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MUTATIONAL MELTDOWN IN LABORATORY YEAST POPULATIONS

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Abstract.—In small or repeatedly bottlenecked populations, mutations are expected to accumulate by genetic drift, causing fitness declines. In mutational meltdown models, such fitness declines further reduce population size, thus accelerating additional mutation accumulation and leading to extinction. Because the rate of mutation accumulation is determined partly by the mutation rate, the risk and rate of meltdown are predicted to increase with increasing mutation rate. We established 12 replicate populations of Saccharomyces cerevisiae from each of two isogenic strains whose genomewide mutation rates differ by approximately two orders of magnitude. Each population was transferred daily by a fixed dilution that resulted in an effective population size near 250. Fitness declines that reduce growth rates were expected to reduce the numbers of cells transferred after dilution, thus reducing population size and leading to mutational meltdown. Through 175 daily transfers and approximately 2900 generations, two extinctions occurred, both in populations with elevated mutation rates. For one of these populations there is direct evidence that extinction resulted from mutational meltdown: Extinction immediately followed a major fitness decline, and it recurred consistently in replicate populations reestablished from a sample frozen after this fitness decline, but not in populations founded from a predecline sample. Wild-type populations showed no trend to decrease in size and, on average, they increased in fitness.

Key words.—Extinction, fitness, mutational meltdown, Saccharomyces cerevisiae.

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A well-established conclusion drawn from many theoretical studies is that slightly deleterious mutations accumulate in small populations and cause their fitness to decline. Small population size hampers selection and increases the role of genetic drift in determining allele frequencies and rates. Some of the deleterious alleles constantly being supplied by mutation are therefore fixed, reducing population mean fitness. Mutation accumulation (MA) was first recognized as a threat to sexual populations, because in the absence of recombination (and if reverse mutation is rare enough to be ignored), offspring carry all of the mutations present in their parent, as well as any newly arisen mutations (Fisher 1930; Muller 1964). Models therefore predict the eventual extinction of small asexual populations. Moreover, extinction may be accelerated if, as mutations accumulate and fitness declines, population size also declines (Lynch and Gabriel 1990; Gabriel et al. 1993). This, in turn, increases the fixation probabilities of future mutations, resulting in a positive-feedback model of MA known as the mutational meltdown. More recent studies have shown that small populations are at risk even if they are sexual, because once a deleterious mutation has been fixed it is unaffected by recombination (Lynch et al. 1995b).

These models of population extinction due to deleterious mutations have serious implications for conservation biology. The effective population sizes (N_e) of endangered species often decline below 100 before conservation efforts begin, while models suggest that the minimal N_e for long-term population viability is “in the neighborhood of a few hundred individuals” (Schultz and Lynch 1997).

Experimental tests of models such as the mutational meltdown are clearly required, but have not been reported. Experimental populations of model organisms have been propagated by the serial transfer of one or a few individuals (Kitada and Lynch 1996; Keightley and Caballero 1997; Shabalina et al. 1997; Vassilieva and Lynch 1999; Zeyl and de Visser 2001). The goal of these studies was to estimate rates and average fitness effects of spontaneous mutation. Populations of Drosophila melanogaster have been maintained at constant sizes of 200 (Shabalina et al. 1997) and 25–500 (Gilligan et al. 1997), with mutation load increasing in the former study but not in the latter. The results of such experiments are certainly relevant to meltdown models, because the rate and probability of extinction are determined in part by the rate at which slightly deleterious mutations increase mutational loads (Bell 1988; Lande 1995; Schultz and Lynch 1997). However, these experiments do not directly address the risk of extinction, because they involve the transfer of a predetermined number of individuals. Mutations accumulate and fitness declines, but population sizes remain constant.

These experiments and other lines of evidence from both laboratory and natural populations (Bell 1988; Lynch et al. 1999) have confirmed that mutations accumulate due to drift. However, there are several reasons why the threat to the long-term viability of small populations may not be as great as indicated by the meltdown models. First, for many organisms the per genome–per generation mutation rate (U) may be considerably lower than the value near one that has often been assumed (e.g., Lynch et al. 1995a,b). Recent estimates of U for organisms as diverse as D. melanogaster, (Fernández and López-Fanjul 1996; Fry et al. 1999), Caenorhabditis elegans (Keightley and Caballero 1997; Vassilieva and Lynch 1999), and Saccharomyces cerevisiae (Zeyl and de Visser 2001) are all orders of magnitude less than one (see also García-Dorado et al. 1999). Many of the endangered species for which there is the greatest public concern are vertebrates.
and flowering plants, and recent evidence still implies that their mutation rates are near or greater than one (Johnston and Schoen 1995; Eyre-Walker and Keightley 1999). Nevertheless, the generality of such high mutation rates is questionable.

Second, whatever the overall rate of mutation, the frequency of slightly deleterious mutations may be low. The mutations that pose the greatest threat to small populations are those with a fitness cost \( s \) near \( 1/2N_e \), because less harmful mutations accumulate relatively easily but have little effect on fitness unless very numerous. More strongly deleterious mutations are fairly efficiently removed by selection, even in small populations (Gabriel et al. 1993). Apart from the low overall mutation rates, an unexpected result of some MA experiments has been the high average fitness cost per mutation (e.g., \( s = 0.21 \) in Keightley and Caballero 1997) and the apparent scarcity of mutations that reduce fitness by 1–2%, previously thought to be the most abundant class (Simmons and Cow 1977).

Third, meltdown models assume not only that reverse mutation is rare enough to be ignored, but also that new mutations do not compensate for any of the deleterious effects of previously fixed mutations. Using geometric models of the phenotypic effects of mutations, Poon and Otto (2000) found that allowing compensatory mutations makes meltdowns very unlikely. Although it seems safe to disregard reverse mutations in small populations, the true role of compensatory mutations is unknown.

Finally, the most pessimistic of the meltdown models assume that the regulation of population sizes is density independent, so that MA reduces not just fitness relative to the ancestral value, but absolute fitness. The resulting decline in population growth rates is what reduces population size and accelerates further MA and fitness decline. Although extinction does eventually occur in models with density dependence or “soft selection,” the expected times to extinction are greatly increased, particularly if mutations with fitness costs in the critical range are rare, as discussed above (Gabriel et al. 1993). Therefore, experimental observations of rates and times of extinction would be informative.

Microbes are ideal test subjects because small populations can be propagated through thousands of generations within several months or a few years. The long-term viability of microbial populations was a subject of much interest in the early 1900s, when the potential immortality of a lineage of microbial populations was a subject of much interest in the early 20th century. The long-term viability of microbial populations was a subject of much interest in the early 20th century. A more ecologically relevant setting by culturing replicate assexual populations in small volumes by daily serial transfer, diluting them by a fixed factor at each transfer. The culture and dilution regimes were calibrated to allow each population to approach stationary phase after 24 h of growth, and then to bottleneck each population to approximately 30 cells at the daily transfer. Any fitness declines that reduced population growth rates would reduce the number of cells transferred each day, accelerating further MA and incorporating the positive feedback element of the meltdown models. Each population therefore underwent a daily feast-and-famine cycle, but over the course of the experiment this cyclical environment was constant except for any effect of declining population size on nutrient availability.

Second, we genetically manipulated mutation rates. Experimental populations were founded by one of two isogenic strains, one with wild-type mutation rates and a mutator whose mutation rates were elevated by deletion of \( MSH2 \), a DNA mismatch repair gene. Estimated genomewide rates of mutation affecting fitness (Zeyl and de Visser 2001) are \( 1 \times 10^{-4} \) in the wild-type strain and approximately 200 times higher in the mutator strain. The mutator strain therefore has a mutation rate comparable to those estimated recently for multicellular organisms such as \( C. elegans \) (Keightley and Caballero 1997; Vassilieva and Lynch 1999) and \( D. melanogaster \) (García-Dorado et al. 1999).

We maintained wild-type and mutator populations at small effective population sizes for close to 3000 generations and monitored population sizes and mean fitness to test the prediction that fitness decline and extinction should occur first and most often in the mutators.

**Materials and Methods**

**Founding Strains and Maintenance of Populations**

Our wild-type ancestor was a diploid, \( leu2 \Delta \) derivative of yeast strain Y55. The mutator was an otherwise identical \( ura3 leu2 \Delta msh2::URA3 \) genotype (Chambers et al. 1996). \( MSH2 \) encodes one of the proteins involved in DNA mismatch repair, and its deletion greatly increases the rates of single-nucleotide substitutions and of one or two nucleotide deletions (Marsischky et al. 1996). The \( MSH2 \) and \( msh2 \) strains were the founders of 12 replicate wild-type and 12 replicate mutator populations, respectively.

To begin the experiment, 0.2 mL cultures of the rich permissive medium YPD (2% yeast extract, 2% peptone, 1% dextrose) in \( 13 \times 100 \) mm borosilicate tubes with metal caps were inoculated with 20-μL aliquots from frozen cultures of each founding strain. After 24 h of growth at 30°C, a 100-μL sample of each founder was diluted by passage through two \( 18 \times 150 \) mm borosilicate tubes containing 9.9 mL sterile water, and 20-μL samples were used to inoculate 0.2 mL of YPD with the glucose concentration reduced from the usual \( 2\% \) to \( 0.2\% \) (0.2% YPD). After another 24 h of growth, replicate populations were established from each ancestor by diluting a 100-μL sample of each strain as above and using 20-μL samples of the final dilution to inoculate 12 tubes of 0.2 mL 0.2% YPD.

Populations were maintained in 0.2 mL of 0.2% YPD at 30°C and transferred at 24-h intervals (± 30 min) to fresh media using the \( 10^2 \)-fold dilution procedure described above.
For the ancestral strains, this regime resulted in the daily transfer of approximately 30 cells (means and standard errors: wild-type 31.5 ± 5.4; mutator 31.7 ± 7.4) and a final population size after 24 h of growth of approximately $3 \times 10^6$ cells. The effective population size, estimated as the harmonic mean of population sizes throughout the 24-h growth cycle, was 255, and the number of generations per day, calculated as the base-2 logarithm of the $10^5$-fold daily dilution, was 16.6.

Throughout the experiment, population sizes at the daily bottleneck were estimated by taking 20-μL samples from the second dilution tube, equivalent to those taken for the daily transfer of the population, and spreading them onto YPD agar. The numbers of colonies visible after two days were used as estimates of population sizes at the bottleneck. These estimates were performed twice a week, at alternating three- and four-day intervals. Any population that produced no colonies on three consecutive plateings and no visible pellet of cells in the culture tube was considered to have gone extinct on the day of the first plating when no colonies appeared. Every seven days, the fraction of each population left behind after the daily transfer was frozen in an equal volume of 30% glycerol. The experiment was run for 175 days, or approximately 2900 generations.

**Fitness Assays**

From the population samples that were frozen on days 57, 113, and 162 (generations 946, 1876, and 2689, respectively) individual genotypes were isolated and their fitness was estimated by competition against a genetically marked version of their ancestor. Six of the 12 wild-type populations and six mutator populations were assayed. The populations to be assayed were chosen arbitrarily as replicates 1–6, but with the knowledge that this subset included a mutator population that went extinct on day 119. To isolate individual genotypes, a 20-μL aliquot of each frozen population was grown overnight in 0.2 mL YPD, diluted, and plated on YPD agar. From each population, five genotypes were randomly chosen as the five colonies that grew nearest to a mark made on the underside of the plate when the cells were spread. Each colony was resuspended in 0.2 mL YPD and re-acclimated to the experimental environment, as described above for the initial establishment of experimental populations. This acclimation procedure was also applied to a strain carrying a neutral genetic marker encoding resistance to the antibiotic geneticin (G418R). The marked strain was a mutator ancestor transformed with the EcoRI-BamHI fragment of plasmid KanMX4, carrying the bacterial kanR gene encoding aminoglycoside phosphotransferase (Wach et al. 1994). This G418R strain was used as a common competitor against all the genotypes being assayed.

Competitions were begun by diluting 24-h cultures of the G418R strain and of each randomly chosen genotype, as described above, to the experimental populations, and mixing them. For each genotype, five replicate tubes containing 0.2 mL of 0.2% YPD were inoculated with 10 μL of that genotype and 10 μL of the G418R competitor. A 5-μL sample of each mixture was added to 50 μL sterile water on YPD agar, spread, and grown for two days. These plates were then replica-plated to YPD agar + G418. The initial frequencies of each competitor were estimated from colony counts of each plate. The competitions ran for two days. After 24 h, each replicate competition was transferred as described above for the experiment, except that the dilution factor was reduced to $10^4$. After a second 24 h, samples of each competition were plated on YPD agar and replica-plated to YPD agar + G418 to estimate the final frequencies of each genotype. The fitness of each genotype was calculated as the ratio of the number of cell divisions of the MA line to that of the marked ancestor (Lenski et al. 1991).

The competitions were performed in three blocks, with the day from which the genotypes were sampled (days 57, 113, and 162) being the block. Parallel competitions between the G418R common competitor and both ancestral strains were included with each block. For the two ancestors, no significant effects of ancestor genotype (ANOVA, $P = 0.33$), block ($P = 0.49$), or block × ancestor interaction ($P = 0.82$) were detected.

We reduced the dilution factor from $10^5$ for the experiment to $10^2$ for the competitions because the experiment was designed to allow genotype frequencies to be determined largely by genetic drift, whereas the competitions were designed to allow genotype frequencies to be controlled by selection. To examine the effect of these procedures, we performed test competitions using the G418R common competitor and a genotype derived from its mutator ancestor in a separate mutation-accumulation experiment (Zeyl and de Visser 2001). The derived genotype was known to have a competitive disadvantage of about 2% in 10-mL YPD cultures. Twenty replicate competitions using each dilution factor were run for 10 days. The final genotype frequencies did not differ between competitions performed with the two dilution factors ($P = 0.33$ in a two-tailed heteroscedastic $t$-test), but were significantly more variable in competitions that were transferred using a dilution factor of $10^2$ ($P = 0.0016$ in a two-tailed $F$-ratio). These results support our assumptions that genetic drift was a more important factor in the meltdown experiment than in the fitness assays we performed and that altering the dilution factor for the competitions did not alter the selective environment.

**Recapitulating a Meltdown**

An experimental population could go extinct because of the fitness decline predicted by mutational meltdown models, or for less interesting reasons unrelated to MA (e.g., a defective aliquot of culture medium or the chance transfer of a dilution volume containing no cells). A major advantage of our experimental system was the opportunity to replay an extinction by retrieving from the freezer the last sample taken before a population went extinct. Replicate populations were reestablished and propagated from that sample to determine whether the extinction was reproducible. For one of the mutator populations, the competitions described above indicated a substantial fitness decline shortly before its extinction after 119 daily transfers (see Results). We used frozen samples taken from this population on day 99 (generation 1643, before the fitness decline) and day 116 (generation 1876, after the fitness decline and immediately prior to extinction) to estab-
cultures, using the same dilution regime, medium, and culture conditions described above for the main experiment. After 24 h of growth, the population size of each culture was estimated by plating diluted samples on YPD agar.

**RESULTS**

**Population Sizes**

During the 175 daily transfers that were performed, two populations went extinct. Both were mutators, but this is not a significant difference between wild-type and mutator populations ($G = 2.59, P = 0.14$ in a likelihood-ratio test). The extinctions occurred on days 119 and 155. The former population, replicate 2 of the mutator populations, was subjected to further study (see below) and is henceforth referred to as population M2. Over the first half of the experiment, mean population sizes (Fig. 1) were similar for the mutators and wild types, but appeared smaller for the mutators thereafter. To test this, we performed separate mixed-model ANOVAs on population sizes from days 1–116 and 119–175, dividing the experiment into two periods defined by the day of the first extinction. As suggested by Figure 1, there was no significant difference between the sizes of mutator and wild-type populations over days 1–116, but mutator populations were significantly smaller over days 119–175 (Table 1). In both periods, there is significant variation among replicate populations founded by the same ancestor.

Trends in population size were also analyzed by linear regression of the twice-weekly estimates of population size at the daily bottleneck on transfer number (day of the experiment). First, regression slopes were estimated for each of the replicate wild-type and mutator populations, and the 12 wild-type slopes were compared with the 12 mutator

**Fitness and Growth Rates**

We compared the growth rates in pure culture of the mutator ancestor and three genotypes that had been isolated from different mutator populations at day 113 for fitness estimates. Each genotype was acclimated to the experimental environment as described above and used to inoculate 10 replicate populations each. If the initial extinction was caused by fitness decline, we expected extinction to recur among populations restarted from day 116, but not among those restarted from day 99.

**Table 1.** ANOVA on population sizes during the mutational meltdown experiment. The experiment was divided into two periods based on the time of the first population extinction (day 119) and on the prediction that an effect of ancestral genotype (wild-type or mutator) on population size would be observed only after enough bottlenecks had occurred to allow substantial mutation accumulation. Ancestor (wild-type or mutator) and day number were fixed factors, and replicate population was a random factor nested within ancestor.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Expected MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days 1–116</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ancestor</td>
<td>1</td>
<td>765.84</td>
<td>765.84</td>
<td>Q(anc, anc × day) + 31.09s_pop + 31.09s_anc × day + 1.96s_pop × day + 1.96s_anc × day</td>
<td>0.97</td>
<td>0.35</td>
</tr>
<tr>
<td>Day</td>
<td>31</td>
<td>52010.96</td>
<td>1677.77</td>
<td>Q(day, anc × day) + 1.96s_pop × day + 1.96s_anc × day</td>
<td>19.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Population (ancestor)</td>
<td>11</td>
<td>8798.15</td>
<td>799.83</td>
<td>31.36s_pop + 31.36s_anc + 8.07</td>
<td>8.079</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ancestor × day</td>
<td>31</td>
<td>4003.57</td>
<td>129.15</td>
<td>Q(anc × day) + s_anc</td>
<td>3.30</td>
<td>0.13</td>
</tr>
<tr>
<td>Population (ancestor)</td>
<td>341</td>
<td>29670.11</td>
<td>87.01</td>
<td>1.98s_pop × day + s_anc</td>
<td>0.879</td>
<td>0.882</td>
</tr>
<tr>
<td>Error</td>
<td>334</td>
<td>33067.09</td>
<td>99.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days 119–175</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ancestor</td>
<td>1</td>
<td>2335.36</td>
<td>2335.36</td>
<td>Q(anc, anc × day) + 15.84</td>
<td>5.46</td>
<td>0.04</td>
</tr>
<tr>
<td>Day</td>
<td>16</td>
<td>9252.96</td>
<td>578.31</td>
<td>Q(day, anc × day) + 1.76</td>
<td>11.23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Population (ancestor)</td>
<td>10</td>
<td>4337.40</td>
<td>433.74</td>
<td>16.10s_pop + 16.10s_anc</td>
<td>7.57</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ancestor × day</td>
<td>16</td>
<td>1117.48</td>
<td>69.84</td>
<td>Q(anc × day) + s_anc</td>
<td>1.22</td>
<td>0.26</td>
</tr>
<tr>
<td>Population (ancestor) × day</td>
<td>175</td>
<td>8947.11</td>
<td>51.13</td>
<td>1.86s_pop × day + s_anc</td>
<td>0.89</td>
<td>0.77</td>
</tr>
<tr>
<td>Error</td>
<td>151</td>
<td>8657.05</td>
<td>57.33</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 2. Regression of population size at bottleneck on daily transfer number. For this analysis the experiment was divided into two time periods defined by the time of the first extinction, which occurred on day 116. Within each time period, separate regressions were performed for the 12 populations founded by the wild-type ancestor and for those founded by the mutator.

<table>
<thead>
<tr>
<th>Populations</th>
<th>Period (days)</th>
<th>Slope (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1–116</td>
<td>0.021 (–0.015, 0.058)</td>
<td>0.250</td>
</tr>
<tr>
<td>Mutator</td>
<td>1–116</td>
<td>–0.027 (–0.066, 0.012)</td>
<td>0.169</td>
</tr>
<tr>
<td>Wild-type</td>
<td>119–175</td>
<td>–0.025 (–0.103, 0.053)</td>
<td>0.523</td>
</tr>
<tr>
<td>Mutator</td>
<td>119–175</td>
<td>–0.123 (–0.211, –0.036)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Slopes. Although few of these individual slopes were significantly different from zero (three of 24, all mutators), there was a highly significant difference between the estimated slopes of wild-type and mutator populations ($P = 0.000514$ in a two-tailed t-test). For a final analysis, we pooled the replicates of each population type (wild-type and mutator) and divided the experiment into the same two time periods as those used for ANOVA. A significantly negative slope was obtained for mutators during the second stage of the experiment; none of the other three regressions was significant (Table 2). Taken together, the regression and ANOVA results indicate that the mutator populations declined in size toward the end of the experiment and that there was no significant change over time in the wild-type populations.

An unexpected observation from early experiments with serially bottlenecked ciliate cultures is that their cell division rates were cyclical, rising and falling with a fairly regular period (Bell 1988). There is similar evidence of cyclical behavior in our populations. Cycles can be illustrated with an autocorrelegram showing the correlations between pairs of population size estimates separated by all the possible intervals of time during the experiment. Peaks in the correlations occur at intervals that are multiples of the period on which the population sizes cycle, whereas troughs represent out-of-phase pairs of population sizes. Figure 2 shows autocorrelegrams for the mean sizes of the replicate wild-type and mutator populations. For much of the duration of the experiment, the mean sizes of both wild-type and mutator populations appear to cycle on a period of about one week (two sample intervals), although from days 60–90 the cycle is less apparent and may have lengthened toward the end of the experiment. As implied by this result, the means of the twice-weekly estimates of wild-type and mutator population sizes (Fig. 1) are significantly correlated ($r = 0.632, P < 0.001$).

Fitness Changes

Five replicate fitness assays were performed on each of five genotypes, sampled on days 57, 113, and 162 from six of the wild-type and six mutator populations. The wild-type populations showed a tendency toward increased fitness: By day 162, the mean fitness of the five sampled genotypes had increased significantly in four populations (as indicated by 95% confidence intervals calculated from the five means) and had not significantly changed in the other two (Fig. 3). Among the six mutator populations assayed, there was no overall trend: Fitness showed no change in two populations, increased in two populations, and decreased in two populations (including M2). ANOVA indicates that neither day of genotype sampling nor ancestor (wild-type or mutator) had a significant fitness effect (Table 3). However, there is significant fitness variation among replicate populations and among sampled genotypes, and there are significant day × ancestor and day × population interactions. Thus, wild-type and mutator populations show different fitness trends over time, and replicate populations founded by each ancestor diverge in fitness.

Further Study of an Extinction

Population M2 went extinct on day 119, and our estimate of its mean fitness on day 113 was the lowest of the populations tested (Fig. 3). Estimates of the size of this population had been made twice weekly as described above, by counting colonies on plates spread with a diluted sample equivalent to the one used for the daily bottleneck and transfer. These counts were averaged across replicates to give the results shown in Figure 1. Considered separately, the counts for population M2 show a great deal of fluctuation, with several particularly small bottlenecks shortly before its extinction (Fig. 4A). To obtain better resolution of the fitness changes in M2 during its final two weeks, we randomly sampled collections of five genotypes from additional samples that had been frozen over this period and performed competitive fitness assays as described above. The results indicate that the fitness decline was not steady (Fig. 4B). Although the overall trend was toward declining fitness, mean fitness fluctuated and genotypes significantly fitter than the ancestor (as indicated by t-tests, data not shown) were recovered from samples frozen as late as day 99. Mean fitness estimates were not
Fig. 3. Fitness changes in (A) six of the wild-type populations and (B) six of the mutator populations. Each point represents the mean of five genotype means, estimated from five replicate competitions of each genotype. The mutator population denoted by ▼ is M2, the population that went extinct on day 119.

significantly correlated with concurrent estimates of population size ($r = 0.585, P = 0.127$).

If extinction occurred because of fitness decline and not because of demographic stochasticity unrelated to mutation accumulation, then it should be recapitulated by replicate populations reestablished from samples that were frozen after the fateful mutation or mutations were fixed. The fitness estimates shown in Figure 4B imply that the fatal fitness decline occurred between days 99 and 116. To test this inference, we ran a repeat of the experiment using the day 99 and day 116 samples as the ancestors for 12 replicate populations each. After 38 daily transfers, five of the 12 populations founded from the day 116 sample had gone extinct, whereas none of the 12 founded by the day 99 sample went extinct. A likelihood-ratio test indicated that this difference is highly significant ($G = 7.24, P < 0.01$). Excluding populations that had gone extinct, population sizes were also smaller for the day 116 descendants (Fig. 5; Table 4). The extinction of M2 therefore appears to have been a bona fide mutational meltdown.

Effects of Mutation on Fitness and Growth

Meltdowns are predicted to occur as accumulating mutations reduce the growth rates of populations and their constituent genotypes. However, it is possible that the relative fitness of mutants in competition with their ancestor could be reduced, as shown above, without significantly affecting the growth rate of an entire population of such mutants. We measured population size after 24 h for pure cultures of the mutator ancestor and three genotypes isolated from separate day 113 mutator populations. Because these genotypes were acclimated and grown under the same conditions used to propagate the experimental populations, population size after 24 h is the demographic variable that is most directly relevant to their risk of meltdown. The three mutated genotypes were chosen from among those that had been randomly isolated for estimates of competitive fitness. There was no correspondence between their competitive fitness and their growth rates over 24 h of pure culture (Table 5): One genotype differed significantly from the ancestor in fitness but not growth rate, a second had higher competitive fitness but a slower growth rate in pure culture than the ancestor, and in a third genotype both fitness and growth rate were significantly reduced. The latter genotype, which behaved as assumed in meltdown models, was isolated from population M2 six days before its demise.

Discussion

After more than 100 daily bottlenecks, yeast populations with effective sizes near 250 and elevated mutation rates showed a tendency to decline in size, whereas the sizes of initially identical populations with wild-type mutation rates remained constant. Among mutator populations, there were two extinctions and no clear fitness trend over time, whereas wild-type populations increased in fitness. The correspondence between elevated mutation rates and the decline of small populations in a constant environment provides general support for models of mutational meltdown.

Although it is perhaps surprising that wild-type populations at $N_e \approx 255$ were able to increase in fitness, this is consistent with our observation that slightly deleterious mutations are very rare in wild-type $S. cerevisiae$ (Zeyl and de Visser 2001). Because slightly deleterious mutations occur much more frequently in mutator populations, we predicted meltdowns only in the latter. Although extinction was not significantly more frequent among our mutator populations than among the wild types, at least one of the two extinctions that did occur immediately followed a fitness decline of approximately 7% and was reproducible.

Our general observation of fitness declines in mutator populations is not surprising. Fitness declines in small populations have been modeled repeatedly, and have been observed in laboratory populations of vesicular stomatitis virus (Elena and Moya 1999), Escherichia coli (Kibota and Lynch 1996), D. melanogaster (Fernández and López-Fanjul 1996; Fry et al. 1999), C. elegans (Keightley and Caballero 1997; Vassileva and Lynch 1999), Daphnia pulex (Lynch et al. 1998), and Arabidopsis thaliana (Schultz et al. 1999; Shaw et al.
Table 3. ANOVA of the fitness estimates shown in Figure 3 from five genotypes sampled randomly from each of six wild-type and six mutator populations at days 0, 57, 113, and 162. Ancestor (wild-type or mutator) and day number were fixed factors; replicate population nested within ancestor and genotype nested within population were random factors.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Expected MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancestor</td>
<td>1</td>
<td>0.099</td>
<td>0.099</td>
<td>Q(anc, anc × day) + 93.196(\sigma^2_\text{ancestor}) + \sigma^2_\text{error}</td>
<td>1.55</td>
<td>0.268</td>
</tr>
<tr>
<td>Day</td>
<td>3</td>
<td>0.308</td>
<td>0.103</td>
<td>Q(day, anc × day) + 46.87(\sigma^2_\text{day}) + \sigma^2_\text{error}</td>
<td>2.34</td>
<td>0.115</td>
</tr>
<tr>
<td>Ancestor × day</td>
<td>3</td>
<td>0.137</td>
<td>0.046</td>
<td>Q(anc × day) + 94.3(\sigma^2_\text{ancestors}) + \sigma^2_\text{error}</td>
<td>29.405</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Population (ancestor)</td>
<td>5</td>
<td>0.321</td>
<td>0.064</td>
<td>38.91(\sigma^2_\text{ancestors}) + \sigma^2_\text{error}</td>
<td>41.347</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Genotype (population)</td>
<td>24</td>
<td>0.063</td>
<td>0.003</td>
<td>48.9(\sigma^2_\text{population}) + \sigma^2_\text{error}</td>
<td>1.699</td>
<td>0.019</td>
</tr>
<tr>
<td>Population (ancestor) × day</td>
<td>15</td>
<td>0.674</td>
<td>0.045</td>
<td>48.9(\sigma^2_\text{population}) + \sigma^2_\text{error}</td>
<td>28.920</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
<td>1111</td>
<td>1.726</td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2000). However, the fact that at least one of two extinctions resulted from MA is not a trivial result, because models predicting mutational meltdown rely on several assumptions of uncertain accuracy. One assumption is that accumulated mutations reduce not just relative fitness but also absolute rates of reproduction. This assumption is supported by the reproducible extinction of population M2 and the declines in both relative fitness (Figs. 3B, 4B) and growth rate (Table 5) of genotypes sampled from this population just prior to its extinction. However, for two genotypes randomly sampled from other mutator populations, there was no correspondence between growth rate and fitness relative to their ancestor (Table 5). Among both adaptive and deleterious mutations there may be some that affect only fitness relative to competing or ancestral genotypes, and others that affect the growth rate of the mutant in isolation. Our results indicate that even with the fairly modest genomic mutation rate \( U < 1 \) of our mutator strain, mutations of the latter type can occur often enough to cause extinction.

The cycles in population size indicated by Figure 2 imply either fluctuation in the laboratory environment or some cyclical feature of growth rates intrinsic to the populations. The cycles may have resulted from using culture conditions and transfer regimes that would barely allow the starting populations to reach stationary phase before each transfer. Growth curves (not shown) indicated that the ancestral genotypes entered stationary phase after 22–23 h. Like most microbes, yeast batch cultures that have entered stationary phase and are then transferred to fresh medium experience a lag phase before resuming logarithmic growth. This lag may be absent if a population was transferred just before entering stationary phase. Such a population could then reach stationary phase earlier in the next day and experience a lag after the subsequent transfer. If this prevented it from reaching stationary phase in the following growth period, the cycle would be repeated. Therefore, slight variation in growth phase at the time of one of the first transfers could have initiated a cycle of population sizes.

We view this experiment as a demonstration that mutational meltdowns do indeed occur, rather than a quantitative test of predicted times or probabilities of extinction. The time to extinction, \( T_e \), due to mutational meltdown can be predicted from Lynch et al. (1993). \( T_e \) is the sum of three phases: \( t_1 \), time for genetic variance to build up to the asymptotic equilibrium value; \( t_2 \), time for mutation-accumulation before it affects population size; and \( t_3 \), the actual meltdown. Because

![Figure 4](image)  
**Fig. 4.** Changes in (A) population size and (B) fitness in mutator population M2. In (A), open circles represent the colony counts from plates used to estimate the numbers of cells transferred during each daily bottleneck, as described in Materials and Methods. In (B), diamonds represent the means of five replicate fitness estimates of single genotypes isolated from frozen samples of population M2.
t2 is by far the most time consuming, assume that $T_e = t_2$. Assuming small $s$ and $Ks \gg 1$ ($K = N_e$; Lynch et al. 1995a), $t_2 = K \ln(R e^{-t})/U$, where $R$ is the rate of reproduction ($R = 2$ in the case of unicellular budding). For our wild-type populations ($U = 0.0001$; Zeyl and de Visser 2001), this leads to $t_2 = T_e = 1.7 \times 10^6$ generations. Our wild-type populations were therefore not expected to melt down due to MA, which is consistent with the observations. For M populations, assuming a 200-fold increase in mutation rate ($U = 0.0023$; Zeyl and de Visser 2001), $t_2 = 8.4 \times 10^5$ generations. However, without knowing the expected distribution of extinction times, we can say only that our observation of two of 12 mutator extinctions does not contradict the expectation that mean times to extinction will exceed 2900 generations.

The population dynamics imposed in this experiment, while reducing effective population size and yielding 16.6 generations per day, also allowed relatively efficient selection during the daily exponential growth phase. An alternative setup for a microbial meltdown experiment might impose a constant small population size using a microchemostat, if one could be devised.

This study examined the risk of extinction due to the fixation of newly arising deleterious mutations, but omitted other genetic risks of repeated bottlenecking. Two of these additional risks are the fixation of previously segregating deleterious mutations (Lynch et al. 1995a) and a diminished potential to respond to environmental changes due to the loss of alleles that might have been adaptive in a changed environment. There was no segregating variation in our initial populations because they were founded by clones of a single cell, and there were no environmental changes. This experiment therefore does not address the increased extinction risk in a changing environment due to small population size.

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**LITERATURE CITED**


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