

Avoiding dead ends: the experimental evolution of constraint as adaptation to environmental variation in *Saccharomyces cerevisiae*

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Article

Keywords:

Posted Date: January 13th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1200104/v1>

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Abstract

Environmental unpredictability results in the evolution of bet-hedging traits, which maximize long-term fitness but are, by definition, suboptimal over short time scales. However, because suboptimal traits are expected to be purged by selection in the shorter term, the persistence of bet hedging remains perplexing. Here, we test the hypothesis that bet hedging persists through the evolution of constraint on short-term adaptation. We experimentally evolve *Saccharomyces cerevisiae* across two sequential treatments in which the frequency of extreme heat shocks decreases. We predict that experimental evolution under lower frequency heat shocks will result in greater adaptive constraint, or “purge-resistant” bet hedging. Constraint is assayed as evolutionary persistence of heat shock tolerance (HST) under constant benign conditions. As predicted, we find the retention of HST only in lines evolved under reduced frequency detrimental conditions. Results help explain the evolution of bet hedging, and challenge the traditional view that evolutionary constraint is inherently maladaptive.

Introduction

The natural environment may be seen as a composite of abiotic and biotic factors that fluctuate unpredictably, both spatially and temporally, causing variance in organismal fitness. Variable fitness response to these dynamics results in evolution at the population level, and in variable extinction risk among higher taxa^{1,2,3}. Bet hedging is a mode of evolutionary response to unpredictable environments that maximizes long-term fitness by reducing a genotype’s variance in fitness across generations. Success over generations is inherently multiplicative, and bet-hedging theory^{4,5} predicts the evolution of traits that maximize the geometric-mean fitness (n^{th} root of the product of n fitness values) rather than arithmetic average, or expected fitness within generations⁶. Bet hedging thus promotes persistence in the face of environmental fluctuations^{3,7}. Because bet-hedging, by definition, results in suboptimality over the shorter term, it is seen as the “sacrifice” of expected fitness for increased fitness in rare but extreme environments.

Although empirical evidence is difficult to obtain⁸, a recent surge of interest^{9,10,11,12,13,14,15} has led to a fuller understanding of the prevalence of bet hedging, and circumstances under which it evolves. This focus on the result of variable selection has masked an enigma of evolutionary process: despite extensive evidence for its existence, the question of *how* bet hedging evolves remains unanswered. Specifically, it is unclear how suboptimal traits persist despite selection acting against them over the short term. Because bet hedging cannot evolve given effective response to selection over the short term, it follows that selection for longer-term bet hedging imposes selection for constraint, or lack of response to selection in the shorter term. Evolutionary constraints would allow for the persistence of bet-hedging traits by preventing adaptive tracking of short-term trends into an evolutionary trap, or “dead-end.” In theory, genetic variation in constraint would be acted on and reinforced by selection during rare but severe events. The idea that bet hedging evolves as a constraint on adaptive tracking has been proposed as a conceptual model¹⁶ but has neither been mathematically modelled nor empirically tested.

Evolutionary constraint has been variously defined, and its sources include, for example, genetic, developmental, and functional mechanisms^{17,18}. For the purposes of this study, evolutionary constraint is interpreted broadly: as an inertial characteristic or trait that impedes adaptation. Constraints are ubiquitous in that, trivially, adaptation is not instantaneous and always lags behind a change in the selective environment because it is limited by, for example, standing genetic variation. Thus, at the extreme, a pre-existing constraint prevents adaptive tracking of benign environments when severe events occur even at high frequency; however, the maximization of geometric-mean fitness becomes a less trivial problem as severe events become rarer, and selection against bet hedging is prolonged. Evolution of a bet-hedging trait against rare events requires genetic variation for constraint. We point out that the source of genetic variation in constraint—although interesting—is immaterial to the evolution of bet hedging, as long as it exists: response to selection on constraint requires only that genetic variants of the focal (quantitative) trait, whether generated by simple polymorphism, number of alleles, pleiotropic interactions among traits, epistasis, etc., are not equally likely to be lost at exactly equal rates. For example, given an underlying tradeoff in performance under high heat and benign “normal” conditions, selection will act against thermotolerance during benign conditions; however, if nonzero genetic variation for the retention of thermotolerance exists, the more constrained genetic variants will be selected for during rare but recurrent extreme heat events. The selected population is then characterized by an increased mean constraint resulting in thermotolerance that is less likely to be lost between extreme events.

Constraints have traditionally been viewed as hinderances to adaptive evolution; here, we take an experimental evolution approach, using the yeast *Saccharomyces cerevisiae*, to test the idea that adaptive evolution of bet hedging evolves as an evolutionary constraint on adaptive tracking. Our basic design first requires the experimental evolution of a conservative bet-hedging trait in lines experiencing low and high-frequency severe environments; then, to test evolved constraint, an assay of the evolved lines will compare their subsequent rates of evolutionary loss of tolerance to severe events when evolved under continuous benign conditions. Only if bet hedging evolves through constraint do we predict lines evolved under a history of lower frequency severe environments to differentially retain tolerance to severe events.

Laboratory experiments using *S. cerevisiae* have shown that tolerance to moderately high temperatures (40°C; “thermotolerance”) evolves at the cost of growth under benign conditions¹⁹. Thermotolerant cells are also resistant to more severe heat shocks (transient exposure to 54°C)²⁰. Moreover, because the evolution of heat shock tolerance trades off as a cost of growth under benign conditions (Supplementary Text S1), this system can be exploited in our study. Specifically, to test the hypothesis that the retention of heat shock tolerance is a bet-hedging strategy²¹ that evolves as a constraint on adaptive tracking of benign environments, we subject a strain of *S. cerevisiae* evolved under high temperature (but not heat shock; see Supplementary Text S3) to a reduction in the frequency of extreme environments (heat shocks) across two sequential selection regimes (Fig. 1). A thermotolerant strain is used to found the experimental evolution lines to minimize extinction risk across sequential regimes of severe heat shocks. A sequential design allows observation of change in evolved constraint over an evolutionary history of

decreasing frequency of severe events. The two evolution regimes impose fluctuating selection using heat shocks (54°C) and benign environments (30°C): Under Regime A (heat shocks applied once every ~8 generations for ~100 generations), *S. cerevisiae* evolves under a frequency of extreme environments high enough to avoid extinction (through evolutionary loss of heat shock tolerance). In Regime B (heat shocks applied once every ~40 generations for ~100 generations), evolution occurs under low frequency extreme environments. The drop in frequency of severe environments from Regime A to Regime B should impose strong selection for enhanced growth at 30°C. However, any improvement in growth results in the loss of heat-shock tolerance required for survival through subsequent rare heat-shock events. Therefore, we predict that a history of low frequency extreme environments will have selected for a constraint on adaptation to benign environments and, by extension, the retention of heat shock tolerance. We compare evolved constraints of replicated lineages with two different evolutionary histories—at the end of Regime A (EoR-A) and at the end of Regime B (EoR-B)—by assaying evolution under constant benign conditions for the loss of heat shock tolerance.

Four possible alternative outcomes of the final trait persistence assays after evolution under benign conditions (Fig. 2) offer either support or refutation of our hypothesis of the evolution of constraint. No support for evolved constraint can be inferred either if lines from EoR-A and EoR-B are indistinguishable (Fig. 2A), or if lines from EoR-A are characterized by retention of heat shock tolerance attributable to higher initial heat shock tolerance (Fig. 2B). In contrast, support for bet hedging through the evolution of constraint on adaptive tracking would be offered by retention of heat shock tolerance in EoR-B relative to that in EoR-A, from any initial tolerance in EoR-B equal to (Fig. 2C) or less than (Fig. 2D) EoR-A. Note that reduced loss of tolerance for EoR-B under the biologically implausible result of higher initial heat shock tolerance (not shown) might be attributable to this high initial tolerance, and could not be taken as support for the constraint hypothesis.

Results And Discussion

In the evolutionary history phase, eight parallel lines were sequentially evolved across two regimes, A and B, for 100 generations each. Selection was successful, in that it resulted in the evolution of significantly greater heat shock tolerance across the sequential regimes (Fig. 3.A). Heat shock tolerance differed significantly across groups with different evolutionary histories ($df=2$, $MS=163.5$, $F_{2,19} = 9.085$, $p=0.0017$, $\eta^2 = 0.49$). Evolved heat shock tolerance in lines from the end of Regime A (EoR-A; mean = 21.92%, $n = 8$) and end of Regime B (EoR-B; mean = 19.77%, $n = 8$) was significantly higher ($p=0.0015$, 0.0125 for EoR-A and EoR-B respectively) than that of the ancestor (T1, Fig. 1) (mean = 12.44%, $n = 6$). Thermotolerance evolution is associated with a reduction in the number of growth-related cellular functions which, while critical to the acquisition of thermotolerance²², is correlated with decreased fitness²² at ancestral temperatures (temperature conditions prior to thermal evolution) in yeast¹⁹. Trade-offs associated with thermal adaptation have also been observed in a variety of systems including of viruses, bacteria and diatoms^{23,24,25,26}. Consistent with the results of these previous studies, we found that evolution in both heat shock regimes resulted in a decrease in relative fitness under benign conditions (the ancestral

condition) compared to the ancestral strain (Fig. 3.B). Relative fitness under benign conditions significantly differed across groups with different evolutionary histories ($df=2$, $MS=0.122$, $F_{2,21}=7.664$, $p=0.0032$, $\eta^2 = 0.42$), with lines from both EoR-A (mean = 0.945, $n = 8$) and EoR-B (mean = 0.951, $n = 8$) having significantly lower ($p=0.0053$, 0.0109 for EoR-A and EoR-B respectively) fitness values relative to the T1 ancestor (mean = 1.016, $n = 6$). Although underlying assumptions for the parametric one-way ANOVA were not violated, the homogeneity of variance assumption was close to being violated (Bartlett test, $p=0.1296$). Therefore, non-parametric tests (Kruskal-Wallis test, Dunn's post hoc test with Benjamini-Hochberg correction, see Supplementary Text S5) were performed and results were found to be qualitatively identical to those from the parametric one-way ANOVA.

Predictions about the direction of change in heat shock tolerance at EoR-B relative to EoR-A are secondary, and difficult to make since this would require estimates of arithmetic and geometric mean fitnesses across all encountered environments. However, despite the higher total number of heat shocks and benign environments encountered in Regime B, lines from EoR-A and EoR-B did not differ significantly either in heat shock tolerance ($p=0.576$) or in relative fitness under benign conditions ($p=0.949$). This could potentially be due to the trade-off between heat shock tolerance and relative fitness because fluctuating selection between benign and heat-shock environmental conditions is expected to prevent the improvement of either trait. Having observed that lines from EoR-A and EoR-B share similar trait values despite having evolved in regimes with different frequencies of heat shocks, we then proceed to the focal question of whether these differences in evolutionary history result in differences in the degree of constraint on adaptive tracking.

The test of the central hypothesis that constraints on adaptive tracking can evolve as a bet-hedging trait requires empirical measurements not of the traits at the termination of the experimental evolution phase, but of the subsequent loss of experimentally evolved traits. This was done by assaying for the persistence of evolved traits under conditions where the selective pressure to maintain these traits is removed. Specifically, the 8 replicate lines resulting from EoR-A and EoR-B were further evolved under constant benign conditions and subsequently assayed for the loss of heat shock tolerance. We found significant main effects of both evolutionary history regime ($df=1$, $MS=113.87$, $F_{1,28}=4.397$, $p=0.045$, $\eta^2 = 0.14$) and subsequent selection under benign conditions ($df=1$, $MS=155.69$, $F_{1,28}=6.012$, $p=0.0207$, $\eta^2 = 0.18$). Most importantly, the interaction between regime and subsequent selection was also significant ($df=1$, $MS=281.14$, $F_{1,28}=10.857$, $p=0.0027$, $\eta^2 = 0.28$), implying that the rate of heat shock tolerance loss differed across regimes. If a constraint on adaptive tracking evolves in response to decreasing frequency of heat shocks, we expect heat shock tolerance to persist in lines from EoR-B (where heat shocks were rarer) longer than in lines from EoR-A. Indeed, replicated lines from EoR-A lost heat shock tolerance more rapidly than did lines from EoR-B (Fig. 4.A). A post-hoc Tukey test revealed a significant reduction in heat shock tolerance by generation 150 for lines from EoR-A ($p = 0.002$) but not in lines from EoR-B ($p = 0.932$), indicating that low-frequency heat shocks of Regime B selected for a constraint on adaptive tracking that prevented the otherwise expected loss of heat shock tolerance. Additionally, the level of heat shock tolerance retained in lines from EoR-B after evolution under benign conditions was significantly

higher than in lines from EoR-A ($p = 0.004$). Maximizing the geometric mean fitness, or long-term evolutionary success, is strongly influenced by fitness in rare but extreme environments. Our results suggest that constraints on adaptive tracking can explain survival under rare detrimental conditions, and adaptation over the long term.

Fitness trade-offs across environments are central to the evolution of bet hedging. Without trade-offs, maximizing long-term fitness would not depend on bet hedging but rather on the independent optimization of all traits under selection. Since heat shock tolerance in the evolutionary history phase of the study evolved at the cost of fitness under benign conditions, we asked if the loss of heat shock tolerance in the trait persistence assay phase is also accompanied by a concomitant gain in relative fitness under benign conditions. Here, only the main effect of subsequent selection was found to be significant ($df=1$, $MS=0.016$, $F_{1,28}=11.58$, $p=0.002$, $\eta^2 = 0.29$) suggesting that the rate of fitness increase did not differ across regimes (Fig. 4.B); however, a post-hoc Tukey test revealed a significant increase in fitness by generation 150 only for lines from EoR-A ($p = 0.044$). In contrast, fitness did not significantly change by generation 150 for lines from EoR-B ($p = 0.204$). Thus, fitness under benign conditions increased for lines from EoR-A, but fitness at generation 150 for lines from EoR-A did not significantly differ from lines from EoR-B ($p = 0.971$). While underlying assumptions for the parametric two-way ANOVA were not violated, the homogeneity of variance assumption was close to being violated (Bartlett test, $p=0.1477$). Therefore, non-parametric tests (Aligned Rank Transform ANOVA, post hoc Tukey test, see Supplementary Text S5) were performed, and results were found to be qualitatively identical to those from the parametric two-way ANOVA. Although a difference in significance cannot be interpreted as a significant difference, this result is consistent with the expectation that lines from EoR-A responded to selection more rapidly than did those from EoR-B under benign conditions. Further evidence that a loss of heat shock tolerance under benign conditions is associated with a gain in relative fitness comes from closer examination of the replicate lines: seven out of eight EoR-A replicate lines (Fig. 5.C) show a positive slope when assayed for fitness after evolution under constant benign conditions, a result unlikely to have occurred by chance alone ($p = 0.0352$, see Supplementary Text S2). In contrast, only five out of eight lines from EoR-B show a positive slope—a result that can be explained by chance ($p = 0.3633$). It is important to note that due to auxotrophies in the reference strain, relative fitness assays were performed in medium enriched with the necessary amino acids (see Methods). We thus make the assumption that genotype-environment interactions between evolved yeast strains and the enriched and experimental media do not alter estimates of relative fitness.

Because of the sequential nature of the experimental design, lines from EoR-B encountered two additional heat shocks, and so the persistence of heat shock tolerance could be explained by alleles for this trait being fixed for lines from EoR-B. We argue that allele fixation is also a type of genetic constraint, albeit a weak one, where the trait for heat shock tolerance persists due to a lack of standing genetic variation. While such weak constraints can be overcome easily under prolonged evolution (given the relatively high mutation rate in yeast²⁷) they are nonetheless pertinent to the evolution of bet hedging because they can act as temporary 'shock absorbers' by retaining fitness under rare but severe events. Such constraints

can, therefore, pave the way for deeper constraints to evolve and can ultimately lead to traits being embedded. Moreover, the greater total number of heat shocks would contribute to the persistence of heat shock tolerance through selection for increased heat shock tolerance because higher trait values take longer to decline under relaxed selection. However, no significant difference in heat shock tolerance was observed between lines from EoR-A and EoR-B. We reiterate here the rationale for using a sequential selection design to observe a change in constraint across the two regimes, as opposed to a parallel selection design—which, while useful to compare evolved constraints across varying frequencies of extreme events, would not have satisfied the aims of this study.

The evolutionary history phase of the experiment began with 8 parallel replicate lines of the T1 ancestor, and was thus homogenous in terms of the genotypic composition in each replicate. Over the course of the imposed evolution regimes, genetic diversity is generated and selected upon, potentially resulting in individual replicate lines having distinct distributions of genotypes. It is important to note that stochastic interactions in replicate heterogeneous populations result in more variable evolutionary outcomes, and thus contribute towards lower experimental repeatability. This variability in evolutionary trajectories is important in this study because the test of the hypothesis demands a comparison of evolutionary trajectories for lines with different evolutionary histories.

A comparison of individual replicate lines from EoR-B (Fig. 5.D) suggested that one replicate (Replicate 4) had a disproportionate influence on mean fitness under benign conditions. At generation 150, this replicate is an outlier in that it had a fitness value more than 5 times the interquartile range away from the 3rd quartile. A plausible explanation is that Replicate 4 contains a distribution of genotypes that is more conducive to rapid adaptation under benign conditions. In further support of this argument, Replicate 4 also had the highest fitness after 150 generations (close to twice the interquartile range away from the 3rd quartile) amongst lines from EoR-A.

Constraints on tracking cannot evolve instantaneously and, like all traits, genetic variation in constraint is a prerequisite for its evolution. Although substantial phenotypic variation in constraint was observed at the end of both Regime A and Regime B, genetic variation for constraint cannot be quantified using this study design. Future study aiming to estimate genetic variation in constraint using our general experimental design would require replication of single genotypes from individual lines and assays for evolved constraints just prior to regime B. Some genetic mechanisms behind bet-hedging traits have been described^{28,29,30,31}. Previous studies have shown that individual-level variability in microbial systems can have varying effects on population-level traits^{32,33}. Specifically, Cerulus *et al.* (2016) show that for a clonal population with a given mean doubling time, increased variability in doubling times increase the overall population growth rate. In yeast, the trait that controls heat shock tolerance has been shown to be a diversification bet-hedging strategy²¹. Clonal yeast populations exhibit a wide distribution of growth rates when cultured in benign environments^{34,35}. Slow growth rates are correlated with elevated intracellular levels of Tsl1, a regulator of trehalose synthesis, and are a reliable predictor of heat shock tolerance²¹. It then follows that the mechanism underlying the evolved bet-hedging strategy across

regimes with reduced frequency of heat shocks could potentially involve changes in growth rate distributions of clonal populations, furthering the case for selection acting on “phenotypic noise”. Physiological responses to elevated temperatures begin with the environmental stress response which entails the transcription of early stress response genes³⁶ followed by the heat shock response which occurs with a change in gene expression and the induction of heat shock proteins (HSPs) and trehalose³⁷. Within the purview of heat stress response, trehalose primarily functions to protect cells against acute heat stress (>45°C)³⁸. On the other hand, HSPs are a diverse family with a wide range of functions across temperatures. However, both trehalose and HSPs are required for survival and growth under heat stress. Because selection along either arm of the heat shock response is expected to come with varying fitness effects, the specific mechanism underlying constrained evolution is also expected to differ. We propose multiple genetic mechanisms through which constraints can manifest. At the simplest level, a constraint could exist due to a trait arising via a multiple-step mutation. In such a scenario, revertants are less likely to arise under relaxed selection compared to single-step mutants. Constraints could also exist due to linkage between an evolved tolerance trait and vital traits. Here, tolerance to extreme events ‘hitchhikes’ across non-selective events due to the strong association with other vital traits. However, despite an understanding of genetic mechanisms underlying putative bet-hedging traits^{21,39,40} themselves, the genetic basis of evolutionary constraints that influence the persistence of bet-hedging traits has not been studied. The present results suggest that genetic studies of evolved constraints are warranted to better understand the enigma of the persistence of bet hedging over the short-term. Long-term selection acting on constrained traits can potentially lead to such traits being phylogenetically embedded and shared between multiple species¹⁶. If so, evolutionarily conserved traits may be a result of long-term selection for constraint as bet hedging. An example of a conserved trait in yeast is orthologous ribosomal protein promoters that show conserved expression even when genomically integrated into other distinct yeast species. This conserved expression was found to be mediated by compensatory changes where the effects of mutations in one site of the core promoter are nullified by mutations in another site⁴¹.

Our study provides qualitative evidence that the evolution of bet hedging occurs through selection for a constraint on adaptive tracking. However, quantitative inferences of the degree of constrained evolution that is adaptive given the frequency of heat shocks is beyond the scope of this study, and would require detailed assessment of the optimal value of the constrained trait that maximizes geometric-mean fitness across fluctuating environments⁸. This study shows that the retention of heat shock tolerance had evolved after an evolutionary history characterized by rare detrimental events (Regime B), demonstrating that selection can act on variation in constraints on adaptive tracking of heat shock tolerance. Although constraints have traditionally been viewed as maladaptive, we show that they may be adaptive and could potentially be selected as bet hedging against unpredictability over the long term.

Materials And Methods

Strains and culture conditions:

The evolved thermotolerant yeast strain T1 (See Supplementary Text S3) with S288C background (*MATa SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*) was used as the ancestor for experimental evolution. Yeast inocula were prepared by growing yeast for 24 h (for competition assays) or 48 h (for heat shock tolerance assays) in 5 ml Synthetic Defined Medium (SDM) broth using a shaker incubator at 30°C. SDM contains 6.7 g of Yeast Nitrogen Base (without amino acids with ammonium sulfate) and 2% dextrose per liter. For competition experiments we used the reference strain YIR044CΔ with a BY4741 background (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), obtained from the yeast non-essential gene deletion array. For competition experiments all focal strains, test populations and the reference strain were grown to saturation in SDM+ Histidine (10 mg/L), Leucine (30 mg/L), Methionine (10 mg/L), Uracil (10 mg/L). Strains were archived every ~20 generations during experimental evolution at -80°C in 15% glycerol.

Heat shock tolerance assays

Cells from an individual colony (in the case of the T1 strain) or from a population aliquot (in the case of test populations) were cultured in SDM at 30°C. At 48 h post inoculation, cells were resuspended in SDM and adjusted by spectrophotometry to an optical density at 600 nm (OD₆₀₀) of 0.05. 250 μL of this cell suspension was heat shocked at 54°C in a water bath for 75 minutes in duplicate. Heat shocked suspensions were kept on ice until plating within 30 minutes. Both heat shocked and non-heat shocked cell suspensions were spread on YPD agar to estimate viable cell counts. Heat shock tolerance was measured as a percentage of cells surviving heat shock relative to the total number of viable cells prior to heat shock.

Competitive fitness assays

Relative fitness under benign conditions (30°C) was measured as competitive fitness using a reference strain, YIR044CΔ. This strain has the pseudogene YIR044C deleted and replaced with a gene conferring G418 resistance—used as a selectable marker for this assay. Cultures were grown to saturation in SDM+ (His, Leu, Met, Ura) at 30°C and resuspended to an OD₆₀₀ of 0.05. Following the resuspension, the reference strain and individual focal strains/test populations were mixed and allowed to compete in SDM+ (His, Leu, Met, Ura) for 48 hours. Proportions of focal strain/test population relative to the reference strain both before and after the competition were determined by plating on YPD agar with and without G418 (200 μg/ml, Sigma-Aldrich). Relative fitness was calculated as previously described⁴², where the selection coefficient was determined using the following equation. Fitness w was calculated as $1 + s$.

$$s = \frac{\ln \frac{\text{focal}_{\text{initial}}}{\text{focal}_{\text{final}}} - \ln \frac{\text{reference}_{\text{initial}}}{\text{reference}_{\text{final}}}}{\text{No. of generations}}$$

Experimental evolution:

Heat shock tolerance was evolved using the thermotolerant T1 strain as the ancestor in 8 replicate lines across two sequential regimes, A and B. Regime A consisted of heat shocks applied every ~8 generations for ~100 generations (11 heat shocks over 32 days, see Supplementary Text S4 for generation time calculation). At the end of Regime A, each of the 8 replicate lines were transferred to Regime B for another ~100 generations. Regime B consisted of heat shocks applied every ~40 generations (2 heat shocks over 26 days). Because heat shocked cells take longer to resume growth, heat shocks in Regime A were applied 72 hours after the preceding heat shocked cells were inoculated in fresh media. In Regime B, cultures were diluted in fresh media five times (~8 generations per dilution) between heat shocks. For the application of heat shocks, cells were first grown in SDM for 48 hours following which a cell suspension was prepared by dilution to an OD₆₀₀ of 0.05. 250 µL of this cell suspension was heat shocked at 54°C for 75 minutes. 100 µL of the heat shocked suspension was used to inoculate 5 ml of fresh SDM.

Constraint estimation:

Evolutionary constraints were measured phenotypically as the retention of heat shock tolerance under opposing selection (growth at 30°C). Cells from the focal strains/test populations were grown in SCM in duplicate for 24 hours and diluted to an OD₆₀₀ of 0.05. This was repeated for 18 days (~150 generations). Heat shock tolerance and fitness under benign conditions were measured at generation 0 and generation 150, following which statistical analyses were performed to gauge the persistence of these traits after 150 generations.

Statistical analyses:

All statistical analyses were performed in R version 3.5.3. Analysis of heat shock tolerance and competitive fitness for the ancestor T1 (6 trait measurements from independent replicate populations) and 8 replicate populations at the end of Regime A (EoR-A lines) and at the end of Regime B (EoR-B lines) were performed using one-way ANOVAs. Post-hoc Tukey tests were performed to make pair-wise comparisons of heat shock tolerance and competitive fitness. Trait measurements of lines from the two regimes A and B at two time points after experimental evolution (generation 0 and generation 150) were used to perform statistical analyses of evolved constraints. A two-way ANOVA was used to model the effect of regimes and time points on heat shock tolerance, following which a post-hoc Tukey test was performed. A similar analysis was performed to model the effect of regimes and time points on competitive fitness. A simpler ANOVA model is used here instead of a repeated measures model as individual replicate lines in this experiment evolve completely independently through time. Effect sizes were measured and reported as partial η^2 values. Parametric tests were used in this study after underlying assumptions were tested (Shapiro-Wilk, Bartlett tests). Any assumption violations are noted, and alternative nonparametric test results provided in Results & Discussion.

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Figures

Selecting for a constraint on adaptive tracking

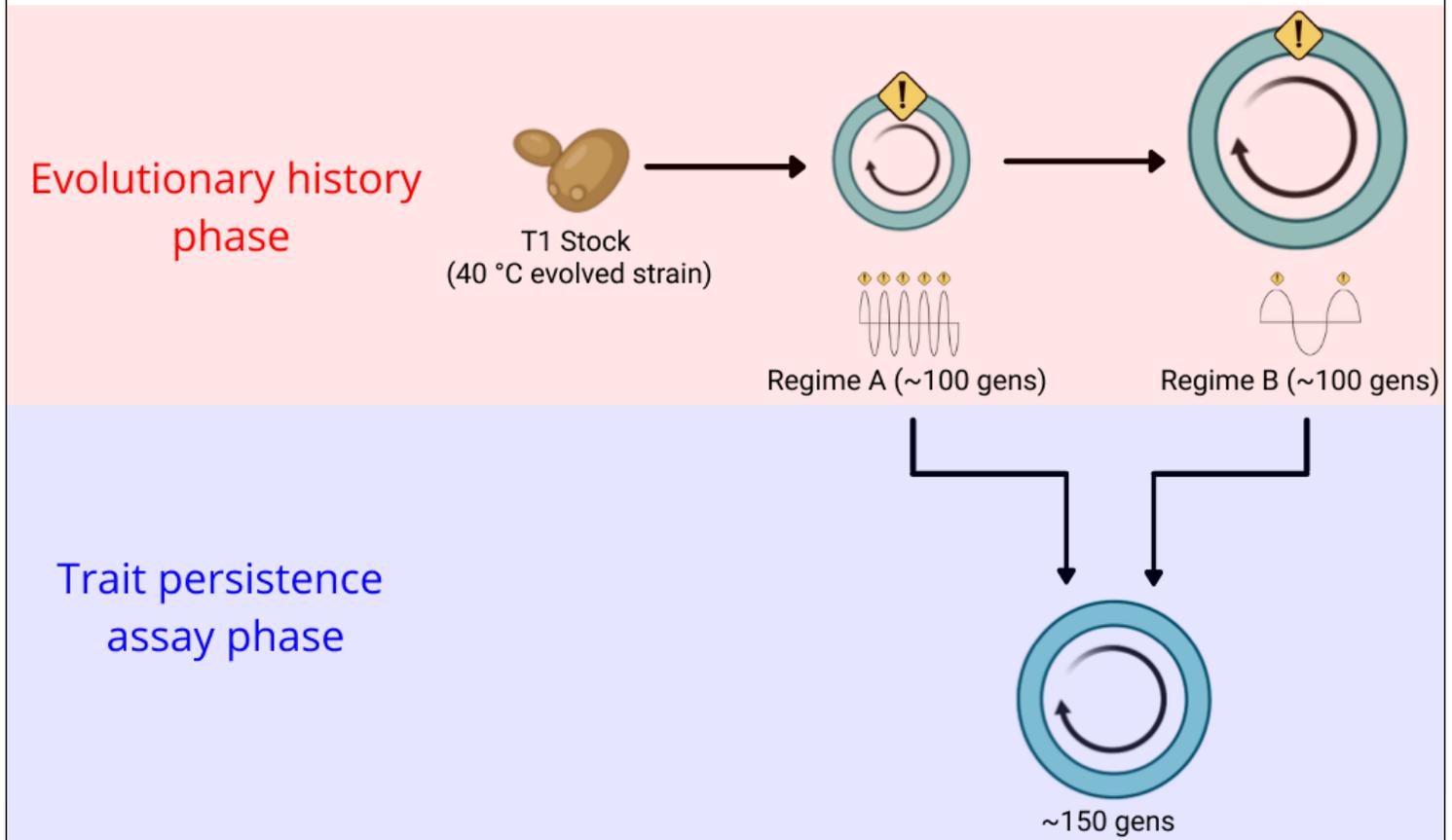
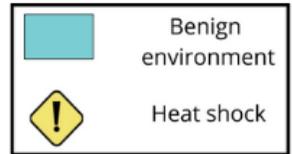


Figure 1

Schematic overview of the experimental design. The evolutionary history phase (top panel) entails evolution of a thermotolerant yeast strain under two sequential regimes, A and B. Regime A consists of high frequency fluctuations (heat shocks applied once every ~8 generations) and Regime B consists of low frequency fluctuations (heat shocks applied once every ~40 generations). Yeast lines were evolved in each regime for ~100 generations. As part of the trait persistence assay phase (bottom panel), lines from the end of Regime A and Regime B are evolved under constant benign conditions for ~150 generations and assayed for the loss of heat shock tolerance.

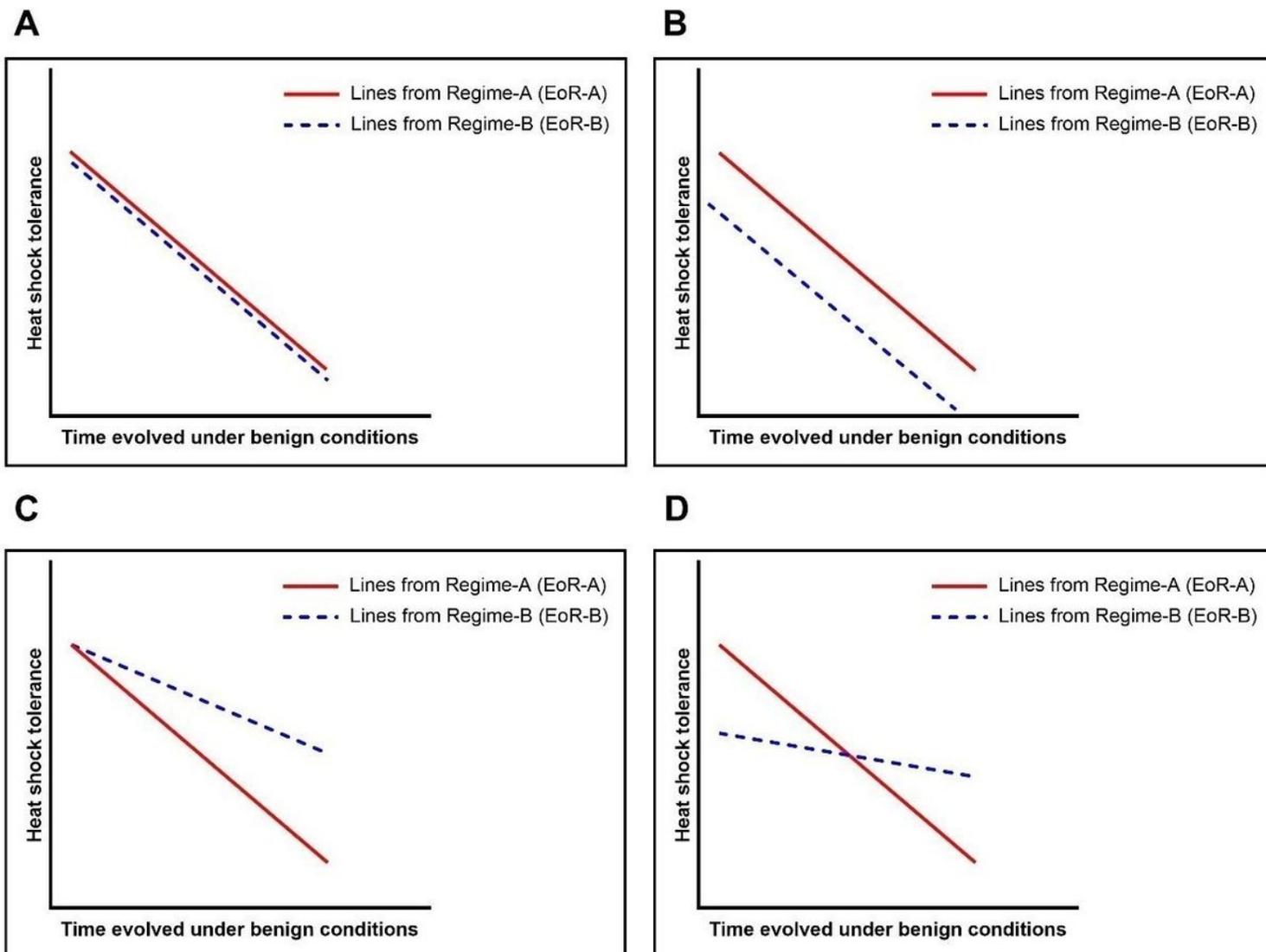


Figure 2

Four possible alternative outcomes of assays of evolved constraints resulting from evolution under high frequency (Regime A, red) and low frequency (Regime B, black) heat shocks. Panels A and B represent the null result that would provide no evidence of evolved constraint: heat shock tolerance is lost at similar rates in Regime B relative to Regime A. Patterns in panels C and D would support the hypothesis of the evolution of constraint as adaptation: the evolutionary rate of loss of heat-shock tolerance is lower in lines from Regime B relative to lines from Regime A.

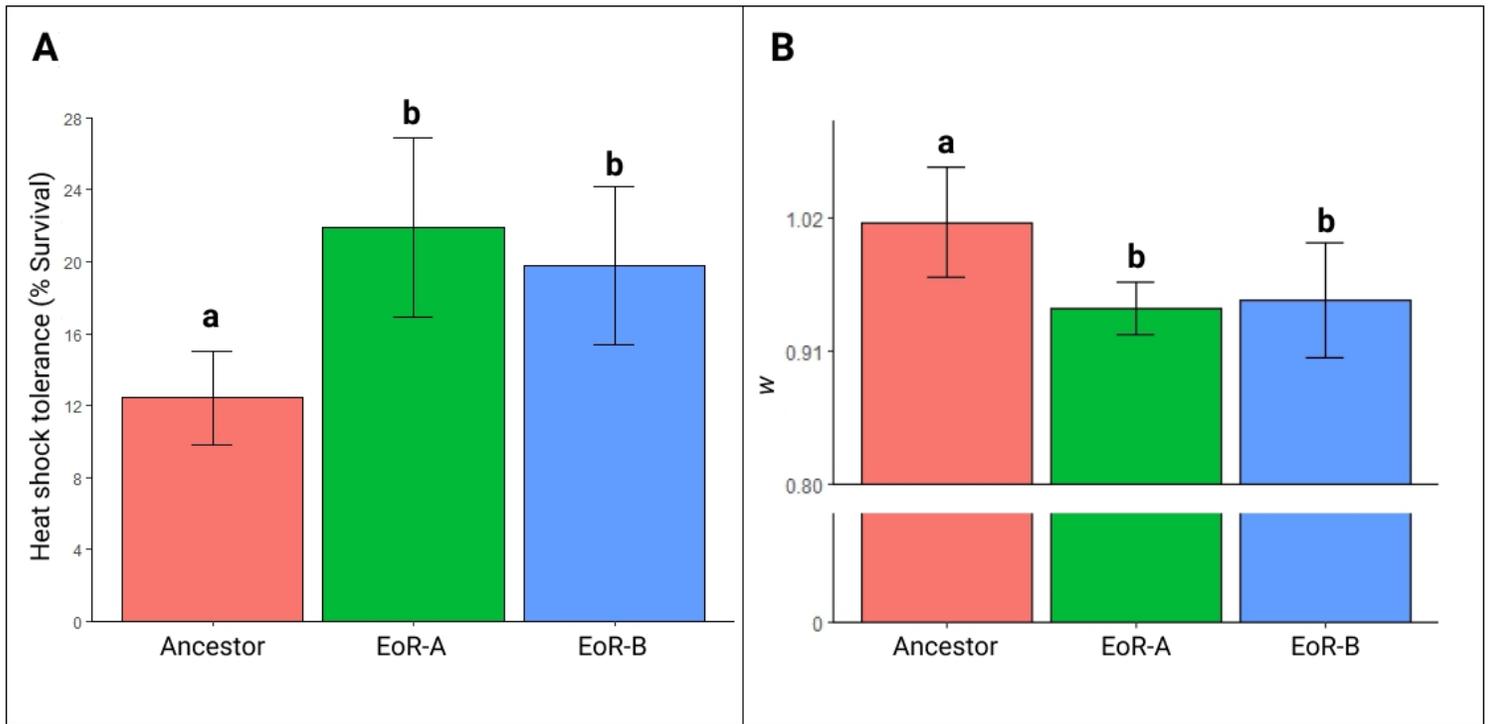


Figure 3

Trait measurements after evolution in Regime A (EoR-A, ~100 generations) and Regime B (EoR-B, ~100 generations) relative to the T1 ancestor. (A) Heat shock tolerance (measured as % Survival) across lines. (B) Competitive fitness (w) under benign conditions across lines. Error bars represent SD. Letters not shared among levels indicates a significant difference in means according to a post-hoc Tukey's test.

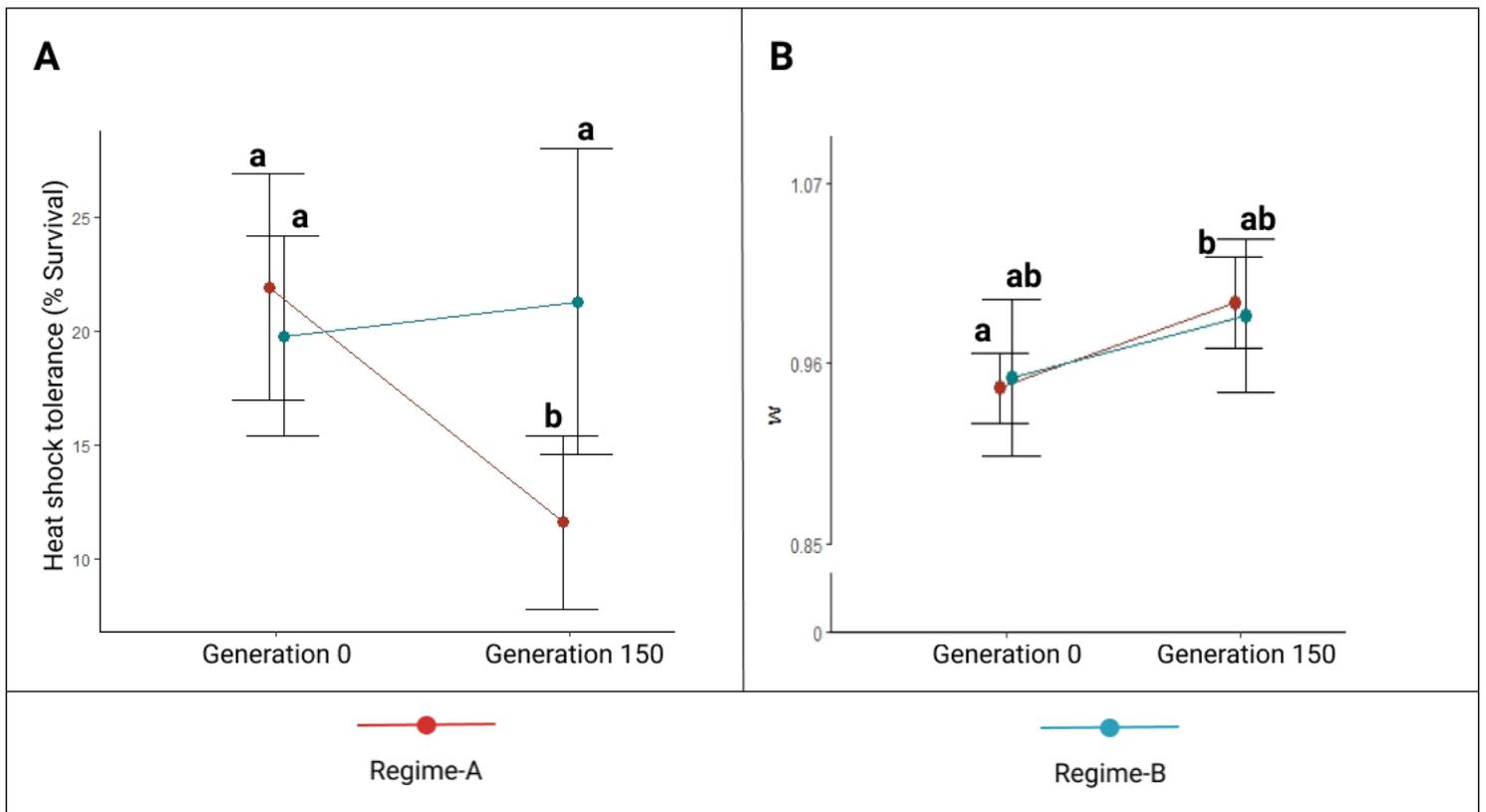


Figure 4

Evolved constraints measured as (A) loss of heat shock tolerance (measured as % Survival) and (B) gain in competitive fitness at 30°C after selection in the evolutionary constraint assay under benign conditions for 150 generations in lines from Regime A (red) and Regime B (blue). Error bars represent SD. Letters not shared among levels indicates a significant difference in means according to a post-hoc Tukey's test.

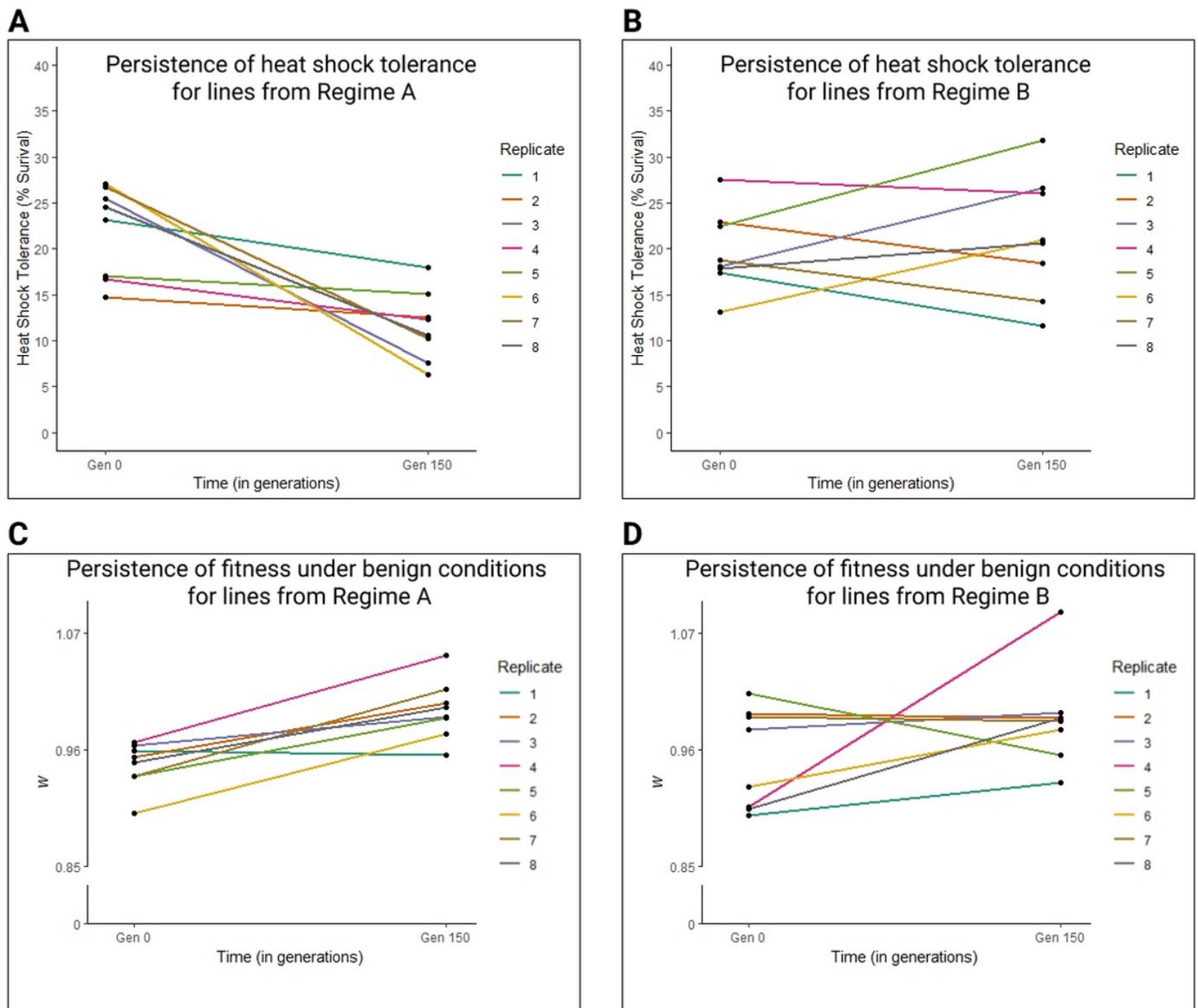


Figure 5

Individual replicate lines from EoR-A and EoR-B in the trait persistence assay phase. (A-B) depict the persistence of heat shock tolerance for individual lines from EoR-A and EoR-B replicate. (C-D) depict the same for fitness under benign conditions.

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