TITLE

**Evolution of no-compromise adaptation to fluctuating selection in yeast**

ABSTRACT

**Background:** Adaptation increases fitness, but there can be different paths to increasing fitness when conditions fluctuate such that multiple outcomes are possible. Specialization with adaptive tracking is one potential evolutionary trajectory, however, specialists to each environment compete with one another and there are restrictive conditions for stable co-existence. Generalization provides another potential adaptive trajectory, where populations gain success across environments by sacrificing depth for breadth of adaptation. Whether generalization or specialization is favored is expected to depend on the costs to each as well as the mutational accessibility of these phenotypes. Both outcomes can increase fitness, at least in the short term, when populations are challenged by novel sources of environmental heterogeneity.

**Results:** In this study, we examine adaptation of 448 asexual populations evolved in constant or fluctuating environments. Specialist phenotypes evolved in constant environments and specialization was sometimes, but not always, accompanied by a cost in the form of fitness tradeoffs between environments. Populations evolved in fluctuating conditions frequently resolved the conflicting environments through increased fitness in both, but suffered a cost relative to populations evolved in constant environments in the form of a trade-off between depth and breadth of adaptation. Despite near-universality of costs at the treatment level for fluctuating evolutionary histories, individual lineages showed notable variation in outcomes. Some populations in each escaped the costs of generalization imposed by fluctuating environments and attained both depth and breadth of adaptation.

**Conclusion:** Adaptation to constant and heterogeneous environments yields repeatable gains in fitness but results in a high diversity of phenotypes across many replicates that may otherwise be missed. While the observed trajectories are just the first step in the adaptive process, they shed light on the types of mutations that may be relevant to evolution in natural populations faced with environmental change.

INTRODUCTION

Environmental conditions vary in space and time in nearly every environment on earth. Heterogeneity, whether driven by abiotic or biotic conditions has been proposed as a driver of diversity and diversification across scales and levels of biological organization (Futuyma & Moreno, 1988; Nevo, 1978; Rosenzweig, 1995; Whittaker & Levin, 1975). For example, heterogeneous environments tend to drive the evolution of wider niche breadths within and among species (Cook & Johnson, 1968; Hulburt, 1985; Janzen, 1967; Stevens, 1989), and to drive an increase in biodiversity at a global scale (Anderson, 1978; Botero et al., 2014; J. H. Brown, 1995; Lomolino et al., 2016; MacArthur, 1964; Nevo, 1978; Rosenzweig, 1995; Tilman, 1982; Weir & Schluter, 2007, 2008).

A wide range of approaches have been utilized to investigate the effects of environmental heterogeneity on diversity and diversification across scales of biological organization. However, causal relationships can be difficult to establish from these studies, as most of them have been limited to drawing conclusions from observed correlations. Experimental evolution provides a powerful approach to isolate and study how environmental heterogeneity relates to adaptive outcomes (Garland & Rose, 2009; Rees Kassen, 2014; Van den Bergh et al., 2018). The advent of cheap, rapid, next-generation sequencing technology and use of robotics has vastly expanded the range of experimental evolution studies that can feasibly be conducted. Thus, it is now possible to examine theories and topics that once could only be studied in natural populations over longer timescales and without the precise control that experimental evolution in a laboratory setting provides.

From these experimental evolution studies, we have gained many important insights into evolutionary processes in complex and heterogeneous environments. For example, we have learned that constant environments tend to promote the evolution of specialists whereas variable ones tend to promote generalization (A. F. Bennett et al., 1992; Rees Kassen & Bell, 1998; Reboud & Bell, 1997; Weaver et al., 1999). Similarly, we now know that the grain of environmental variation (Levins, 1968) matters to evolutionary outcomes (G. Bell & Reboud, 1997; Bradshaw, 1965; Crill et al., 2000; Scheiner, 1993; Via & Lande, 1985). Specifically, studies have shown that spatial heterogeneity often allows the evolution and coexistence of multiple specialists (Dykhuizen & Davies, 1980; Garcia-Dorado et al., 1991; Joshi & Thompson, 1997; Silver & Mateles, 1969; Taplitz & Coffin, 1997; Verdonck, 1987; Wasserman & Futuyma, 1981), whereas temporal heterogeneity tends to favor generalists (Reboud & Bell, 1997). The capacity to adapt to degrading and rapidly changing environments (G. Bell & Gonzalez, 2011; Dallinger, 1887; Gonzalez & Bell, 2013; Gorter et al., 2017; Low-Décarie et al., 2015; Swings et al., 2017) has also been shown to depend on the dynamics of environmental change (Gorter et al., 2017; Gorter, Aarts, et al., 2016), population history (Gonzalez & Bell, 2013; Mongold et al., 1999), migration rate (J. S. Brown & Pavlovic, 1992; Holt, 1996; Kawecki, 2000; Low-Décarie et al., 2015), and the degree of difference from ancestral conditions (A. F. Bennett et al., 1992; Mongold et al., 1996). Additionally, spatial structure (Kryazhimskiy et al., 2012), environmental complexity (Boyer et al., 2021; Dhar et al., 2013; Gao et al., 1992, 1994; Merlo et al., 2020; White et al., 2020), and the predictability of the environment (Graham et al., 2014; S. M. Karve et al., 2016; Shraddha M. Karve et al., 2018; Manenti et al., 2015; Serra et al., 2020; Sorensen et al., 2018; Tarazona et al., 2017, 2019) have been shown to impact rates of adaptation and adaptive outcomes. Finally, evolutionary studies in coevolving populations (Ferris et al., 2020; Gorter, Scanlan, et al., 2016; Jordt et al., 2020; Kloock et al., 2020; Papkou et al., 2019; Quintero-Galvis et al., 2018; Rafaluk-Mohr et al., 2018; Vidal & Segraves, 2021; Zhao et al., 2017) have established that ecological outcomes can be more complex in when constructed assemblages are larger (Kerr et al., 2002; Marchal et al., 2017; Nahum et al., 2011; Prado & Kerr, 2008).

Basic questions remain regarding the role of environmental heterogeneity in promoting the evolution of generalization versus specialization. For example, experimental studies have shown that generalist phenotypes do not always evolve, either because of a lack of genetic variation in the founding population (Riddle et al., 1986), or because of strong negative genetic correlations in fitness among environments (G. A. C. Bell, 1997). Furthermore, cases abound where environmental heterogeneity is associated with no response to selection in any treatment for traits of interest (Choo et al., 1980; Ehiobu & Goddard, 1989; Joshi & Thompson, 1997; Scheiner & Yampolsky, 1998), and there are cases where responses are observed in lines from constant but not variable treatments (G. A. C. Bell, 1997; Gao et al., 1992, 1994; Hodges et al., 1992). In short, results are mixed and studies tend to be constrained in size, complexity, and number of treatments or comparisons (Condon et al., 2014; Hughes et al., 2007; S. M. Karve et al., 2015; Shraddha M. Karve et al., 2018; Shraddha Madhav Karve et al., 2016; T. Ketola & Saarinen, 2015; Tarmo Ketola et al., 2013; New et al., 2014; Razinkov et al., 2013). Consequently, we still know little about the associated costs, consequences, and outcomes of adaptation under heterogeneous conditions and, in comparison to static environments, there is a distinct need for complex, replicated studies of adaptation in that context (R. Kassen, 2002). Additionally, little is known about the process of adaptation to heterogeneous environments, particularly about how the initial steps of adaptation proceed. Understanding these initial adaptive responses to environmental fluctuation is critical in the present context given that many of the stresses that ecosystems face today are related to increased environmental fluctuation and environmental extremes (Diffenbaugh, 2020).

In this study we examine evolution in constant and temporally variable environments. We evolved 448 genetically barcoded *Saccharomyces cerevisae* lineages across 14 treatments that vary in the concentration, dynamics, and identity of a chemical stress. We examine the costs and consequences of adaptation and additional costs associated with evolution in fluctuating environments. We find that adaptation in a constant environment results in fitness costs under alternative conditions for some, but not all, populations. Treatments evolved in fluctuating environments pay an additional cost by trading depth for breadth of adaptation. However, the cost associated with evolution in fluctuating environments is not universal; some populations pay no cost, while others go one step further and acquire both breadth and depth of adaptation. Our experiment was short, just 500 generations, and focused on evaluating the first steps of adaptation in heterogeneous environments, but it is possible that the populations without cost are the material through which evolution proceeds when environmental conditions fluctuate. These observations may represent adaptive trajectories that would be viable in natural populations.

**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) (Sniegowski et al., 2002): YJF153 (MATa, *HO*::dsdAMX4) and YJF154 (MATalpha, *HO*::dsdAMX4) (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 (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).

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, read as: **E**volutionary **H**istory 0%), 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. Yeast lines 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 with and without additional chemical stress. Yeast lines evolved in microplates with NaCl treatments were assayed in NaCl chemical stress and yeast lines 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 line prior to evolution (Day-0 assays) and after evolution (Day-50 assays) was quantified and the resulting values were used to assess change in fitness for each line in each environment relative to the static ancestral reference (see *Fitness Calculations*, below).

**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 as well as any contamination that may have occurred during sample processing. 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). To avoid noise due to low counts entries with <= 20 reads were treated as missing data and removed prior to read summary reporting and downstream analyses.

*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) (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%).

*Fitness calculations:* The Malthusian fitness of focal barcoded line *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) (Chevin, 2011; Hartl & Clark, 1997). We use the standard equation *m=ln(w)* to convert Malthusian fitness values to Wrightian fitness values (Orr, 2009; Passagem-Santos & Perfeito, 2018; Wu et al., 2013). Hereafter, fitness, denoted by a *w* will refer to Wrightian fitness. The change in fitness of line *i* between Day-0 and Day-50, *Δwi*, was therefore computed as,

Equation 2.

Where wi day-0 and wi day-50 are the line’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:* Lines 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. There were no cases in which both barcodes in a microplate well went extinct: extinction of one barcode resulted in fixation for its competitor.

**Statistical analysis**

*Analysis & visualization tools:* R version 4.0.2 was used for all calculations, analyses, and figure generation (Core R Team, 2020). Data processing uses base-R functionality supplemented with methods from the plyr package (Wickham, 2011). Statistical models with linear mixed effects utilize the lme4 (Bates et al., 2015) and lmerTest packages (Kuznetsova et al., 2017). Power analyses were conducted using the pwr package (Champely, 2018). Figures and tables were generated with ggplot2 (Wickham, 2009) and sjPlot (Ludecke, 2019); multi-panel figures were built using methods from grid (Core R Team, 2020), gridExtra (Auguie, 2017), and cowPlot (Wilke, 2020).

*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. Consequently, we have 80% power to discern fitness differences of 0.306% between treatments and 80% power to identify fitness deviations from zero of 0.634% (Figure S2 B, Table S3). We have 80% power to detect a fitness change (increase or decrease) of 2.163% for any individual barcode (Figure S2 A, Table S4).

*Fitness change in 50 days of experimental evolution:* Using the 80% power cutoff, we called individual barcoded yeast lines that increased (>= 2.163%) or decreased (<= 2.163%) in fitness over the 50-day experimental evolution. The effect of evolutionary treatment on fitness change was assessed using linear mixed-effects models with change in fitness as the response variable and treatment as the predictor variable. A random effect of line ID was placed on the model intercept (fitness change ~ treatment + (1|line ID) + 0; family = gaussian). Separate models were run for each dataset by environment (NaCl lines in CM, NaCl lines in CM+NaCl, CuSO4 lines in CM, CuSO4 lines in CM+ CuSO4). This set of linear mixed effects models was repeated without the intercept term to assess fitness differences among treatments (rather than versus zero fitness change).

**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 (https://github.com/VinceFasanello/FS\_Code\_Supplement). 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.” Yeast lines are available from the Justin C. Fay Lab at The University of Rochester(e: justin.fay@rochester.edu).

**RESULTS**

We evolved 448 yeast lines for 50 days (ca. 500 generations) in environments that varied in the concentration, dynamics, and identity of a chemical stress. Thirty-two barcodes were initialized into each of seven treatments with two barcodes per microplate well. Three treatments were subject to constant NaCl or CuSO4 concentrations equal to 0%, 40%, or 80% the ancestral lethal limit for that chemical, hereafter referred to as treatments EH0, EH40, and EH80, respectively. Four treatments 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) line against a static reference strain in 0%, 40%, and 80% stress. From these data we calculated change in fitness for each barcoded yeast line in each assay environment. Datasets for NaCl stress and CuSO4 stress are hereafter referred to as “NaCl data” and “CuSO4 data”.

**Fitness gains in 500-generations of experimental evolution:** We expected yeast lineages to adapt to the chemical stress concentrations to which they were exposed in evolution. Indeed, although most strains exhibited some fitness gains under 0% *or* 80% chemical stress (Figure 1), lineages that experienced less chemical stress during experimental evolution tended to exhibit greater fitness gains in the 0% environment (Figure 1., A & B), whereas those that were exposed to higher stress concentrations during evolution tended to exhibit greater fitness gains in media with 80% chemical stress (Figure 1., C & D).

Adaptation to a particular set of environmental conditions is likely to impact fitness in alternative environmental conditions. These effects can be complementary, neutral, or antagonistic. We observed all three cases in our data. In the NaCl dataset, adaptation to 0% chemical stress was associated with negative fitness change in 80% stress (Figure 1., A & C - EH0) and adaptation to 80% stress was associated with negative fitness change in the 0% stress environment (Figure 1., A & C – EH80, EH40\_80, EH0\_80). In the CuSO4 dataset, adaptation to 0% chemical stress frequently resulted in a fitness increase in 80% stress (Figure 1., B & D - EH0) as well, while adaptation to 80% stress had neither a positive nor negative effect on fitness change in the 0% chemical stress environment (Figure 1., A & C – EH80, EH40\_80, EH0\_80).

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**Figure 1:** Fitness change in in 0%, 80% chemical stress after 500-generations experimental evolution. (A) Fitness change in 0% stress for lineages from the NaCl dataset. (B) Fitness change in 0% stress for the CuSO4 dataset. (C) Fitness change in 80% NaCl stress. (D) Fitness change in 80% CuSO4 stress. Lower-triangle insets illustrate fitness differences among treatments; significant associations have beta-values, non-significant associations in grey. Asterisks denote treatment differences from 0 (no fitness change). Black open circles are treatment median fitness. Black Closed circles are treatment mean fitness with standard error bars depicted. Panels A, C and B, D depict data from two, separate, evolution experiments: A, C depict data from the “NaCl” experiment; B, D depict data from the “CuSO4” experiment. Lineages in A, C were assayed only in CM (A) and 80% NaCl (C); lineages in B, D were assayed only in CM (B) and 80% CuSO4 (D).

**Genetic correlation in fitness:** Selection under uniform environmental conditions favors individuals whose fitness is highest in that environment and should therefore result in the evolution of specialism (A. F. Bennett et al., 1992; Rees Kassen & Bell, 1998; Reboud & Bell, 1997; Weaver et al., 1999). Consequently, cross-environment genetic correlation in fitness should evolve to become negative if adaptive mutations perform better in their evolutionary environment than in other environments. Positive genetic correlations in fitness arise when mutations perform equally or better in an alternative environmental compared to lineages with evolutionary histories in that alternative environment itself (R. Kassen, 2002). To test for cross-environment genetic correlation in fitness we compared the fitness of strains evolved under different experimental treatments in media with 0%, 40% and 80% stress.

As in Kassen (R. Kassen, 2002), we identified cross-environment genetic correlations in fitness as the slope of the line connecting the fitness of the specialist treatments for that pair of environmental conditions (Figure 2). We observed negative cross-environment genetic correlations in fitness between 0% and 40% chemical stress (Figure 2, A) and between 0% and 80% chemical stress (Figure 2, B). The slope of this relationship became more negative with increasing environmental dissimilarity (Figure 2, A vs. B). In contrast, we found a positive cross-environment genetic correlation in fitness between the 40% stress and 80% stress environments in both chemicals (Figure 2, C). Despite noteworthy differences in patterns of adaptation in the NaCl and CuSO4 datasets (Figure 1), patterns of cross-environment genetic correlation were qualitatively similar under both chemical stressors.

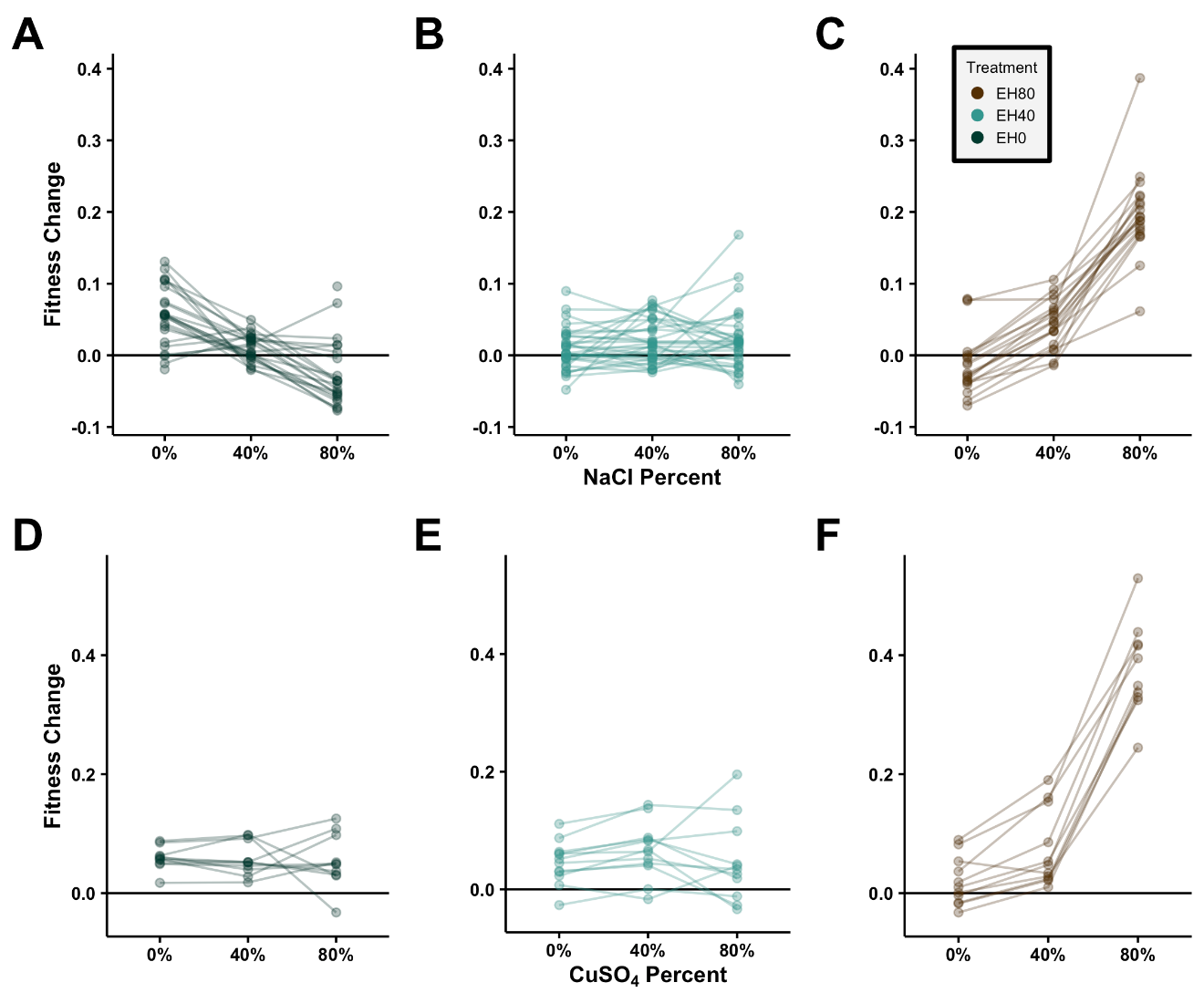
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**Figure 2:** Cross -environment genetic correlation in fitness. Fitness change for each treatment is depicted in a two-dimensional space with fitness change in the lower stress environment on the x-axis and fitness change in the higher stress environment on the y-axis. Negative slopes indicate the presence of a negative cross-environment genetic correlation in fitness change. Positive slopes indicate the presence of a positive correlation. (A) Blue. EH40, EH0 in 0%, 40% stress; (B) Purple. EH0, EH80 in 0%, 80% stress; (C) Red. EH40, EH80 in 0%, 80% stress. NaCl dataset depicted with circle endcaps; CuSO4 dataset depicted with triangular endcaps. Dotted lines included for visual comparison of slopes. Axes have the same scaling in A, B, C to allow comparison of cross-environment genetic correlation in fitness between pairs of environments.

**Costs of adaptation in constant conditions:** Negative cross-environment genetic correlation in fitness can evolve due to fitness trade-offs, in which adaptation to one environment has a fitness cost in others such that fitness increases in the home environment are associated with fitness decreases in other environments. Alternatively, negative cross-environment genetic correlation in fitness can arise in the absence of strict costs if direct responses to selection are reciprocally larger than correlated responses in other environments, i.e.- each treatment adapts more to its home environment than to other conditions (Reboud & Bell, 1997).

To evaluate the prevalence of costs of adaptation in our data, we assessed whether strains evolved under constant conditions exhibited fitness gains in their respective treatment environments and fitness losses in other environments. At the treatment level, we observed costs in the NaCl dataset, but not the CuSO4 dataset when we examined the extremes: treatments EH0 vs EH80 in 0%, 80% chemical stress (Figure 2). Results for individual lineages reciprocated these findings: costs were relatively common in the NaCl data (Figure 3 A, C; Table 1), but rare in the CuSO4 dataset (Figure 3 D, F; Table 1). Results for the EH0 and EH80 treatments in 40% stress were intermediate (Figure 3 A, C, D, F; Table S5). The majority of lineages from the EH40 treatments did not exhibit evidence of costs in 0% nor 80% stress, regardless of chemical identity (Figure 3 B, E; Table S5).



**Figure 3:** Fitness relationships for individual barcodes. Fitness change in CM plus 0%, 40%, and 80% chemical stress for treatments exposed to constant chemical stress at 0% (EH0), 40% (EH40), and 80% (EH80) the ancestral lethal limit. Each line depicts the fitness change in three assay environments for a single barcoded yeast strain. Top row shows data from our NaCl dataset: (A) EH0, (B) EH40, (C) EH80. Bottom row shows data from our CuSO4 dataset: (D) EH0, (E) EH40, (F) EH80. One barcoded strain with low fitness and missing data removed from (A) and one strain with low fitness removed from (C) for visualization purposes only.

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| --- | --- | --- | --- |
| ***Chemical Identity*** | ***Treatment*** | ***0% Stress*** | ***80% Stress*** |
| **NaCl** | EH0 | **17/23 (74%)** | 14/17 (82%) |
|  | EH80 | 12/20 (60%) | **20/21 (95%)** |
| **CuSO4** | EH0 | **9/10 (90%)** | 1/9 (11%) |
|  | EH80 | 2/11 (18%) | **11/11 (100%)** |

**Table 1:** Number of lineages with positive or negative changes in fitness. Number of positive changes in evolutionary environment (bold) and negative fitness change in alternate environment (non-bolded) in 0%, 80% chemical stress for the NaCl and CuSO4 datasets. To quantify cost, the numerator in the evolutionary environment entry becomes the denominator for the other entry in the row such that the alternate (“non-home”) environment reports the number (percentage) of lineages that increased at home *and* decreased in the non-home environment. Fitness change of 2.16% was used as a cutoff for positive and negative changes.

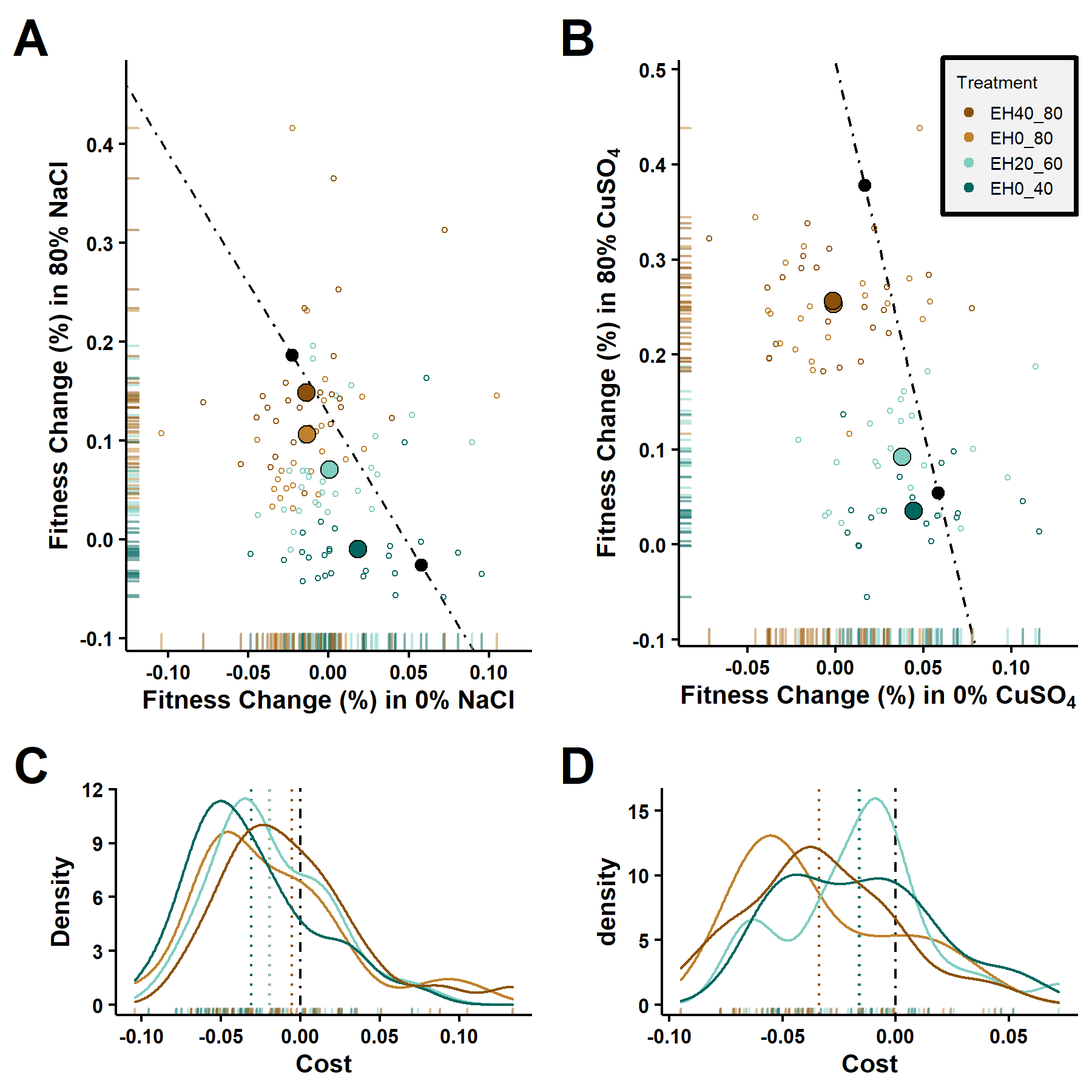
**Costs of adaptation in fluctuating conditions**

Adaptation to environmental fluctuation over short time scales often involves generalization (Bradshaw, 1965; Levins, 1968), particularly in cases where negative cross-environment genetic correlations in fitness that are not driven by strong trade-offs in adaptation to different environmental conditions and when specialist and generalist mutations are both reasonably accessible. Our evolutionary conditions meet these requirements. Therefore, adaptation to environmental fluctuation in our experiment could have favored strategies that trade-off mean performance for breadth of adaptation as is the expectation under the classic “jack of all trades, master of none” paradigm (DeWitt et al., 1998; Fry, 1996; Joshi & Thompson, 1995; Via et al., 1995; Whitlock, 1996).

To evaluate whether this proposed trade-off was evident in our data, we assayed the fitness of lines selected in constant (EH0, EH80) and fluctuating conditions (EH0\_40, EH20\_60, EH0\_80, EH40\_80) in environments with 0% and 80% chemical stress—i.e., the conditions used in constant selection treatments. Under this model, a scenario in which breadth and depth of adaptation do not trade off against each other can be inferred if strains from fluctuating environments lie on the line connecting the performance of EH0 and EH80 treatments. Alternatively, if breadth of adaptation increases at the expense of mean performance, then the performance of lineages evolved under fluctuating treatments should fall below this line. Finally, if evolution in fluctuating chemical stress concentration results in jack of all trades, master of all” generalist phenotypes, then the performance of lineages evolved in these treatments should fall above the “no cost” line (R. Kassen, 2002).

*Costs are universal at the treatment level:* All treatments with evolutionary histories in fluctuating environments (EH0\_40, EH20\_60, EH0\_80, EH40\_80) traded mean performance for breadth of adaptation in the CuSO4 data, i.e.- these treatments fell significantly below of the no-cost line at a detection limit of 0.634% fitness change (Figure 4 B, D; Table S6). Treatments EH0\_40, EH20\_60, and EH0\_80 exhibited this trade-off in the NaCl dataset; results were non-significant for the EH40\_80 treatment (Figure 4 A, C; Table S7). The magnitude of realized cost (distance below the no-cost line) was negatively associated with the amount of chemical stress experienced during evolution in the NaCl dataset (Figure 4, A; Table S7). The opposite pattern exists in the CuSO4 data: cost was positively associated with the amount of chemical stress experienced (Figure 4, B; Table S6). Costs were also observed for the EH0\_40 treatment relative to the EH0 and EH40 specialists in both chemicals (Figure S3).

*A diversity of strategies exists within each treatment:* Coarse, treatment-level, results suggest the general existence of a trade-off between breadth of adaption and mean performance but fail to capture the full range of adaptive outcomes that are present in each treatment. Examination of fitness change for individual lineages revealed a rich diversity of phenotypes within each treatment and uncovered broad overlap in fitness phenotypes among treatments (Figure 4, small open circles). In the NaCl data, 58/105 (55%) of populations across the EH0\_40, EH20\_60, EH0\_80, and EH40\_80 exhibited significant costs at a detection limit of 2.163% fitness change; 46/88 (82%) showed costs in the CuSO4 dataset. These represent classical “jack of all trades, master of none” generalist trajectories. Each treatment contained a subset of replicates whose performance was not significantly different from the no-cost line connecting the EH0 and EH80 treatments (i.e., showed no evidence of a trade-off). Across the fluctuating treatments, 30/105 (29%) exhibited no cost nor benefit of adaptation in fluctuation in the NaCl data and 34/88 (39%) exhibited no cost nor benefit in the CuSO4 data. The trade-off of mean performance for breadth of adaptation (i.e., the “cost of generalism”) is, therefore, not a universal outcome. Furthermore, all treatments contained at least some lineages that fell significantly above the no cost line, indicating that some lineages may have developed adaptations that allow them to increase *both* mean fitness and breadth of adaptation, i.e.- “jack of all trades, master of all” generalist trajectories; 17/105 (16%) populations in the NaCl data, and 8/88 (9%) in the CusO4 data fell into this class. Cost classes broken down by treatment are available in Table S8.



**Figure 4:** Lineages with evolutionary histories in fluctuating chemical stress environments do not always trade depth for breadth of adaptation. (A, B) Black circles are the treatment mean fitness for the constant chemical stress lineages EH0 (bottom, right) and EH80 (top, left). Orthogonal distance below- and to the left of the dot-dash line connecting the EH0 and EH80 indicates a fitness cost in the form of a trade-off of depth for breadth of adaptation. Lineages on the line pay no cost of generalization. Lineages orthogonally above- or to the right of the dot-dash line enjoy additional fitness benefits in the form of depth and breadth of adaptation. (A,B) Cost of generalism for all fluctuating chemical stress treatments (EH0\_40, EH20\_60, EH0\_80, EH40\_80) relative to the constant 0% (EH0) and 80% chemical stress (EH80) treatments. (C,D) Corresponds to (A,B) data; density plots depict cost, distance above (+), below (-) the dot-dash line. (A,C) data for the NaCl dataset; (B,D) CuSO4 dataset.

**DISCUSSION**

Microbial experimental evolution provides a tool with which we can examine adaptation to fluctuating environments and explore how evolution may proceed in the natural world, where mechanisms are difficult to isolate and replicated evolutionary experiments are frequently out of reach. In this work, we examined adaptation in constant and fluctuating environments. Our results show most populations are consistent with expectations: selection in constant conditions results in the evolution of negative cross-environment correlations in fitness that are sometimes associated with a fitness cost in alternate environments. Evolution in temporally fluctuating environments typically results in the sacrifice of adaptive depth for breadth of adaptation. Despite the repeatability of these results, they are not universal. With sufficient replication, we uncover a broad diversity of adaptive trajectories both in terms of the costs of adaptation to constant environments and in the expected breadth-for-depth trade-off for lineages exposed to fluctuating conditions. We explore these topics in more depth below.

**TOPIC 1: The Population Genetics of Specialization**

*Cross-environment genetic correlation in fitness*Selection in a constant environment should result in the evolution of specialization. Consequently, negative genetic correlations in fitness across environments should evolve if mutations are environment-specific, do not perform equally in all environments, or if mutation accumulation is prominent (G. Bell & Reboud, 1997; A. F. Bennett et al., 1992; Deatherage et al., 2017; Gao et al., 1992, 1994; Rees Kassen & Bell, 1998; Mongold et al., 1996; Shiotsugu et al., 1997; Shirley & Sibly, 1999; Tisdale et al., 1995; Weaver et al., 1999). The cross-environment fitness correlations uncovered in our NaCl and CuSO4 datasets are consistent with these expectations: negative correlations are common when environments with and without added chemical stress are assessed (Figure 2., A, B), and the strength of this negative correlation depends on the degree of difference in environmental conditions (Figure 2., A v. B.).

If the specific selective pressures presented by two environments are similar (e.g., different in amount but not kind), measured cross-environment genetic correlation in fitness may be positive (at least in the short term) (Reboud & Bell, 1997). We see this in our data when lineages are assayed in environments with different sublethal chemical stress concentrations (Figure 2., C). Mutations that confer fitness benefits in 40% chemical stress also confer fitness gains in 80% chemical stress, and visa versa. However, adaptive trajectories are not identical. Evolution in 80% chemical stress confers a greater benefit in 40% stress than evolution in 40% stress does in the 80% chemical stress environment (Figure 3, B v. C, E v. F). It is possible that genetic correlations in fitness would again become negative at higher stress concentrations because adaptations that confer benefits in sublethal stress concentrations do not always confer benefits in lethal stress concentrations, where a different class of mutations may underlie survival (Mongold et al., 1999).

*Costs of adaptation*

*Fitness trade-offs v. Correlated Responses:* Two, non-mutually exclusive, drivers can underlie the evolution of negative cross-environment correlation in fitness. Adaptation to one environment can come with a pleiotropic cost of adaptation in other conditions. This phenomenon is traditionally referred to as a “fitness trade-off” in which fitness increases under one condition directly result in fitness decreases under one or more other contexts. However, the evolution of negative cross-environment genetic correlation in fitness need not involve trade-offs. Negative fitness correlations can also evolve if direct responses to selection tend to be larger than correlated responses such that specialists reciprocally perform better in their evolutionary environment than in the alternate condition (G. Bell & Reboud, 1997).

The traditional view is that negative cross-environment genetic correlation in fitness primarily evolves due to trade-offs in fitness among environments (i.e., not correlated responses). Most studies support this prediction (G. Bell & Reboud, 1997; Cooper & Lenski, 2000; Dallinger, 1887; Mongold et al., 1996; Reboud & Bell, 1997; M. Travisano et al., 1995; Michael Travisano & Lenski, 1996; Weaver et al., 1999). We found evidence for fitness trade-offs at the treatment level in the EH0, EH80 treatments assayed in CM and CM + 80% chemical stress in the NaCl dataset (Figure 2, B), but not when comparing the EH0 and EH40 treatments in CM and CM + 40% chemical stress (Figure 2, A). We did not uncover evidence for fitness trade-offs at the treatment level in our CuSO4 dataset (Figure 2 A, B). Instead, correlated responses, in which specialists increase in fitness in both environments but reciprocally perform better in their evolutionary environment, tend to drive negative cross-environment fitness correlations in these latter comparisons. Correlated responses like the ones we uncover here are infrequently uncovered as drivers of negative cross-environment genetic correlations in fitness; trade-offs tend to be the rule (A. F. Bennett et al., 1992; Albert F. Bennett & Lenski, 1993).

Interestingly, the presence and magnitude of trade-offs within treatments both exhibit notable variation at the individual barcode level. In fact, the percentage of populations with a realized cost exhibited wide variation among treatments and chemicals and was never present in greater than 82% of replicates within a treatment (Figure 3, Table 1, table S5). We observed fitness costs in a fair proportion of lineages from the EH0 and EH80 treatments in the NaCl dataset (Figure 3 A, C; Table 1; Table S5), while costs were rare amongst replicates from the same treatments in the CuSO4 dataset (Figure 3 D, F; Table 1; Table S5). Our findings indicate that adaptation, even in constant environmental conditions, does not lead to homogeneity in adaptive trajectories. There are likely multiple feasible adaptive trajectories in any environment and these trajectories can differ markedly in cost when challenged with environmental conditions dissimilar to the evolutionary environment. Additional evidence of heterogeneity in fitness for specialist treatments is apparent in Figure 1., where we observe a wide range of negative, neutral, positive fitness change in the alternate environment for lineages from constant environments (Figure 1., EH80 in A, B; EH0 in C, D).

Taken together, our treatment- and replicate-level findings indicate that mutations that confer benefits in CM or in CuSO4 are likely to have some cross-environment benefit, while the same is not true in CM versus NaCl where the mutations that seem to be favored (or accessible) can have negative pleiotropic effects in the alternate environment. These results highlight the importance of utilizing multiple stressors to assess questions of adaptation rather than basing results on adaptation to a single chemical stress.

*Trade-offs are greater when environments are more dissimilar:* When Trade-offs underlie negative genetic correlations in fitness between environments, trade-off intensity is commonly associated with the magnitude of the difference between the evolutionary environment and the assay environment (Mongold et al., 1996; Michael Travisano & Lenski, 1996). Our results support this assertion (Figure 3 A:C; Table 1; Table S5). Replicates from the EH0 treatment do not pay a significant fitness cost in CM + 40% chemical stress, but most exhibit significant fitness costs in CM + 80% chemical stress (Figure 3, A). Mirroring these results, replicates from the EH80 treatment do not exhibit evidence of fitness costs in CM + 40% chemical stress, but do show evidence of significant costs in CM without added chemical stress (Figure 3, C). Results are mixed in the EH40 treatment. Regardless, the magnitude of costs paid by the EH40 lineages in both CM and CM + 80% chemical stress are less than those paid by the EH0 lineages in CM + 80% chemical stress and those paid by the EH80 lineages when assayed in CM.

*Fitness trade-offs driven by antagonistic pleiotropy v. mutation accumulation:* When present, trade-offs in fitness among environments can stem from two sources: Antagonistic pleiotropy—i.e., genes favorable in one environment are deleterious in others—and mutation accumulation—i.e., accumulation of mutations that are neutral in the environment of selection but deleterious elsewhere. Antagonistic pleiotropy is the largest contributor to the cost of adaptation and the maintenance of specialization in the short-term, while mutation accumulation is thought to be the largest contributor to the cost of adaptation in the long term (Reboud & Bell, 1997). Both are likely to co-occur as drivers of fitness trade-offs in longer experimental evolution trials. However, the expectation is that antagonistic pleiotropy dominates if the length of experiment is short, simply because there is no time for multiple mutations to accumulate (Michael Travisano & Lenski, 1996). Antagonistic pleiotropy and mutation accumulation have been observed in experimental evolution studies, however, reports of antagonistic pleiotropy are more common (see (Cooper & Lenski, 2000; M. Travisano et al., 1995; Michael Travisano & Lenski, 1996) v. (Reboud & Bell, 1997)), likely because most experimental evolution projects do not run for long enough or do not have sufficient supply of mutations to allow mutation accumulation to contribute noticeably to costs.

Our study was relatively short (500 generations) compared to the supply of mutations (re: population size x mutation rate), so we expected antagonistic pleiotropy to be the underlying driver of observed costs of adaptation in our lines (when costs were present). If mutation accumulation were the primary driver of fitness trade-offs among environments, we would have expected lineages from fluctuating environments to perform similarly to those from constant environments in the evolutionary environments of the constant selection lines. We do not see this; Most replicates from our fluctuating environmental treatments perform worse than constant selection lineages in the evolutionary environment of the latter (Figure 1). Again, however, there is marked variation among populations within a treatment in all cases. Some replicates from fluctuating treatments outperform replicates from the constant selection treatments in the environment in which the constant selection lines were evolved (Figure 1). It is possible that we would have uncovered a stronger role for mutation accumulation as a driver of fitness trade-offs if we ran our experiment for many more generations.

**TOPIC 2: Costs of Generalization**

Conditions for coexistence of specialists in heterogeneous environments are strict (Rees Kassen et al., 2000; Smith & Hoekstra, 1980; van Tienderen, 1997). Consequently, ecological generalists have the opportunity to evolve when the environment is spatially or temporally heterogeneous.

*Sources of cost:* Evolution of generalization is not without constraint. If it were, generalists would arise and dominate under nearly all circumstances. Evolution of generalist strategies can be constrained by two non-mutually exclusive sources: one possibility is that the existence of intrinsic functional interference in performing two qualitatively different tasks equally well leads to a negative cross-environment genetic correlation in fitness, making it impossible to adapt to one environment without losing ground in others. This cost underlies the classic idea that generalists are the “jack of all trades, but master of none”. Another cost associated with generalization, is that generalism may not evolve, at least in the short-term, if more accessible strategies for success are attainable by specialist phenotypes. This latter constraint would lead to rapid evolution of specialism relative to comparatively slow evolution of generalist phenotypes (Futuyma & Moreno, 1988). Given their prevalence, costs associated with adaptation in fluctuating environmental conditions likely stem from negative cross-environment correlations in fitness in our system. We see some evidence that lineages in the EH0\_40 fluctuating treatments have fitness phenotypes similar to lineages from the EH0 constant selection treatment (Figure 4), indicating a potential role for mutation accessibility in the adaptive trajectories observed in our study. Despite no obvious evidence for similar patterns at the top end of our stress gradient, i.e.- between the EH0\_80 & EH80 or EH40\_80 & EH80 treatments (Figure 4), we cannot completely rule out additional constraints derived from differences in the accessibility of mutations that confer breadth over depth of adaptation in higher chemical stress environments.

*Generalists trade depth for breadth of adaptation:* Costs of generalization can manifest as a trade-off in depth of adaptation for breadth of adaptation. Consequently, generalists will have lower-than-expected fitness when compared against specialists in a two-dimensional fitness space depicting performance of specialist lineages in their evolutionary environments (sensu R. Kassen, 2002, Figure 2.), i.e.- lineages from fluctuating evolutionary histories should fall below the no-cost line connecting the performance of the EH0, EH80 lineages in our Figure 4. Indeed, evidence for this sort of trade-off is sometimes uncovered in the literature (A. F. Bennett et al., 1992), but it is by no means universally observed (Rees Kassen & Bell, 1998; Reboud & Bell, 1997; Weaver et al., 1999). We uncover trade-offs between depth and breadth of adaptation in each of our fluctuating treatments when assessed under a hypothetical scenario of fluctuating exposure to 0% and 80% chemical stress (Figure 4). The magnitude of observed costs are similar in our NaCl and CuSO4 datasets and are universal at the treatment level. As stated above, these results are intuitive given the negative cross-environment correlation in fitness between the 0% and 80% chemical stress environments in both datasets.

*Costs of generalization may be associated with the range of environmental fluctuations:* The costs of generalization should be greater for lineages that experienced a wider range of conditions during evolution, especially if negative cross-environment genetic correlation in fitness drives the observed costs of generalization rather than maintenance or accessibility of generalist-specific mutations. This has rarely been assessed in experimental evolution studies, and existing examples provide mixed support (Rees Kassen & Bell, 1998; Scheiner & Yampolsky, 1998). We do not find convincing evidence to support the assertion that costs of generalization increase with fluctuation range. In our NaCl data, costs were equivalent for the EH0\_40 treatment and the EH0\_80 treatment when assayed against the relevant specialists (Figure S3, A, B, C). Additionally, costs were greater for the EH0\_40 treatment when the EH0\_40 treatment and EH0\_80 treatment were assayed against the EH0 and EH80 specialists (Figure 4 A, C). In fact, costs appear to decrease with chemical stress concentration in the NaCl data rather than showing any convincing association with the range of fluctuation in stress. CuSO4 results are quite different. Here, the EH0\_40 treatment exhibits a lower cost relative to the EH0 and EH40 specialists than does the EH0\_80 treatment when assayed against the EH0 and EH80 specialists (seeming to support the hypothesis that wider flux is associated with greater cost) (Figure S3, D, E, F). However, when all fluctuating and intermediate treatments are assayed against the EH0 and EH80 specialists in 0%, 80% chemical stress, it becomes apparent that cost increases with chemical stress concentration in the CuSO4 data rather than showing an association with fluctuation range per say (Figure 4 B, D). These results are likely driven by differences in the strength of selection between 0% and 80% NaCl stress and by asymmetry in the pleiotropic benefit of CM vs. CuSO4 mutations in the CuSO4 data.

*Costs of generalization are not universal:* Costs of generalization are not always detected (R. Kassen, 2002). Lack of detectable trade-offs of depth of adaptation for breadth of adaptation in lineages selected for generalization may indicate that populations are far from equilibrium (Rees Kassen & Bell, 1998), that negative correlation in performance across environments is driven by mutation accumulation rather than antagonistic pleiotropy (Reboud & Bell, 1997), or that some generalists are truly without cost at least when costs are assessed as a trade-off between breadth and depth of adaptation (R. Kassen, 2002).

Despite strong evidence for negative cross-environment correlations in fitness between our CM and CM + 80% chemical stress environments, we found quite a few lineages in each of our fluctuating treatments that exhibited no evidence of trade-offs between depth and breadth of adaptation when assayed under these conditions (Figure 4, Figure S3). Some of these lineages had fitness phenotypes similar to the constant selection treatments (in x, y space), while others fell far from the EH0 & EH80 treatment means, but still fell along the no-cost line. The replicates that fell in this class exhibited no evidence of costs, but no particular benefit of evolution in fluctuating conditions either. Surprisingly, we also observed a small number of lineages in each treatment that not only appeared to escape the costs of generalization but also achieved a “jack of all trades, master of all status” with elevated mean fitness and increased breadth of performance relative to the constant selection treatments. The frequency of adaptive trajectories consistent with the “jack of all trades, master of none” paradigm v. the rarity of “jack of all trades, master of all” outcomes indicates that mutation availability and chance play an important role in the first steps of adaptation to fluctuating environments. Given sufficient replication, it becomes apparent that a wide diversity of adaptive trajectories are possible in fluctuating environmental conditions. Despite their rarity, the “jack of all trades, master of all” trajectories that we observe here are likely disproportionately important to evolutionary outcomes observed in natural populations. If they were to arise, “jack of all trades, master of all” adaptive strategies should be able to invade natural populations containing “jack of all trades, master of none” generalists as well as populations dominated by specialists. Perhaps evolution of generalization is not always associated with a trade-off of depth for breadth – maybe you really can have it all.

**CONCLUSIONS**

Adaptation is a complex, historically contingent, process even under simple, static, environmental conditions. Paths to success are legion and not all phenotypes incur costs even when costs are commonplace. We show that a diversity of outcomes exist and can succeed in static and cycling conditions. Furthermore, phenotypes are not binary, specialist vs. generalist, but instead exist as a range of adaptive trajectories. All outcomes are not equal – relatively rare phenotypes can escape the costs of adaptation and generalization and it may be these lineages that contribute disproportionately to patterns observed in natural populations. Genomic study of these lines is a crucial next step as is evaluation of the long-term fate of these lineages.

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