**File S1 (Supplemental Material, Kozela and Johnston)**

1. **Estimation of Cell Number (Population Size) and Number of Generations from Colony Size During MA**

We used colony area as a proxy for population number by deriving the empirical relationship between the two for the ancestral genotype. This was used as an initial guide for tracking the approximate generation number during the course of MA. We could not rule out that the MA treatments themselves may have affected the relationship between the two with the possible result that one treatment would undergo many more divisions than the other and thus skewing our estimates of mutation parameters. We therefore wished to directly address the effect of the mutation accumulation conditions we used in our experiment on the derived relationship between colony area and population size. We generated 6 additional lines from the frozen ancestral population and cultured three on YPD and three on 1.0 M NaCl YPD in an identical fashion to our main MA experiment. At different time points, we measured colonies at the relevant sizes picked in our main MA experiment. One group of colonies was chosen in the early phase of the experiment while a second group of colonies was chosen in a later phase, in an attempt to detect any effect with a significant latency.

After a colony was haphazardly chosen and re-streaked on a fresh plate to continue the MA procedure, additional colonies from the same plate were chosen for analysis. These colonies were measured for colony area from images taken using a CCD camera (COHU 4915-2000, Cohu Inc., USA) mounted on a stereo dissection microscope (Olympus SZH-ILLK, Olympus Optical Co. Ltd., Japan)). The photographed colonies were subsequently excised from the agar with minimal disruption of the colony and placed in 100μl of fixative (3.7% formaldehyde in PBS). Colonies were stored at 4°C until cell counts were taken. Just prior to counting, samples were briefly sonicated to disrupt cell clumps. We used the Countess® automated cell counter (Invitrogen) to count cells in these excised colonies according to the manufacturer’s instructions. Accurate readings from this device depend on the concentration of cells in a given sample. Large colonies that contained cell numbers beyond the reliable limit of the machine were diluted by an appropriate factor (10X or 100X). Small colonies that contained numbers of cells below the reliability limit of the machine were concentrated by centrifugation and resuspension in one-fourth the volume, prior to counting. We plotted cell number against colony area for these colonies and fitted curves of several different forms to establish the relationship between the two measures. We used randomization tests to test for differences in the coefficients of each term for various polynomials between YPD and high salt treatments. No significant differences were detected between MA treatments. We therefore pooled our data together in order to determine a single relationship between colony area and cell number. We ultimately choose to use a quartic function fit through zero because it accounted for a high proportion of the variability (*R2*=0.93) and had no discernible pattern upon visual inspection of residual values. The relationship was determined to be:

Cell Number = 2.3×106Area – 4.6×105Area2 + 1.1×107Area3 – 3.3×106Area4

This relationship was then used to derive colony population sizes for colonies we transferred during the main MA experiment. Colony population values during MA were then used to estimate population parameters including generation number and effective population size during the main experiment.

1. **Procedures for sporulation, haploid isolation and scoring mating types**

Yeast were sporulated for two different purposes necessitating two variants of the sporulation procedure. The first was used in the isolation of our haploid strains. The second was used in our assessment of sporulation as a fitness measure and is described in S3. For haploid isolation, we inoculated 3 ml of presporulation medium (1% yeast extract, 2% peptone, 1% potassium acetate, 0.05% dextrose) from frozen diploid stocks and incubated cultures for 3 days shaking in the dark at room temperature. Cells were centrifuged at 1,500 g for 5 min, washed with an equal volume of sterile distilled water, centrifuged again, and resuspended in 3 ml of sporulation medium (1% yeast extract, 1% potassium acetate). Cells were returned to the shaker for three days prior to tetrad dissection. After incubation, 0.1 ml of sporulated culture was spread in a narrow streak on a 2% peptone, 1% dextrose agar slab. Tetrads were identified by microscopic examination and haploids were separated using a micromanipulator. We picked five tetrads per MA line. After dissection, the slab was transferred to a fresh YPD plate, incubated at 27°C until colonies appeared and then photographed for later scoring of haploid viability. To confirm that our dissections produced haploids, we scored mating types of our newly isolated strains. Confirmed haploids were grown in replicate 0.75ml cultures at 27C to which was added an equal volume of 40% glycerol. Cultures were then frozen at -80C for later fitness assays.

Alongside the sporulation and dissection of a single or pair of MA lines from each treatment we performed the same procedure on the ancestral genotype. This was performed for two reasons. First, our ancestral strain may have contained some amount of heterozygosity. This variation could be expressed among our MA lines but will be treated as residual variation that is present in the ancestor. Second, due to the labor involved in haploid isolation, we could only isolate a few strains at a time. Therefore, environmental differences from one episode of haploid isolation to another could have confounded effects we might otherwise ascribe to mutation. By culturing lines from each treatment along with the ancestor in parallel, we reduced the effects of different isolation environments on our subsequent analyses.

We scored the mating type of our MA line-derived haploids first to confirm that they were in fact haploid and second to check for errors in our dissection technique. We spread a lawn of one of a pair of *S. cerevisiae* tester strains, XBH8(mat α) and J4383(mat a) across each of a pair of YPD petri plates. We then spot arrayed our MA-derived haploid strains in the same approximate position on both the α-plate and the a-plate. Haploid strains will inhibit the mitotic growth of the opposite mating type. After incubation at 27° C, the relatively high-density inoculum of our unknown MA strain spot should produce a clear zone of inhibition on the plate bearing the opposite mating type but no clearing zone on the plate containing the same mating type. This allows us to gain some information on the relative success of our haploid isolation. For example, it is possible that diploids could unintentionally be isolated from the mixed-ploidy sporulation culture. If this were the case, no zone of inhibition would be observed on either test plate. This was only observed in one case where all four products of a dissection showed no inhibition of both tester strains. These four strains were excluded from further study. All other tetrad products from the same diploid MA line behaved normally producing both ‘α’ and ‘a’ haploid types. Mating types did not differ in any fitness component.

This screening procedure also controlled for a second kind of error during haploid isolation. It is possible that haploids other than the four products of a single dissection could have been inadvertently spotted on the agar slab. These would produce a zone of inhibition on one of the pair of test plates. If this kind of contamination were common, we would expect to see frequent deviations from the expected ratio of 2:2 mating types found when all four products were recovered or other unusual ratios e.g., 3:0 when only three products were recovered. We found zero cases of unusual segregation ratios of mating type in all our dissections.

1. **Fitness Assay Procedures: Diploid and Haploid Growth Curve Analysis, Including Corrections for Growth Rate and Sporulation Rate Environmental Effects.**

The large numbers of strains involved in this study combined with the generally small expected effect sizes, required the use of large sample sizes. This led us to use microplate formats for culturing yeast whenever possible. We devised a series of randomized well arrangements such that a pair of 96-well plates contained a complete set of surviving MA lines (approximately 90 lines) along with 20 replicate ancestral cultures. Each plate within a pair thus contained 25 lines from each treatment and 10 ancestral replicates for a total of 60 cultures per plate randomly assigned to the 60 positions within the interior of a plate (edge wells not used because of growth differences). Five separate plate-pair randomizations were used (detailed procedures in Kozela 2012).

**Diploid Growth Rate:**

For diploid maximal growth rate, OD was measured at set intervals over a 48-hr period in 96-well plates at 28±0.5° with shaking. The average value of the yeast-free wells at the plate margins at each time period was subtracted from each well at the corresponding times. Growth rates were estimated by finding the slope of the log2-transformed OD values. We used a sliding-window procedure to find the region of maximal growth for each curve, where the window size chosen minimized the between-replicate variance (Hill and Otto 2007). In our experiment this corresponded to the growth rate in 7-hr windows. For diploids, two complete sets of measurements were taken, each consisting of the same five plate-pair randomizations (20 plates total) resulting in a total of 10 point estimates for each extant MA line and 200 estimates for the ancestor. We found minor positional patterns within plates as well as an effect of individual plates on ancestral genotype growth rates. We used this information to remove the effects of these factors on the measurements for each MA line estimate, reducing the environmental variation in the data. Variance components were calculated using restricted maximum likelihood (REML) in JMP 9.0.2 statistical software (SAS Institute Inc.).

 For sporulation as a fitness measure we used a modified protocol similar to that which was used during haploid isolation except that cultures were manipulated in 96-well plates. We used the same set of MA line-well arrangements as used for the diploid growth rate estimates. We inoculated plates containing 150 μl of presporulation medium and incubated them at room temperature for 48 hr before cells were washed with sterile distilled H2O and resuspended in 150 μl sporulation medium. Culture densities were measured by spectrophotometry and incubated at room temperature for 48 hr with shaking. Cells were then observed and photographed using an inverted compound microscope. Sporulation rates were obtained by counting a minimum of 200 cells per replicate and scoring tetrads versus unsporulated cells. All counts were conducted in a blind fashion, with the line identity unknown to the counter. This was repeated four times each with a different set of line-well arrangements giving a total of 100 ancestral sporulation rate estimates and five estimates for each MA line.

All analyses to remove environmental effects were performed using JMP 9.0.2 (SAS Institute Inc.) statistical software. We used the ancestral genotype that was replicated 10 times on each assay plate as the standard with which to investigate environmental effects during our assays. We investigated several factors, including position within a plate, individual plate, year (growth rate only) and culture density (sporulation only). To investigate plate position, we created a coordinate system that allowed us to assign a numerical position to every well on a plate. For culture density, we used OD measurements taken after the presporulation cultures had been transferred to sporulation medium. We investigated a series of models using ANOVA to determine which factors significantly affected the fitness of the ancestor.

 We found that the following model best described the growth of the ancestor:

Growth Rate = x + y + x2 + plate + error

 We found the following model best described sporulation in the ancestor:

Sporulation = plate + optical density + error

We then asked JMP to calculate a predicted value for each well in each experiment based on these models. We then subtracted these predicted values from the actual values obtained in our replicate fitness measurements to arrive at relative fitness values corrected for environmental effects.

**Haploid Growth Rate:**

We used procedures described above for diploids except that haploids derived from each possible diploid parent were included on every microplate and measurements were taken every 10 minutes over a 48-hour period. Either eight or 10 haploid strains derived from the ancestral genotype were also included, depending on the MA set combination on a given plate. Haploid strains were recovered from frozen culture and arrayed randomly in a 96-well microplate containing YPD agar. These agar plates were incubated at room temperature in the dark for 3 days prior to inoculation of the assay plates. Cells were transferred between microplates using a flame-sterilized 96-pin apparatus. We used an intermediate dilution step to prevent high initial OD readings resulting from the transfer of excessive numbers of cells by the pin apparatus directly from some agar-supported cultures to liquid. Specifically, cells were transferred from the agar plate to a sterilized, custom-made aluminum 96-well plate containing 150 μl of YPD broth. From this plate, cells were transferred to a 96-well cell culture assay plate using the pin apparatus. Two separate dips of the apparatus were made from the dilution plate to the assay plate to ensure an adequate inoculum.