Chance and necessity in the pleiotropic consequences of adaptation for budding yeast

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Mutations that a population accumulates during evolution in one ‘home’ environment may cause fitness gains or losses in other environments. Such pleiotropic fitness effects determine the evolutionary fate of the population in variable environments and can lead to ecological specialization. It is unclear how the pleiotropic outcomes of evolution are shaped by the intrinsic randomness of the evolutionary process and by the deterministic variation in selection pressures across environments. Here, to address this question, we evolved 20 replicate populations of the yeast *Saccharomyces cerevisiae* in 11 laboratory environments and measured their fitness across multiple conditions. We found that evolution led to diverse pleiotropic fitness gains and losses, driven by multiple types of mutations. Approximately 60% of this variation is explained by the home environment of a clone and the most common parallel genetic changes, whereas about 40% is attributed to the stochastic accumulation of mutations whose pleiotropic effects are unpredictable. Although populations are typically specialized to their home environment, generalists also evolved in almost all of the conditions. Our results suggest that the mutations that accumulate during evolution incur a variety of pleiotropic costs and benefits with different probabilities. Thus, whether a population evolves towards a specialist or a generalist phenotype is heavily influenced by chance.

To explain widespread ecological specialization and local adaptation in nature, pleiotropy was originally assumed to be mostly antagonistic, such that fitness benefits in one environment must come at the cost in others16–19. However, recent field studies have found that locally adaptive alleles confer pleiotropic fitness defects much less frequently than anticipated12–14,18. Although ecological specialization and local adaptation can arise without trade-offs1–5,13–16, it is also possible that field studies provide a skewed view of the structure of pleiotropy owing to statistical complications and confounding factors, such as migration and unknown environmental variation13–17.

Laboratory microbial and viral populations are powerful model systems in which the structure of pleiotropy can be investigated under controlled conditions and with a degree of replication seldom achievable in natural systems19–31 (reviewed recently in refs. 13,14,21). Experimental studies generally support the conclusions from the field that fitness trade-offs exist12–15,20–25,30–34,41–44,46 but are not ubiquitous29,31,33,36,45,41,43,44. However, why generalists or specialists evolve in different evolution experiments is not entirely clear13,15,16. One possibility is that adaptation to each home environment leads to the accumulation of mutations that have typical, home-environment-dependent pleiotropic fitness effects, such that the pleiotropic outcomes of evolution depend primarily on the differences in selection pressures between environments16–18. The set of home and non-home environments then determines whether specialists or generalists evolve in each specific case.

It is also possible that chance events have an important role13,15,16. As independently evolving populations stochastically acquire different sets of mutations that could have dramatically different pleiotropic effects19, even populations evolving in the same condition may reach pleiotropically different states. Thus, in addition to differences in selection pressures between environments, random chance may determine whether a population evolves towards a specialist or a generalist phenotype.

Disentangling and quantifying the contributions of selection and chance to pleiotropy requires observing evolution in many replicate populations and measuring their fitness in many other conditions. To this end, we evolved populations of the yeast *S. cerevisiae* in a variety of laboratory environments, sequenced their full genomes and measured the fitness of the evolved clones in multiple panels of non-home conditions. To quantify the contribution of natural selection and evolutionary stochasticity to pleiotropy, we estimated the variance in the pleiotropic fitness gains and losses explained by these two factors. We also examined how pleiotropic outcomes depend on the similarity between the new and the home environments.

Results

To investigate the pleiotropic consequences of adaptation, we experimentally evolved 20 replicate *S. cerevisiae* populations in 11 different laboratory environments (a total of 220 populations). Each
population was founded from a single colony that was isolated from a common clonal stock of a laboratory strain. We chose the 11 laboratory environments to represent various degrees of several types of physiological stresses (such as osmotic stress and temperature stress). A complete list of all 11 evolution conditions, in addition to two conditions used only for assays, is provided in Table 1.

We evolved each population in batch culture at an effective size of about $N_e \approx 2 \times 10^5$ for about 700 generations using our standard methods for laboratory evolution (see Methods). Seven populations were lost owing to pipetting errors during evolution, leaving a total of 213 evolved lines. We randomly selected a single clone from each evolved population for further analysis.

**Specialization is the typical outcome of adaptation.** To understand how adaptation to one home environment alters the fitness of the organism in other non-home environments, we measured the competitive fitness of each evolved clone relative to their common ancestor across multiple conditions (see Methods). We first focused on a diagnostic panel of eight conditions that represent different types of physiological stresses (see Table 1).

Figure 1 shows the median change in fitness of these clones across the eight diagnostic conditions. As expected, clones that evolved in all of the environments typically gained fitness in their home environment, although the magnitude of these gains varied between conditions (Fig. 1, diagonal entries). We quantified the degree of specialization as the average fraction of non-home environments (such as osmotic stress and temperature stress) to represent various degrees (for example, compare the populations that evolved at low temperature and high salt with idiosyncratic patterns of fitness variation across environments. To discriminate between these two possibilities, we quantified the variation in the patterns of pleiotropic fitness gains and losses. We calculated the pleiotropic profile of each clone—the eight-dimensional vector containing its fitness changes (relative to the ancestor) in the eight diagnostic environments. If clones isolated from the same home environment cluster together in this eight-dimensional space, it would indicate that evolution in this environment leaves a stereotypical pleiotropic signature. Lack of clustering would suggest that the patterns in the median pleiotropic profiles shown in Fig. 1 are driven by evolutionary stochasticity and idiosyncratic pleiotropy.

**Evolution leads to diverse but environment-specific pleiotropic outcomes.** The patterns of median fitness gains and losses shown in Fig. 1 may be driven by differences in selection pressure between environments, such that mutations acquired in different environments have systematically different pleiotropic effects in other conditions. Alternatively, these patterns could have arisen because different clones stochastically acquired different sets of mutations with idiosyncratic patterns of fitness variation across environments.

We conclude that adaptive evolution typically leads populations to specialize to their home environment, and the evolved specialists are typically able to resist invasions from populations that evolved elsewhere. As expected, the specific set of conditions in which an evolved population gains and loses fitness depends on the home environment of the population. One exception, which we discuss below, is the unexpected similarity between pleiotropic consequences of evolution in three apparently unrelated conditions—adaptation to high salt, pH 3 and pH 7.3 led to similar and large median fitness losses in synthetic complete medium (SC), galactose (Gal) and at low temperature.

**Methods.** Figure 1 (left) shows that, by this definition, populations that evolved in all of the environments typically specialized to various degrees (for example, compare the populations that evolved at pH 3 with the populations that evolved at low temperature).

For the long-term survival of an ecological specialist, its fitness in the home environment must be greater than the fitness of populations that evolved elsewhere. To test whether adaptation leads to a ‘resident’ population that is fitter than ‘invader’ populations that evolved elsewhere, we estimated the proportion of pairwise competitions between residents and invaders in which the resident wins (see Methods). We found that, in most home environments, an average resident is able to outcompete most or all invaders from other environments (Fig. 1, bottom). The exception to this rule was the pH3 environment, in which residents lost in more than half of competitions.

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**Table 1** Environmental conditions used in this study

| Environment | Evolution condition | Diagnostic | Salt | pH | Temp | Formulation |
|-------------|---------------------|------------|------|----|------|-------------|
| SC          | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Glu, 30°C |
| Low salt    | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Glu + 0.2 M NaCl, 30°C |
| Med salt    | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Glu + 0.4 M NaCl, 30°C |
| High salt   | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Glu + 0.8 M NaCl, 30°C |
| pH 3        | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Glu, buffered to pH3, 30°C |
| pH 3.8      | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Glu, buffered to pH3.8, 30°C |
| pH 6        | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Glu, buffered to pH6, 30°C |
| pH 7.3      | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Glu, buffered to pH7.3, 30°C |
| Low temp    | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Glu, 21°C |
| Med temp    | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Glu, 34°C |
| High temp   | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Glu, 37°C |
| Low Glu     | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 0.07% Glu, 30°C |
| Gal         | ✓                   | ✓          | ✓    | ✓  | ✓    | SC + 2% Gal, 30°C |

SC, synthetic complete medium; Med, medium; temp, temperature; Glu, glucose; Gal, galactose.
The t-SNE embedding reveals that there are two large and clearly separated clusters, both of which contain clones from all of the home environments (Fig. 2a). The main features that discriminate between the two clusters are the fitness in SC, Gal and at low temperature (Fig. 2b,d). Clones that belong to one cluster lost 10–40% in these conditions, whereas clones that belong to the other cluster did not (Fig. 2b,d). We refer to these two phenotypes as $V^-$ and $V^+$, respectively, for reasons that we describe in the section ‘The genetic basis of pleiotropic outcomes’.

Clones that evolved in different conditions are not distributed identically in the t-SNE space. First, clones from different home environments have different likelihoods of evolving the $V^-$ phenotype ($\chi^2, P = 6.8 \times 10^{-4}$), for example, high temperature versus Gal-evolved clones. In fact, this variation explains the large median fitness losses in the SC, Gal and low-temperature conditions that we observed in Fig. 1 (Extended Data Fig. 1). Second, within the large $V^+$ and $V^-$ clusters, clones from some environments form tight smaller clusters (for example, high-salt clones; Fig. 2a). More generally, 2.8 out of the 5 nearest neighbours of a typical clone are from the same environment, compared with 0.60 ± 0.12 under random permutation.

We next set out to quantify the extent to which the observed variation in pleiotropic profiles is explained by the deterministic differences in selection pressures between environments versus the intrinsic randomness of the evolutionary process. Using a nested linear model, we estimated the fraction of observed variance in fitness in each diagnostic environment that is attributed to the identity of the home environment of a clone and to measurement noise. We attribute the remaining unexplained variance to evolutionary stochasticity, that is, the fact that each clone acquired a unique set of mutations that have idiosyncratic pleiotropic effects. We found that the home environment accounts for 20–51% of the variance in fitness, depending on the diagnostic environment (Fig. 2c). Measurement noise accounts for less than 4% of variance, leaving 48–77% attributable to evolutionary stochasticity (Fig. 2c). If the status of a clone with respect to the $V^+/V^-$ phenotype becomes known (for example, after measuring its fitness at low temperature), the fraction of unexplained variance decreases to 16–70% (Fig. 2c).

Taked together, these observations show that the home environment leaves a distinct signature in the pleiotropic profile of a clone, such that clones that evolve in the same condition tend to be more similar to each other than clones that evolve in different conditions. However, these deterministic differences are generally less important than the randomness of the evolutionary process, accounting for on average 34% of the variance in pleiotropic outcomes, compared with 65% for stochastic effects.

The genetic basis of pleiotropic outcomes. Next, we sought to determine the genetic basis that underlies the diverse pleiotropic outcomes that we observed above using two approaches. First, we used DNA staining and flow cytometry (see Methods) to look for ploidy changes, because this is a common mode of adaptation in yeast47–51. Second, we sequenced the full genomes of the evolved clones. We performed these analyses on all 213 clones, that is, those evolved in the diagnostic conditions considered above as well as other intermediate-stress environments listed in Table 1. In 15 cases, sequencing failed at the library preparation stage or due to insufficient coverage, leaving us with 198 sequenced clones. Using standard bioinformatic methods for calling SNPs and small indels (see Methods), we identified a total of 1,925 de novo mutations. We note that, because our sequencing and analysis pipeline can result in false negatives (that is, certain mutations are difficult to confidently identify), our results represent a subset of all of the mutations in each sequenced clone.

Loss of killer virus causes the $V^-$ phenotype. We began by looking for the genetic differences between the $V^+$ and $V^-$ clones. We found no association between $V^+$ and $V^-$ phenotypes and ploidy or any of the mutations identified in the sequencing data. Instead, multiple lines of evidence demonstrate that the $V^-$ phenotype was caused by the loss of the yeast killer virus, a toxin–antitoxin system encoded by a ~2 kb cytoplasmic double-stranded RNA52–56 that was present in the ancestor of our experiment (and was retained in the $V^+$ clones). First, we found that both the ancestor and 7 out of 7 randomly selected $V^+$ clones displayed the killer-virus band in a gel electrophoresis assay (see Methods), whereas all of the 7 randomly selected $V^-$ clones did not (Fig. 3a). Second, we competed the evolved clones against the reference strain that was cured of the virus (see Methods). We performed this assay at low temperature because $V^-$ clones have the largest fitness defect in this condition in competitions against their direct virus-carrying ancestor (Fig. 2d). As expected, this severe fitness defect entirely disappeared in competitions against the cured ancestor (Fig. 3b). We obtained several additional pieces of evidence that support the conclusion that the loss of the killer virus is the cause of the $V^-$ phenotype (see Methods; Supplementary Figs. 1 and 2).

Our results suggest that the severe fitness defects in the SC, low temperature and Gal environments (Figs. 1 and 2d) are not due to an inherent growth disadvantage. Rather, $V^-$ clones suffer large losses of fitness in competitions against the virus-carrying ancestor because they succumb to the virus expressed by the ancestor. As a consequence, these fitness losses are frequency dependent (Extended Data Fig. 2); they are particularly severe in the SC, low temperature and Gal conditions probably because virus activity is higher in these conditions57. Nevertheless, virus loss evolved even in these environments (Fig. 2d). This initially puzzling observation could be explained if the virulence of the virus was lost first and resistance was lost second, after non-virulent genotypes dominated the population. In support of this explanation, we found that
some of the evolved clones have similar fitness relative to both the virus-carrying and virus-cured ancestors (Fig. 3b, horizontal lines), suggesting that they are resistant but non-virulent\(^5\)\(^8\). A recent study examining the co-evolution of yeast and its killer virus also reported such stepwise progression towards virus loss and showed that virus loss probably provides no fitness benefit to the host\(^5\)\(^9\).

Diversity at the genetic level underlies diversity of pleiotropic outcomes. We next looked for the genetic basis of the fine-scale phenotypic variation between clones that we observed in our t-SNE plot (Fig. 2a,b). We found that 35 out of 213 clones became diploid during evolution. Diploid clones evolved more often in some environments than in others (\(\chi^2, P = 1.3 \times 10^{-9}\)) and 24 out of 35 diploid clones retained the killer virus, whereas 11 lost the killer virus (Fig. 2b). Moreover, 13 V\(^+\) diploid clones that evolved in the low temperature and Gal conditions formed a small cluster in the t-SNE space (Fig. 2a, inverted triangles), suggesting that a change in ploidy—irrespective of where it evolved—leads to certain characteristic changes in the pleiotropic profile, perhaps in conjunction with other mutations.

We next used our full-genome sequencing data to call putatively beneficial SNPs and indels. We identified such mutations as non-synonymous, nonsense or frameshift changes within multi-hit genes, which we define here as genes that were mutated in four or more clones from the same home environment (see Methods; Fig. 4a). In total, we identified 176 such mutations in 42 multi-hit genes (Fig. 4a). Only three individual multi-hit genes (SIR3, HNM1 and PDE2) were significantly associated with one home environment (\(P < 0.01\), Bonferroni-corrected permutation test; see Methods). Mutations in other multi-hit genes arose in multiple home environments, but with significantly different frequencies (\(P < 10^{-4}\); see Methods; Fig. 4a).

To quantify the extent to which this genetic information improves our ability to statistically predict the fitness of a clone in a diagnostic
understand what determines whether a clone that evolved in one environment, we expanded the list of predictor variables in the nested linear model described in the previous section to include the presence or absence of multi-hit mutations shown in Fig. 4a and the ploidy status. We found that mutations in multi-hit genes account for 11–30% of the variance in pleiotropic effects (Fig. 4b), and all of the genetic factors combined account for 15–60% of variance. The top (~2 kb) band is consistent with the killer virus, and the bottom (~4 kb) band is consistent with the helper virus. Fitness of all of the evolved clones relative to the ancestor and to the ancestor cured of the killer virus. Classification of clones into V+ (blue) and V− (red) is based on the t-SNE plot shown in Fig. 2b.

Fitness trade-offs are not inevitable, but their frequency increases with dissimilarity between environments. We next sought to understand what determines whether a clone that evolved in one condition gains or loses fitness in another. Our hypothesis is that the pleiotropic outcomes depend on the dissimilarity between the test condition and the clone’s home environment\(^{35,42}\). As it is unclear how to measure similarity among conditions in our original diagnostic panel, we tested this hypothesis in three additional panels of
environments, in which yeast is exposed to different intensities of a particular type of physiological stress (salt, temperature and pH; see Table 1). To simplify interpretation, we restricted this analysis to V+ haploid clones (see Methods).

Consistent with results for the diagnostic panel (Fig. 1, Extended Data Fig. 1), clones typically gained more fitness in their home environment than clones that evolved in other conditions in the same panel (Fig. 5a–c). The mean fitness of a clone was lower in conditions that were more dissimilar to its home environment, consistent with our hypothesis. Higher moments of the distribution of pleiotropic outcomes might also depend on the similarity between conditions, but the patterns are less clear (Extended Data Fig. 3). The fact that clones that evolved at one extreme of a panel lost fitness on average at the other extreme suggests that there may be inherent physiological trade-offs between fitness in dissimilar environments. However, we found that many clones that evolved at one extreme of each panel actually gained fitness at the other extreme (Fig. 5d–f). The only exceptions were the clones that evolved in the more acidic environments—all of which lost fitness in the most basic conditions (Fig. 5b,e). However, some of the clones

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**Fig. 5 | Specialization across salt, pH and temperature panels of environments.** a–c, Average fitness of clones from each home environment (colours) across the following test conditions: salt concentration (a), pH (b) and temperature (c). The squares represent the fitness of individual clones. Note that in b, two measurements that fell below −10% are not displayed (one clone evolved at pH 6 and was measured at pH 3.8, and one clone evolved at pH 7.3 and was measured at pH 4.5). d–f, The fraction of clones from each home environment (colours) that gained fitness in the following test conditions: salt concentration (d), pH (e) and temperature (f). The error bars represent ±1 s.e. g–i, The fraction of clones from each home environment (salt concentration (g), pH (h) and temperature (i)) that gained fitness across all of the test conditions in the panel. The error bars represent ±1 s.e.
that evolved in the more basic environments gained fitness in the more acidic conditions, suggesting that mutations beneficial at both extremes are available. In fact, generalists—clones of which fitness increased across the entire panel—arose in almost all of the environments (Fig. 5g–i).

These results demonstrate that mutations exist that are beneficial across the entire range of environments that vary along one physicochemical axis. Thus, the trade-offs between fitness, even in the most dissimilar conditions along such axis, are not physiologically inevitable. To further corroborate this conclusion, we measured the correlation between the fitness of clones in pairs of environments in each panel (Extended Data Figs. 4–6). If fitness trade-offs between a pair of conditions were physiologically inevitable, we would expect a negative correlation between fitness measured in these conditions. Instead, we observed diverse and complex fitness covariation patterns, but there is a notable lack of strong negative correlations between clone fitness even in the most dissimilar pairs of environments. In conclusion, our results suggest that whether a population evolves towards being a specialist or a generalist depends on the specific set of mutations that it accumulates, that is, this outcome is largely stochastic.

Discussion

To assess how chance and necessity in evolution affect the fitness of an organism across multiple environments, we evolved populations of budding yeast in a variety of laboratory home conditions. We characterized each population by its pleiotropic profile—the vector of fitness gains and losses in an array of diagnostic environments. We found that a diverse set of pleiotropic profiles arose during evolution in all of the home conditions. Underlying this phenotypic diversity, we found a diversity of evolutionary outcomes at the genetic level. Nevertheless, home environments leave statistically distinct signatures in the genome that, in turn, lead to statistically distinct pleiotropic profiles for clones evolved in different conditions. We estimated that a clone's home environment and the set of most common genetic changes together explain about 60% of variance in the pleiotropic fitness gains and losses of the clone. The remaining ~40% are attributable to evolutionary stochasticity, that is, the accumulation of hitchhikers or rare beneficial variants of which pleiotropic effects are unpredictable.

Despite the fact that the pleiotropic outcomes of evolution in any individual population are to a large degree governed by chance, clear and repeatable patterns emerge when we consider ensembles of populations that evolved in the same home environment. On average, evolution leads to specialization such that pleiotropic fitness gains are typically smaller or turn into losses in environments that are less similar to the home environment (Fig. 5). The most obvious explanation for these patterns is that different environments exert different selection pressures on the organism, but variations in the spectra and rates of mutations across environments may also have a role36.

Our results help us to better understand the evolution of specialists and generalists, a long-standing problem in evolutionary ecology31,32,40. To explain the ubiquity of specialists, many models require physiological trade-offs or antagonistic pleiotropy34,38,42. By contrast, it has long been appreciated that fitness losses in non-home environments can arise without physiological trade-offs if the population accumulates mutations that are neutral in the home environment and deleterious elsewhere36,42. However, field and experimental studies to date do not clearly favour one model over another34,38,39,41,42.

To explain the existing data, Bono et al. recently proposed a model that unifies the antagonistic pleiotropy and mutation accumulation perspectives17. In their model, the fitness effects of mutations form a continuum, such that the mutations accumulated in the home environment may provide a range of pleiotropic fitness costs and benefits in a non-home condition (see figure 1 in ref. 17).

If mutations that incur pleiotropic costs are more common and/or more beneficial than those that provide pleiotropic benefits, the population will tend to lose fitness in the non-home condition and evolve into a specialist. Our results are consistent with this model. Moreover, they indicate that the probabilities of acquiring mutations with various pleiotropic effects depend on the similarity between conditions. As the physicochemical similarity between conditions declines, more mutations that are beneficial in one become deleterious in the other. As a result, populations are more likely to suffer pleiotropic fitness costs in conditions that are more dissimilar to the home environment.

Here we examined the statistics of pleiotropy among mutations that arose in populations of a particular size descended from one particular ancestral yeast genotype. These statistics probably depend on the population size, because populations of different size sample different sets of adaptive mutations42. Furthermore, different genotypes probably have access to beneficial mutations with different statistics of pleiotropy47. To understand these broader patterns, we need to know the joint distribution of the fitness effects of new mutations and how this distribution varies across genotypes.

Assuming that the structure of pleiotropy does not change substantially between closely related genotypes, our results suggest that longer periods of evolution in a constant environment should lead to further specialization, simply because pleiotropically costly mutations are more abundant and generalists have no advantage. In nature, most populations live in fluctuating environments in which generalist mutations are favoured by selection. Why then do ‘jacks of all traits’ not evolve? Our results suggest that generalist genotypes may not be physiologically impossible but are simply unlikely to evolve because mutations that are beneficial in increasingly larger sets of distinct conditions become exceedingly rare.

Methods

Experimental evolution. The S. cerevisiae strain yGIL104 (derived from W303, genotype MATα, URA3, leu2-3,112, trp1-1, CAN1, ade2-1, his3, bar1::ADE2 (ref. 62)) was used to found 220 populations for evolution. Each population was founded from a single colony that was picked from an agar plate. The populations were propagated in batch culture in 96-well polystyrene plates (Corning, VWR, 29445-154), with 12 μl of medium per well. Populations evolving in the same environment were grown in wells B2–B11 and E2–E11 on the same plate. Except for the Gal and low-glucose conditions, all medium contained 2% dextrose (BD, VWR, 90000-904), 0.67% Yeast Nitrogen Base (YNB) with nitrogen (Sunrise Science, 1501-500) and 0.2% Synthetic Complete Medium (SC; Sunrise Science, 1300-030). The Gal condition contained 2% Gal (Sigma-Aldrich, G0625) instead of dextrose, and the low-glucose condition contained 0.07% dextrose. Other conditions contained the following in addition to SC complete: low salt, 0.2 M sodium chloride; medium salt, 0.4 M sodium chloride; high salt, 0.8 M sodium chloride; pH 3, 0.035 M disodium phosphate and 0.032 M citric acid; pH 6, 0.064 M disodium phosphate and 0.0179 M citric acid; and pH 7.3, 0.0936 M disodium phosphate and 0.0032 M citric acid. Buffered medium was filter sterilized; all other media were autoclaved.

All of the populations were grown at 30 °C, except for the high-temperature lines (37 °C) and the low-temperature lines (room temperature (21 ± 0.5 °C)). In the SC, high-temperature, medium-salt, low-glucose, pH 3, pH 3.8 and pH 6 conditions, dilutions were carried out once every 24 h. In the Gal, low-temperature and high-salt conditions, dilutions were performed every 36 h. All dilutions were performed using a Biomek-FX pipetting robot (Beckman-Coulter). Before each transfer, cells were resuspended by shaking on a Titramax 100 orbital plate shaker at 1,200 r.p.m. for at least 1 min. In the pH 7.3 condition, dilutions were performed every 48 h. At each transfer, all of the populations were diluted 1:512 except for the low-glucose populations, which were diluted 1:64. This maintained a bottleneck size of about 10 cells in all of the conditions. Populations underwent approximately the following numbers of generations (doublings): SC, high temperature, medium salt: 820; low glucose: 730; pH 3, pH 3.8 and pH 6: 755; and high salt, Gal, low temperature: 612. Every seven transfers, populations were mixed with glycerol to a final concentration 25% (w/v) and stored at −80 °C. Each 96-well plate contained blank wells; no contamination of blank wells was observed during the evolution. Over the course of evolution, 7 populations were lost owing to pipetting errors, leaving 213 evolved lines.
To pick clones for further analysis, each final population was streaked onto SC-complete medium with 2% agar. One colony per population was picked, grown in 128 μl of SC at 30°C, mixed with 25% (v/v) glycerol and stored at −80°C.

**Competitive fitness assays.** We conducted flow-cytometry-based competitive fitness assays against yGIL104-1c, a fluorescently labelled derivative of the common ancestor, yGIL104. To construct the fluorescent reference strain, we amplified the HIS3MX6-ymCitrineM233I construct from genomic DNA of strain YK111 (courtesy of M. Muller, J. Koschwaszne and A. Murray, Department of Molecular and cellular Biology, Harvard University) using the primers oGW137 and oGW138 and integrating it into the his3 locus. The fitness effect of the fluorescent marker is less than 1% in all of the environments (Supplementary Fig. 5).

Fitness assays were conducted as described previously\(^7\). In brief, we grew all of the test strains and the reference strain from frozen stock in SC medium at 30°C. After 24 h, we diluted all of the lines into the assay environment for growth over a second generation of preconditioning. We then mixed the reference strain and the test strains 50/50. We monitored the relative numbers of the reference and test strain over 3 d in co-culture. We measured fitness as \( f = \frac{n_r}{n_t} \) where \( n_r \) is the number of generations between timepoints, \( n_t \) is the count of the test strain at the initial timepoint, \( n_r \) is the count of the test strain at the final timepoint, and \( n_r \) and \( n_t \) are the counts for the reference. Fitness gains and losses are reported per 700 generations of evolution.

**Library preparation and whole-genome sequencing.** Libraries were prepared for sequencing as described previously\(^7\). In brief, genomic DNA was extracted from each of the 213 clones using the PureLink Pro 96 Genomic Purification Kit (Life Technologies, K1821-04A) and quantified using the Qubit platform. The multiplexed sequencing library for the Illumina platform was prepared using the Nextera kit (Illumina, FC-121-1031 and FC-121-1012) and a modified version of the Illumina-recommended protocol\(^7\). Libraries were sequenced on a Nextera HiSeq 2500 in rapid-run mode with paired-end 150-bp reads.

**Nucleic acid staining for ploidy.** Clones were grown to saturation in YPD (2% dextrose, 2% peptone and 1% yeast extract). Saturated cultures were diluted 1:10 into 120 μl of sterile water in a 96-well plate. The plate was centrifuged and cultures were resuspended in 50 μl of fresh water. Then, 100 μl of ethanol was added to each well, and the wells were mixed slowly. Plates were incubated for 1 h at room temperature or overnight at 4°C. Cells were centrifuged, ethanol solution was removed and 65 μl RNAse solution added (2 mg ml\(^{-1}\) in 10 mM Tris-HCl, pH 8.0 and 15 mM NaCl). The samples were incubated at 37°C for 2 h. To stain, 65 μl of 300 nM SYTOX green (Thermo Fisher Scientific, S-34860) in 10 mM Tris-HCl, pH 3.8 (15) and medium salt (19), pH 3.8 (15) and high temperature (19), pH 3.8 (15) and medium salt (19).

**t-SNE and clustering analysis.** We used the sklearn.manifold.t-SNE class in the Python package scikit-learn 0.2, with 2 dimensions and perplexity 30, to project the eight-dimensional fitness vectors into a two-dimensional t-SNE space. We then used the sklearn.cluster.KMeans class to perform k-means clustering with k = 2 in the t-SNE space. We used this cluster assignment to call V+ and V− phenotypes. These clusters correspond to those that are identifiable visually in Fig. 2. The number of clones from each diagnostic environment was as follows: SC (19), high salt (20), high temperature (18), low Glu (17), Gal (18), low temperature (18), pH 3.17, pH 3.7 (18), pH 6.19, pH 3.8 (15) and medium salt (19).

**Specialization and competitiveness summary statistics.** To assess the degree of specialization of a clone, we counted the number of non-home environments in which the fitness of the clone relative to its ancestor was >2 s.e.m. below zero, Fig. 1 (left) shows the proportion of such conditions averaged over all of the clones from the same home environment. To assess the competitiveness of resident clones in their home environment relative to clones evolved elsewhere, we estimated the proportion of all of the clones evolved in other conditions with fitness lower than a randomly chosen resident clone (Fig. 1, bottom). For both statistics, we measured the proportion of all of the clones evolved in other conditions with fitness lower than a randomly chosen resident clone (Fig. 1, bottom). For both statistics, we measured 95% confidence intervals on the basis of a bootstrap over clones in each evolution environment.

**Nested linear models for analysis of variance.** To evaluate the fraction of the variance in pleiotropic effects attributable to the evolution condition versus stochastic evolutionary effects, we fit the following series of nested linear models for each of the diagnostic measurement conditions:

\[
Y_i = \alpha + \sum_j \beta_j E_j + \epsilon_i \quad (2)
\]

\[
Y_i = \alpha + \sum_j \beta_j E_j + \gamma V_j + \epsilon_i \quad (3)
\]

where \(Y_i\) is the fitness of clone \(i\); \(E_j\) = 1 if clone \(i\) evolved in environment \(j\) 0 otherwise; \(V_j = 1\) if clone \(i\) is \(V^+\), 0 otherwise; \(\epsilon_i\) is the measurement noise, \(\alpha, \beta_j, \gamma\) are the regression coefficients. Note that we excluded clones from their own home environment to focus on pleiotropic effects, as opposed to adaptation to the home condition. Note also that we restricted analysis to clones measured in all eight diagnostic conditions to maintain comparability between environments. We fit the models using the sklearn.linear_model.LinearRegression class in Python, and used the score method of this class to calculate \(R^2\).

Fig. 2e shows the partitioning of the total variance in \(Y_i\), as follows. We measured the variance due to measurement error as:

\[
V = \frac{1}{n} \sum_i \frac{V_i}{n_i} \quad (4)
\]

where \(n_i\) is the number of clones, \(n_i\) is the number of replicate measurements of each clone and \(V_i\) is the estimate of the variance across replicate fitness measurements of clone \(i\). We attribute the variance explained by model (2) to home environment. We attribute the variance not explained by model (2) but explained by model (3) to \(V^+/V^-\) phenotype. We attributed leftover variance not accounted for by model (3) and not attributed to measurement noise to additional stochastic effects, which we label other pleiotropy.
To evaluate the contributions of most common genetic factors to the pleiotropic effects (Fig. 4b), we performed an analogous analysis of variance using the following series of nested linear models:

\[ Y_i = a + \sum_j m_j M_j + e_i \]

(5)

\[ Y_i = a + \sum_j m_j M_j + 6D + e_i \]

(6)

\[ Y_i = a + \sum_j m_j M_j + 6D + y + V_i + e_i \]

(7)

\[ Y_i = a + \sum_j m_j M_j + 6D + y + V_i + \sum_j \beta E_j + e_i \]

(8)

where \( M_i = 1 \) if clone \( i \) has at least 1 mutation in biological process \( j \) of 10 biological processes shown in Fig. 4a (other ‘Other’); \( D = 1 \) if clone \( i \) is a diploid, 0 otherwise; \( e_i \) is a regression coefficient.

Extraction of double-stranded RNA and gel electrophoresis. Yeast cell pellets from 1.5 ml of an overnight culture were resuspended in 50 µl of a Zymolase-enzyme-based enzymatic digest to lyse the cells (5 mg/ml Zymolase 20T, 100 mM sodium phosphate buffer pH 7.4, 10 mM EDTA, 1 M sorbitol, 20 mM dithiothreitol, 200 µg/ml RNase A, 0.5% 3-(N,N-Dimethylglylylamino)propanesulfonate) and incubated at 37°C for 1 h. The spheroplasts were then lysed with 200 µl of lysis/binding buffer (4.125 M guanidine thiocyanate, 25% isopropanol, 100 mM MES pH 5). After vortexing briefly, the clear solution was passed through a standard silica column for DNA purification, and washed twice with 600 µl of wash buffer (20 mM Tris-Cl pH 7.4, 80% ethanol). After drying the column, the DNA and double-stranded RNA were eluted using a low-salt elution buffer (10 mM Tris-Cl pH 8.5).

Total extracted genomic material was processed for standard gel electrophoresis (1% agarose gel, Tris-acetate EDTA (TAE) running buffer, stained with 0.5 µg/ml ethidium bromide).

Curing the killer virus. Strain yGIL104-cit-V– was constructed from yGIL104-cit as follows. yGIL104-cit was grown from frozen stock overnight in YPD. A colony was grown in SC-Ura dropout medium with 2% sucrose overnight. Then, 1 ml of culture was centrifuged and resuspended in SG-Ura dropout medium (2% Gal) to induce. Cells were plated on SC-Leu dropout medium directly after transfer to SG-Ura and, 60 min later, colonies were streaked on SD complete + 5FOA to eliminate the plasmid. MATα versions of evolved lines were constructed using the same method. After mating, diploids were selected on CSM-Ura-His dropout medium. Diploids were sporulated in Spoo++ medium supplemented with 0.5% dextrose at 21°C for 3–5 days. Tetrad were dissected according to standard yeast genetics methods. Four-spore complete tetrad from each mating were grown in SC, which was mixed with glycerol to final concentration 25% and frozen at ~80°C. Fitness assays of four-spore complete tetrad from each mating, competed against yERJ104-cit, were conducted as described above at 21°C. We also constructed a mitochondrial-cured (p–) version of the reference and of the evolved lines; the fitness of spores from crosses involving these lines were not distinguishable from the corresponding p+ crosses, so spore fitness were pooled.

To determine whether the defect was caused by a mutation that we did not detect during sequencing, we back-crossed three evolved clones that displayed the characteristic large fitness defect at low temperature. A colony was grown in SC-Ura dropout medium with 2% sucrose overnight. Then, 1 ml of culture was centrifuged and resuspended in SG-Ura dropout medium (2% Gal) to induce. Cells were plated on SC-Leu dropout medium directly after transfer to SG-Ura and, 60 min later, colonies were streaked on SD complete + 5FOA to eliminate the plasmid. MATα versions of evolved lines were constructed using the same method. After mating, diploids were selected on CSM-Ura-His dropout medium. Diploids were sporulated in Spoo++ medium supplemented with 0.5% dextrose at 21°C for 3–5 days. Tetrad were dissected according to standard yeast genetics methods. Four-spore complete tetrad from each mating, competed against yERJ104-cit, were conducted as described above at 21°C. We also constructed a mitochondrial-cured (p–) version of the reference and of the evolved lines; the fitness of spores from crosses involving these lines were not distinguishable from the corresponding p+ crosses, so spore fitness were pooled.

For one mutation, in the gene CUE4, we used fitness relative to the original and cured ancestor to classify clones. The code used for analysis and figure generation is available at https://github.com/erjerison/pleiotropy.

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**Author contributions**

E.R.J., M.M.D. and S.K. designed the research. E.R.J., S.K. and A.N.N.B. performed experiments and analysis. E.R.J., M.M.D. and S.K. wrote the paper.

**Competing interests**

The authors declare no competing interests.

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**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s41559-020-1128-3.

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Extended Data Fig. 1 | Median fitness gains and losses, restricted to V⁺ clones. Median fitness gains and losses among groups of clones from the same home environment, excluding V⁻ clones. Notations as in Fig. 1.
Extended Data Fig. 2 | Frequency-dependence of competition between V⁺ and V⁻. Fitness of a V⁻ clone relative to the ancestor at Low Temp, initiated at different initial frequencies. The frequency dependence of the relative fitness suggests that the fitness defect might be caused by a direct interaction between the competitors. Error bars show ± 1 s.e.m.
Extended Data Fig. 3 | Variance in fitness across environmental panels. As in Fig. 5a–c, but variance in fitness across groups of clones rather than means. Error bars represent ± 1 standard error of the variance.
Extended Data Fig. 4 | Correlations between clone fitness in different salt conditions. Each panel below the diagonal shows clone fitness in a particular pair of environments. (Error bars: ± 1 s.e.m. on clone fitness.) The diagonal shows the correlation between technical replicates in the fitness assay in each condition. Panels above the diagonal are colored by and display the Pearson correlation coefficient between clone fitness in the corresponding pair of environments.
Extended Data Fig. 5 | Correlations between clone fitness in different pH conditions. Each panel below the diagonal shows clone fitness in a particular pair of environments. (Error bars: ±1 s.e.m. on clone fitness.) The diagonal shows the correlation between technical replicates in the fitness assay in each condition. Panels above the diagonal are colored by and display the Pearson correlation coefficient between clone fitness in the corresponding pair of environments.
Extended Data Fig. 6 | Correlations between clone fitness in different temperature conditions. Each panel below the diagonal shows clone fitness in a particular pair of environments. (Error bars: ± 1 s.e.m on clone fitness.) The diagonal shows the correlation between technical replicates in the fitness assay in each condition. Panels above the diagonal are colored by and display the Pearson correlation coefficient between clone fitness in the corresponding pair of environments.
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Software and code

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Data collection
BD Diva software was used for flow cytometry data collection.

Data analysis
FlowJo was used for the initial analysis of raw flow cytometry data. Custom software written using Python 3.6.7 was used for further data analysis. All data analysis code is available at https://github.com/erjerison/pleiotropy

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- **Sample size**: No sample size calculation was performed. The number of independent populations evolved per environment was fixed based on the maximum feasible number.
- **Data exclusions**: No data was excluded from analysis of fitness changes. Clones were excluded from sequence data analysis if they had less than 5x coverage.
- **Replication**: The experiment was carried out with n=20 independently evolved populations per environment. Fitness measurements were performed in triplicate.
- **Randomization**: All populations were founded from independently-picked clones from the same strain stock.
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**Methodology**

- **Sample preparation**: Flow cytometry was used to determine the relative abundance of two yeast populations: ymCitrine+ and ymCitrine-. (Note that flow cytometry was used for analysis only, not sorting.) No flow cytometry plots are shown in the manuscript (this is why boxes 1-4 are unchecked).
- **Instrument**: BD LSRFortessa, BD LSRII
- **Software**: BD Diva software was used on the instruments. FlowJo was used for analysis
- **Cell population abundance**: To estimate the fitness of a query strain relative to a reference strain, an initially 1:1 mixed culture of the two strains was subject to flow cytometry analysis at two time points separated by about 20 generations. At each time point, the relative abundance of
Two cell populations (query, ymCitrine−, and reference, ymCitrine+) was estimated based on typically 10 to 50 thousand events.

Gating strategy

Gating of the two populations was performed manually in FlowJo for each sample in a two-dimensional plot of SSC vs ymCitrine.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.