**METHODS**

**Strains, media and culture methods.**

Barcoded yeast strains were constructed using two isogenic haploid derivatives of a strain collected from an oak tree in Pennsylvania (YPS163) (REF – Sniegowski, Dombrowski, & Fingerman, 2002): YJF153 (MATa, *HO*::dsdAMX4) and YJF154 (MATalpha, *HO*::dsdAMX4) (REF – Li & Fay, 2017). 113 diploid strains (Table S1) were constructed such that each contained a unique 20bp barcode-sequence flanking KAN, inserted in the HO locus (REF - Fasanello et al., 2020). A single barcoded strain (d1H10) was arbitrarily selected from this set to serve as the “ancestral reference strain”; the remaining 112 barcoded strains were subject to 50 days of experimental evolution (See *Experimental design*, below). Strain construction is straightforward (REF - Fasanello et al., 2020) and additional strains can be constructed from the MoBY plasmid collection (REF – Ho et al., 2009).

Yeast were cultured in complete medium (CM; 20 g/l dextrose, 1.7 g/l yeast nitrogen base without amino acid and ammonium sulfate, 5.0 g/l ammonium sulfate, 1.3 g/l dropout mix complete without yeast nitrogen base) with or without additional chemical stress in 96-deep well plates (2.2-ml poly-propylene plates, square well, v-conical bottoms; Abgene AB-0932) covered with rayon acrylate breathable membranes (Thermo Scientific, 1257605). Growth plates were incubated at 30°C for 24 hours inside an incubator (VWR, Forced Air Incubator, basic, 120v, 7 cu. ft.) with agitation using a horizontal electromagnetic microplate shaker (Union Scientific LLC, 9779-TC). Saturated (stationary phase) 24-hour culture was diluted (1:1000) into fresh medium at the same time each day to initialize the next round of growth.

**Experimental design**

The experimental design included a 50-day experimental evolution with subsequent fitness quantification of ancestral (Day-0) and evolved (Day-50) yeast via competition-based fitness assay.

*Experimental Evolution:* 112 barcoded yeast strains were divided evenly among seven treatments variable for chemical stress concentration and temporal dynamics. “Constant” chemical stress treatments were evolved for 50 days in Complete Medium (CM) plus chemical stress at 0% (EH0), 40% (EH40), or 80% (EH80) of the lethal limit for unevolved yeast strains in our library; chemical stress concentration did not change from transfer-to-transfer for these treatments. Fluctuating treatments were evolved for 50 days in chemical stress that alternated daily between two concentrations: 0%-40% (EH0\_40), 20%-60% (EH20\_60), 40%-80% (EH40\_80), or 0%-80% (EH0\_80) of the ancestral limit. This design was copied to create four microplates which were evolved in parallel for 50 days: two were exposed to NaCl stress (ancestral lethal limit = 20g/l) and two were exposed to CuSO4 stress (ancestral lethal limit = 8um). Stress concentrations were selected such that they were comparable between chemical stressors and such that the 80% stress treatment reduced growth but did not result in extinction (from transfer to transfer) for an average ancestral strain. Samples were collected from the initial mixtures (Starting material for plate copies, Day-0) and from the final overnight cultures (on Day-50). These samples served as the starting material for the Day-0 and Day-50 fitness assays, respectively.

Fitness Assays: Sequencing based competition assays, hereafter fitness assays, were subsequently conducted on Day-0 (ancestral) and Day-50 (evolved) yeast to assess fitness relative to the ancestral reference strain. Briefly, yeast strains from Day-0 and Day-50 of the experimental evolution were revived from stocks and mixed, separately, in equal proportions to create five pools. A single pool was created from each [evolutionary microplate] X [day] for a total of 1 Day-0 sample (the template for the evolution) and 4 Day-50 samples (2 NaCl evolved and 2 evolved in CuSO4). The ancestral reference strain was then spiked into each pool at a high proportion (~70%). Pools were diluted into fresh medium and cultured for two rounds of growth to allow competition to occur. Fitness assays were conducted in CM without additional chemical stress and in CM with added stress at 80% of the lethal limit for the ancestral population. Yeast strains evolved in microplates with NaCl treatments were assayed in NaCl chemical stress and yeast strains evolved in CuSO4 treated microplates were assayed in the presence of CuSO4; ancestral strains were assayed in both NaCl and CuSO4. Day-0 and Day-50 Fitness assays were run in quadruplicate and initial measures (barcode starting proportions) for each were taken in quintuplicate. Samples were collected from the initial mixtures (fitness assay starting material) and from the final cultures (ca. 20 generations later). From these data, the fitness of each barcoded strain prior to evolution (Day-0 assays) and after evolution (Day-50 assays) was quantified. The resulting values were used to assess change in fitness for each strain in each environment relative to the static ancestral reference.

**Library construction and sequencing.**

DNA was isolated using a ZR Fungal/Bacterial DNA Kit (Zymo Research D6005) in individual 2.0 mL screw-cap tubes following the manufacturer’s instructions. Physical cell disruption by bead-beating was conducted in a mixer mill (Retsch, MM 300) at 30 Hz (1800 min-1) for ten minutes (1-minute on, 1-minute off, times ten cycles). MoBY barcodes were then amplified with forward/reverse Ion Torrent adapters containing a 9-12 bp index for multiplex sequencing (Table S2). PCR products for library construction were generated using 25 cycles and were subsequently quantified with a Qubit 3.0 Flourometer (ThermoFisher Scientific, Q33216) using the high sensitivity assay kit (ThermoFisher Scientific, Q32851). Products were combined at equimolar concentrations and purified using a Zymo DNA Clean & Concentrator kit (Zymo Research D4014) to create a single multiplexed library for sequencing. Additional control samples were included in the library to track barcode cross-contamination. An aliquot of the library was sequenced using an Ion Torrent sequencer (Ion Proton System, Ion Torrent) at the Genomics Core Facility at Saint Louis University with a customized parameter to assess polyclonality after 31bp (the start of the forward Ion Torrent adapter index sequence). A second aliquot was sequenced to augment read depth following preliminary assessment of data quality.

**Sequence data processing & calculations**

*Sequence datasets:* Sequence data in FASTQ format were parsed and demultiplexed using custom scripts in R. 96,807,316 reads were retained for analysis that perfectly matched a forward adapter index (9-12 bp), a reverse adapter index (9-12 bp), and a MoBY genetic barcode (20 bp) included in the full experimental design. Of these, 52,853,350 (54.6%) mapped to non-reference barcodes. The average number of reads per non-reference barcode per sample was 5,655 and the median value was 3,442 (Figure S1). Entries with <= 20 reads were treated as noise and removed prior to read summary reporting.

*Reads:*Because count data are ultimately handled as relative frequencies (proportions), variation in sample size (re. underlying number of counts recovered; confidence) was accounted for by utilizing weights in summary calculations and by including weights in all statistical models (REF - See Fasanello et al., 2020).

*Contamination rate:*No instances of culture contamination were observed. Barcode cross-contamination rate was also measured and is defined as the total number of counts mapping to barcodes included in the full experimental design (library) but not expected to be present in that particular sample (given pair of forward/reverse Ion Torrent adapter Indices) (REF - Modified from Fasanello et al., 2020). The rate of barcode cross-contamination was tracked using samples seeded with a single pair of barcoded strains, such that the subsequent presence of other barcodes in these wells could be identified and quantified by sequencing. Barcode cross-contamination was low overall (0.269% +/- 0.619%) and exhibited minor variation among sample sets (Day-0, 0.01% +/- 0.007%; Day-50 NaCl, 0.030% +/- 0.012%; Day-50 CuSO4, 0.765% +/- 0.990%) (Table S3).

*Fitness calculations:* The Malthusian fitness of focal barcoded strain *i,* relative to the ancestral reference (d1H10), at experimental evolution generation *gn*, *mi gn*, was measured as,

Equation 1.

Where *Ci* and *R* refer to barcode counts for the focal barcode and reference barcode at fitness assay time 0-hours (initial mixtures) and time 48-hours (final overnight cultures), and 20 is the number of generations over 48 hours (two overnight cultures at 9.97 generations each – calculated from number of doublings based on optical density data) (REF – Hartl & Clark, 1997; REF – Chevin 2011). We use the standard equation *m=ln(w)* to convert Malthusian fitness values to Wrightian fitness values (REF - Orr, 2009; REF - Wu et al., 2013; REF - Passagem-Santos & Perfeito, 2018). Hereafter, fitness, denoted by a *w* will refer to Wrightian fitness. The change in fitness of strain *i* between Day-0 and Day-50, *Δwi*, was therefore computed as,

Equation 2.

Where wi day-0 and wi day-50 are the strain’s fitness relative to the ancestral reference at day-0 (before the experimental evolution) and at day-50 (end of experimental evolution) as measured from Equation 1. We assume no frequency dependent selection.

*Extinction:* Strains were deemed extinct if they were not present at the initiation of the Day-50 fitness assays or if they fell below the detection limit over the course of the 48-hour Day-50 fitness assay. Fitness is not reported for extinct barcodes.

**Statistical analysis**

*Analysis & visualization tools:* R version 3.5.2 was used for all calculations, analyses, and figure generation (REF - R Core Team, 2012). Data processing uses base-R functionality supplemented with methods from the plyr package (REF – Hadley, 2011). Statistical models with linear mixed effects utilize the lme4 (REF – Bates et al., 2015) and lmerTest packages (REF – Kuznetsova, Brockhoff & Christensen, 2017). Power analyses were conducted using the pwr package (REF – Champely, 2018). Figures and tables were generated with ggplot2 (REF – Wikham, 2009) and sjPlot (REF – Ludecke, 2019); multi-panel figures were built using methods from grid (REF – GRID REF), gridExtra (REF - Auguie, 2017), and cowPlot (REF – COWPLOT REF).

*Power analysis:* Power analyses were conducted using root mean squared error (RMSE) and population standard deviation (PSD). RMSE for fitness change was 2.419 and PSD was 0.009. Consequentially, we have 80% power to detect a fitness change (increase or decrease) of 2.163% for any individual barcode (Figure S2., A). We have 80% power to discern fitness differences of 0.306% between treatments assuming no extinction and 0.634% assuming 50% extinction (Figure S2., B).

*Fitness change in 50 days of experimental evolution:* Individual barcoded yeast strains that increased or decreased in fitness over the 50-day experimental evolution were identified using weighted linear models with change in fitness as the response variable and strain ID as the predictor variable (fitness change ~ strain ID; family = gaussian). The effect of evolutionary treatment on fitness change was assessed using weighted linear mixed-effects models with change in fitness as the response variable and treatment as the predictor variable. A random effect of strain ID was placed on the model intercept (fitness change ~ treatment + (1|strain ID); family = gaussian). Separate models were run for each dataset by environment (NaCl strains in CM, NaCl strains in CM+NaCl, CuSO4 strains in CM, CuSO4 strains in CM+CuSO4). Using the same single-environment fitness-assay data, two additional sets of weighted linear mixed-effects models were conducted to assess the effects of treatment on geometric mean fitness and absolute fitness difference between environments under a hypothetical scenario of 50:50 exposure to CM and CM plus chemical stress at 80% of the ancestral lethal limit.

**Availability of data and materials**

The dataset supporting the conclusions of this article is available in the NCBI Sequence Read Archive (SRA) repository, BioProject Number XXXXXXXXXXXX, BIOPROJECT LINK (REF – Bioproject REF). Data formatted for analysis and custom R scripts utilized for all data processing, statistical analyses, and figure generation are available from GitHub (GITHUB LINK). A static version of the repository is available from Zenodo (REF – Zenodo REF); instructions to reproduce the analyses and to confirm the results presented in this article are provided within. Supplementary figures, tables, and files referenced throughout the main text are available as “Supplemental Files.” Strains are available from the Justin C. Fay Lab at The University of Rochester; contact James Miller (e: j.h.miller@rochester.edu).

**RESULTS**

**Single-environment results**

We evolved 448 yeast strains for 50 days (ca. 500 generations) in environments variable for chemical stress concentration and dynamics. Three evolutionary histories (treatments) were subject to constant NaCl or CuSO4 concentrations equal to 0% (EH0), 40% (EH40), or 80% (EH80) the ancestral lethal limit for that chemical. Four evolutionary histories were subject to daily-alternating chemical concentrations: 0% & 40% (EH0\_40), 20% & 60% (EH20\_60), 40% & 80% (EH40\_80), or 0% & 80% (EH0\_80) the ancestral lethal limit. We subsequently conducted competition-based fitness assays to assess the fitness of each ancestral (Day-0) and evolved (Day-50) strain against a static reference in complete media (CM) and in CM plus chemical stress at a concentration equal to 80% the ancestral lethal limit. From these data we calculate change in fitness in each assay environment for each barcoded yeast strain.

We observe significant fitness change in most treatments in CM (5/7 NaCl dataset, 4/7 CuSO4 dataset) and CM plus chemical stress (5/7 NaCl dataset, 6/7 CuSO4 dataset) (Table S4). In the NaCl dataset, EH0 (5.5%, p<<<0.05) and EH0\_40 (1.6%, p=0.017) increase in fitness in CM, while EH0\_80 (-1.5%, p=0.040), EH40\_80 (-1.6%, p=0.025), and EH80 (-2.1%, p=0.008) decrease in fitness (Table S4). The opposite pattern is apparent when the same lineages are assayed in CM plus NaCl: EH0 (-3.3%, p=0.013) decreases in fitness while EH20\_60 (6.7%, p<<<0.05), EH0\_80 (10.1%, p<<<0.05), EH40\_80 (14.3%, p<<<0.05), and EH80 (18.5%, p<<<0.05) increase (Table S4). Fitness gains in CM plus NaCl stress are greater than those in CM without added stress (BASIC STAT HERE) (Table S4). The trend for fitness change is different in the CuSO4 dataset and the overall magnitude of fitness change tends to be greater (BASIC STAT HERE) (Table S4). Here, no treatments significantly decrease in fitness in CM nor CM plus CuSO4. Instead, EH0 (5.7%, p<<<0.05), EH0\_40 (4.1%, p<<<0.05), EH40 (4.1%, p<<0.05), and EH20\_60 (3.7%, p<<<0.05) increase in fitness in CM, and all treatments (EH0: 4.3%, p=0.03; EH40: 4.3%, p=0.02; EH20\_60: 8.4%, p<<<0.05; EH0\_80: 25.3%, p<<<0.05; EH40\_80: 25.7%, p<<<0.05; EH80: 36.6%, p<<<0.05), except EH0\_40 (2.6%, p = 0.07), exhibit fitness increases in CM plus CuSO4 (Table S4). Again, the magnitude of fitness change is greater in CM plus stress than in CM alone (Table S4). Finally, the magnitude of fitness gains for treatments that increase in fitness are greater than the magnitude of fitness decreases for treatments that decrease in fitness for both the NaCl and CuSO4 datasets (BASIC STAT HERE) (Table S4, positive entries vs negative entries).

Subsequent assessment of variation in fitness change among treatments (i.e., rather than vs. zero) reveals broad similarities between the NaCl and CuSO4 datasets and suggests that both mean- and maximum-stress concentration experienced during evolution shape fitness in CM and CM plus chemical stress (Figure 1). Intuitively, treatments evolved in lower mean- and maximum-stress concentrations tend to rank higher in fitness in CM for both the NaCl (Figure 1A) and CuSO4 datasets (Figure 1B). The opposite trend is found in CM plus chemical stress. Here, treatments evolved in higher mean- and maximum-stress concentrations tend to rank higher in fitness for both the NaCl (Figure 1C) and CuSO4 datasets (Figure 1D). Fitness differences among treatments change gradually with treatment mean- and maximum-stress for the NaCl evolved treatments (Figure 1A, C; note gradient in model estimates); the pattern is more discrete in the CuSO4 data (Figure 1B, D; note discrete blocks in model estimates). Additionally, the magnitude of fitness difference between treatments evolved in no stress (EH0) versus high stress (EH80) is greater in CM + stress than in CM with no added stress for both the NaCl (22%, p<<<0.05 vs. 8%, p <<<0.05, Figure 1 C vs. A) and the CuSO4 datasets (32%, p <<<0.05 vs. 6%, p<<<0.05, Figure 1 B vs. D) and the magnitude of difference between the EH0 and EH80 treatments is greater in the CuSO4 data (32%, p<<<0.05, Figure 1 D) than in the NaCl data (22%, p<<<0.05, Figure 1 C) when fitness is assayed in CM + 80% stress. There are no obvious qualitative differences in fitness change between constant and fluctuating chemical stress treatments when CM and CM + chemical stress data are assessed separately (Figure 1; EH0, EH40, EH80 vs. EH0\_40, EH20\_60, EH0\_80, EH40\_80).

**Joint-environment results**

We assayed the fitness of lines selected for specialization (EH0, EH80) and generalization (EH0\_40, EH20\_60, EH0\_80, EH40\_80) in the evolutionary environments experienced by the EH0 and EH80 treatments: CM and CM plus chemical stress at 80% the ancestral lethal limit, respectively. We then assessed joint performance in these environments (assuming 1:1 exposure) as geometric mean fitness, absolute fitness difference, and in the context of the cost of generalism (sensu. Kassen, 2002).

We observe significant positive change in geometric fitness for nearly all treatments (5/7 NaCl dataset, 7/7 CuSO4 dataset; Table S5). For the NaCl Dataset, geometric mean fitness increases significantly in EH40 (1.5%, p = 0.02), EH20\_60 (3.6%, p <<< 0.05), EH0\_80 (4.4%, p <<< 0.05), EH40\_80 (6.5%, p <<< 0.05), and EH80 (8.1%, p <<< 0.05) (Table S5). In the CuSO4 data, geometric mean fitness increases in all treatments: EH0 (5.6%, p <<< 0.05), EH0\_40 (4.0%, p <<< 0.05), EH40 (4.5%, p <<< 0.05), EH20\_60 (6.5%, p <<< 0.05), EH0\_80 (11.9%, p <<< 0.05), EH40\_80 (12.0%, p <<< 0.05), EH80 (18.6%, p <<< 0.05) (Table S5). Additionally, absolute fitness difference between the CM and CM plus chemical stress is significantly greater than zero in all treatments (7/7 NaCl dataset, 7/7 CuSO4 dataset; Table S6). Geometric mean fitness change is positively associated with treatment mean- and maximum-stress concentration in the NaCl (Figure S3, A) and CuSO4 datasets (Figure S3, B). Results for absolute fitness difference between CM and CM with added stress also suggest a positive association between fitness difference and treatment mean- and maximum-stress (Figure S3, B & D). Interestingly, evolution in CM with no added stress (Figure S3, B, EH0 treatment) results in elevated fitness difference between assay environments as well.

Generalists may trade-off mean performance for breadth of adaptation (REF – Joshi & Thompson, 1995; REF – Via et al., 1995; REF – Fry 1996; REF – Whitlock, 1996; REF – DeWitt et al., 1998). To detect this potential cost of generalism we depict the fitness change for treatments in the NaCl dataset as heavy points at the intersection of their geometric mean fitness in CM (x-axis) and geometric mean fitness in CM plus chemical stress (y-axis) in Figure 2A; points for treatments EH0 (x-axis specialist) and EH80 (y-axis specialist) are connected with a heavy black line. Treatments that fall below this line pay a cost (re: trade mean performance for breadth of adaptation). Treatments that fall on or above the line do not exhibit evidence of this type of trade-off (REF – Kassen, 2002). Treatments EH0\_40, EH40, EH20\_60, EH0\_80, and EH40\_80 all exhibit evidence of a trade-off (Figure 2A). Results are qualitatively similar for the CuSo4 dataset (Figure S4 A). The magnitude of realized cost (distance below- and to-the-left-of the line) appears to decrease with increasing mean- and maximum-stress in NaCl (Figure 2A) but not CuSO4 (Figure S4 A).

Further examination of fitness change for individual strains (rather than treatment means) prompts several additional observations (NaCl data: figure 2, B:H). First, the endpoints of the line connecting the EH0 and EH80 treatments (Figure 2) are generous with respect to the overall tendency of those treatments, resulting in a tendency towards type II error: failure to detect a cost when one exists. In other words, utilizing only the core data for these treatments (See figure 2, B & H) would shift the line connecting the specialists up and to the right, further exaggerating the cost of generalism already evident in Figure 2A. Second, evaluating the data at the level of individual lineages illustrates that there are lines that deviate markedly from the central tendency of the treatment in all evolutionary histories (Figure 2, B:H). Interestingly, Many evolutionary histories produce lineages with fitness values that fall in close proximity to the “no-cost” line connecting the EH0 and EH80 treatments (Figure 2, D:G). Finally, every generalist treatment includes several lineages that fall far above or to the right of the EH0-EH80 line, indicating that there are outcomes in each that result in no-trade-off adaptation in which lineages appear to enjoy elevated mean fitness *and* breadth of adaptation. Results are qualitatively similar in the CuSO4 dataset (Figure S4, B:H).

**DISCUSSION**

**Result 1: Fitness change relative to zero**

* Topic 1:
  + In NaCl, high stress concentration treatments increase in fitness in CM plus chemical stress and decrease in CM without added chemical stress while no/low-stress treatments increase in fitness in CM but decrease in CM with added chemical stress (Table S4, Left).
    - First, the basic result makes sense: yeast increase in fitness in environments similar to those experienced in their evolutionary history and decrease in fitness in environments that are dissimilar.
    - However, we only expect to observe this pattern if negative pleiotropy (REF – Pleiotropy Theory) exists such that mutations that increase fitness in CM decrease fitness in CM with added NaCl (and visa-versa).
    - CITE AND DISCUSS SUPPORTING / DISSENTING NaCl adaptation LITERATURE HERE.
  + In CuSO4, the tendency is for all treatments to increase in fitness in both CM and CM with added chemical stress (Table S4, Right).
    - This result suggests that there exists positive pleiotropy such that mutations that increase fitness in CM also increase fitness in CM plus CuSO4 stress.
    - CITE AND DISCUSS SUPPORTING / DISSENTING CuSO4 adaptation LITERATURE HERE.
* Topic 2
  + Magnitude of fitness change for treatments that increase in fitness is greater than the magnitude of fitness decrease for treatments that decrease in fitness in every fitness assay environment (Table S4).
  + Observation makes sense: physiological tolerance envelope expands faster than it contracts (REF, REF, REF – Increase fit breadth by greater rate of expansion on leading edge than contraction on lagging edge).
  + Mutations that increase fitness in one environment do not ALWAYS lead to decreases in fitness in the opposite environment (REF – types of mutations) -- could drive a pattern of greater fitness increases than decreases at the treatment level.
  + CITE AND DISCUSS SUPPORTING / DISSENTING experimental evolution of physiological tolerance LITERATURE HERE.
* Topic 3
  + Among treatments that increase in fitness, the magnitude of fitness gain in CM plus stress (for both NaCl and CuSO4 data) is greater than the magnitude of fitness gain in CM alone (Table S4).
  + There are several non-mutually exclusive explanations for this observation:
    - Possible that there are more / more accessible mutations that lead to fitness increase in CM plus stress than in CM alone (REF – available mutations in stress vs no stress).
    - Mutation rate could be elevated in environments with added stress (REF – mutation rates in stress vs. no stress)
    - Likely that there are differences in strength of selection between the environments. Expect strength of selection to be greater in the CM plus stress environment than in CM without added stress (REF – strength of selection in stress vs. no stress)
  + Might expect there to be differences in extinction rate between treatments if there is variation in the strength of selection between CM and CM with added chemical stress (REF – extinction rate as function of strength of selection).
    - Two barcodes were evolved in each well, therefore, fitness gains in one barcode population but not the other could lead to extinction of the lagging barcode population in any well at any time in evolution.
    - However, extinction results do not support this conclusion; treatments EH0 and EH80 exhibit similar levels of extinction in CM and CM with added stress in both the NaCl (EH0: 10/32 extinct, EH80: 11/30 extinct) and CuSO4 (EH0: 22/32 extinct, EH80: 20/30 extinct) datasets (Table S7).
    - It is possible that we do not see differences in extinction rate between CM and CM with added stress because stress concentrations were relatively low overall in order to prevent widespread extinction of lineages during the first few transfers of the evolution experiment, but this should not impact the ability for barcodes to drive one-another extinct (REF – extinction rates via competition across environments variable for stress).
* Topic 4
  + The Magnitude of fitness gain for treatments with positive fitness change in CM plus CuSO4 is greater than the magnitude of fitness gain for treatments with positive fitness change in CM plus NaCl (Table S4).
  + The same non-mutually exclusive explanations for differences between CM and CM plus stress apply here to explain discrepancies between outcomes in NaCl and CuSO4 stress: differences in availability / accessibility of mutations (same REFS), differences in mutation rate (same REFS), and differences in strength of selection are all possible (same REFS).
  + Extinction rate was higher for the EH80 treatment in the CuSO4 dataset than in the NaCl dataset (67% vs. 37%)(Table S7). This pattern holds for the next three highest stress treatments in CuSO4 (EH20\_60: 22%, EH0\_80: 33%, EH40\_80: 25%) versus NaCl (EH20\_60: 12%, EH0\_80: 20%, EH40\_80: 19%) as well (Table S7), suggesting that the strength of selection may have been overall stronger in CuSO4 than NaCl.
  + However, because fitness assays were conducted in a multiplexed fashion, the presence of very high fitness lineages in the CuSO4 treatments (e.g., see the EH80 treatment; Figure 1D; Figure S4, H) may have resulted in elevated rates of extinction for the CuSO4 data overall. This is best evidenced by the difference in extinction rates between chemicals within the EH0 (extinction rate in the CuSO4 dataset is 69%; it is 31% in the NaCl data) and EH40 treatments (CuSO4: 66%; NaCl: 3%).
  + It is likely that the mechanism of adaptation and the exact challenge imposed by the NaCl stress and CuSO4 stress differed in both amount and kind (REF – mechanism of adaptation to NaCl; REF – Mechanism of adaptation to CuSO4).

**Result 2: Fitness change among treatments**

* Topic 1:
  + The pattern of relative fitness difference among treatments in CM and CM with added chemical stress is concordant between the NaCl and CuSO4 datasets despite notable differences between the two chemicals when fitness is assessed relative to 0 (Table S4 vs. Figure 1; note dotted vertical line at 0).
  + This is promising and suggests that the following observations and discussion points regarding adaptation to constant and fluctuating stress may be general.
* Topic 2
  + Overall, evolutionary histories with higher mean- and maximum-stress concentrations (EH20\_60, EH0\_80, EH40\_80, & EH80) rank higher for fitness in the CM with added stress environment and lower in the CM without added stress environment, while the opposite is true for treatments with mean- and maximum-stress concentrations on the low end of the stress concentration spectrum (EH0, EH0\_40, EH40) (Figure 1 C,D vs. A,B).
  + This result is intuitive: relative performance in the presence of chemical stress is associated with the relative degree of exposure to that chemical in evolutionary history.
  + CITE AND DISCUSS SUPPORTING / DISSENTING fitness in env associated with exposure to env LITERATURE HERE.
* Topic 3
  + Echoing the previous results, fitness differences among treatments are more pronounced in CM plus chemical stress than in CM alone for both NaCl (Figure 1 C vs. A) and CuSO4 (Figure 1 B vs D).
  + And, again, the difference in fitness between the evolutionary histories with the most positive and most negative fitness change is more pronounced in CuSO4 than NaCl when the lineages are assayed in CM with added chemical stress (Figure 1. C vs D).
* Topic 4
  + increasing mean- or maximum-chemical stress in evolutionary history results in notably different patterns of adaptation in CuSO4 and NaCl (Figure 1 C vs. D, but see also Table S4).
  + Treatment mean fitness in CM plus NaCl increases at a relatively constant rate as NaCl concentration increases in evolutionary history (Figure 1 A). The trend is reversed in CM without added NaCl: fitness increases steadily with decreasing mean- and maximum-stress (Figure 1 C).
  + Variation in treatment mean fitness in the CuSO4 dataset is more discretized. There is a clear distinction between the EH0, EH0\_40, & EH40 treatments and the EH0\_80, EH40\_80, & EH80 treatments in CM with added stress (Figure 1 D), and to a lesser extent in CM without added chemical stress (Figure 1 B).
  + CITE AND DISCUSS SUPPORTING / DISSENTING adaptation to NaCl=smooth and adaptation to CuSO4=stepwise or binary LITERATURE HERE.
* Topic 5 (Segue to second half of results)
  + There is no obvious difference between evolutionary histories expected to select for specialization (EH0, EH40, EH80) and evolutionary histories expected to select for generalization (EH0\_40, EH20\_60, EH0\_80, EH40\_80) when evaluated in CM and CM with added stress individually (Figure 1).

**Result 3: generalization and specialization**

* Topic 1
  + There does not appear to be any obvious advantage or disadvantage to evolution in constant versus daily-alternating stress when assessing geometric fitness change in CM and CM plus chemical stress either (Figure S3 A, C).
  + As in the single-environment data, there is a directional trend where fitness change is associated with mean- and maximum-stress during evolution; higher mean- and maximum-stress histories are associated with greater (more positive) geometric fitness change (Figure S3 A, C).
  + This latter result reinforces the earlier indication that there is variation in strength of selection between the CM and CM with added stress environments, suggesting that a strategy of increasing geometric mean fitness via a disproportionate increase in CM plus chemical stress (potentially at the cost of performance in CM alone) is prominent in our data.
  + Indeed, this can be seen clearly in all panels of Figure 2 for the NaCl data (and Figure S4 for the CuSO4 data).
  + CITE AND DISCUSS SUPPORTING / DISSENTING geometric mean fitness change tied to harsher environment LITERATURE HERE.
  + Comparison of the magnitude of geometric mean fitness differences among treatments in the NaCl versus CuSO4 datasets (Figure S3, A vs. C) reveals that, much like it was in the single-environment data, the strength of selection was also likely stronger in CuSO4 than NaCl.
* Topic 2
  + The real first indication of variation in kind (rather than amount) between the evolutionary histories expected to select for specialization (EH0, EH40, EH80) and evolutionary histories expected to select for generalization (EH0\_40, EH20\_60, EH0\_80, EH40\_80) is seen in Figure S3, Panel B.
  + Here, the absolute difference in fitness change between CM and CM plus chemical stress is clearly positively associated with mean- and maximum-stress concentration in evolutionary history, but there is also evidence that the specialist treatments, EH0 and EH80, are different than the more intermediate treatments (EH0\_40, EH40).
  + CITE AND DISCUSS SUPPORTING / DISSENTING variance in fitness between generalists and specialists LITERATURE HERE.
* Topic 3
  + *how* geometric mean fitness evolves is also of interest (REF – how does geomean fitness evolve mean and variation in fitness).
  + In our data, geometric mean fitness in CM and CM with added stress (1:1) increases via an increase in variation between performance between environments for most lineages in both NaCl and CuSO4 (Figure S5, A & B).
  + There are lineages that exhibit very little change in geometric mean fitness but accumulate a noticeable difference in fitness between the two environments (Figure S5, especially A). These lineages are tuning their fitness in each environment with little overall change in geometric mean fitness (seen also in REF, REF, REF).
  + There are also lineages that increase geometric mean fitness without a concomitant increase in fitness difference among environments (Figure S5). these lineages are the same that map to the 1st quadrant in Figure 2 (and Figure S4) – lineages with similarly positive increases in fitness in both CM and CM with added stress (seen also in REF, REF, REF).
  + While each treatment occupies a slightly different position in the geometric mean fitness by fitness difference space, there are no obvious differences in the relationship among the treatments nor does there appear to be any overall acceleration nor deceleration of the positive trend in each environment (Figure S5).
* Topic 4
  + Figure 2A provides a clear view of the real difference in kind between evolutionary histories expected to select for specialization and evolutionary histories expected to select for generalization (REF REF REF, expectations for difference in kind for generalists versus specialists).
  + As explained briefly above, this figure depicts the geometric mean fitness change in CM (x-axis) by geometric mean fitness change in CM plus chemical stress (y-axis) for each evolutionary history as heavy points.
  + The points for the “Specialist” treatments EH0 (x-axis specialist) and EH80 (y-axis specialist) are connected with a thick black line, denoting the line of “no cost” (sensu. Kassen, 2002).
  + Generalists (that is, individuals evolved in some environment intermediate to EH0 and EH80) that fall on or above this line are said to exhibit no cost, i.e., exhibit no trade-off between mean performance and breadth of adaptation (REF trade-off in generalists).
  + Lineages that fall below the line do pay a cost: they trade mean performance for breadth of adaptation such that they minimize variation in fitness change among environments (See also Figure S3, B & D) but do not enjoy particularly high geometric mean fitness (See also Figure S3 A & C)
  + All treatments expected to select for generalization with respect to the CM and CM plus 80% chemical stress environments exhibited evidence of this trade-off in both NaCl (Figure 2, A) and CuSO4 (Figure S4, A).
  + CITE AND DISCUSS SUPPORTING / DISSENTING examples of cost / no cost generalism in exp evo LITERATURE HERE.
  + There is additional evidence that the magnitude of this cost (re: distance below and to-the-left of the no-cost line) decreases with increasing mean- and maximum-stress in evolutionary history in the NaCl dataset, to the point where the cost paid by the EH40\_80 treatment is quite close to the detection limit in our system (Figure 2, A). No such evidence is found in CuSO4 (Figure S4, A).
* Topic 5
  + It is, however, important to consider that evolution and the process of adaptation are not necessarily governed by means.
  + Frequently, evolution proceeds via the success of mutations that arise in single individuals / lineages such that populations adapt through overlapping and sequential soft and hard genetic sweeps in which these beneficial mutations rise to high frequency in the population (REF, REF– genetic sweeps, REF REF – mutations in individuals drive adaptation not means). These individual lineages are frequently the focus of experimental evolution research (REF, REF – mutant screens, etc…)
  + We see evidence of this in our data for lineages evolved in a range of NaCl stress concentrations and dynamics (Figure 2 B:H) and in our corresponding CuSO4 dataset (Figure S4 B:H) when we look at fitness in CM (x-axis) versus fitness in CM plus chemical stress (y-axis).
  + First, while it is clear that some evolutionary histories have a prominent central tendency (e.g., Figure 2 D, EH20\_60), this is certainly not the rule in the NaCl (Figure 2 B:H) nor CuSO4 treatments (Figure S4 B:H). Most evolutionary histories exhibit quite a bit of variation among the <= 32 lineages that were evolved under identical treatment conditions; this is a common observation in experimental evolution studies (REF, REF, REF – treatments have a lot of spread in exp evo).
  + In each of the treatments expected to select for generalization (EH0\_40, EH20\_60, EH0\_80, EH40\_80) there exist at least some lineages that do not exhibit evidence of trade-offs between mean fitness and breadth of performance (i.e., fall along the no-cost line) (REF, REF, REF – costless generalists in experimental evolution).
  + The location at which these points fall along the line appears to be associated with the mean- and maximum-stress in each treatment such that treatments with higher means and maximums fall further up and to the left, while those evolved in treatments with lower mean and maximum stress fall on towards lower-right extreme of the line. This makes intuitive sense as the treatments were exposed to challenges that varied in chemical stress amount between the two extremes.
  + Many treatments (e.g., Figure 2 E, EH20\_60; F, EH0\_80; and G, EH40\_80; see also Figure S4, same treatments) also have at least a handful of points that fall above and to the right of the line connecting the specialists, indicating that these generalists have some additional “special quality” (REF, REF, REF – master of all generalists and the like) that allows them to not only escape the cost of generalism but actually enjoy increased mean fitness and breadth of fitness because of it. Again, observation of mutants with particularly high fitness and/or rare phenotypes is common in experimental evolution (REF, REF, REF – mutants and high fitness lines in exp evo).
  + It is possible that a class of mutations exist which result in increased mean fitness and breadth of fitness (as we see here) and that this class of mutations is more accessible or more likely to rise to fixation in treatments from fluctuating evolutionary histories (REF, REF, REF – mutation access differs in different environments).
  + It is also possible that separate mutations increased fitness in CM and CM plus stress within the same lineage; this is, however, unlikely as fixation of multiple mutations is rare in experimental evolution studies of this duration (REF, REF, REF – number of mutations expected in exp evo).
  + Finally, It is important to note that the prevalence of points on and above the no-cost line in the NaCl dataset could be exaggerated by the tendency for the no-cost line to be conservative (biased down and left) in these data (Figure 2). The “real” frequency of low-cost, costless, and potentially master-of-all generalists is likely lower than we observe here. There is, however, no evidence that the CuSO4 dataset suffers from the same bias (Figure S4).
* Topic 6
  + Finally, extinction is more prevalent among lineages in the specialist treatments, EH0 (31% in NaCl data; 69% in CuSO4 data) and EH80 (NaCl: 37%; CuSO4: 67%), than in the generalist treatments, EH0\_40 (NaCl: 16%; CuSO4: 41%), EH20\_60 (NaCl: 12%, CuSO4: 22%), EH0\_80 (NaCl: 20%, CuSO4: 33%), EH40\_80 (NaCl: 19% , CuSO4: 25%) (Table S7#).
  + This could indicate stronger selection in constant versus fluctuating environments (REF, REF, REF – strength of selection associated with extinction rate); the consequences of not acquiring a beneficial mutation are more dire and lead to extinction more frequently in a constantly stressful environment than in one where less harsh conditions are present on alternate days.
  + It is also possible that mutations of large effect are more common in constant versus fluctuating environments (REF, REF, REF – frequency of mutations of large effect associated with stress / dissimilarity from evo hist env.), such that barcodes are likely to be driven extinct when a mutation of positive effect arises in the barcode they share a well with.
  + Alternatively, fluctuation itself could allow more phenotypes / genotypes to persist if certain strategies do relatively well in one environment but do relatively poorly in the alternate-day’s conditions – maybe success only half the time is enough to persist in an environment where success is temporally variable on a relatively short timescale (REF, REF, REF – theory on maintenance of diversity in evo, maybe community ecology too).
    - CITE AND DISCUSS SUPPORTING / DISSENTING examples of flux / complexity allowing diversity to persist in exp evo LITERATURE HERE.

**Conclusion**

* We provide a detailed examination of adaptation to chemical stress in yeast by evolving yeast in seven evolutionary histories variable for stress concentration and dynamics (constant vs. daily-alternating) for 50-days (ca. 500 generations) and subsequently assessing fitness change in the absence and presence of chemical stress.
* We describe a range of similarities and differences between adaptation to NaCl chemical stress and CuSO4 chemical stress and examine at length variation in adaptation among evolutionary histories expected to select for specialization (constant chemical stress treatments) versus generalization (fluctuating chemical stress treatments).
* We consistently recover evidence for a cost of generalism in the form of a trade-off between mean fitness change and breadth of performance, while simultaneously discovering a wealth of variation in strategy and “quality” among individual lineages within each treatment. The cost of generalism is not universal.
* Genomic analyses and additional phenotyping are the next logical steps to examine more deeply the differences between generalists and specialists in these evolved populations.
* Phenotyping across a gradient of chemical stress will allow us to better understand the full physiological tolerance profile for our ancestral and evolved lines, leading to a deeper understanding of how this profile changes during evolution.
* Furthermore, phenotyping in the alternate chemical stress (NaCl evolved lines in CuSO4 stress and visa-versa) will allow us to better explore whether or not generalist strategies translate to fitness advantages in the presence of novel types of stress.
* Genomic analyses are needed in order look at the mechanism by which strains from fluctuating and constant environments adapt to chemical stress. It is likely that different evolutionary histories favor the success of alternative strategies of adaptation.
* Genomic analyses and phenotyping will both also allow us to examine exceptional lineages to understand what makes these lines different from the majority of the population evolved in each environment.