

Mild environmental stress elicits mutations affecting fitness in *Chlamydomonas*

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Cultures of *Chlamydomonas* were exposed to a range of relatively mild stresses for a period of 24 h. These stresses comprised high and low temperatures, osmotic stress, low pH, starvation and toxic stress. They were then allowed to recuperate for around ten vegetative generations under near-optimal conditions in unmodified minimal medium. Fitness was then assayed as the rate of division of isolated cells on agar. We found that there was a strong tendency for stressed cultures to have lower mean fitness and greater standardized variance in fitness than the negative controls which had been cultured throughout in unmodified minimal medium. The same tendency was shown, as expected, by positive controls which received mutagenic doses of ultraviolet irradiation. We concluded that the most reasonable interpretation of these observations is that mild stress increases the genomic rate of mutation. This appears to be the first time that this phenomenon has been noticed in eukaryotes. The response might be adaptive because lineages in which higher mutation rates are elicited by stress can be favourably selected through the production of a few mutants which are fortuitously well adapted to the stressful environment. Other interpretations are not excluded, however. Regardless of the mechanism involved, the elevation of mutation rates under stress will affect the rate of evolutionary response to environmental change and also the maintenance of sexuality.

Keywords: fitness; mutation rate; polygenic mutation; environmental stress; Chlamydomonas

1. INTRODUCTION

Mutation rates vary among natural populations (Muller 1928; Demerec 1937; Ives 1950) and genes which cause elevated rates of mutation ('mutator genes') have been identified in many species (Demerec 1937; Neel 1942; Ives 1950; Smith 1992). Transposable elements, which are responsible for a large fraction of spontaneous mutations (Sankaranarayanan 1988), also vary in frequency and activity within and between populations (Anxolabehere *et al.* 1989; Berg & Howe 1989). The rate of mutation is therefore a characteristic which can respond to selection and different rates of mutation will evolve in different circumstances.

When a population becomes well adapted to long-term conditions of growth, mutation is nearly always deleterious. The rate of mutation will evolve towards lower values until further improvements in replication and repair become too costly (Leigh 1973; Drake 1991). Higher rates of mutation are expected to evolve in variable environments (Levins 1967; Ishii et al. 1989) where from time to time populations experience stressful conditions in which novel mutations are selected. This is because the alleles which cause elevated rates of mutation will spread, provided that they are sufficiently frequent (so that one such allele is likely to be responsible for a rare beneficial mutation) and that the population is asexual (so that mutator alleles remain linked to the beneficial mutations they generate). This process has been been demonstrated in chemostat populations of bacteria (Cox & Gibson 1974; Chao & Cox 1983). It drives a transient increase in the average rate of mutation. Selection for an increased mutation rate in novel, stressful environments has been documented in computer simulations of large, asexual populations (Taddei *et al.* 1997) and in chemostat populations of bacteria (Sniegowski *et al.* 1997). Exceptionally high frequencies of mutators are also found among pathogenic bacteria exposed to the rapidly fluctuating conditions created by host immune response (LeClerc *et al.* 1996).

A constitutive elevation of the mutation rate, as seen in the bacterial populations of LeClerc et al. (1996) and Sniegowski et al. (1997), is costly because it generates deleterious mutations once adaptation to the changed conditions of growth has evolved. For this reason, several authors (Echols 1981; Wills 1984; McDonald 1987) have proposed that some form of stress-induced increase in the mutation rate might be favoured by natural selection. Genes that direct an inducible response to stress will generate beneficial mutations following an environmental change without being penalized by the continued generation of deleterious mutations thereafter. This will require systems for detecting and responding to environmental stress. Several detection systems have been described (Hengge-Aronis 1993; Huisman & Kolter 1994) and there is some evidence for the regulation of mutator activity in Escherichia coli (LeClerc et al. 1996). Moreover, previously dormant transposable elements may be activated by physical stresses such as temperature (Strand & McDonald 1985; Ratner et al. 1992) or infection (Pouteau et al. 1994) and the new genetic variation created by transposon insertion might then support a more rapid response to selection (see Mackay 1985).

Most experimental investigations of mutation rates have concerned a single gene or a few genes only (Smith 1992) from whose behaviour the response of the whole genome is inferred (Drake 1991). However, mutation rates vary greatly over loci (DeMarini *et al.* 1989; Smith 1992) and mutations of large effect at single loci are not

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necessarily responsible for adaptive responses to stress. Stress might instead induce a modest increase in the rates of mutation at thousands of loci. This would be very difficult to detect by studying individual loci, but would nevertheless have a substantial effect on fitness. An alternative procedure for exploring this possibility is to measure fitness itself or a rate of increase closely associated with fitness without regard for the particular genetic mechanisms involved. We then expect to observe a heritable decrease in mean fitness, coupled with an increase in the variance in fitness, when a population is exposed to stress. Here we report on experiments which use this approach in experimental populations of the eukaryotic microbe *Chlamydomonas reinhardtii*.

2. METHODS

(a) Stress treatments

Our experimental organism was the unicellular chlorophyte C. reinhardtii, which can readily be propagated vegetatively with a generation time of 4-5 h in optimal conditions. We exposed cultures of two genotypes (CC-1952 (Harris 1989) and CC-2938 (Sack *et al.* 1994)) to a pulse of a non-lethal stress. The stressful environment was in all cases a modification of Bold's minimal medium (Harris 1989), which at room temperature is a nearly optimal environment. Moreover, strains are well adapted to growth on Bold's medium after three years' maintenance in the laboratory on agar slants. The treatments we employed were as follows.

- (i) Negative control. Unmodified minimal medium at room temperature.
- (ii) Low temperature. Unmodified minimal medium at 5 °C.
- (iii) High temperature. Unmodified minimal medium at 40 °C.
- (iv) Low pH. Minimal medium at pH 6.
- $\left(v\right)$ Osmotic stress. Minimal medium with a fivefold greater concentration of sodium chloride.
- $\left(vi\right)$ Starvation. Double-distilled water.
- (vii) Toxic stress. Minimal medium with $5\,\mu g\,l^{-1}$ cyclohexamide.
- (viii) Positive control. Irradiated with $3000 \,\mu J \,cm^{-2}$ ultraviolet

(UV) light in minimal medium.

The strains were subcultured before being stressed to ensure that they possessed as little genetic variation as possible. Single small colonies were isolated, used to inoculate flasks containing unmodified medium and allowed to grow for four days (*ca.* 12 doublings) with aeration and continuous illumination. We then established replicate lines by transferring 1ml of culture (containing *ca.* 0.6×10^6 cells) from these flasks to each of two replicate tubes containing 20 ml of medium representing a given treatment. After 24 h these cultures were allowed to recuperate before any measurements of fitness were made. The cultures were centrifuged and resuspended twice in unmodified medium before being transferred to tubes containing 20 ml of unmodified medium and allowed to grow for three days, representing at least ten vegetative generations.

We established that all these treatments (except (i)) are stressful in the sense of causing an immediate decline in the division rate of cells by making up agar plates with stressful medium, inoculating them directly and scoring growth after 24 h. The positive control, UV radiation, caused a drastic decline to less than one division per day. Temperature stress and toxic stress had similar effects. Osmotic and pH stress were less severe, with cultures maintaining the four to five divisions per day typical of unstressed cultures. Starved cultures also went through around four divisions per day, but in this case large colonies consisted of very small cells which had apparently divided without intervening growth.

(b) Fitness assay

Fitness was estimated by transferring the cultures to a solid minimal medium and counting the number of divisions they went through in 24 h. We spread 25 μ l of culture from each tube onto each of five plates containing Bold's minimal medium solidified with $1.5 \, gl^{-1}$ of agar. These were held for 24 h on shelves illuminated with cool-white fluorescent tubes, then fixed with Lugol's solution (Harris 1989). The number of cells in 100 colonies was counted on each plate so that we could calculate the number of divisions that had taken place; this was usually an integer. The mean and variance of the number of divisions per colony were then estimated for each plate. Two replicate experiments were performed four months apart using the same protocol.

(c) Analysis

The data were analysed using Proc GLM in SAS 6.12 (SAS Institute, Inc. 1996) with strain and replicate experiments as random effects and treatment or stress as a fixed effect. The variance of replicate lines was used as the error term in hypothesis testing.

3. RESULTS

(a) Effect on mean fitness

Stress caused a heritable reduction in mean fitness in most cases (figure 1). The mean of the negative controls for strain CC-2938 in the first experiment was 4.46 divisions day⁻¹ (standard deviation (s.d.) among plates 0.23) and that of the mildly stressed cultures (i.e. excluding the positive control) 4.22 divisions $day^{-1}(s.d.$ 0.39); for strain CC-1952 the mean of the negative controls was 4.65 divisions day^{-1} (s.d. 0.33) and that of the stressed cultures 4.21 divisions day^{-1} (s.d. 0.49). In the second experiment, the mean of the negative controls for CC-2938 was 4.72 divisions $day^{-1}(s.d. 0.17)$ and that of the mildly stressed cultures 4.37 divisions $day^{-1}(s.d. 0.30)$; for strain CC-1952 the mean of the negative controls was 4.65 divisions day^{-1} (s.d. 0.13) and that of the stressed cultures 4.37 divisions day⁻¹ (s.d. 0.15). The overall effect of mild stress was highly significant (table 1, $F_{1.9} = 28.9$ and p < 0.0001). This is consistent with the positive control, which also caused a highly significant reduction in mean fitness ($F_{1,9} = 41.0$ and p < 0.0001). There was no consistent difference between mildly stressed cultures and the positive control ($F_{1,9} = 2.7$ and p = 0.10).

(b) Effect on the variance in fitness

The variance in fitness of the stressed lines often exceeded that of the controls (figure 2). In the first replicate experiment the mean of the negative controls for strain CC-2938 was 0.6475 and that of the stressed cultures (i.e. excluding the positive control) 0.8569; for strain CC-1952 the mean of the negative controls was 0.7179 and that of the stressed cultures 0.8001. In the second replicate experiment, the mean of the negative controls for strain CC-2938 was 0.7505 and that of the stressed cultures 0.9075; for strain CC-1952 the mean of the negative controls was 0.6175 and that

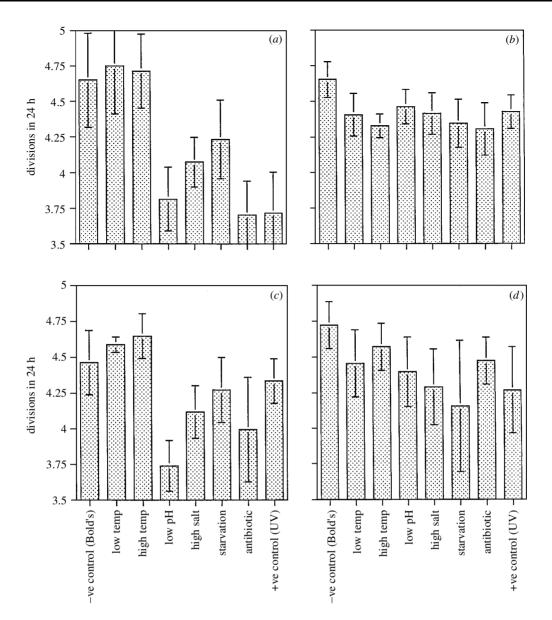


Figure 1. Mean fitness. The *y*-axis indicates the mean number of cell divisions in 24 h of 100 colonies as the average of two lines (five replicate plates per line) per strain-treatment combination. Error bars are ± 1 s.e. of the mean (based on n = 2). (*a*) CC-1952 experiment 1, (*b*) CC-1952 experiment 2, (*c*) CC2938 experiment 1 and (*d*) CC-2938 experiment 2.

of the stressed cultures 0.6970. Although consistent in direction, these effects are not formally significant (table 1, $F_{1,9}=3.4$ and p=0.09), although they would become so if a one-tailed test were allowed. This would be consistent with the significant difference between the negative and positive controls ($F_{1,9}=5.5$ and p=0.02).

Moreover, the use of the variance as a measure of variability is conservative because the variance is generally correlated with the mean and the stressed lines had lower mean fitness. A more appropriate measure might be the standardized variance, the variance divided by the squared mean fitness. This is an index of the opportunity for selection: Fisher's (1930) 'fundamental theorem of natural selection' states that the rate of response to selection is equal to this standardized genetic variance in fitness. In the first experiment all treatments except high and low temperature resulted in significant (testwise p < 0.05 by t-test) increases in the scaled variance for both genotypes. In the second experiment, all treatments of

CC-1952 and all but cyclohexamide of CC-2938 resulted in significant (p < 0.05) increases in the scaled variance. Thus, the standardized variance increased under stress in nine out of 12 lines in the first replicate (p = 0.06, binomial test) and in 12 out of 12 lines in the second replicate (p = 0.0002), the combined probability of these results being p < 0.001. The overall effect as evaluated by analysis of variance is highly significant (table 1, $F_{1,9} = 30.5$ and p < 0.0001).

4. DISCUSSION

(a) Persistent physiological effects

Two independent experiments involving two unrelated genotypes showed that the mean fitness tended to fall and the variance in fitness tended to rise as a consequence of brief exposure to any of a range of mildly stressful conditions. The fitness assays were carried out in log-phase cultures at least ten generations after removal from the stress and we consider it unlikely that our results can be

Table 1. Analysis of variance for the effect of stress

(Stress treatments are compared with the negative control; the positive control (UV irradiation) is omitted. The estimates reported are based on SAS Proc GLM Type III SS, with strain and replicate experiment as the random variables. The error term of the *F*-test for the effect of stress is based on the variance in replicate lines, i.e. line (plate), except where the residual MS is greater. Interactions were not significant at p = 0.1 and have been included in the residual MS. n.s., no significance.)

source	d.f.	mean			variance ($\times 100$)			variance/mean ² ($\times 1000$)		
		MS	F	þ	MS	F	þ	MS	F	þ
strain	1	0.001	0.0	n.s.	3.122	2.4	0.10	3.037	16.6	0.0001
experiment	1	1.568	13.3	0.0002	0.594	0.6	0.50	2.303	12.6	0.0005
stress	1	3.408	28.9	0.0001	7.239	3.4	0.09	5.560	30.5	0.0001
line (plate)	9	0.060	0.5	n.s.	2.152	1.7	0.10	0.162	0.9	n.s.
residual	260	0.118	_		1.290	_		0.183	_	

explained by any persistent physiological effects of the stress. It is true that the longer the period of recuperation, the less likely any lingering physiological effect becomes. Unfortunately, it is also true that, as the recuperation period is extended, evolutionary effects become more important. Any depression of mean fitness caused by mutation will be transient because selection will act to restore the prior level of fitness, removing the mutational variance in the process. Thus, an assay which is not carried out immediately will underestimate the genetic effect of stress. We chose around ten generations as a reasonable compromise; long enough so that any conventional environmental effect would long since have disappeared, but not so long that selection would have entirely effaced any effects on the mean fitness and variance in fitness. Furthermore, any persistent physiological effect would have to be responsible not only for a decline in mean fitness but also for an increase in the variance in fitness. It remains possible that the effects that we observed were epigenetic rather than truly genetic in nature.

(b) Antagonistic pleiotropy

One explanation for our results is that a range of different mutations conferring adaptation to the stress spread through the populations and collectively reached high frequency. These mutations had the side-effect of reducing the fitness in permissive conditions to varying degrees. When the cultures were assayed under permissive conditions, they expressed lower mean fitness (because of antagonistic pleiotropy) and a greater variance in fitness (because many different mutations donating convergent adaptation to stress were expressed differently in the permissive conditions). The cultures were exposed to the stress for only 24 h, representing approximately two to three generations in these conditions, so this rather complicated argument seems implausible. A rough quantitative assessment can be made as follows. The same assay of colony growth on agar that we used in the present experiments yielded an estimate of the rate of increase in genetic variance for fitness of 5×10^{-3} per generation, in units of environmental variance (S. Goho and G. Bell, unpublished data). The mutational heritability after four days' culture before the stress was applied would then be around 5%. The response to selection during stress would then be 0.05D per generation, where D is the selection differential, leading to a total

response over three generations of approximately R = 0.15D. The correlated response, ignoring differences in phenotypic variances, will be rR or 0.15rD, where r is the genetic correlation. To explain a reduction in mean fitness of 15% requires that rD = -1. (For comparison, the average reductions in mean fitness were 5.3 and 7.0% for CC-2938 and 9.4 and 5.9% for CC-1952, respectively; these are minimum estimates because of selection during recuperation.) Taking the mean fitness before stress to be 1, this implies that D = 1 (i.e. in the stressful environment, in each generation the survivors were twice as fit as their parents) and r = -1 (i.e. gain in fitness in the stressful environment is accompanied by an equivalent loss of fitness in the permissive environment). Relaxing the condition on the genetic correlation would require even more intense selection. We conclude that this explanation of our results, while conceivable, requires values for population parameters so extreme as to be unreasonable.

(c) Magnitude of stress-induced elevation of mutation rate

We concluded that stress caused a general elevation of the mutation rates, resulting in the heritable reduction in mean fitness and increase in the standardized variance that we observed. A rough minimum estimate of the magnitude of this effect can be obtained as follows. A population which is initially genetically uniform will accumulate genetic variance for fitness through novel mutations at a rate of around $5 \times 10^{-3} \sigma_{\rm E}^2$ per generation. Approximately 20 generations elapsed between subculturing and assay (ten generations before stress and ten after), so the mutational heritability at assay should have been around 0.1. For strain CC-2938, the average variance of the negative controls in the first experiment was 0.6475, consisting of a component 0.5828 (=0.6475 \times 0.9) of environmental variance and a component 0.0647 of new genetic variance. Thus, new genetic variance was arising under permissive conditions at a rate of around 0.0647/ 20 = 0.0032 per generation. The overall mean of the variances in the six stress treatments (excluding the positive controls) was 0.8557, so the difference between stressed and unstressed cultures was 0.2082. This had arisen over an average of approximately three generations, so the variance arising through new mutation under stress is around $0.0694/0.5828 = 0.1191\sigma_E^2$ per generation. The increase in mutational variance for fitness was therefore

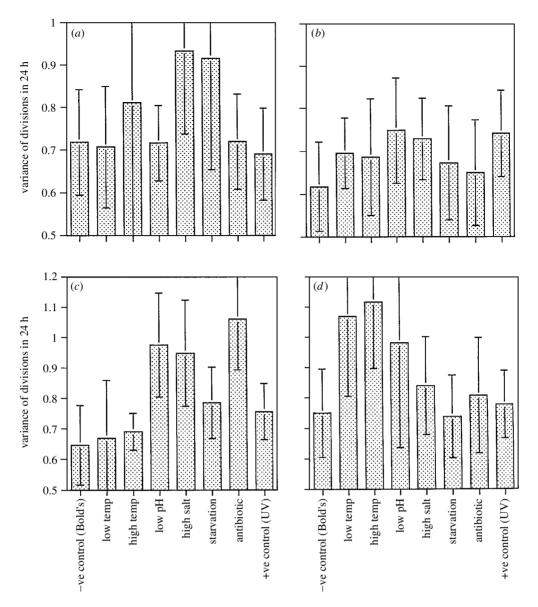


Figure 2. Variance in fitness. The y-axis indicates the variance in the number of cell divisions in 24 h of 100 colonies as the average of two lines (five replicate plates per line) per strain-treatment combination. Error bars are ± 1 s.e. of the mean (based on n = 2). (a) CC-1952 experiment 1, (b) CC-1952 experiment 2, (c) CC2938 experiment 1 and (d) CC-2938 experiment 2.

elevated under stress by a factor of 0.1191/0.0032 = 37.2. The corresponding estimate for the second experiment was 22.9. The estimates for strain CC-1952 in the two experiments were 11.8 and 15.5. These estimates are minimal, in particular because they do not allow for selection against deleterious mutations during the recuperation period, nor are they corrected for the overall decrease in mean fitness. We concluded that the relatively mild stresses that we used increased the rate at which new mutational variance for fitness is generated by at least an order of magnitude. It seems reasonable to argue that the underlying mutation rates will be increased by the same factor.

It has been known for some time that mutation rates are elevated in bacterial populations grown under stressful conditions. The clearest examples include lactose use in *Escherichia coli* (Shapiro 1984; Cairns *et al.* 1988; Hall 1988) and phenol use in *Pseudomonas* (Kasak *et al.* 1997); there is a brief recent review of the phenomenon by Cairns (1998). Similar phenomena have been reported in the eukaryote *Saccharomyces* (Hall 1992; Steele & Jinks-

Robertson 1992). In these cases, the populations were stressed by starvation caused by their inability to exploit the only carbon source available and the mutation rates were elevated at loci which restore this ability. Starvation was also effective in increasing the variance in fitness in our experiments. Following the argument in the previous paragraph, culture for 24 h in distilled water increased the rate at which mutational variance for fitness was generated by factors of 24.8 (experiment 1) in strain CC-2938 and by factors of 28.3 (experiment 1) and 7.3 (experiment 2) in CC-1952; in the second experiment, unstressed and starved cultures of CC-2938 had almost identical variances (0.7505 versus 0.7381). Our experiments therefore suggest that the locus-specific events observed in bacteria can be extrapolated to genomic mutation rates in eukaryotic microbes.

(d) Adaptive and non-adaptive interpretations

Three interpretations of a stress-induced elevation of the mutation rate might be advanced: it might be non-adaptive, it might be an adaptive response by replicators which cause mutation, but not for the organism as a whole or it might be adaptive at the level of the whole organism.

An elevated mutation rate need not be adaptive. An obvious non-adaptive explanation is that the stresses we used caused some breakdown of cellular processes, resulting in physical damage to the genome. It is noteworthy that their effect was comparable to that of UV radiation, a known mutagen, and was apparently not repaired effectively.

The second possibility is that an increased mutation rate might be selected through its effects on the genetic elements causing mutations. Transposons might escape from host-encoded regulation or mutator alleles might be downregulated from transrepression. Elements which behave like this in stressful conditions will be linked to the beneficial mutations they cause and will thereby tend to increase in frequency. In this case, inducible mutation is adaptive at the level of particular genetic elements.

The third possibility is that lineages of cells which are able to increase mutation when stressed will usually be the ancestors of the types selected as a consequence of the stress. Alternatively, if the increased mutation rate is the direct effect of environmental stress, lineages which fail to increase the level of repair might be selected (S. Hekimi, personal communication). In either case, inducible mutation or the absence of inducible repair is adaptive at the level of whole organisms. In variable environments, organisms are stressed from time to time by the advent of conditions to which they are not well adapted. A lineage in which the mutation rate is elevated by stress will be likely to be successful because it is more likely to give rise to variants which by chance happen to flourish in the new conditions of growth. The number of deleterious mutations produced is irrelevant if the population eventually becomes fixed for one or a few superior genotypes. This process will be most effective in asexual populations, where the elements controlling mutation rates remain linked to the few beneficial mutations they create. However, it might also occur in sexual populations if adaptation requires mutations of small effect at many loci, although the advantage of generating high mutation rates will diminish through subsequent generations of out-crossing. Stressinduced mutation will be selected most effectively, of course, if it is restricted to the loci responsible for restoring function in the stressful environment; our experiments provide no evidence for or against a hypothesis of 'adaptive mutation' (Cairns et al. 1988; Hall 1988).

(e) Mutation and the maintenance of sex

At least one other behaviour of *Chlaymdomonas* which has the effect of increasing variability is elicited by stress: vegetative cells enter the sexual cycle as gametes when starved of nitrogen (Harris 1989). This is undoubtedly an adaptive response. There are two leading theories of how sex is maintained in natural populations, both based on the fact that mutations arising independently in different lineages can be recombined into the same lineage in outcrossing populations. If these mutations are deleterious, sex can generate a process of mutation clearance which preserves adaptedness in a stable environment (Kondrashov 1984). If they are beneficial, sex makes possible a process of mutation assembly which accelerates adaptedness to a changed environment (Weismann 1889; see the reviews in Michod & Levin (1989)). In many organisms, sex is an occasional event in a life cycle which consists of vegetative reproduction most of the time. It is usually induced as the alternative to continued vegetative propagation by the stress associated with crowding or starvation (Bell 1982). Our experiments have shown that this stress is itself mutagenic. The interpretation of sexuality is then coupled with the interpretation of mutation. If stress merely causes damage in a non-adaptive fashion, then sex will act as a mechanism to clear the population of deleterious mutations. This interpretation envisages that favourable conditions of growth will eventually be restored and, therefore, applies to transient stress, such as the cold and hunger experienced at the end of the growing season. On the other hand, an elevated rate of mutation may be an adaptive response, increasing the supply of beneficial mutations. Sex will then act as a mechanism for assembling multilocus genotypes when stress heralds permanently altered conditions of growth, such as the appearance of new strains of pathogens. The interpretation of mutation and sex as variance-generating devices therefore hinges on the frequency and duration of stressful episodes. Natural populations will be exposed to both short- and long-term stress and it is entirely possible that both interpretations are correct.

(f) The genomic mutation rate

In either case, if stress-induced mutation turns out to be a general phenomenon, it will throw some doubt on current estimates of mutation rates. Most estimates of the overall mutation rate for loci affecting fitness lie in the region of 0.1-1 per genome per generation or somewhat less (Mukai 1964; Mukai et al. 1972; Charlesworth et al. 1990; Johnston & Schoen 1995; Deng & Lynch 1997; Drake et al. 1998). The rate at which new mutational variance for fitness appears is found to be around $10^{-3}\sigma_{\rm F}^2$ per generation or somewhat less in most studies (Lynch 1988; Houle et al. 1996). In both cases, mutation accumulation and measurement are usually carried out in nearly optimal conditions. A general process of stress-induced mutation would have important implications for evolutionary biology, regardless of the particular genetic and evolutionary mechanisms involved. It will no longer be possible to identify a single characteristic mutation rate, but in most conditions the rates will be higher, perhaps by approximately an order of magnitude, than those currently accepted. This implies that the rate of response to selection following an environmental perturbation is a function of the perturbation itself and will be more rapid than would be anticipated from measurements of mutation rates in non-stressful conditions. We emphasize that this does not necessarily involve any sort of directed mutation, but only a general elevation of mutation rates through conventional processes.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.