Accelerating Mutational Load Is Not Due to Synergistic Epistasis or Mutator Alleles in Mutation Accumulation Lines of Yeast

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ABSTRACT Much of our knowledge about the fitness effects of new mutations has been gained from mutation accumulation (MA) experiments. Yet the fitness effect of single mutations is rarely measured in MA experiments. This raises several issues, notably for inferring epistasis for fitness. The acceleration of fitness decline in MA lines has been taken as evidence for synergistic epistasis, but establishing the role of epistasis requires measuring the fitness of genotypes carrying known numbers of mutations. Otherwise, accelerating fitness loss could be explained by increased genetic mutation rates. Here we segregated mutations accumulated over 4800 generations in haploid and diploid MA lines of the yeast *Saccharomyces cerevisiae*. We found no correspondence between an accelerated fitness decline and synergistic epistasis among deleterious mutations in haploid lines. Pairs of mutations showed no overall epistasis. Furthermore, several lines of evidence indicate that genetic mutation rates did not increase in the MA lines. Crucially, segregant fitness analyses revealed that MA accelerated in both haploid and diploid lines, even though the fitness of diploid lines was nearly constant during the MA experiment. This suggests that the accelerated fitness decline in haploids was caused by cryptic environmental factors that increased mutation rates in all lines during the last third of the lines' transfers. In addition, we provide new estimates of deleterious mutation rates, including lethal mutations, and highlight that nearly all the mutational load we observed was due to one or two mutations having a large effect on fitness.

KEYWORDS fitness effects; mutation-rate plasticity; experimental evolution; genetic drift; *Saccharomyces cerevisiae*

MUTATIONS that sicken and kill segregate in populations and are created anew each generation (Agrawal and Whitlock 2012). The rate at which mutations appear and how these mutations interact to determine fitness tell us how maladapted populations are expected to be in comparison to some optimum (Poon and Otto 2000; Silander *et al.* 2007). This fitness load is especially heavy on genes experiencing little or no recombination (*i.e.*, asexual genomes and sex chromosomes) (Roze and Blanckaert 2014; Hussin *et al.* 2015) and even can threaten the survival of asexual lineages (Lande 1994; Schwander and Crespi 2009). Data on the fitness effects of new mutations have been obtained primarily from mutation accumulation (MA) experiments, in which the

efficacy of selection is minimized to favor genetic drift and the rapid fixation of deleterious and beneficial mutations (Mukai 1964; Halligan and Keightley 2009). However, few of these experiments isolated the effect of single mutations. Most work relied instead on assumptions regarding the variance of fitness effects among mutations to infer mean mutation rate and effect (Halligan and Keightley 2009). Here we present a detailed analysis of the fitness effect of mutations isolated from a MA experiment using the yeast *Saccharomyces cerevisiae* (Dickinson 2008).

MA experiments were popularized by Terumi Mukai, who reported a high genomic mutation rate and synergistic epistasis among deleterious mutations affecting viability in *Drosophila* (Mukai 1964, 1969; Mukai and Yamazaki 1968; Fry 2004). Synergistic epistasis was inferred from the accelerating decline of fitness traits during MA (see also Fry 2004; Ávila *et al.* 2006; Kouyos *et al.* 2007; Dickinson 2008; Schaack *et al.* 2012). It was later shown that an accelerating fitness decline in MA lines also can result from a positive relationship between mutational load and mutation rates.

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This association can happen (1) if being in poor physiologic condition elevates mutation rates (Matsuba *et al.* 2012; Sharp and Agrawal 2012), (2) because loaded genotypes have a higher probability of carrying mutator alleles (e.g., transposable elements) (Ávila *et al.* 2006), and (3) if transposable elements spread in sexual MA lines (Nuzhdin *et al.* 1997). In principle, mutation rates also could increase without genetic evolution if uncontrolled aspects of the growth environment raise mutation rates over time (an instance of mutation-rate plasticity; Krasovec *et al.* 2014). Because non-linear fitness decline in MA lines can have many causes, inferences about epistatic interactions among mutations accumulated in MA lines require direct determination of these epistatic effects.

Here we experimentally address two aspects of fitness and epistatic effects of new mutations in MA experiments. First, we segregated mutations from haploid and diploid MA lines of the yeast *S. cerevisiae* as previously published by Dickinson (2008), who observed an accelerating fitness decline in these lines. We found that load in these lines is due to one or two mutations of large effect and that synergistic epistasis does not explain the trend of accelerating fitness decline. Second, we ask whether loaded haploid lines have higher genetic mutation rates than their ancestor. We found that this is not the case; mutation rates to canavanine resistance were not higher in loaded strains, and loaded strains did not accumulate more load at an increased rate. Moreover, there was no heterogeneity in the number of large-effect mutations accumulated among replicate lines. We explain the accelerated rates of MA and fitness decline as the effect of cryptic environmental factors that increased mutation rates during the last third of the line transfers relative to the first two-thirds. This interpretation is supported by the fact that the number of large-effect mutations accumulated per generation increased over the course of the MA transfers in both haploid and diploid lines, even though only the fitness of haploid parental lines declined at an accelerating rate. Our results have important implications for the interpretation and design of MA experiments and provide further support for the idea that most of the fitness loss in small populations is due to a few mutations of large fitness effect.

Materials and Methods

MA lines

The MA lines analyzed here were provided by W. J. Dickinson (Dickinson 2008) [see also Lynch *et al.* (2008) for genomic analyses of some of the strains]. Dickinson (2008) propagated 48 lines, composed of 32 haploid lines labeled Ai, Bi, Ci, and Di (Ai and Bi are *MATa*, and Ci and Di are *MATα*) and 16 diploid lines labeled Ei and Fi, where *i* is for replicate line numbers 1–8. All Ai–Ei genotypes were derived from FY10 (S288c *ura3-52*) (Winston *et al.* 1995). The lines were propagated through 200 single-cell bottlenecks on YPD plates (20 g/liter glucose, 20 g/liter bacteriological peptone, 10 g/liter

yeast extract, and 20 g/liter agar) incubated at 30°. Between each bottleneck, lineages expanded from a single cell to $\sim 2 \times 10^7$ cells in ~ 24 doublings (3–4 days). Dickinson (2008) eliminated respiratory-deficient mutations by periodically screening the lines on YPG plates (same as YPD plates but with 20 g/liter glycerol instead of glucose) [see Zeyl and De Visser (2001) about respiratory-deficient mutants in yeast MA lines]. Here we worked with the genotypes isolated after 130 (~ 3100 doublings) and 200 (~ 4800 doublings) bottlenecks, for a total of 96 evolved clones (48 lines $\times$ 2 time points) and 6 ancestral clones. Dickinson (2008) estimated that the ancestor divided 24 times per cycle, but this number probably declined as mutations accumulated. As a consequence, the true acceleration of fitness loss per generation is probably larger than the one we report. The observed rate of MA suffers from an analogous underestimation at later time points. We estimated the fitness effect of accumulated mutations using tetrad dissection and competition experiments between segregant genotypes and fluorescent-labeled ancestors.

Ploidy determination

Ploidy level was determined for each of the 96 evolved clones using flow cytometry (FACS). Clonal cultures were harvested in exponential phase by transferring 10 μ l of overnight cultures into 1 ml of YPD liquid medium, incubating the fresh cultures for 6–8 hr at 30°, and resuspending the cells in 1 ml of 70% ethanol. Following overnight incubation with shaking, cells were centrifuged, washed in 1 ml of 50 mM sodium citrate, and incubated overnight at 37° with proteinase K and RNase A in 0.5 ml of 50 mM sodium citrate with shaking. Samples were then sonicated. Finally, cells were resuspended in 0.5 ml of 50 mM sodium citrate with 0.5 μ g/ml of SYTOX Green dye, incubated for 2–3 hr at room temperature, and processed with a BD FACSCalibur (Becton-Dickson, Le Pont-de-Claix, France). The two modes of the distribution of fluorescence (height of FL1 values) were used to determine ploidy (cells in G_1 and G_2 each form a mode) (Delobel and Tesnière 2014). These measurements were replicated on two different days.

Segregating mutations

We dissected eight complete meiotic tetrads for all 16 evolved diploid clones from generation 4800 (16 tetrads for strains carrying a lethal mutation). For the 12 lines that carried at least one large-effect mutation at $t = 4800$ (detectable among haploid segregants following two replicate fitness measures), we also dissected 8 tetrads (16 tetrads when a lethal mutation was present) from the clone from generation 3100. Fitness was assayed in duplicate for each haploid segregant [32 segregants $\times$ (16 + 12) evolved diploids = 896 segregants]. All tetrad dissections were done on YPD plates, and after 2 days of incubation at 30°, each plate was replica plated on minimal medium (20 g/liter glucose, 5 g/liter ammonium sulfate, 5 g/liter yeast nitrogen base without nitrogen, 60 mg/liter uracil, and 20 g/liter agar) to test for the viability of segregants in that environment.

Table 1 Competitive fitness of clones from the haploid lines at two time points (3100 and 4800 generations) and fitness classes among 32 segregants (average $\pm$ 95% CI)

Line	W at 3100	W at 4800	W AB	W aB	W Ab	W ab	R ² model	SS _{mut.} SS _{model}	SS _{seg.} SS _{model}	P seg.
A2	0.985 $\pm$ 0.001	0.830 $\pm$ 0.003	0.997 $\pm$ 0.003	0.832 $\pm$ 0.003	NA	NA	0.995	0.992	0.003	0.24
A3	1.000 $\pm$ 0.001	0.845 $\pm$ 0.020	0.999 $\pm$ 0.001	0.850 $\pm$ 0.007	NA	NA	0.996	0.982	0.003	0.11
D5	0.940 $\pm$ 0.002	0.943 $\pm$ 0.001	0.993 $\pm$ 0.003	0.949 $\pm$ 0.005	NA	NA	0.956	0.912	0.044	0.02
A4	1.000 $\pm$ 0.001	0.809 $\pm$ 0.003	1.019 $\pm$ 0.012	0.887 $\pm$ 0.007	0.967 $\pm$ 0.001	0.790 $\pm$ 0.053	0.989	0.937	0.010	0.02
A7	0.862 $\pm$ 0.010	0.817 $\pm$ 0.014	1.000 $\pm$ 0.007	0.880 $\pm$ 0.007	0.969 $\pm$ 0.001	0.824 $\pm$ 0.031	0.974	0.942	0.020	0.10
B4	0.919 $\pm$ 0.003	0.916 $\pm$ 0.001	1.000 $\pm$ 0.003	0.967 $\pm$ 0.001	0.985 $\pm$ 0.001	0.941 $\pm$ 0.004	0.998	0.955	0.033	0.0001
B6	1.000 $\pm$ 0.003	0.853 $\pm$ 0.005	1.008 $\pm$ 0.003	0.863 $\pm$ 0.010	0.985 $\pm$ 0.006	0.853 $\pm$ 0.001	0.996	0.987	0.007	0.0002
B7	0.995 $\pm$ 0.001	0.966 $\pm$ 0.001	0.998 $\pm$ 0.001	0.976 $\pm$ 0.005	0.982 $\pm$ 0.001	0.963 $\pm$ 0.016	0.904	0.793	0.096	0.03
C3	0.968 $\pm$ 0.001	0.930 $\pm$ 0.002	0.995 $\pm$ 0.002	0.989 $\pm$ 0.002	0.974 $\pm$ 0.003	0.964 $\pm$ 0.003	0.885	0.832	0.099	0.09
D1	0.756 $\pm$ 0.008	0.741 $\pm$ 0.011	0.979 $\pm$ 0.001	0.946 $\pm$ 0.001	0.900 $\pm$ 0.002	0.868 $\pm$ 0.002	0.997	0.835	0.059	0.0001
C5	0.923 $\pm$ 0.004	0.949 $\pm$ 0.003	Range: 0.933–1.031; average = 0.983; median = 0.987; SD = 0.031							
C7	1.000 $\pm$ 0.002	0.979 $\pm$ 0.002	Range: 0.991–1.009; average = 0.999; median = 0.999; SD = 0.004							

AB represents the fitness class most similar to the ancestor, aB and Ab carry a single large-effect mutation, and ab is the double mutant. The last four columns present outputs from an ANOVA of segregants' fitness including mutation classes (mut.), days (not shown), mutation $\times$ days interaction (not shown), and segregant (seg.) effects. By definition, W = 1 at $t = 0$. NA = not applicable because the genotypes do not exist.

Because our ability to analyze mutations with selection coefficient $-0.02 < s < 0.02$ is limited given the large number of genotypes assayed, we analyzed only haploid lines for which clones from either generation 3100 or 4800 have $s < -0.02$ relative to the ancestor, yielding 13 haploid lines of 32. However, the final genotype from line B5 (fitness 0.93 at generation 4800) could not be mated to the ancestor and was not analyzed further.

The analysis of haploid genotypes started by eliminating the mitochondrial genome from the evolved strains prior to mating them to the ancestral genotypes. This was done to prevent mitochondrial heteroplasmy, recombination, and segregation (all common in yeast; Thrailkill *et al.* 1980), which could have inflated variance in fitness among segregants given that mitochondrial mutations have accumulated in the lines (Lynch *et al.* 2008). Eliminating the mitochondrial genome was done by transferring 10 μ l of overnight cultures in 1 ml of minimal medium supplemented with ethidium bromide (20 g/liter dextrose, 5 g/liter ammonium sulfate, 1.7 g/liter yeast nitrogen base, 60 mg/liter uracil, and 25 mg/liter ethidium bromide). The cultures were grown in the presence of the mutagen for two daily cycles (10 μ l transferred to 1 ml of medium with incubation at 30°) and plated on YPGD medium (20 g/liter glycerol, 1 g/liter glucose, 20 g/liter bacteriological peptone, 10 g/liter yeast extract, and 20 g/liter agar). Following 2 days of incubation at 30°, colonies from the cultures exposed to ethidium bromide were small relative to respiratory-competent control colonies. These small colonies (*petites*) are formed by cells lacking mitochondrial DNA, or p_0 cells (Spencer and Spencer 1993). We mated these p_0 evolved strains to each haploid ancestral genotype (Ai p_0 and Bi p_0 with C and Ci p_0 and Di p_0 with A) to isolate diploid hybrids that were heterozygous for accumulated nuclear mutations, but all sharing the ancestral mitochondrial genome. We then sporulated these diploid hybrids to segregate accumulated mutations. Eight tetrads were dissected for each of the 12 hybrids (8 tetrads $\times$ 4 spores $\times$ 12 lines = 384 segregants). We can reject the possibility that ethidium bromide mutagenesis generated new nuclear muta-

tions by comparing the fitness of the least-fit segregants in each cross with that of the corresponding genotype from $t = 4800$. If a given p_0 $t = 4800$ genotype carried new deleterious mutations as a result of mutagenesis, it would generate segregants that are less fit than the mitochondrial DNA (mtDNA)-carrying $t = 4800$ parent, but we never observed this (Table 1).

Fitness assays

The fitness of haploid and diploid evolved clones and all segregants was estimated by head-to-head competitions in liquid YPD medium. Segregants from a given evolved clone were always analyzed together and with 20–80 replicates of the appropriate mutation-free ancestor (A–D). Strains maintained frozen in 15% glycerol were inoculated in 150 μ l of YPD medium on 96-microwell plates and incubated overnight at 30° without shaking. Two days later, cultures were streaked onto YPD plates and incubated for 2 days at 30°. For each genotype assayed, a single colony was inoculated into 1 ml of liquid YPD medium on 24-well plates. These cultures were incubated for 24 hr, after which 3 μ l of each culture was mixed independently with 1 μ l of a competitor's culture of the appropriate mating type (described later) in 1 ml of YPD medium. After 24 hr of incubation, 4 μ l was transferred to fresh wells containing 1 ml of YPD medium. Plates with 1-day-old mixtures were kept at 4° (for up to 3 hr) until FACS analyses for estimating the initial frequencies of focal genotypes and competitors in each well. The fresh cultures were incubated for 24 hr before FACS analysis to estimate the final frequencies of the two genotypes. Fitness was estimated by the change in the focal strain's frequency over one cycle [$\log_2(1000/4) = 7.97$ generations] as $1 + \ln\{[p_1(1 - p_1)]/[p_0(1 - p_0)]\}/7.97$, where p_0 and p_1 are the frequency of the focal strain at the beginning and the end of the competition cycle, respectively. The fitness of focal strains against the fluorescent-labeled ancestor was corrected using ancestral fitness (also competed against the fluorescent-labeled ancestor) independently for each day. On a given day, if ancestral fitness was

$w_a = 1 + c = 0.955$, with c being the advantage of the ancestor against the fluorescent ancestor ($-c$ is the marker cost), and the fitness of a focal strain was $w = 1 + c + s = 0.954$, with s being the selection coefficient of that focal strain, then the corrected fitness value of the focal strain was $W = 1 + w - w_a = 0.999$. The ancestor has $W = 1$ by definition. This correction addresses marker cost by assuming that fitness is transitive among all strains. The assumption of fitness transitivity is justified in laboratory strains of *S. cerevisiae* given that competitive fitness is generally well correlated with maximal growth rates in monocultures (Bell 2010; Jasmin *et al.* 2012), indicating weak genotype $\times$ genotype interactions among strains with small genetic divergence, such as those analyzed here.

FACS analyses were carried on a BD FACSCalibur (Becton-Dickson, Le Pont-de-Claix, France) by transferring 20 μ l of overnight YPD cultures in 1 ml of FACS flow and reading events for 35 sec (yielding 4×10^6 to 8×10^6 events). The frequency of each clone was estimated by transforming the bivariate values of FL1-H and FSC-H into a specific fluorescence index defined by FL1-H⁴/FSC-H. This fluorescence index was bimodal with a wide valley between fluorescent and nonfluorescent cells. Control wells of either fluorescent or nonfluorescent cells contained fewer than 1 in 10,000 cells with a mistaken attribution.

The common competitor was a direct derivative of the ancestor expressing the fluorescent protein mCitrine under the strong promoter of the actin gene *ACT1*. It was obtained by PCR amplifying *ura3Δ::P_{ACT1}-ymCitrine-tADH1-URA3* from strain yJHK226 of Koschwanez *et al.* (2011) and transforming the PCR product into the A ancestor (S288c *ura3-52 MATa*) using the *URA3* gene as a selectable marker and a standard lithium acetate/single-stranded carrier DNA/polyethylene glycol protocol (Gietz and Woods 1991). The transformed allele then was moved to the *MATα* mating type by mating the transformant with the C ancestor, sporulating the resulting diploid, and dissecting tetrads.

Mutation rates to canavanine resistance

For the parental haploid strains from $t = 4800$ (carrying evolved mitochondrial genomes), we determined single-locus mutation rates using a fluctuation test for canavanine resistance, coded by a null allele at the *CAN1* locus. We used liquid cultures and the p_0 method (Luria and Delbrück 1943; Rosche and Foster 2000) to estimate the mutation rate at *CAN1*. This consisted of inoculating a small volume (3 μ l) of diluted (1:100) overnight cultures in replicate wells from 96-well plates containing 100 μ l of DM minimal medium supplemented with uracil. The cultures then were incubated for 2 days to reach their limiting cell density, after which the entire cultures (~ 100 μ l) were transferred to 100 μ l of DM + uracil supplemented with 0.6 g/liter canavanine, for a final concentration of canavanine that was five times the usual dose (0.3 g/liter) (Lang and Murray 2008). These cultures were incubated for 2 days to select for resistant clones. After that, 20 μ l from each culture was transferred to 180 μ l of DM + uracil supplemented with 0.3 g/liter canavanine to test for the presence of resistant mutants. After 2 days of incuba-

Figure 1 Competitive fitness for (A) 32 haploid and (B) 16 diploid MA lines (dotted lines). Thick solid and dashed lines, respectively, show the median and average among lines. The haploid line that drops out of scale has fitness ~ 0.75 at both time points. Similar data are presented by Dickinson (2008, Figure 1), although he did not separate haploid and diploid lines in his analyses.

tion, we scored whether each well contained resistant mutants. Throughout these experiments, evaporation was prevented with Breathe-Easy films. The mutation rate is given by $-\ln(p_0)$, with p_0 being the proportion of cultures without mutations that corresponds to the zero term from the Poisson-distributed number of mutations for each replicate culture. Mutations rates per cell were calculated for each strain by dividing $-\ln(p_0)$ by the number of cells transferred earlier into 100 μ l canavanine medium (cell densities in DM + uracil were counted using a CASY Cell Counter). The protocol described earlier was calibrated in pre-experiments to obtain $0.1 < p_0 < 0.7$ (Rosche and Foster 2000).

Results

MA lines

The median fitness change per generation in the haploid lines was six times steeper during the last 1700 generations than during the first 3100 generations (-9.0×10^{-7} vs. -5.5×10^{-6} ; Wilcoxon signed-rank test, $Z = 1.66$, $P = 0.0002$)

Figure 2 Fitness changes over time (A, C, and E) and fitness effects of accumulated mutations (B, D, and F) in three haploid MA lines. The fitness of 32 segregants is shown (eight complete tetrads from hybrids between clones from $t = 4800$ and the ancestor). A single mutation segregates in the top row (line A3), while two mutations segregate in the bottom two rows (line A4 in the middle and line B6 at the bottom).

(Figure 1A). Note that this acceleration of fitness loss is conservative because we assumed that the number of generations that lines underwent between each transfer cycle was constant (at 24 generations per transfer cycle achieved by the ancestors) (Dickinson 2008), even though many lines probably accumulated mutations that slowed down their growth and thus reduced this number. Because this overestimation must have been larger at later time points owing to mutations accumulating over time, we are probably underestimating fitness changes per generation for later transfers.

In the diploid lines, conversely, fitness changes were positive, small, and not different for both time periods (medians are 1.6×10^{-7} vs. 2.6×10^{-7} ; Wilcoxon signed-rank test, $Z = 0.27$, $P = 0.39$) (Figure 1B). At generation 4800, median haploid fitness was about 2% lower than median diploid fitness (0.983 vs. 1.001; Wilcoxon-Kruskal-Wallis test, $Z = 2.2$, $P = 0.02$). The ploidy level increased in 3 of the 32 haploid lines (lines A4 and D4 had near-diploid values; B1 was intermediate), but these changes were not associated with consistent fitness changes. These ploidy-level changes are likely to be large-scale structural variants known to exist in these (Lynch *et al.* 2008) and other *S. cerevisiae* MA lines (Nishant *et al.* 2010).

Fitness effect and timing of accumulated mutations

We segregated the mutations accumulated in the 12 haploid lines with a $>2\%$ fitness difference relative to the ancestor at

either time point (3100 or 4800 generations). Mutations were segregated by mating evolved haploids lacking mtDNA (p_0) to the ancestral strain of the opposite mating type and dissecting meiotic tetrads of the resulting hybrids. Three lines carried a single large mutation, as indicated by 2:2 fitness patterns in each tetrad (Figure 2A); seven lines carried two large-effect mutations, as indicated by four discrete fitness classes among segregants (Figure 2, B and C), altogether cumulating 17 single mutations (Table 1); whereas the remaining two lines did not carry identifiable mutations with large effect.

In the seven lines carrying two large mutations, there are only three possible tetrad types: tetratypes (ab, aB, Ab, AB), nonparental ditypes (aB, aB, Ab, Ab), and parental ditypes (ab, ab, AB, AB). For the 10 strains carrying one or two large-effect mutations, mutation-free segregants (AB) were identified as those having fitness similar to the ancestors, and whole tetrads then were used to estimate the fitness of each genotype (Table 1). These large-effect mutations explain nearly all the variance in fitness among segregants in 8 of the 10 lines (Figure 3), although other mutations make a small ($<5\%$) but statistically significant ($P < 0.05$) contribution to fitness variation in 6 of the 10 lines [segregant (seg.) effect in Table 1]. Such segregant effects indicate the presence of at least one mutation with $s \leq 1\%$. Segregants carrying the large-effect mutations have fitness similar to that of

Figure 3 Fitness of haploid strains isolated at the end of the MA experiment in comparison with the fitness of segregants carrying one (empty circles) or two (filled circles) large-effect mutations identified in the line. The dashed line indicates equivalent fitness. Error bars, ± 2 SE.

the strain isolated at $t = 4800$, except for three lines (B4, C3, and D1), for which double mutants are slightly fitter than the $t = 4800$ parents (Figure 3, Table 1; see also *Discussion*).

The two lines without discernible large-effect mutations are (1) line C7, the fittest haploid studied here, which nevertheless carried small mutations evidenced by a strong segregant effect in a model, including also a day effect ($R^2 = 0.760$, $SS_{\text{seg}}/SS_{\text{model}} = 0.947$, $F_{31,95} = 6.0$, $P < 0.0001$) (Supporting Information, Figure S1A), and (2) line C5, which carried several large mutations, including at least one beneficial mutation, forming a near-continuous range of fitness also well explained by segregants ($R^2 = 0.989$, $SS_{\text{seg}}/SS_{\text{model}} = 0.995$, $F_{31,95} = 173$, $P < 0.0001$) (Figure S1B).

In most cases, we were able to determine whether large-effect mutations occurred before or after $t = 3100$ and thus attribute each mutation to a time period by comparing fitness classes among segregants to the fitness of parent genotypes at $t = 3100$ and $t = 4800$ (Table 1). We thus attribute seven large mutations to the first 3100 generations and 10 mutations to the last 1700 generations (Table 1 and Table 3). The timing of mutations in line C5 is unclear; they occurred in both time periods but were too numerous to be counted. Hereafter we consider that line C5 has accumulated two or three large-effect mutations in each period. The overall rate of mutations affecting fitness in the haploid lines was at least $(17 \text{ large} + 6 \text{ small} + 1 \text{ in line C7} + 5 \text{ in line C5}) / (32 \times 4800) = 1.89 \times 10^{-4}$ mutations per haploid genome replication, conservatively assuming that

Table 2 Fitness classes among 32 segregants from the diploid lines after 3100 and 4800 generations (average $\pm$ 95% CI)

Period	Line	Time	W AB	W aB	W Ab	W ab	R^2 model	$\frac{SS_{\text{mut}}}{SS_{\text{model}}}$	$\frac{SS_{\text{seg}}}{SS_{\text{model}}}$	P seg.	$\frac{SS_{\text{mut}} \times \text{seg.}}{SS_{\text{model}}}$	P mut. $\times$ seg.
First 3100 only	E5	3100×	1.000 $\pm$ 0.001	NA	NA	NA	0.663	NA	0.840	0.08	NA	NA
		4800×	0.998 $\pm$ 0.001	NA	NA	NA	0.787	NA	0.533	0.03	NA	NA
	F3	3100×	0.967 $\pm$ 0.003	0.867 $\pm$ 0.005	NA	NA	0.993	0.903	0.004	0.9	0.02	0.0001
		4800×	0.967 $\pm$ 0.004	0.871 $\pm$ 0.005	NA	NA	0.987	0.930	0.042	0.002	0.01	0.0001
	F6	3100	1.000 $\pm$ 0.001	0.887 $\pm$ 0.004	NA	NA	0.996	0.971	0.005	0.5	0.003	0.0001
		4800	1.003 $\pm$ 0.002	0.936 $\pm$ 0.001	NA	NA	0.992	0.986	0.013	0.0001	0.0008	0.06
Both	E8	3100	0.997 $\pm$ 0.004	0.905 $\pm$ 0.003	0.747 $\pm$ 0.006	0.745 $\pm$ 0.004	0.999	0.997	0.003	0.02	0.0001	0.27
		4800	0.995 $\pm$ 0.003	0.904 $\pm$ 0.004	0.763 $\pm$ 0.013	0.754 $\pm$ 0.008	0.998	0.982	0.006	0.006	0.007	0.0001
	F7	3100	0.994 $\pm$ 0.002	0.986 $\pm$ 0.002	0.841 $\pm$ 0.002	0.834 $\pm$ 0.003	0.999	0.997	0.002	0.002	0.0008	0.0001
		4800	0.999 $\pm$ 0.003	0.985 $\pm$ 0.003	0.840 $\pm$ 0.004	0.831 $\pm$ 0.004	0.999	0.995	0.003	0.03	0.002	0.0001
	E2	3100	0.983 $\pm$ 0.009	0.877 $\pm$ 0.004	NA	NA	0.994	0.962	0.013	0.01	0.02	0.0001
		4800×	0.994 $\pm$ 0.004	0.877 $\pm$ 0.006	0.759 $\pm$ 0.006	0.717 $\pm$ 0.004	0.995	0.987	0.007	0.0002	0.001	0.02
Last 1700 only	F5	3100×	0.982 $\pm$ 0.003	0.962 $\pm$ 0.004	0.942 $\pm$ 0.003	0.928 $\pm$ 0.002	0.989	0.858	0.048	0.0008	0.006	0.008
		4800×	NA	NA	NA	NA	0.991	NA	0.999	0.0001	NA	NA
	E1	3100	1.009 $\pm$ 0.002	NA	NA	NA	0.907	NA	0.425	0.0001	NA	NA
		4800	0.994 $\pm$ 0.002	0.969 $\pm$ 0.002	NA	NA	0.969	0.846	0.105	0.0001	0.01	0.0004
	F1	3100	1.001 $\pm$ 0.002	NA	NA	NA	0.757	NA	0.094	0.2	NA	NA
		4800	0.983 $\pm$ 0.002	0.972 $\pm$ 0.003	NA	NA	0.886	0.399	0.268	0.02	0.07	0.0005
None	F8	3100	0.999 $\pm$ 0.002	NA	NA	NA	0.659	NA	0.999	0.04	NA	NA
		4800×	0.993 $\pm$ 0.003	0.779 $\pm$ 0.002	NA	NA	0.999	0.998	0.002	0.1	0	0.77
	E3	3100×	1.001 $\pm$ 0.009	NA	NA	NA	0.551	NA	0.180	0.4	NA	NA
		4800×	0.995 $\pm$ 0.009	0.920 $\pm$ 0.003	0.852 $\pm$ 0.002	0.802 $\pm$ 0.003	0.999	0.995	0.001	0.3	0.0001	0.4
	E7	3100	1.010 $\pm$ 0.002	NA	NA	NA	0.878	NA	0.262	0.09	NA	NA
		4800×	1.010 $\pm$ 0.003	0.988 $\pm$ 0.007	0.958 $\pm$ 0.004	0.906 $\pm$ 0.005	0.998	0.962	0.006	0.0003	0.0002	0.3
None	E4	4800	1.001 $\pm$ 0.002	NA	NA	NA	0.642	NA	0.788	0.4	NA	NA
		4800	0.998 $\pm$ 0.002	NA	NA	NA	0.898	NA	0.646	0.003	NA	NA
	F2	4800	0.999 $\pm$ 0.002	NA	NA	NA	0.713	NA	0.194	0.7	NA	NA
		4800	1.003 $\pm$ 0.001	NA	NA	NA	0.843	NA	0.255	0.2	NA	NA

Rows show lines that acquired large mutations only during the first 3100 generations, during both time periods, only during the last 1700 generations, or that did not gain any large-effect mutations. Crosses (×) indicate the number of lethal mutations carried by each strain. AB represents the fitness class closest to the ancestor, aB and Ab carry a single large-effect mutation, and ab is the double mutant. The last four columns present outputs from an ANOVA model of segregant fitness including mutation classes (mut.; P -value < 0.0001 in all cases), days (not shown), mutations $\times$ days interaction (not shown), and segregant (seg.) effects. A model with only days and segregant effects was used in cases for which there were no fitness classes. NA = not applicable because the genotypes required to fill the cell do not exist.

Figure 4 Fitness effect of single mutations identified among segregants of (A) haploids at 4800 generations crossed to the ancestor and (B) diploid clones from both 3100 and 4800 generations. For single mutations segregating in haploids, we used fitness trajectories during MA to attribute mutations to the first 3100 generations or last 1700 generations of the MA experiment. Note, however, that the two distributions should not be compared directly because whereas mutations from all 16 diploid lines are included here, only 12 (of 32) haploid lines with large fitness changes are shown.

segregant effects are caused by a single mutation and that the 20 haploid lines not studied here did not carry mutations affecting fitness. However, mutation rates are likely overestimated because we assumed that all lines consistently underwent 24 generations per transfer cycle, even though this number probably declined as lines accumulated deleterious mutations.

We also segregated mutations present in the 16 diploid lines at $t = 4800$, as well as $t = 3100$ for 12 diploid lines with large mutations at $t = 4800$. The 16 diploid lines accumulated eight lethal mutations over the course of the experiment (Table 2). Four lethal mutations appeared in the first 3100 generations and four more in the last 1700 generations. Fitness assays further revealed nine nonlethal large-effect mutations accumulated in the first 3100 generations and eight more in the last 1700 generations. Line F5 had two clear mutations at generation 3100 but too many mutations to observe fitness classes by generation 4800; we attribute line F5 one more mutation, for a total of nine in the second period. Finally, significant segregant effects were present in five lines by generation 3100 and five more lines by generation 4800 (Table 2). Figure 4 compares large mutations in diploid vs. haploid lines (Table 2 and Table 3).

Summing 8 lethal mutations, 18 nonlethal large-effect mutations, and 10 segregant effects together, we obtain a rate of $(8 + 18 + 10)/(4800 \times 16 \times 2) = 2.34 \times 10^{-4}$ mutations per haploid genome replication in the diploid lines. The number of mutations accumulated in haploid and diploid lines is summarized in Table 3. Late mutations did not have a stronger effect than earlier ones (Wilcoxon-Kruskal-Wallis test, $Z = -0.23$, $P = 0.8$) (Figure 4B). Finally, no conditionally lethal mutations were found when tetrads from diploid lines were replica plated on minimal medium in which metabolites must be synthesized *de novo*.

We analyzed rates of lethal and large-effect mutations in haploid and diploid lines using a likelihood model that assumes that mutations are independently drawn from a Poisson distribution (data in Table 3). We compared several models constraining mutation rates between time periods and ploidy levels (Table 4). The best model [*i.e.*, with the lowest Akaike information criterion (AIC)] indicates a basal mutation rate $\mu = 1.1 \times 10^{-4}$ (support limits 0.7×10^{-4} to 1.6×10^{-4}), which is the same in haploids and diploids but increases in the second period by a factor $f = 2.1$ (support limits 1.2–3.7). This strong period effect indicates that the observed mutation rate per haploid genome replication during the last 1700 generations was about twice as high as during the first 3100 generations in both haploid and diploid lines. Furthermore, the distribution of the number of mutations per line shows no underlying heterogeneity among lines for the

Table 3 Counts and rate of occurrence (per generation per haploid genome replication) of lethal, large-effect, and small-effect mutations accumulated during the first (from 0 to 3100 generations) and second (from 3100 to 4800 generations) period of mutation accumulation

Ploidy (number of haploid genomes)	Mutation type Time period	Lethal		Large effect		Total
		First	Second	First	Second	
Haploid (32)	Count	0	0	7 + 2.5 ^a	10 + 2.5 ^a	17 + 5 ^b
	Rate ($\times 10^{-5}$)	—	—	9.6	23.0	18.9
Diploid (16 $\times$ 2)	Count	4	4	9.0	8 + 1 ^c	36.0
	Rate ($\times 10^{-5}$)	4.0	7.4	9.1	16.5	23.4

^a +2.5 is for line C5, for which mutations were numerous but could not be dated; we assume that 2 or 3 (hence the notation 2.5) mutations accumulated in each period.

^b +5 counts five mutations in C5.

^c F5 had two clear mutations at generation 3100, but too many mutations to observe fitness classes by generation 4800; we conservatively assume that this was due to one extra mutation.

Table 4 Model selection for the overall haploid mutation rate μ , acceleration factor f , and ploidy effect on mutation rate d applied to large-effect mutations assuming a Poisson distribution of the number of mutations

{Haploids 1, haploids 2}, {diploids 1, diploids 2}	Number of parameters	Deviance	AIC
$\{\mu, \mu\}, \{\mu, \mu\}$	1	21.3	23.3
$\{\mu, \mu\}, \{d \mu, d \mu\}$	2	21.0	25.0
$\{\mu, f \mu\}, \{\mu, f \mu\}$	2	15.1	19.1
$\{\mu, f_1 \mu\}, \{\mu, f_2 \mu\}$	3	15.1	21.1
$\{\mu, f \mu\}, \{d \mu, f d \mu\}$	3	14.8	20.8
$\{\mu_1, \mu_2\}, \{\mu_3, \mu_4\}$	4	14.6	22.6

Parameters are shown as {haploids first period, haploids second period}, {diploids first period, diploids second period}. The model with the lowest AIC is shown in bold.

mutation rate (Table 5). Assuming that the mutation rate is gamma distributed among strains, and computing the likelihood of the data with a gamma-Poisson mixture, we found no significant heterogeneity among strains for both haploid and diploid lines and no trend indicating higher heterogeneity in mutation rates in the second period of the experiment.

We studied epistasis in lines with two segregating mutations of large effect by comparing the observed fitness of double mutants with their expected fitness if single mutants had multiplicative fitness effects (Figure 5). Five pairs showed synergistic epistasis, of which two were from diploid lines, two were from haploid lines with decelerating fitness decline, and the last pair was from a haploid line with accelerating fitness decline. Thus, accelerating fitness decline is not due to synergistic epistasis in these lines. Lastly, three of six pairs of mutations from diploid lines showed antagonistic epistasis.

Mutation rates to canavanine resistance

We tested whether genetic mutation rates were higher for parental genotypes from $t = 4800$ generations (carrying the evolved mitochondrial genomes) than for the ancestor. To do this, we estimated mutation rates for a subset of haploid genotypes isolated from the MA lines using the p_0 method (Rosche and Foster 2000). We found that low-fitness strains have a lower mutation rate than do strains with fitness similar to the ancestor (Wilcoxon-Kruskal-Wallis test comparing

strains with fitness higher vs. lower than 0.95, $Z = -3.8$, $P < 0.0001$) (Figure 6).

Discussion

Our aims were to test whether accelerating fitness decline in MA lines of yeast was due to synergistic epistasis among deleterious mutations and, more broadly, to estimate selection coefficients and epistatic interactions among deleterious mutations in haploid and diploid MA lines. We found no association between synergistic epistasis and an accelerating fitness decline in MA lines (Table 1 and Figure 5). Synergistic epistasis in haploid lines was rare and present in both lines with decelerating or accelerating fitness decline. Consequently, we conclude that these MA lines do not support synergistic epistasis among deleterious mutations, which has now become rarer (but see Tong *et al.* 2004; Constanzo *et al.* 2010; Szappanos *et al.* 2011; Jakubowska and Korona 2012). Later we discuss our estimates of lethal and other large-effect mutation rates, as well as possible causes of accelerating fitness loss and MA in these lines.

The second common explanation for accelerating fitness decline in MA lines is that mutation rates increase over time owing to the appearance of mutator alleles (Ávila *et al.* 2006). We tested this possibility using a fluctuation test and observed a positive association between mutation rates to canavanine resistance and fitness in haploid lines (Figure 6), the opposite of what is expected if mutators explain accelerating fitness decline. This pattern probably results from fit strains having a higher basal tolerance to canavanine than loaded strains. Nevertheless, the threefold difference in mutation rates observed between fit and unfit strains makes mutator alleles unlikely in these lines. Mutator alleles are also unlikely because large-effect mutations were evenly (Poisson) distributed among lines, without any indication that mutation rates toward large-effect mutations were heterogeneous among strains or that heterogeneity increased over time (Table 5). Moreover, at least 7 of 32 haploid lines showed a clear acceleration of fitness decline (Figure 1 and Table 1). If we assume that 524 mutations accumulated in the 32 haploid lines (16.4 nonsynonymous or intergenic base substitutions, indels, or large-scale changes per line, on average; Lynch *et al.* 2008)

Table 5 Mutation-rate heterogeneity among haploid and diploid lines for the first and second periods of the MA experiment

Ploidy	Period	Number of strains with k new mutations				ν	Support limits	LRT P -value
		0	1	2	3			
Haploid	First	26	3	2.5 ^a	0.5 ^a	0.22	0–5.3	0.10
	Second	24	4	3.5 ^a	0.5 ^a	0.22	0–3.4	0.17
Diploid	First	8	4	3	1	0.13	0–3.8	0.72
	Second	8	3 + 1 ^b	3	1	0.13	0–3.8	0.72

Mutation-rate heterogeneity was estimated by maximum likelihood, assuming that mutation rates are gamma distributed among strains and that the number of observed mutations per strain is Poisson distributed (gamma-Poisson mixture). The parameter ν corresponds to the variance of the gamma distribution aforementioned, with support limits within two likelihood units. LRT P -value refers to the likelihood-ratio test (LRT) given the null hypothesis $\nu = 0$ (i.e., no rate heterogeneity among strains). No ν estimates are significantly different from zero. Data as in Table 3, compiling the number of strains having 0, 1, 2, or 3 large mutations in either period.

^a C5 strain in haploids was assumed to carry either two or three mutations in each period, and the likelihood was computed taking this uncertainty into account.

^b +1 is for line F5.

Figure 5 Observed fitness of double mutants (W_{ab}) vs. their expectation in a multiplicative model ($W_{Ab}W_{aB}$). Circles are mutations from diploid lines, and triangles are from haploid lines showing accelerating (filled) or decelerating (empty) fitness decline during MA. The dashed line indicates additive mutational effects, and mutants above and below that line, respectively, show antagonistic and synergistic epistasis. Error bars, 95% CIs.

and that 0.7% of these mutations can increase mutation rates [a conservative estimate obtained for whole-gene deletions by Huang *et al.* (2003)], then the probability of gaining more than seven mutations that increase mutation rates is only 0.033 (with binomial sampling). Finally, one of the four haploid lines sequenced by Lynch *et al.* (2008) showed an accelerating fitness loss but did not accumulate more base substitutions than two lines showing little fitness change (and thus not analyzed here) and one line with decelerating fitness loss. Thus, the presence of mutators should be rejected as an explanation for the acceleration of fitness loss in most lines.

Mutation rates also can increase in MA lines without the accumulation of mutator alleles. Previous experiments with *Drosophila* have shown that genotypes in poor physiologic condition (because of deleterious mutations) accumulate new deleterious mutations at higher rates than do wild-type genotypes (Sharp and Agrawal 2012). In this case, increased genetic mutation rates can be interpreted as a pleiotropic effect of mutations altering condition. This hypothesis would be consistent with an accelerating fitness decline in Dickinson's haploid lines if those lines that accumulated one or more large-effect mutations (potentially altering condition) during the first 3100 generations also accumulated an even greater load during the last 1700 generations. However, none of the haploid lines that contribute to the accelerating fitness decline has accumulated a large-effect mutation in the first 3100 generations of MA (Figure 1 and Table 1).

Another possibility—besides synergistic epistasis and elevated genetic mutation rates—is that mitochondrial mutations played a key role in the acceleration of fitness loss in these lines. Mitochondrial mutations are known to be pervasive in Dickinson's haploid lines (Lynch *et al.* 2008), and they

Figure 6 Relationship between mutation rates and competitive fitness for parental haploid clones from $t = 4800$ (circles) and for the haploid ancestors (square, with 95% CI).

could contribute to the acceleration of fitness loss if there is synergistic epistasis between nuclear and mitochondrial mutations (common in yeast; Zeyl *et al.* 2005; Paliwa *et al.* 2014) or if mitochondrial mutations are more likely to appear during the last 1700 generations of MA. Our experimental design precludes us from studying mitochondrial mutations directly because we experimentally removed mtDNA from the parental haploid genotypes prior to segregating their nuclear mutations (this was done to avoid heteroplasmy and vegetative segregation of mitochondrial mutations). Consequently, all segregants carry the ancestral mitochondrial genome. Nevertheless, we can gain some information about the fitness effects of mitochondrial mutations by comparing the fitness of a set of segregants to the fitness of their parent line at $t = 4800$. If a $t = 4800$ genotype carries mitochondrial mutations affecting fitness, we should be unable to find segregants (all carrying the mutation-free mitochondrial genome) with the same fitness as the $t = 4800$ parent. Indeed, we found that the least-fit segregants in haploid lines B4, C3, and D1 are fitter than their respective $t = 4800$ genotype, suggesting that these lines accumulated deleterious mitochondrial mutations. Critically, fitness loss decelerates in these three lines (Table 1). Thus, these putative deleterious mitochondrial mutations do not contribute to the accelerating fitness loss observed in the haploid lines.

Mitochondrial mutations also can affect MA by modifying nuclear mutation rates (Veatch *et al.* 2009). This could explain our results if mitochondrial mutations occurring during the first 3100 generations of MA increased nuclear mutation rates during the last 1700 generations. The direct fitness effect of these mitochondrial mutations would need to be small ($<1\%$) to be consistent with our results, but known mitochondrial modifiers increase nuclear mutation rates by altering major cell physiology components (Rasmussen *et al.* 2003; Veatch *et al.* 2009; Dirick *et al.* 2012) and are likely to have large fitness effects in our test environment (e.g., Zeyl

Table 6 Rates of recessive lethal mutations in *S. cerevisiae* expressed per haploid genome replication

Number of diploid MA lines	Diploid divisions	Haploid replications	Recessive lethals	Rate ($\times 10^{-5}$)	Reference
16	4800	153600	8	5.2	This study
1036	61	126392	19	15	Wloch <i>et al.</i> (2001) ^a
20	1740	69600	3	4.3	Nishant <i>et al.</i> (2010)
151	2062	622724	10	1.6	Zhu <i>et al.</i> (2014)
		972316	40	4.11	Total

The number of haploid replications is twice the product of the number of diploid lines by the number of diploid divisions in each line.

^a We pooled the two controls and calculated the average number of diploid division per line as (64 generations $\times$ 508 lines + 58 generations $\times$ 528 lines)/1036 lines = 61 generations. We also removed one lethal of 20 to account for random spore mortality, as discussed in Wloch *et al.* (2001).

and De Visser 2001). Moreover, only two haploid strains from $t = 4800$ (and thus carrying evolved mitochondrial genomes) have nuclear mutation rates potentially higher than the ancestor in our fluctuation tests (Figure 6), and neither of these two strains (A1 and B1; not dissected) showed a significant fitness loss. Consequently, we also reject mitochondrial mutators as an explanation for our results.

The acceleration of load accumulation (Figure 1A) (Dickinson 2008) and of the number of large-effect mutations accumulated in the haploid lines thus needs to be explained without evoking synergistic epistasis, mitochondrial mutations, and increased genetic mutation rates. A fourth possibility is that cryptic environmental effects increased mutation rates during the last 1700 generations of MA relative to the first 3100 generations. Cryptic environmental effects on mutation rates can be tested with the diploid lines because they shared the same environment (growth medium and incubator) as the haploid lines, and consequently, they too should have been affected. We indeed found that the rate of MA was 2.1 times greater in the last 1700 generations of MA in the diploid lines. This result provides further evidence against the presence of mutator alleles, which are generally recessive (Wloch *et al.* 2001) and should not have accelerated fitness loss in diploid lines. Cryptic environmental effects thus are a plausible explanation for the acceleration of fitness decline and MA of the haploid MA lines.

The nature of these potential cryptic environmental effects is unknown. Temperature is known to affect mutation rates; for example, Matsuba *et al.* (2012) reported a significant increase in deleterious mutation rates in MA lines of *Caenorhabditis briggsae* and *C. elegans* at 26° relative to 18°. In *S. cerevisiae*, reversion rates of an amber mutation at the *TRP1* locus were about twice higher at 30° than at 24° (Gralla and Valentine 1991). However, such large variations in incubation temperatures seem unlikely to have happened for months without notice during the MA experiment. A more likely source of environmental variation affecting mutation rates is the amount of time cells spent in a starved state [e.g., in *S. cerevisiae*, see Halas *et al.* (2002) and Achili *et al.* (2004)]. In microbial MA experiments, this is determined by the timing of transfers, which happened every 3–4 days in these MA lines (Dickinson 2008). However, the transfer cycles were performed at constant intervals throughout the MA experi-

ment (W. J. Dickinson, personal communication). A third possibility is that a single event [e.g., elevated temperature, a batch of medium contaminated by a mutagen, or freeze-thaw, as shown in Todorova *et al.* (2012)] during the last third of the MA experiment was solely responsible for the increased mutation rates.

Examples of cryptic environmental effects are easier to detect and have been reported more often for traits related to fitness than for mutation rates. For example, Hall *et al.* (2008) and Joseph and Hall (2004) found that seemingly identical experiments involving the same *S. cerevisiae* strains a few years apart gave wildly varying fitness estimates: the environmental variance for fitness was approximately four times higher in the 2008 study compared to the 2004 study. Such effects are also illustrated by strong day (or block) or day $\times$ genotype interaction effects in statistical models analyzing variation in fitness in microbial competition experiments (Ostrowski *et al.* 2005; Gerstein *et al.* 2006; Spor *et al.* 2008, 2014; this study). Unfortunately, the role of cryptic environmental effects on mutation rates cannot be tested directly with current data.

Instead of revealing the role of epistasis for the shape of fitness decline in MA lines, our study highlights the importance of mutations with large fitness effects for the mutational load in MA experiments. Indeed, we found that nearly all the variation in fitness among segregants from diploid and haploid lines was explained by one or two large-effect mutations, which implies that variance in fitness among lines is largely due to variance in mutational effects. This is expected when lines are propagated for a short period of time (Wloch *et al.* 2001) but is perhaps more surprising in long-term lines such as Dickinson's [although for similar results, see also Caballero *et al.* (2002), Halligan *et al.* (2003), Estes *et al.* (2004), Heilbron *et al.* (2014), and Katju *et al.* (2015)].

We observed a high rate of lethal mutations in diploid lines, which yields a bimodal distribution of mutation effect in those lines (but not in the haploid lines) (Figure 4). Previous studies have similarly reported a bimodal distribution of fitness effects, one mode for lethal and the other for nonlethal mutations (Mukai *et al.* 1972; Sanjuán and Elena 2004). The available rates of recessive lethal mutations in *S. cerevisiae* lie in a 10-fold range (ours being intermediate) and provide an overall estimate of 4.1×10^{-5} recessive lethal mutations

per haploid genome replication (Table 6). In comparison, rates of recessive lethal mutations of 1% per haploid genome division have been reported in *Drosophila melanogaster* (Crow and Temin 1964), about 250 times higher than our estimate in yeast. Considering the 10-fold difference in genome size between these two organisms (120 vs. 12 Mb), this leaves a 25-fold excess of recessive lethal mutations in *Drosophila*. This difference vanishes if we consider that there are ~25 germ-line cell divisions per generation in *Drosophila* (Drost and Lee 1995). Despite considerable differences in developmental biology, genome architecture, and mating systems between these two organisms, it thus appears that they have quite similar rates of recessive lethal mutations per base-pair replication. The rate of recessive lethal mutations also can be compared to the rate of nonlethal mutations detected in the diploid lines. We obtained 8 lethal vs. 28 nonlethal mutations (22%, summing both large and small mutations). This figure is similar to the 29% value obtained by Wloch *et al.* (2001), who also found 42% lethal mutations among deleterious mutations from mutagenized populations. However, ~25% lethal mutations seems very high when considering that the proportion of essential ORFs in *S. cerevisiae* is 18% (Dowell *et al.* 2010). The proportion of lethal mutations among all ORF deletions is not a good comparison for our estimate because it includes deletions having no effect on fitness, whereas our experiments focus on deleterious mutations. If, instead, we calculate the number of essential genes relative to the number of ORFs affecting fitness [*i.e.*, the number of lethals/(lethal + deleterious)], we find that 45% of ORF deletions affecting fitness are lethal ($W \leq 0.99$ was used as the cutoff for deleterious mutations; we get 49% if $W \leq 0.98$ is used instead) (data from Deutschbauer *et al.* 2005). This suggests that ORF deletions are lethal about twice as often as random mutations affecting fitness. Nevertheless, a very large number of mutations with small effects were missed here and in the study by Wloch *et al.* (2001) (see also Thatcher *et al.* 1998), and these would decrease the proportion of lethal mutations among deleterious mutations in MA experiments. Unfortunately, there are currently no estimates of the rate of deleterious mutations with $s < 0.01$, which would provide a better estimate of the proportion of lethal mutations among random mutations.

Environmental effects and unwanted evolution in controls were obvious problems in MA experiments carried out with organisms such as *Drosophila*, which cannot be preserved free of evolution, *e.g.*, frozen (Keightley 1996; Fry *et al.* 1999). Such organisms make it difficult to obtain an appropriate control for the lines experiencing Muller's ratchet. This issue was thought to be resolved by working with microorganisms and roundworms that can be cryopreserved. Our study shows that microbial mutation rates might be more vulnerable to slight environmental variation than previously assumed, as revealed recently in the bacterium *Escherichia coli* (*e.g.*, Krasovec *et al.* 2014). This idea will need to be tested with specific MA lines.

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Accelerating Mutational Load Is Not Due to Synergistic Epistasis or Mutator Alleles in Mutation Accumulation Lines of Yeast

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Figure S1. Variation in fitness among segregants in haploid lines C7 (A) and C5 (B) as shown in replicate assays. Fitness is equal between replicates on the dashed line.