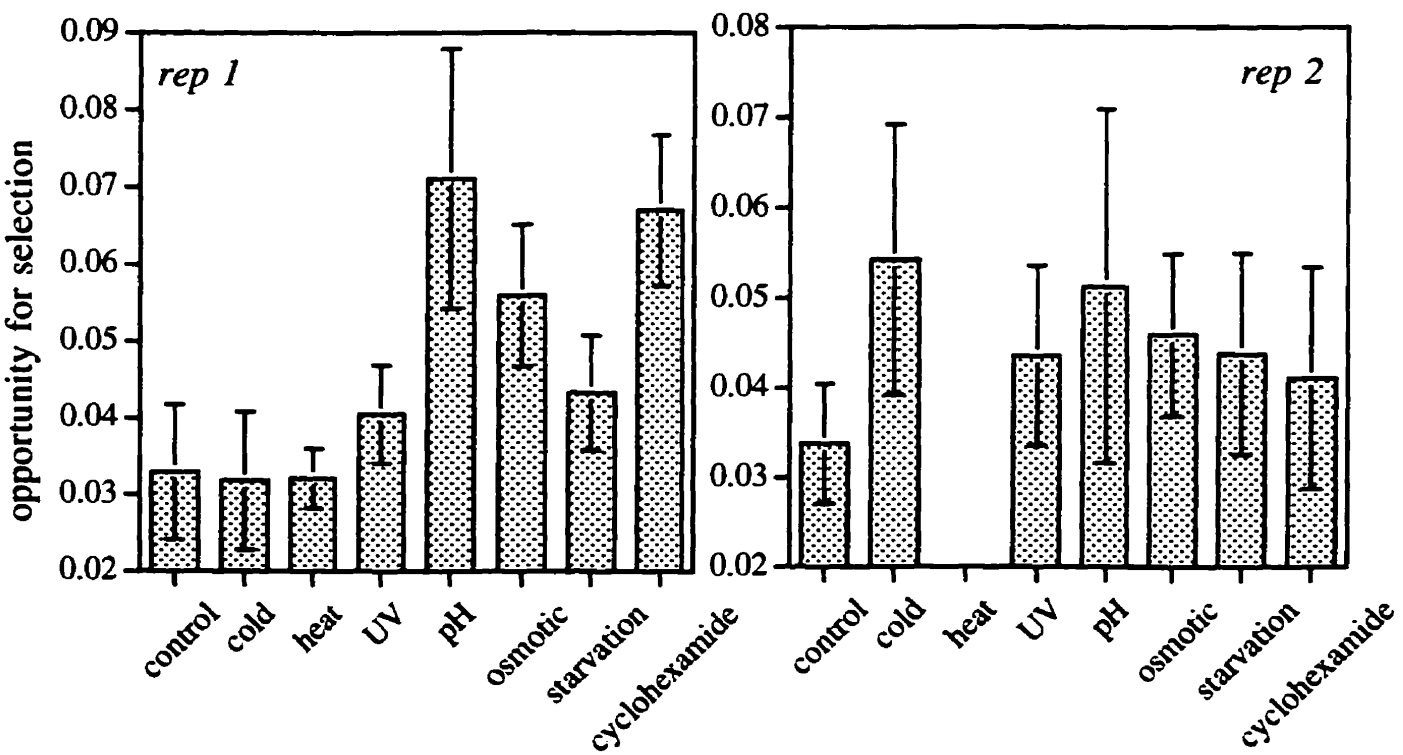
Discussion

Both plate assays produced similar results. The stressed cultures demonstrated a general decrease in mean fitness and an increase in the scaled

figure 16: Opportunity for selection in plate assays. The y-axis indicates the variance of the number of cell divisions in 24 hours of 100 colonies divided by the square of the mean number of cell divisions; the x-axis indicates the treatment (see table 1). **a**. Results from first plate assay ("replicate 1") for CC-1952 and CC-2938. **b**. Results from second plate assay ("replicate 2") for CC-1952 and CC-2938.


rep 1
rep 2
opportunity for selection
0.07
0.05
0.03
0.01
0.05
0.04
0.03
0.02
control
cold
heat
UV
pH
osmotic
starvation
cyclohexamide

a b


rep 1
rep 2
opportunity for selection
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.08
0.07
0.06
0.05
0.04
0.03
0.02
control
cold
heat
UV
pH
osmotic
starvation
cyclohexamide

variance in fitness. However, the results of the tube assay were inconclusive. There was in no case any significant difference between the control and stressed cultures. I will first discuss the interpretation of the plate assays, and then explain why the tube assay could not reproduce these results.

Plate assay results as evidence of increased mutation rate.

I consider the plate assay results to indicate that the stressed cultures have accumulated more deleterious mutations than the control cultures. The fitness assays were carried out about 10 generations after the cultures were removed from stressful conditions. The cultures used to inoculate the plates were all in log-phase growth. It therefore seems unlikely that the observed effects were the result of any physiological adaptation to the stressful conditions that had the side-effect of decreasing fitness in non-stressful conditions, or that the cells were still physiologically stressed.

Though the basic nature of the phenomenon we are observing seems unquestionably to be an increase in the mutational load of stressed lineages, there are several possible mechanisms underlying it. These can be grouped into three types of explanation. First, it may be the result of a general, non-adaptive deterioration of cellular functions. Alternately, it might be an adaptive response. Within this category, a further subdivision is possible: such a response could be beneficial either for the cell, or for genetic elements which it bears.

Self-regulated release of mutagenic genetic elements.

Transposons and mutator alleles are both potentially "mutagenic" genetic elements which can persist in the genomes of cellular hosts through autoselection (Chao and Cox 1983, Chao et al. 1983). However, the ways in

which they each do so are slightly different. Mutator alleles can persist in asexual populations provided that they occasionally produce favourable mutations. Most of the mutations they produce will be deleterious, and therefore the mean fitness of carriers will be lower than that of those who do not bear the allele. However, the bearers of the rare favourable mutations may take over the population, as the mutator "hitch-hikes" with them.

In asexual populations, selection on transposons will be identical to that on mutator alleles. Since genomes are selected as a unit because of the lack of sex or recombination, the transpositional ability of transposons is irrelevant. However, transposons, unlike mutator alleles, can be maintained in sexual populations. There are two reasons for this.

The first, and most important, is that transposons can replicate independently of the host's replicatory apparatus. By being present in a genome as several copies, a transposon can ensure that it is passed on to virtually all offspring resulting from a cycle of meiosis and sexual fusion. This ability to infect new lineages can more than compensate for selection against hosts bearing them due to the production of deleterious mutations by transposition.

The second reason is that transposons can hitch-hike with the favourable mutations they produce even in sexual populations. Mutator alleles will quickly be separated from the favourable mutations they produce by meiosis. Transposons, because they are physically present at the location where the favourable mutation is produced, are not subject to this.

This brief overview of the autoselection of mutator alleles and transposons reveals an important fact: either can persist in populations despite reducing the mean fitness of their hosts. It is also possible that the

activation of such genetic elements by stress, and the subsequent production of mutations, could be selectively favourable from the perspective of the elements, while still be harmful to the majority of their hosts.

Self-regulated transposons or mutator alleles could "hitch-hike" through populations on the favourable mutations they produce, just as unregulated ones did in the experiments of Chao and colleagues (Chao and Cox 1983, Chao et al. 1983). It might be possible to design a simulation that will test this hypothesis. The main difficulty would be deciding on the appropriate parameter values for the rate of production of favourable mutations in different environments.

It could be that in these experiments previously quiescent transposons underwent a burst of transposition in response to the stress. As outlined above, there is a considerable literature describing the activation of transposons by the exposure of the host to stressful conditions. *Chlamydomonas reinhardtii* is host to several transposons, including *Gulliver* (Ferris 1989) and *TOC1* (Day et al. 1988). In most strains, including those used for these experiments, transposition is rare (Zeyl et al. 1994). However, this certainly does not rule out the possibility of their activation by the stresses applied to the cultures.

This possibility could be easily examined in future experiments. "Before and after" Southern blots of the strains used in the experiment should suffice to establish whether known transposons have moved to new sites. This sort of experiment has the weakness of only being able to detect transposition by known categories of transposons, while there undoubtedly remain transposons in *Chlamydomonas* which are as yet undetected [Bureau, de la

Torre and Bell (unpublished) have recently detected a new transposon in *Chlamydomonas reinhardtii*]. Other sorts of experiments suggest themselves in order to test more generally for transposon activation. In order to detect the activity of retrotransposons, one could measure levels of reverse transcriptase protein or RNA transcripts.

Selection on cells for increased mutation rate.

Another type of explanation is that selection at the level of the cells has favoured an increase in the mutation rate during exposure to stressful conditions. Mechanistically, this could take a similar form to the previous explanation. Transposons could be released from host-encoded regulation. Mutator alleles could be upregulated from trans-repression.

Another mechanism is suggested by some observations made in the assay of stressfulness. On some of the stressful plates, particularly those made from distilled water (ie, starvation stress), it was observed that some colonies were made up of exceptionally small cells. The cells seemed to have undergone several rounds of cell division with little, if any, intervening period of growth. Such a phenomenon has also been observed in our lab in other cultures, when they were transferred from light to dark conditions (R. Kassen, unpublished data). Such a response would increase the number of mutations available in a population for selection, even if the mutation rate per cell division remained constant. In effect, the lineages are increasing the mutation rate per unit time not by increasing the rate per generation, but by decreasing generation time. If one also considers that DNA replication under such conditions is likely to be less accurate due to a lack of precursors or nutrients, one sees a simple way for cultures of asexually dividing unicells to increase their variability in times of stress.

It might seem unlikely that selection at the level of the cells could favour an increased mutation rate since, even in a novel environment, most mutations will be deleterious. However, the populations studied here are reproducing clonally. Therefore, all members of the population are genetically identical with the exception of novel mutations. In this situation, kin selection can be acting. It could be favourable for the cells to increase their mutation rates if it makes it more likely that another cell in their lineage will have an adaptive mutation. As with selection on genetic elements, it should also be possible to model this situation.

Non-adaptive deterioration.

The stresses applied may be so severe, though not lethal, that they lead to a general breakdown of cellular function, and consequent damage to DNA. This could me manifested in several ways. Either DNA replication or repair could be less accurate. Transposons or mutator alleles that are normally repressed could be freed by the breakdown of repressor proteins. Loss of cell cycle control could lead to rapid cell division with insufficient intervening growth. In other words, the proposed mechanisms might be the result of dysfunction, rather than selectively favoured adaptations to coping with stress.

While less exciting to evolutionary biologists, such an explanation does not in any way decrease the importance of the observed phenomenon. Whether adaptive or not, if an increased mutation rate is a response to general stress, then this fact has profound implications for organismal evolution.

Negative tube assay results.

There are three main reasons that I suspect are responsible for the tube assay's lack of confirmation of the plate results. These are selection, a power failure, and an insufficient sample size.

One benefit of the plate assay was its speed. Cultures were stressed, removed from the stress for approximately 8-12 generations, and then assayed. While this length of time permitted me to rule out physiological effects, it was also short enough that selection could not reduce the variation present in the stressed populations to the level of the controls. Most of the novel mutations were deleterious in Bold's medium, as demonstrated by the reduced mean fitness of the stressed populations in the plate assay. Therefore, given enough time, selection would act to eliminate most of the novel types in the stressed population.

Compared to the plate assay, the cultures underwent 15 to 20 additional cell divisions before the beginning of the tube assay. Most of this time was spent on agar plates. Only visible colonies could be chosen to inoculate tubes. Therefore, any colonies that were dividing very slowly would have been excluded from the tube assay.

In addition to this, the small sample size (2 replicates of 20 tubes) made differences in variance very hard to detect. Each replicate had 20 measures of fitness parameters(r or K), but only one of the variability in these parameters. Therefore the tube assay was really only an adequate test for differences in mean fitness, and not for variance.

Besides these considerations of experimental design, one unforeseen circumstance seriously disrupted the course of the tube assay. In September 1996, for a period of 48 hours, the McGill campus, including the Stewart Building in which these experiments were taking place, was deprived of

electricity. As described above, the tubes were only exposed to indirect, natural light during this time. This undoubtedly disrupted the growth of the cultures, particularly as they had only been inoculated into the tubes one or two days before the power failure. The strongly significant shelf position and rack colour effects detected in the analysis are probably the result of this, as such effects have never been detected before in similar assays carried out in our laboratory. As well, many tubes showed unusually long lags (up to two weeks) before their transmittance began to decrease, and some tubes never showed signs of growth. Suggestively, these effects were much more pronounced in the cultures of CC-2938 than in CC-1952; these were the cultures that were inoculated only the day before the power failure.

Upon analysis of the data from the tube assay, it became clear that it had been seriously perturbed by the power failure. Because of this, and the contrastingly significant results of the plate assay, it was deemed important to carry out a repeat of one of the assays, so as to see if the plate results were replicable. However, due to the other problematic aspects of the tube assay, as described above, the decision was taken to repeat the plate assay rather than undertake a new tube assay.

General implications of results.

When a population is in a constant environment to which it is well-adapted, virtually all mutations will be deleterious (Leigh jr. 1973). However, when faced with a change to an unfavourable environment, favourable mutations will be more likely to occur. It is then, when exposed to a novel stress, that mutations are most needed in a population. The results presented here suggest that in such moments the mutation rate of *Chlamydomonas reinhardtii* is in fact increased.

When faced with an environment in which their fitness is low, the algae are responding by increasing their variability, thus increasing the likelihood that a mutant will arise that has increased fitness in the stressful environment. Such a seemingly risky strategy may present the best opportunity for preserving the survival of the clone under adverse conditions.

Other such methods for increasing variability in times of stress are not unknown in these organisms. Indeed the standard laboratory protocol for inducing gametogenesis and sexual fusion in facultatively sexual algae such as *Chlamydomonas* is to place them in media lacking nitrogen. Thus sex is a response to starvation stress in *Chlamydomonas*.

Wills (1984) rightly emphasized that it was not enough to state that mutation rates increased in times of stress in order to conclude that this could increase the rate of evolution in response to the changing environment. What matters is not only the number of mutations produced, but also the kind of mutations. This is one reason why the experiments described herein, which examine the effect of mutation rate on fitness, are superior to experiments that only consider the mutation rate at a single locus. However, we considered here the fitness of lines when returned to a normal, non-stressful environment. What really matters is fitness in the stressful environment itself.

An appealing way to examine this would be to compete stressed and unstressed lines in a stressful medium. If the greater variability in the stressed lines included a few extreme variants with the greatest fitness, then the stressed lines should displace the controls. Such an experiment would be analogous to those of Chao and Cox (1983) and Chao et al. (1983) demonstrating

the selective advantage of mutator- and transposon-bearing lines in chemostats. However, the experiment seems to be problematic in this case, since if the stressful environment under examination is in fact stressful, then the mutation rate of the control lines should also increase as soon as they are exposed to the stress at the beginning of competition.

Despite these problematic aspects of experimental design, the phenomenon described here is interesting and novel, and deserves further attention.

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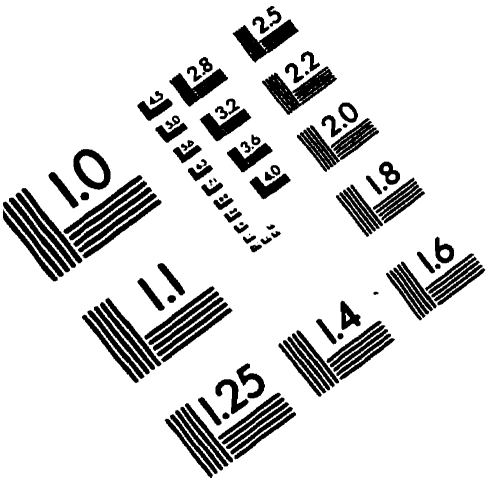
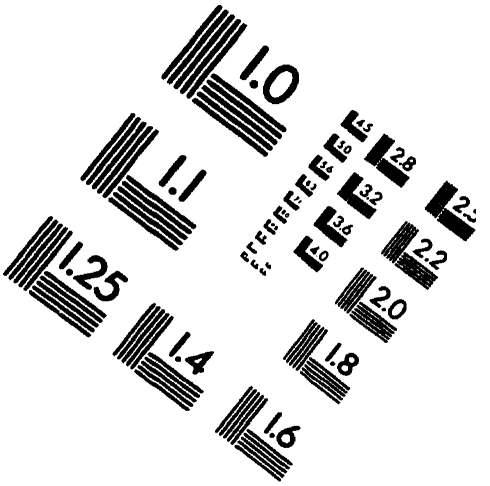
Conclusions

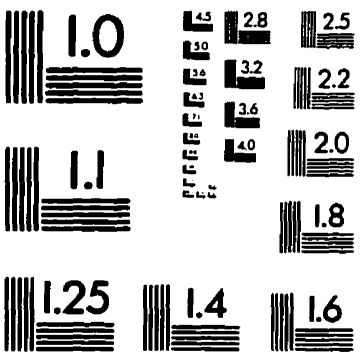
The results presented here give an overview of the mutation rate for fitness in *Chlamydomonas reinhardtii* in different environments. First, the mutational heritability of fitness was estimated in chemostat culture. The estimates obtained for this parameter were within the range of estimates obtained from other organisms and traits. However, they were greater than those obtained from the studies that were most directly comparable. The hypothesis that this discrepancy was due to disruptive selection based on the environmental heterogeneity of the chemostat culturing vessel was investigated, but not supported by the results. Other hypotheses, and experimental tests of them, were suggested.

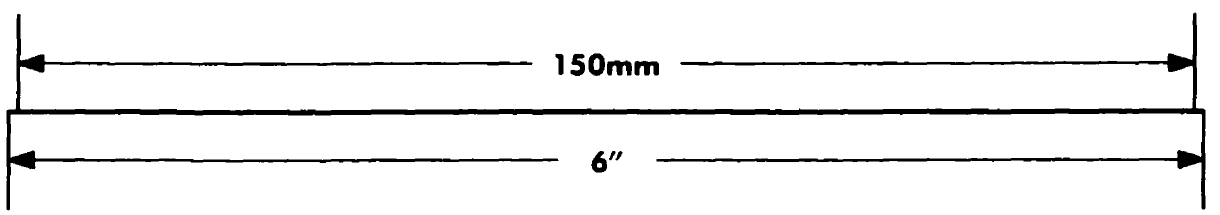
In the second chapter, the examination of mutation rate for fitness in *C. reinhardtii* was extended to stressful environments. It was found that a variety of environmental stresses, not normally though of as mutagenic, resulted in an increase in the variance of, and a decrease in the mean of, fitness. This was interpreted as evidence for an increased mutation rate. Some mechanistic explanations of this phenomenon were suggested, along with possible paths for experimental investigation.

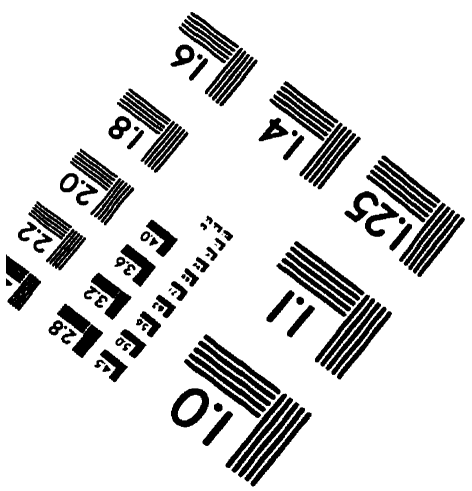
IMAGE EVALUATION TEST TARGET (QA-3)

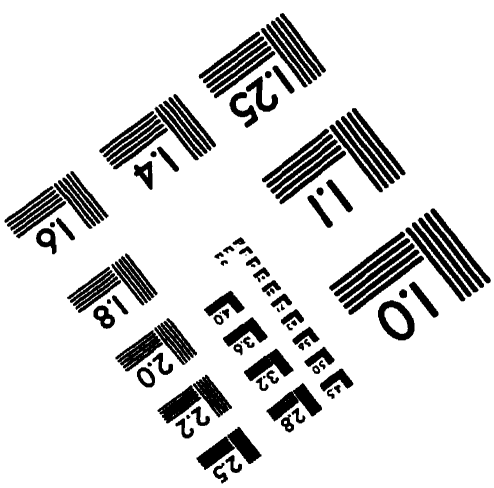












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