Experimental Evolution with *Escherichia coli* in Diverse Resource Environments: are 'Jacks of all Trades' truly 'Masters' of None?

A Thesis Presented to the Faculty of the Department of Biology and Biochemistry

University of Houston

In Partial Fulfillment of the Requirements for the Degree

Master of Science

By

Rebecca Satterwhite

August 2013

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ABSTRACT

Can a generalist population, evolved in two distinct resources, reach the same fitness in both as specialist populations, evolved in each resource individually, or, is a 'jack of all trades' truly 'master' of none? This question is relevant to theories of ecological specialization, the maintenance of genetic variation, and sympatric speciation, yet its answer remains uncertain, despite a wealth of experiments aimed at elucidating the limits of adaptation. To test whether bacterial jacks of all trades truly are masters of none, I measured the fitness, relative to a common ancestor, of replicate *Escherichia coli* populations evolved for 6,000 generations in the presence of either glucose or lactose alone (specialists), or in varying combinations (generalists). I found that all populations had significantly increased their fitness in both glucose and lactose, though the rate and magnitude of the increases differed. The generalists were masters of all trades for the first 4,000 generations; specifically, the geometric mean fitness of most generalist populations in both single-resource environments was not significantly different from the geometric mean fitness of the specialist populations measured in their selective environments. Subsequently, however, the generalists were masters of none as their geometric mean fitness fell increasingly behind the specialists at 5,000 and 6,000 generations. My results indicate that costs of adaptation are ultimately unavoidable, even if they fail to constrain the evolution of generalists for several thousand generations.

CONTENTS

1 INTRODUCTION

The limits of adaptation are of central importance to any general theory of adaptive evolution. A well-established example of this is the continued effort of ecologists and evolutionary biologists to explain costs of adaptation and their influence on evolutionary trajectories. This effort has produced a substantial body of literature aimed at evaluating the role of adaptive costs in sympatric speciation (Felsenstein 1981; Hawthorne and Via 2001; Berlocher and Feder 2002), the maintenance of genetic variation (Hedrick 1986; Kassen 2002; Clavel et al. 2011; Colautti et al. 2012; Draghi and Whitlock 2012), the evolution of life history traits (Rose and Charlesworth 1980; Frank 1996; Birand et al. 2012; Selman et al. 2012), the evolution of virulence (Lenski and May 1994; Alizon and van Baalen 2005; Brown et al. 2012; Berngruber et al. 2013), and the evolution of specialization (Futuyma and Moreno 1988; Wilson and Yoshimura 1994; Van Tienderen 1997; Ravigné et al. 2009; Remold 2012). An intuitive way to consider how costs of adaptation may arise stems from Fisher's geometric model, which posits that mutations are likely to have pleiotropic effects, such that each affects several traits (Fisher 1930). Evidence of pervasive pleiotropy in a number of model systems (e.g., social amoeba: Foster et al. 2004, Gram-negative bacteria: Batallion et al. 2011, Lenski 1988a, MacLean et al. 2004, Travisano and Vasi 1995, Ostrowski et al. 2005; fruit flies: Service and Rose 1995; mice: Wagner et al. 2008; DNA and RNA viruses: Bull et al. 2000, Duffy et al. 2005, Elena et al. 2009; and yeast: Featherstone and Broadie 2002, Cooper et al. 2007; Jasmin et al. 2012) lends support to this position.

Traditionally, costs of adaptation are inferred from negative correlations between fitness in the selective environment (direct response) and fitness in at least one non-selective environment (correlated response); for example, in reciprocal transplant experiments which assay the fitness of replicate populations (or individuals from the same population) in their own, and in each other's selective environments (reviewed in Fry 2003; Kassen 2002). Such inferences are based on the assumption that deleterious pleiotropic effects associated with selectively beneficial mutations have caused fitness to improve in one environment, but decrease in another. However, a negative relationship in fitness across environments does not necessarily indicate the presence of a cost; for example, the same pattern may arise by way of adaptive specificity, such that organisms are simply more fit in the environments in which they evolved (Lenski and Travisano 1996; Kassen and Bell 1998; Cooper and Lenski 2010). Moreover, any of three population genetic mechanisms could underlie a negative relationship: 1) beneficial pleiotropy, i.e., mutations that are beneficial to different extents in different environments, 2) mutation accumulation (MA), i.e., selectively neutral mutations that have deleterious effects in different environments, or 3) antagonistic pleiotropy (AP), i.e., selectively beneficial mutations that have deleterious effects in different environments (Roff 1996; Sgrò and Hoffmann 2004; Roff and Fairbain 2007). The distinction between the first two mechanisms and AP is important, because only AP can impose a true cost of adaptation, such that organisms experience a necessary trade-off in fitness that increases with the fixation of each adaptive mutation. It can

be difficult to disentangle the contributions of the different mechanisms to the overall adaptive pattern, in part because of the potentially confounding effects of mutations that cause general adaptation across environments (Service and Rose 1985; Bennett et al. 1992; Fry 1993; Ostrowski et al. 2005). If populations fix generally beneficial mutations, in addition to those with deleterious pleiotropic effects, the relative influence of each type of mutation will determine the slope and intercept of those populations' correlations in fitness across environments. Monitoring this relationship as it changes in response to selection can provide a more comprehensive picture of the underlying mechanisms than evaluating the relationship at any single point in time. The development of a convenient lab-based approach for observing real-time evolution (Elena and Lenski 2003; Buckling et al. 2009) has provided the opportunity for a long-term study to test how costs ultimately influence the trajectory of evolution.

Experimental evolution with microorganisms offers several benefits to researchers seeking to observe adaptation and elucidate its genetic basis. Microbes have short generations times and can be maintained at large populations sizes, making it possible to observe the continued effects of evolutionary processes. Ancestral populations can be frozen in a non-evolving state and later revived, a key factor that allows fitness (and the effects of individual mutations) to be determined through direct comparisons between ancestral and evolved lines. Replicate populations founded from a single clone effectively 'replay' evolution, which increases statistical

power and serves as a means to distinguish between chance and repeatable outcomes. Finally, the ability to control environmental conditions provides considerable flexibility in experimental design, and allows researchers to test for costs directly by comparing the fitness of generalist populations, evolved in dual-resource environments, to specialist populations, each evolved in a single-resource environment. This is the approach that I used to determine the role of adaptive costs in constraining generalists from simultaneously optimizing their fitness in two environments (relative to specialists evolved in those environments).

Previous studies have used microbial systems to test for costs of adaptation to environments differing in photosynthetic opportunity (Bell and Reboud 1997; Reboud and Bell 1997; Kassen and Bell 1998), temperature (Bennett et al. 1992; Bull et al. 2000), number of resources (Velicer and Lenski 1999; Dykhuizen and Dean 2004; Jasmin and Kassen 2007; Cooper and Lenski 2010; Bailey and Kassen 2012), number of pathogens (Lennon et al. 2007; Koskella et al. 2012), and host type (Weaver et al. 1999; Turner and Elena 2000; Crill et al. 2000; Duffy et al. 2005; Remold et al. 2008). Each of the studies listed here identified at least one negative association between direct and correlated responses, but they disagreed on the underlying genetic bases. The majority found evidence of AP by confirming the existence of a trade-off in fitness (advance in one environment caused regress in another, e.g., Bell and Reboud 1997; Bull et al. 2000; Turner and Elena 2000; Dykhuizen and Dean 2004; Duffy et al. 2005; Remold et al. 2008; Ward et al. 2009;

Kassen and Bailey 2012), though in some cases AP was present in only a small number of the populations evaluated (Velicer and Lenski 1999; Lennon et al. 2007; Cooper and Lenski 2010). Others found evidence for MA (Funchain et al. 2000; MacLean and Bell 2002) or a combination of mechanisms, e.g., AP and MA (Reboud and Bell 1997; Remold 2008), or differences in the specificity of beneficial mutations (Bennett et al. 1992; Jasmin and Kassen 2007; Cooper and Lenski 2010; Koskella et al. 2012). Taken together, the results of these studies indicate that AP is common, though not ubiquitous.

It may therefore seem reasonable to predict that generalists would be constrained by the aggregation of deleterious pleiotropic effects as they attempt to adapt simultaneously to multiple selective pressures. That is, a generalist experiencing selection in two environments will be constrained from increasing fitness in both as much as distinct specialist populations, each experiencing selection in one of the two environments (i.e., 'jacks of all trades' should be 'masters of none'). Support for this expectation has been found (Ward et al. 2009, Koskella et al. 2012), but several instances of low or no-cost generalization (Reboud and Bell 1997; Kassen and Bell 1998; Turner and Elena 2000; Crill et al. 2000; Trindade et al. 2009; Cooper and Lenski 2010) confound this prediction. One reason for the disparity in results is the progressive nature of adaptation; failure to detect does not prove their absence, and may stem from subsequent mutational events. The most common of these is the fixation of beneficial, second-site mutations that compensate for the original fitness

decrease. Epistatic (gene by gene) interactions between mutant alleles can determine their individual effects, such that a genotype having a single 'costly' mutation suffers a greater decrease in fitness than one having multiple individually 'costly' mutations. Several examples of such compensatory mutations have been identified in microbial systems: e.g., Gram-negative bacteria (Lenski 1988b, Schrag et al. 1997, Björkman et al. 1999, Trindade et al. 2009), and DNA and RNA viruses (Burch and Chao 1999; Bull et al. 2000, Poon and Chao 2006). Another, less common event that yields a similar effect is an adaptive reversion (Cairns and Foster 1991; Crill et al. 2000), such that the mutant allele reverts to the wild-type, and the associated decrease in fitness disappears. Both reversals and compensatory mutations generally require a dramatic change in the selective environment subsequent to the initial mutation, such that the acquired fitness decrease is no longer selectively neutral. Detecting any type of adjustment to an initial decrease in fitness, and moreover, whether such adjustments can counter continuously amassing decreases, requires an extended experimental timescale.

1.1 Experimental Overview

A previous study has shown that deleterious pleiotropic effects were present, and indeed common, in bacteria evolved in constant environments, and more often arose by AP than MA (Cooper and Lenski 2000). I aim to extend earlier work by identifying the extent to which costs influence the evolutionary trajectories of bacterial populations evolved in constant and fluctuating environments. I tested the

fit of standard AP and MA models to the patterns of adaptation in my system by examining the relationship between fitness in two environments for each group of populations, and by making between group fitness comparisons, at regular intervals over the course of 6,000 generations of evolution.

Cooper and Lenski (2010) investigated patterns of adaptation and divergence in populations of *E. coli* founded from a single ancestral strain and experimentally evolved in diverse resource environments for 2,000 generations. Here, I examine a subset of the same populations, and additional populations derived from them, after 6,000 generations of evolution. Six replicate populations were evolved in each of six environments differing only in the identity and presentation of the limiting resource(s): glucose (Glu), lactose (Lac), glucose and lactose together (G+L), glucose and lactose fluctuating daily (G/L), and two environments initially containing glucose or lactose alone, fluctuating between the two every 2000 generations (G-L and L-G). Hereafter, replicate populations evolved in the same environment are referred to as groups. Groups evolved in environments containing one or two resources are termed specialists and generalists, respectively.

My tests of the AP and MA models rely on detectable differences in the response of populations to the resource regimes prevailing in their selective environments. In general, there is an expectation that the antagonistic effects of mutations will be (roughly) inversely proportional to the degree of similarity between environments;

for example, if environments contain distinct resources that have a high degree of overlap in the cellular pathways of import and metabolism, mutations causing adaptation to either environment are expected to have low or no cost in the other (Travisano 1997, Ostrowski et al. 2005). The relationship of *E. coli* to the resources used in this experiment is well understood; for example, glucose and lactose are imported through different inner membrane transport systems and produce different metabolic byproducts (Postma et al. 1993). Knowing that *E. coli* have distinct physiological responses to the two resources, I assumed that adaptation would be specific to the individual resource regimes, such that each group would have its highest fitness increase in its own evolution environment. The specificity of adaptation was most important to establish at 6,000 generations, as my overall conclusions depended most heavily on adaptive patterns observed at the experimental endpoint.

I used two approaches to examine the basis for any fitness costs incurred during adaptation. First, I sought to assess the contributions of AP and MA to adaptation by analyzing the relationship between fitness in glucose and lactose for the replicate populations within each group. Mutations should be selected based on their effect in one resource in specialist populations, but in two resources in the generalist populations. Note that expectations for the long-term fluctuating groups are distinct, in that for these generalists, most mutations will be selected based on their effect in one environment. In general, I expected that specialists would be more likely than

generalists to adapt using mutations with AP and to accumulate costs due to MA, because generalists should fail to fix mutations that are deleterious in either resource (unless they confer a substantial benefit in the other). AP models predict a trade-off in fitness that increases with the fixation of every adaptive mutation; therefore, the fitness of each specialist group measured in an alternative environment should be negatively correlated with its direct response. In contrast, MA models predict costs that occur independently of adaptive mutations, which would manifest as a negative Y-intercept and a zero slope. A negative slope and intercept would indicate that both MA and AP have contributed to a cost of adaptation. In addition to comparing the direct and correlated responses of the specialists, I examined the relationship between glucose and lactose fitness for the replicate lines within the generalist groups as well. I did this to get an overall picture of the adaptive dynamics in my system, despite my expectation that costs would be less common in the generalists.

My second approach involved comparing the mean fitness of all groups measured in glucose and lactose, to determine whether generalists ('jacks of all trades') truly were 'masters' of no environment. The results of my first analysis will indicate whether specialists experienced costs; as all populations should experience the same types of mutations (on average), I can then use the results of this analysis to infer whether the majority of costs in my system as a whole are due to MA or AP. If the specialists experience costs due to MA, then I expect generalists will also, but the generalists should be able to overcome the constraints of these costs as they fix

adaptive mutations. Thus, the generalists should eventually be masters of all trades; i.e., reach the same fitness in two environments as the specialists evolved in those environments. If the specialists adapt using AP, then the act of adapting should constrain generalists from being masters of all trades in one of two ways: 1) generalists will fail to fix mutations with AP and thereby have access to a smaller number of mutations than the specialists (who can substitute all mutations that provide a benefit in their selective environment, irrespective of antagonistic effects), in which case the generalists will adapt to the two single-resource environments at a slower rate than the corresponding specialists, or 2) generalists will adapt with AP and will thereby accrue costs in both single-resource environments, which will constrain their rate of adaptation relative to the specialists evolved in those environments. The first scenario predicts that specialists, *but not* generalists, will adapt using mutations with AP. The second scenario predicts that specialists *and* generalists will fix mutations with AP.

One caveat to the predictions set forth by the AP model is that they assume a limited supply of mutations contributes to the constraint on generalist adaptation. All populations were founded from the same clone and thus began the experiment with the same mutation rate, but *E. coli* populations have evolved increased mutation rates within the timescales used in this experiment (e.g., Sniegowski et al. 1997). If this occurred in a generalist group, it would violate the assumption set forth by the model's prediction and could allow a generalist to be a master of all trades even in

the presence of AP. If this occurred in a specialist group, it could cause generalists to be masters of no environment despite the absence of costs. I did not perform a comprehensive survey for mutator phenotypes in my populations, however, comparisons between specialists and generalists rely on the mean group fitness (mean of six replicate populations) so it is unlikely that a few mutator populations would significantly skew the overall results. Also, populations that are extreme outliers in terms of fitness could be easily identified and flagged for follow-up. Another caveat to my predictions is that they only apply when populations are at equilibrium. Additional factors must be taken into account to predict the relative success of generalist populations at intermediate time points; the most obvious of these is that, at the same time point, generalist groups have evolved in the presence of each resource for only half the time of specialist groups. Even in the absence of AP, this will cause a generalist population to have a lower fitness in each environment than the corresponding specialist population (Whitlock 1996). To overcome this complication, I also measured the fitness of specialist and generalist populations at an earlier time point.

2 METHODS

2.1 Strains & Environments

The strains used to found the populations in this study were derived from the same ancestor, REL606, a clone of *E. coli* B that was unable to metabolize the sugar arabinose (Ara-); previously, a mutant able to grow on arabinose (REL607, Ara+) was isolated from this strain (Lenski et al. 1991). Three populations of each Ara marker type make up the six replicate populations within each group; the Ara marker is neutral with respect to growth on any of the carbon sources used in this study (Cooper and Lenski 2010) but allows strains to be distinguished when plated on tetrazolium arabinose (TA) medium. The ancestor used to found these populations was strictly asexual, and all populations were initially identical (except for the Ara marker), thus *de novo* mutation was the only source of genetic variation. In relative fitness assays, I used a GFP expressing derivative of REL606 as a reference strain. This strain, TC1233, expresses a fast maturing GFP protein from a strong P_{A1} promoter (Gallet et al., 2012), which allows ancestral and evolved cells to be distinguished in a flow cytometer.

The environments used here and precautions taken to screen for external- or cross-contamination occurring during propagation have been described previously (Cooper and Lenski 2010). Briefly, the environments consisted of a base Davis minimal medium to which different limiting carbon source(s) were added to a final concentration that supported equivalent cell densities of ~3.5×10^8 cfu/ml.

Resource concentrations were: glucose 175 μg/ml, lactose 210 μg/ml, and glucose 87.5 μg/ml and lactose 105 μg/ml together. Cultures were propagated by transferring 10 μl from each population into 1 ml of the corresponding fresh medium each day.

2.2 Relative Fitness Competitions

The fitness of all strains, relative to the ancestor, was assayed in their selective environment (direct response) and in glucose and lactose; i.e., I obtained at least one correlated response (fitness in a non-selective environment) for each population. For example, the Glu group was assayed in glucose (direct response) and in lactose (correlated response). Fitness was determined by competing evolved strains against the GFP-labeled ancestor, and the density of each competitor was measured using flow cytometry (Zhang et al. 2012). Before each fitness assay, competing populations were grown separately for one complete propagation cycle in the assay environment, allowing competitors to reach comparable cell densities and physiological conditions; populations were incubated at 37°C in 96 well blocks. Competitors were mixed in equal proportions and diluted 1:200 into a mix of purified water, Davis minimal medium, and SYTO 17, a red fluorescent nucleic acid stain used to distinguish bacterial cells from background noise (used at a final concentration of 200 nM). Flow cytometry was performed with an Accuri C6 Flow Cytometer (BD Biosciences), and for each sample a total of 5,000 events were captured at a rate of 500-2,000 events/s.

All competitions were carried out for one day; samples were taken immediately to estimate the initial densities of the competing strains, and again after 24 hours to obtain the final density of each competitor. The only exception to this was measuring the direct response of the daily fluctuating (G/L) group, which required competitors to have a full 24-cycle in both glucose and lactose; in those cases samples to determine final densities were taken after 48 hours. For all, the fitness of evolved strains relative to the ancestor was calculated as $\ln(N_{E1}/N_{E0}) / \ln(N_{A1}/N_{A0})$, where N_{E0} and N_{A0} represent the initial densities of the evolved and ancestral strains, respectively, and N_{E1} and N_{A1} represent their corresponding densities at the end of the competition. Fitness assays involving the first 4,000 generations were performed with nine-fold replication; assays for later time points and direct competitions were replicated four times.

For direct competitions between evolved strains, populations with opposite Ara markers were competed. Competitors were preconditioned and mixed in equal proportions as described above, and plated on TA agar immediately and after 24 hours to determine densities (Cooper and Lenski 2010).

2.3 Experimental Blocks

Competitions for generations 1,000–4,000 were organized in blocks that allowed two replicate lines from each group to be run simultaneously in both environments

(glucose and lactose); on collection days I ran three blocks consecutively to collect fitness estimates for all populations. Competitions for generations 5,000–6,000 were organized such that three replicate lines from each group were run simultaneously in both environments (two blocks were run consecutively on collection days to collect estimates for all populations). Competitions to measure the direct response of the G+L and G/L groups were run in independent blocks that included two replicate measures for all replicate lines of the respective group at generations 1,000–6,000. A few additional blocks were run to repeat individual competitions that had been dropped due to experimental error (e.g., pipetting error resulted in anomalously low cell counts).

All blocks contained the same controls: blank wells, REL607 alone, TC1233 alone, and REL607 competed against TC1233. All blocks run subsequent to the initial 1,000–4,000 set contained additional controls: TC1233 competed against each of the four strains which had the highest and lowest mean fitness estimates in glucose and lactose at 4,000 generations. In all cases, no significant difference was found between new and previous fitness estimates for these strains (base on Student's *t*-tests, $p < 0.05$).

2.4 Statistical Analyses

Fitness estimates were calculated in Excel. All other statistical analyses were performed in R version 2.13.0 with $\alpha=0.05$ as a significance cut-off. For analyses of

variance, population was tested as a random factor and environment was tested as a fixed factor. In cases where overall trends were interpreted from the outcome of multiple tests or comparisons (to determine adaptive specificity, Table 1) sequential Bonferroni corrections were performed (Rice 1989). For the Master of All Trades analysis, which compares the mean fitness of all groups, mean fitness estimates are geometric, i.e., the *n*th root of the product of the total number of (*n*) replicated measures; this was done to allow comparisons between fitness in constant and temporally fluctuating environments (Bell 1997; Gillespie 1998).

The mean effect of the GFP marker over all relative fitness assays was a cost of 2% in glucose, and a cost of 2.3% in lactose. To account for the effect of the marker, data was normalized based on the average marker effect per environment for all estimates collected in the same experimental block.

2.5 Master of All Trades Analysis

For the generalists to qualify as masters of all trades at any given time point, they needed to have geometric mean fitness (averaged across glucose and lactose) equal to the geometric mean direct response of the specialists (averaged together). To visualize the master of all trades fitness level for any given time point, I first combined the geometric mean direct response of the specialists to create a new, two dimensional point in fitness space (red point, Figure 1.A.). I then averaged the coordinates of this point to get an estimate of fitness *across* environments, which

served as the master of all trades fitness level. Finally, I extended this point to all other points in fitness space having the same geometric mean fitness *across* environments, forming a fitness isocline (red dashed line, Figure 1.B.). This fitness isocline, hereafter referred to as the master of all trades (MAT) isocline, represents the threshold geometric mean fitness at which generalists are considered masters of all trades. Using an isocline as the master criterion, rather than a single point, allowed generalists to qualify as masters without requiring that adaptation must have occurred to the exact extent in each resource as it did for the specialists (i.e., generalists could increase fitness more or less in either resource as long as their geometric mean fitness across environments was equal to the isocline level).

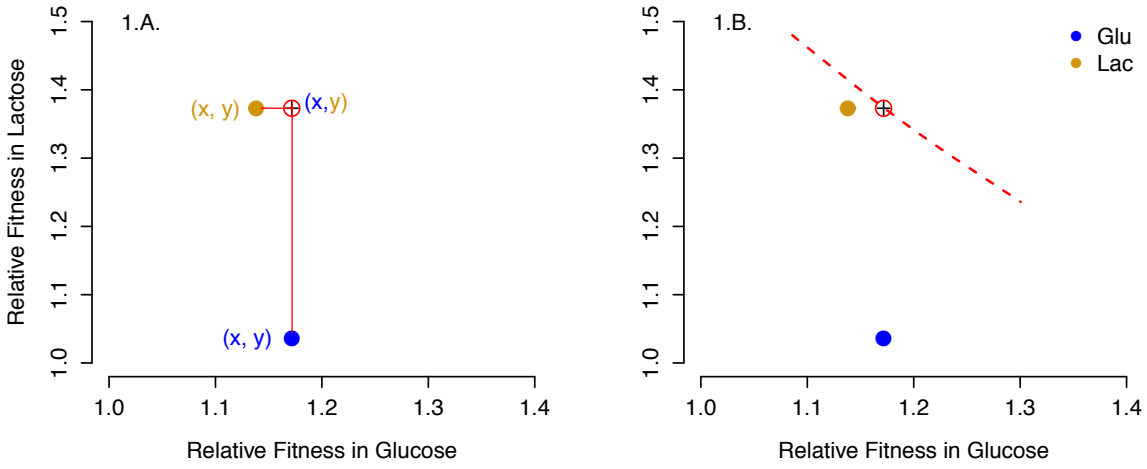


Figure 1 (A-B). The MAT isocline (red dashed line) is extrapolated from the geometric mean direct response of the specialist groups (red open circle). At any given time point, generalist groups with geometric mean relative fitness not significantly different from the MAT isocline are designated masters of all trades.

The movement of the MAT isocline over time reflects the direct response of the specialist groups, and therefore the level of fitness to which the generalist groups were compared. To determine whether generalist groups were masters at any given time point, I compared the geometric mean fitness of each generalist group to the MAT isocline (Figure 5) using a bootstrap analysis in which I resampled with replacement from relative fitness estimates and recalculated the geometric mean for the generalists in both environments, and the specialists in their selective environments, 1,000 times. 95% confidence intervals were calculated based on the 1,000 resampled estimates. Figure 5 shows the distance in fitness space between the MAT isocline and each of the generalist groups at all time points; if at any point the error bars of the points representing mean fitness for any generalist group overlap the isocline, that generalist group qualifies as a master.

2.6 Direct Fitness Competitions

I performed two sets of direct competitions between evolved lines to better interpret the results of between-group comparisons, and a third set to examine unique within-group dynamics. In all cases, the results of direct competitions were interpreted with respect to the results previously obtained through indirect competitions between the ancestor and evolved lines. I compared the fitness estimates obtained by each using the following bootstrap analysis. First, I resampled with replacement from replicate direct fitness estimates and recalculated the mean and 95% confidence intervals for fitness, 10,000 times. I then performed the same

analysis using indirect fitness estimates for each of the evolved strains that were competed, measured relative to the ancestor, and created a ratio from the bootstrapped values [(evolved/ancestor) / (evolved/ancestor)]; the mean and 95% confidence intervals were calculated from this ratio.

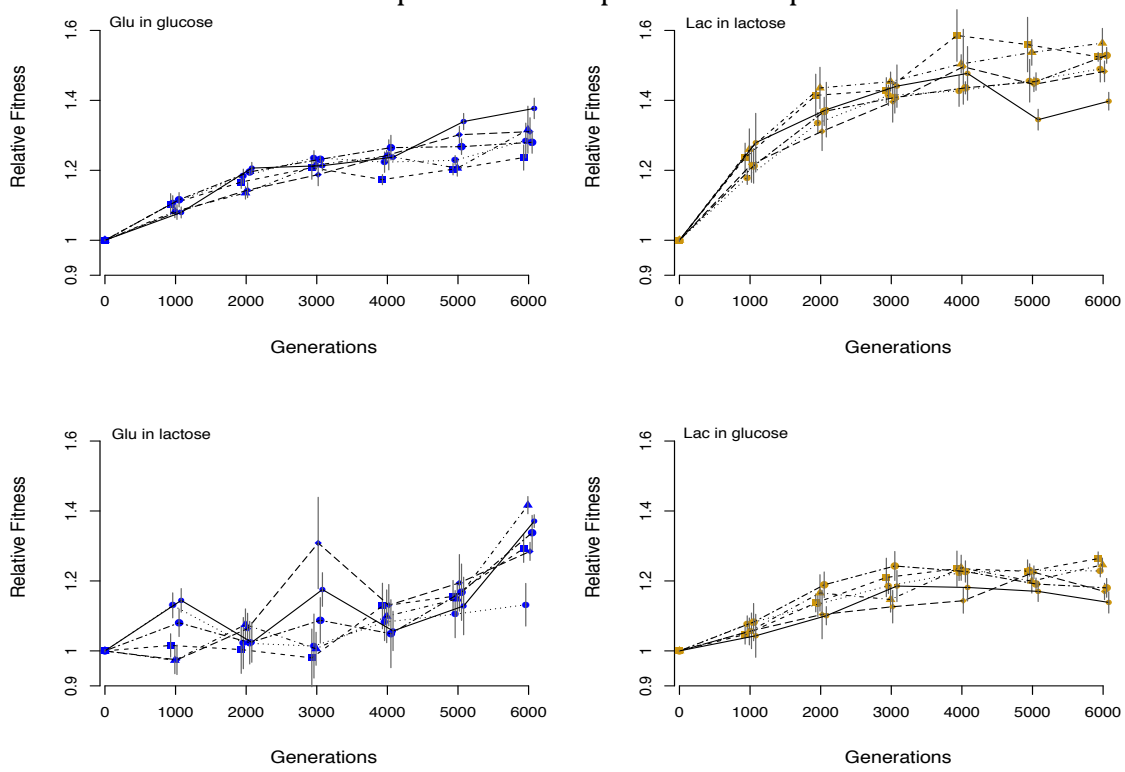
3 RESULTS

3.1 Specificity of Adaptation

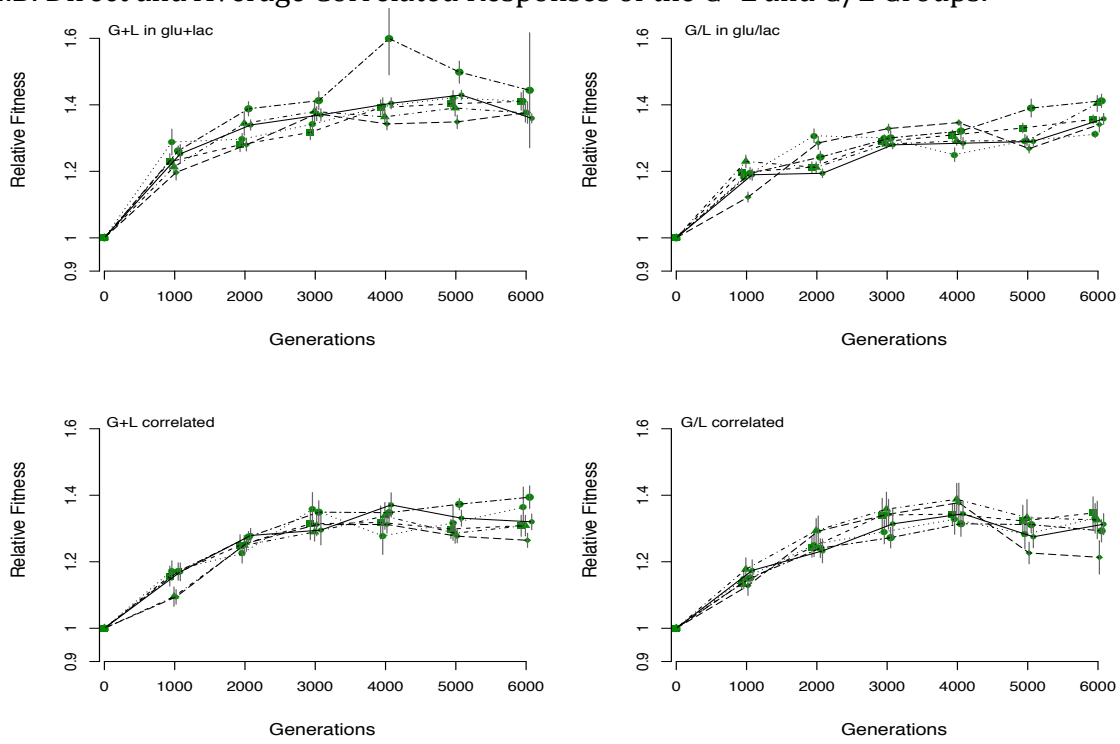
All populations adapted to their selective environments (Figure 2). Moreover, all populations significantly increased their fitness in both the glucose and lactose environments, even if one of these resources was absent from their selective environment (Figure 2).

Figure 2 (A-D). Fitness of evolved groups relative to the ancestor over the course of the experiment. Points and error bars represent the mean and 95% confidence interval of replicate fitness measures for each population in a group. Shaded regions indicate periods of selection in lactose for the G-L and L-G groups.

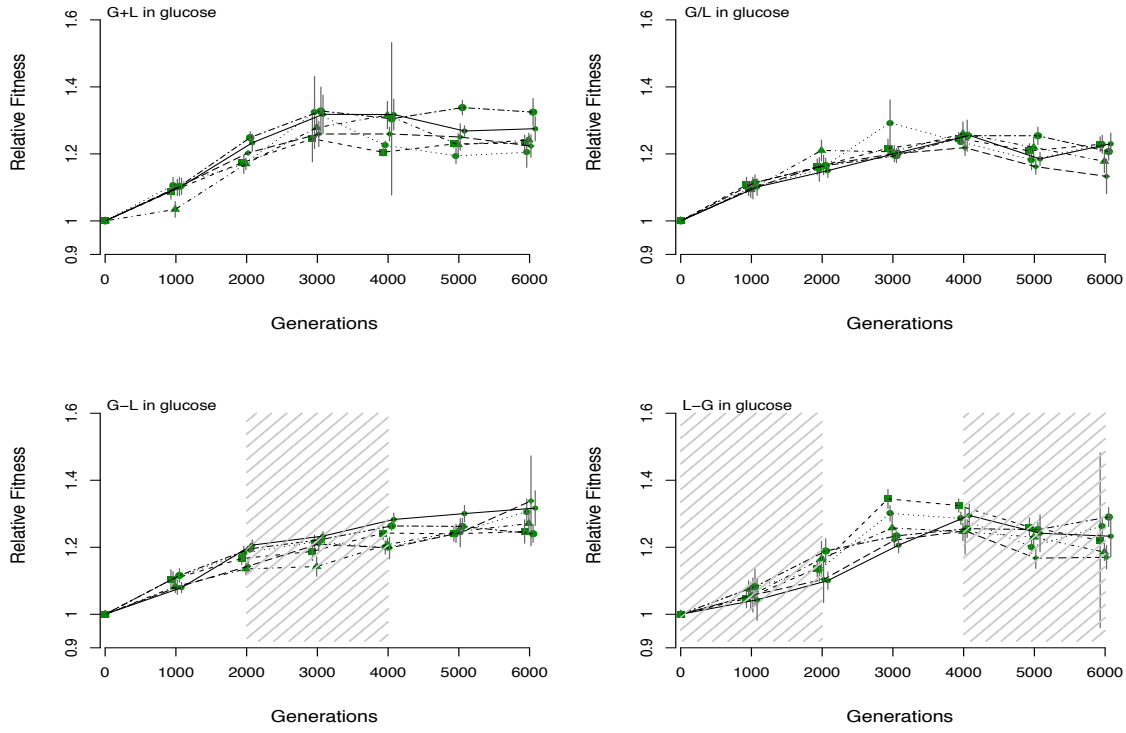
2.A. Direct and Correlated Responses of the Specialist Groups.



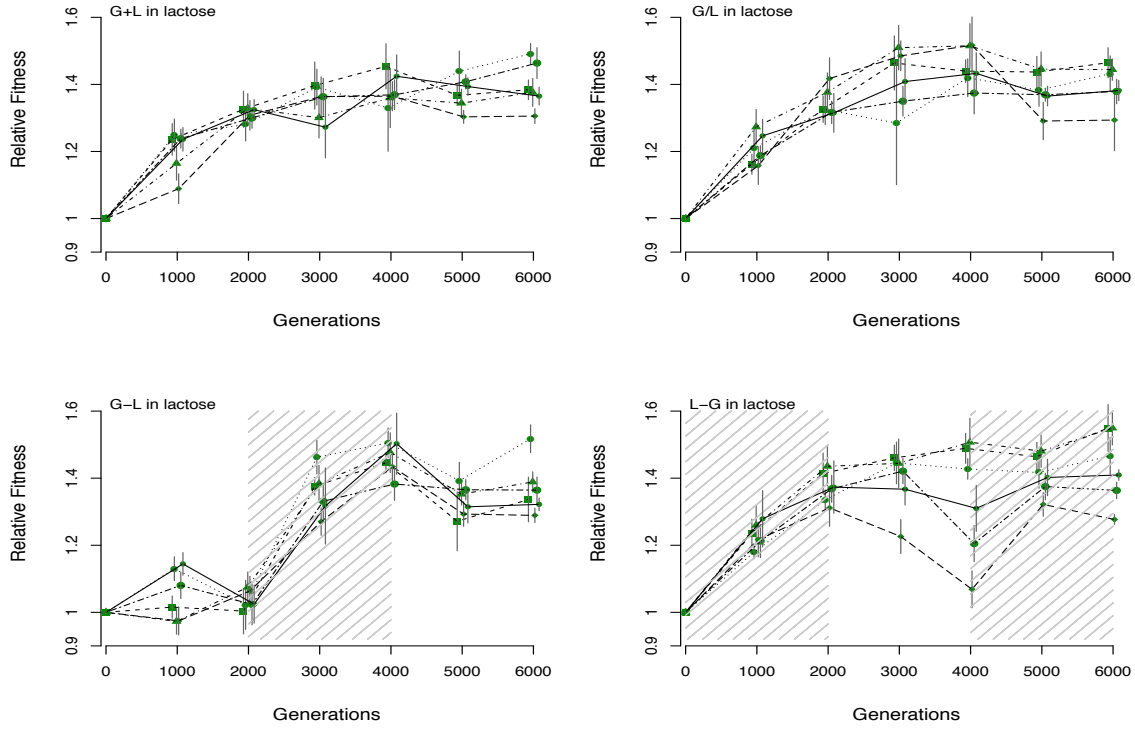
2.B. Direct and Average Correlated Responses of the G+L and G/L Groups.



2.C. Relative Fitness of the G-L and L-G Groups in Glucose.



2.D. Relative Fitness of the G-L and L-G Groups in Lactose.



These results suggests that groups had adapted to aspects of the environments common to all resource regimes (e.g., temperature or minimal media). Testing my central question is only tenable if the selective environments are distinct, therefore, I needed to confirm that the majority of adaptation was specific to environment. I did this by comparing the mean endpoint fitness of each group in its own environment to the fitness of other groups in the same environment (Table 1); the direct responses of the G+L and G/L groups were excluded from this analysis as other groups were not assayed in their environments. A majority of the comparisons were positive, such that groups tended to increase their fitness most in their own selective environment. This was true for all groups except L-G; three groups had correlated responses that were either not significantly different from, or larger than,

its own mean fitness in lactose at 6,000 generations. Taken together, these results indicate that a substantial portion of the overall adaptation was specific to the particular resource regime imposed in the environments, so I had some confidence that it was appropriate to apply the following analyses to my system.

Group	Direct Response	Difference in Response		p-value
Glu	1.30	Lac	0.10	**< 0.001**
		G+L	0.05	**0.005**
		G/L	0.10	**< 0.001**
		G-L	0.02	0.225
		L-G	0.07	**< 0.001**
Lac	1.50	Glu	0.19	**< 0.001**
		G+L	0.10	**< 0.001**
		G/L	0.10	**< 0.001**
		G-L	0.13	**< 0.001**
		L-G	0.06	**0.007**
G-L	1.29	Glu	-0.02	0.776
		Lac	0.08	**< 0.001**
		G+L	0.09	0.079
		G/L	0.09	**< 0.001**
		L-G	0.06	**0.009**
L-G	1.43	Glu	0.13	**< 0.001**
		Lac	-0.06	0.994
		G+L	0.04	0.073
		G/L	0.04	0.086
		G-L	0.07	**0.005**

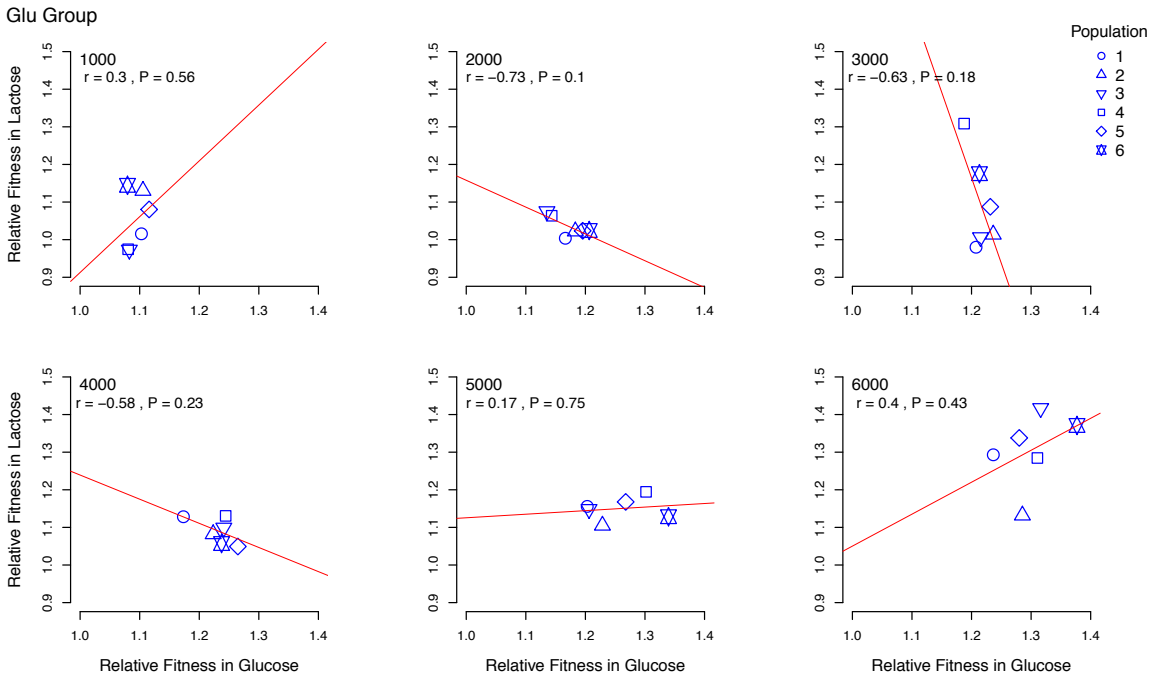
Table 1. Mean direct response of each group compared to the mean correlated responses of other groups measured in the first group's selective environment. Comparisons were done at 6,000 generations. Difference in response is the mean direct response of the first group minus the mean correlated response of the comparison group. Welch two-sample *t*-tests following F-tests for equal variance; bolded p-values indicate that direct response was larger after correction for multiple comparisons.

3.2 Costs of Adaptation

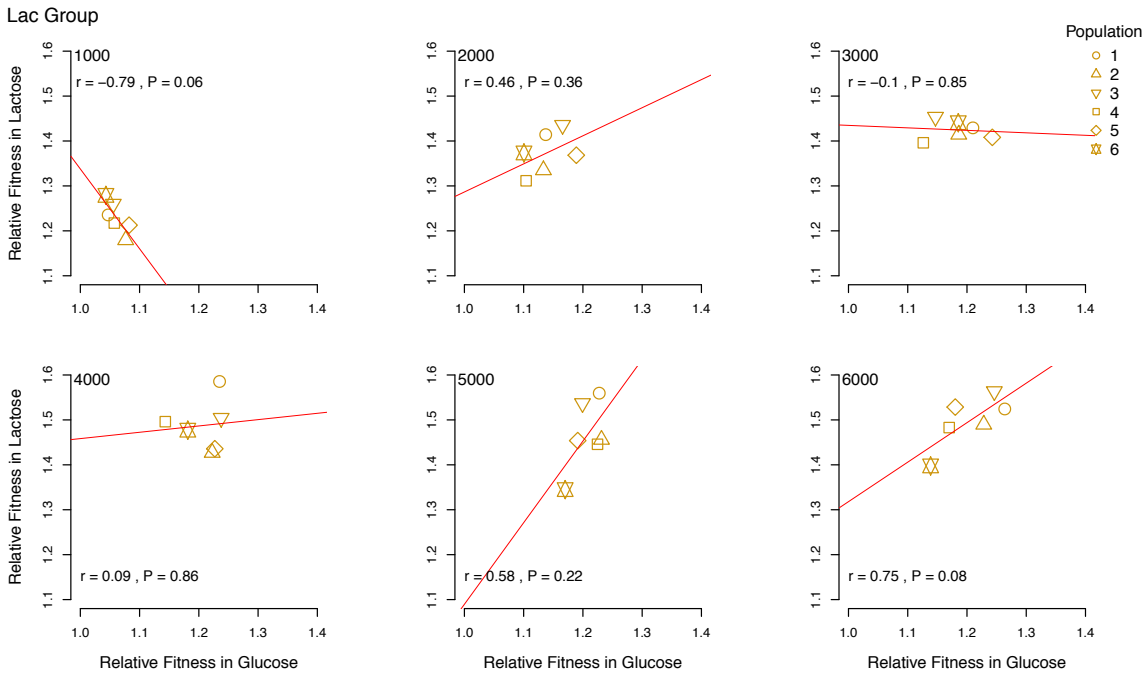
Having established the specificity of adaptation, I next sought to determine which genetic mechanism could best explain this specificity. Though I expected costs would be more likely to occur in the specialist populations, I wanted to examine the basis of any constraints that may be detected, so I included the generalists in this analysis as well. Figure 3 shows the relationship between fitness in glucose and lactose for the replicate populations making up each group at all time points.

Figure 3 (A-F). Relationship between glucose and lactose fitness for the replicate populations of each group. Points represent the mean fitness of replicate lines in both environments, at the time (in generations) indicated. P-values and Pearson correlation coefficients (r) are indicated.

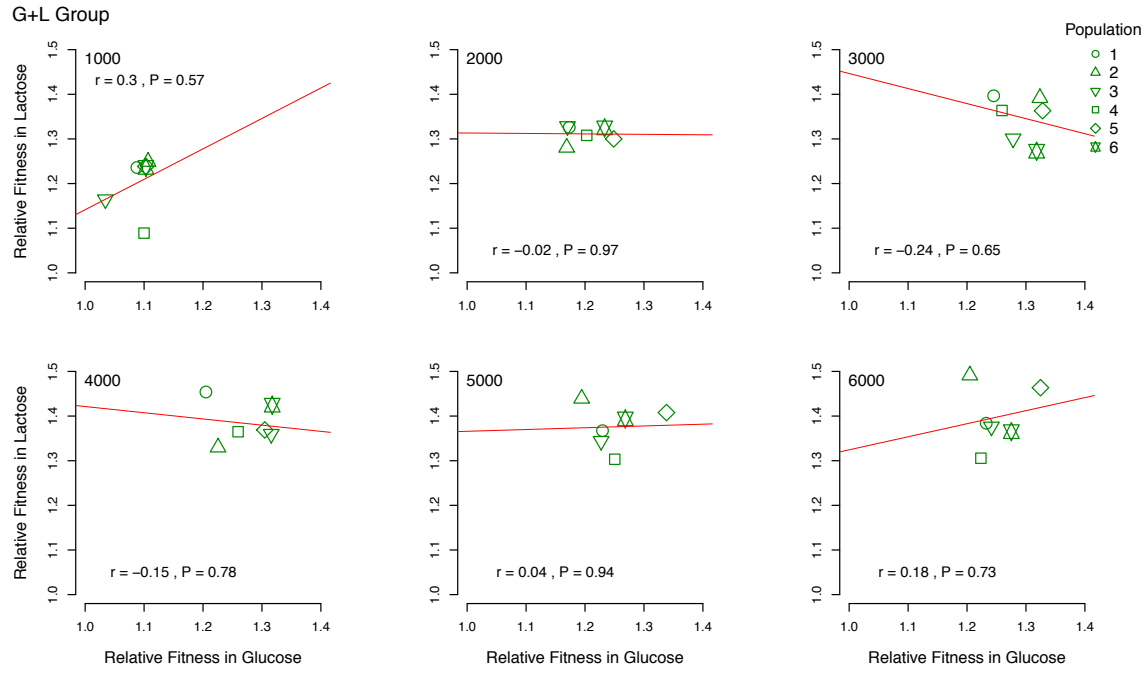
3.A. Relationship Between Glucose and Lactose Fitness for the Glu Group.



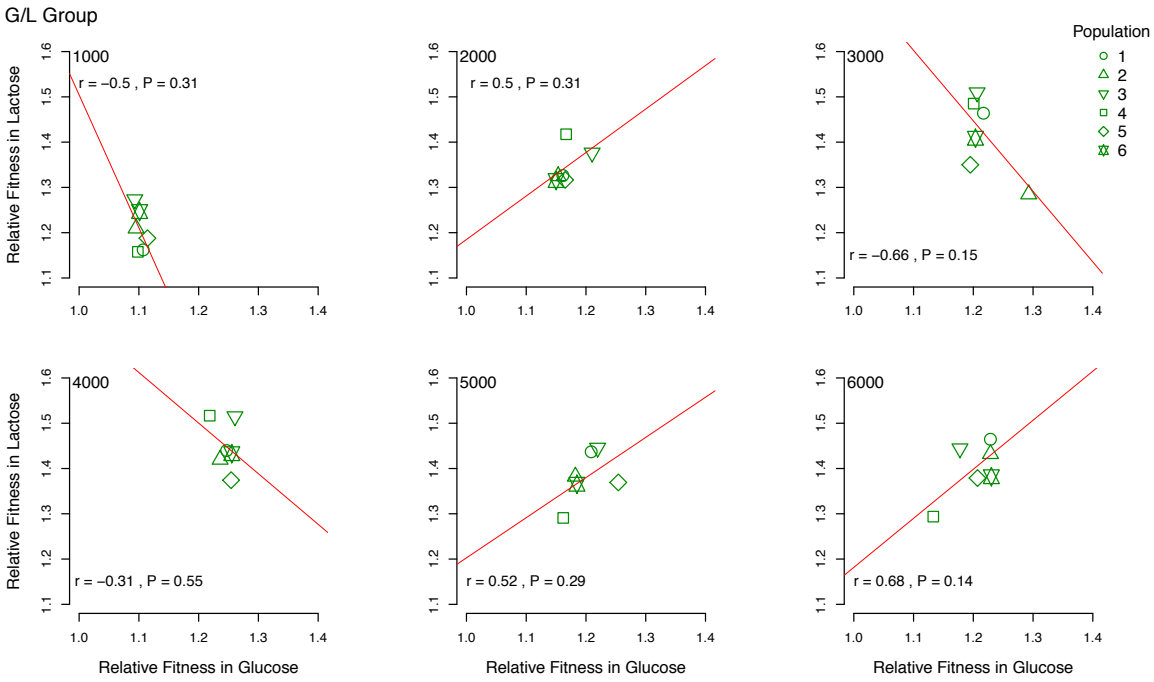
3.B. Relationship Between Glucose and Lactose Fitness for the Lac Group.



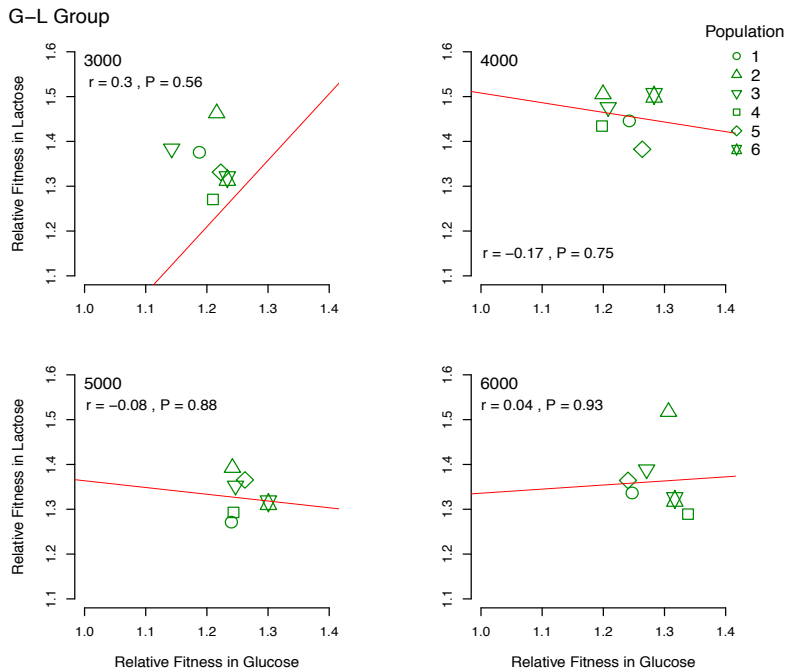
3.C. Relationship Between Glucose and Lactose Fitness for the G+L Group.



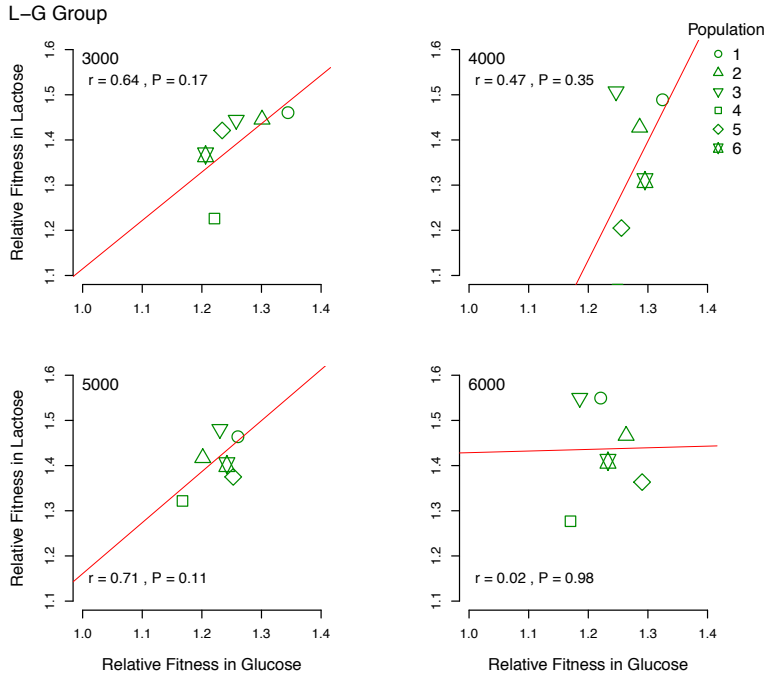
3.D. Relationship Between Glucose and Lactose Fitness for the G/L Group.



3.E. Relationship Between Glucose and Lactose Fitness for the G-L Group.



3.F. Relationship Between Glucose and Lactose Fitness for the L-G Group.



No correlations for any group at any time were significant, which limits the strength of conclusions; still, some informative patterns emerged. For the specialist groups, which demonstrated a striking contrast in long-term patterns, the relationship between fitness in glucose and lactose is equivalent to the relationship between their direct and correlated responses. Negative correlations would be consistent with the action of AP, and negative Y-intercepts with the action of MA, in causing the observed specificity in adaptation. After 1,000 generations of evolution, the Lac group had a marginally non-significant (p=0.06) negative correlation between direct and correlated response (Figure 3.B.), though all replicate lines had increased fitness in both environments. This pattern is consistent with AP in combination with generally beneficial mutations, though it could also be explained by beneficial

pleiotropy. For the same group at subsequent time points, most correlations were positive; the overall pattern indicates that mutations with deleterious effects in glucose were not a predominant mechanism by which adaptation to lactose occurred.

In contrast, the Glu group had a (nonsignificant) positive correlation between direct and correlated response at 1,000 generations (Figure 3.A.). All replicate lines had increased fitness in glucose, but some had decreased fitness in lactose; the negative Y-intercept and positive correlation suggest the presence of MA. At subsequent time points the Glu group had negative correlations with positive Y-intercepts, which suggests a mix of adaptive mechanisms including AP. This interpretation is corroborated by the pattern of correlated response seen in Figure 2.A.; for the first 3,000 generations, Glu group populations adapted to glucose while experiencing fluctuations in lactose fitness that indicate the presence of multiple competing mutations with deleterious pleiotropic effects. During the latter portion of the experiment, positive correlations and a pattern of steady increase in lactose fitness indicate that costs accrued by the Glu group during the earlier stages of adaptation were eventually overcome, at least in part, by the fixation of mutations with beneficial pleiotropy. In fact, there was no significant difference in the mean fitness of the Glu group in glucose or lactose at 6,000 generations (Welch two-sample *t*-test, $p > 0.5$). The specialist groups were each selected in a single resource, but they had distinct adaptive patterns; the Lac group may have experienced a mild cost of

adaptation that was quickly overcome, whereas evidence of costs were relatively strong in the Glu group during the first half of the experiment.

Evidence of costs is present in the generalist groups as well, though they demonstrated a mix of (nonsignificant) positive and negative correlations between fitness in glucose and lactose, all with positive Y-intercepts. A contrast in the adaptive trajectories of the mix and daily fluctuating groups is evident; the G/L group increased fitness in glucose significantly less (Welch two-sample *t*-test, $p = 0.001$), than the G+L group (Figure 2.C.). This suggests that the mix regime more efficiently selected against mutations with deleterious effects in glucose or lactose than the daily fluctuating regime. This is not surprising, as mutations occurring in the daily fluctuating regime would experience up to 6.4 generations of selection in a single resource before the environment changed, so mutations with AP would have had time to rise to high frequency before their associated costs would be selected against. In contrast, mutations in the mix regime would experience a maximum of half that number of generations in the presence of a single resource (as cells preferentially metabolize the glucose first) before the environment changed. Both groups spent the same average number of generations of selection in both resources, and indeed there was no significant difference between the G+L and G/L groups' average correlated responses (Welch two-sample *t*-test, $p > 0.2$). However, the lower endpoint fitness of the G/L group in glucose, coupled with its negative correlations, strongly suggests the influence of AP.

The adaptive patterns of the long-term fluctuating groups also indicate the presence of costs. The L-G group (evolved in lactose for 2,000 generations before switching to glucose) had positive correlations between fitness in glucose and lactose for the majority of the experiment (Figure 3.F.), but two replicate populations significantly decreased their fitness in lactose after switching to a glucose selective environment (Welch two-sample *t*-tests, $p < 0.01$). During the same time period, neither population had a significant fitness increase in the selective environment, so the costs were independent of any (detectable) adaptive benefit and therefore, most likely due to MA. Finally, both long-term fluctuating groups had significant decreases in mean fitness in the alternate environment after switching back to their original selective environment between 4,000 and 5,000 generations (Welch two-sample *t*-tests, $p \leq 0.001$). However, during the same time period, both groups significantly increased mean fitness in their selective environments (Welch two-sample *t*-tests, $p < 0.05$). The fact that costs accrued while the groups were adapting implicates AP as the underlying mechanism.

Though correlations between fitness in the single-resource environments were non-significant, I was able to infer that groups adapted using a combination of mechanisms. The results of this analysis are most consistent with beneficial pleiotropy; allele replacement experiments are needed to identify the effects of the specific mutations present, and to confirm the presence of those with deleterious pleiotropic effects. Though I did not find strong evidence of 'costly' mutations, I next

sought to determine whether costs of adaptation could be inferred from comparing the long-term adaptive patterns of specialist and generalist groups.

3.3 Specialists vs. Generalists

Analyzing the relationship between direct and correlated response in the specialist groups did not reveal a consistent pattern of cost. An alternative explanation for the adaptive specificity observed in the specialist groups is the fixation of beneficially pleiotropic mutations that conferred greater benefits in the environments in which they occurred. Such mutations would be accessible to generalist populations; therefore, if beneficial pleiotropy was common enough to be the main underlying cause of specificity in the Glu and Lac groups, I expected it would manifest as generalists and specialists maintaining comparable levels of fitness in the single-resource environments throughout the experiment. The possibility remained that MA or AP were the underlying cause of specificity in the Glu and Lac groups, but that their deleterious effects had, for the most part, been masked by additional beneficial mutations. If the majority of costs were due to MA, I expected that generalists would be able to overcome these costs and eventually catch up the specialists, as adaptive mutations fixed. If AP was the underlying mechanism, I expected that the generalists would experience an insurmountable constraint on their abilities to increase fitness in both environments.

I tested these predictions by comparing the geometric mean relative fitness of specialist and generalist groups in glucose and lactose over the course of the experiment. If generalists were masters of all trades, they would have fitness not significantly different from the MAT isocline, representing the combined direct response of the specialist groups. Figure 4 shows the geometric mean fitness of all groups, in both environments, at all time points making up the experiment. Figure 5 shows the Euclidean distance between the MAT isocline and the geometric mean relative fitness of each generalist group at all time points; I considered generalist groups masters of all trades if the 95% confidence intervals for their geometric mean fitness overlapped the MAT isocline.

The G+L and G/L groups were masters of all trades for the first two time points making up the experiment, and in fact, by 3,000 generations, the majority of generalists were masters of all trades (Figure 5). This marked the half-way point of the experiment, and the first time point at which the long-term alternating groups could be considered generalists. A similar pattern was observed at 4,000 generations, when again most generalists were masters of all trades. Thus, for the first two-thirds of the experiment, the generalists were not constrained by costs from increasing their fitness relative to the specialists. At subsequent time points, however, the generalists' rates of adaptation fell increasingly behind the specialists', and no generalists were masters of all trades at 5,000 or 6,000 generations (Figure 5).

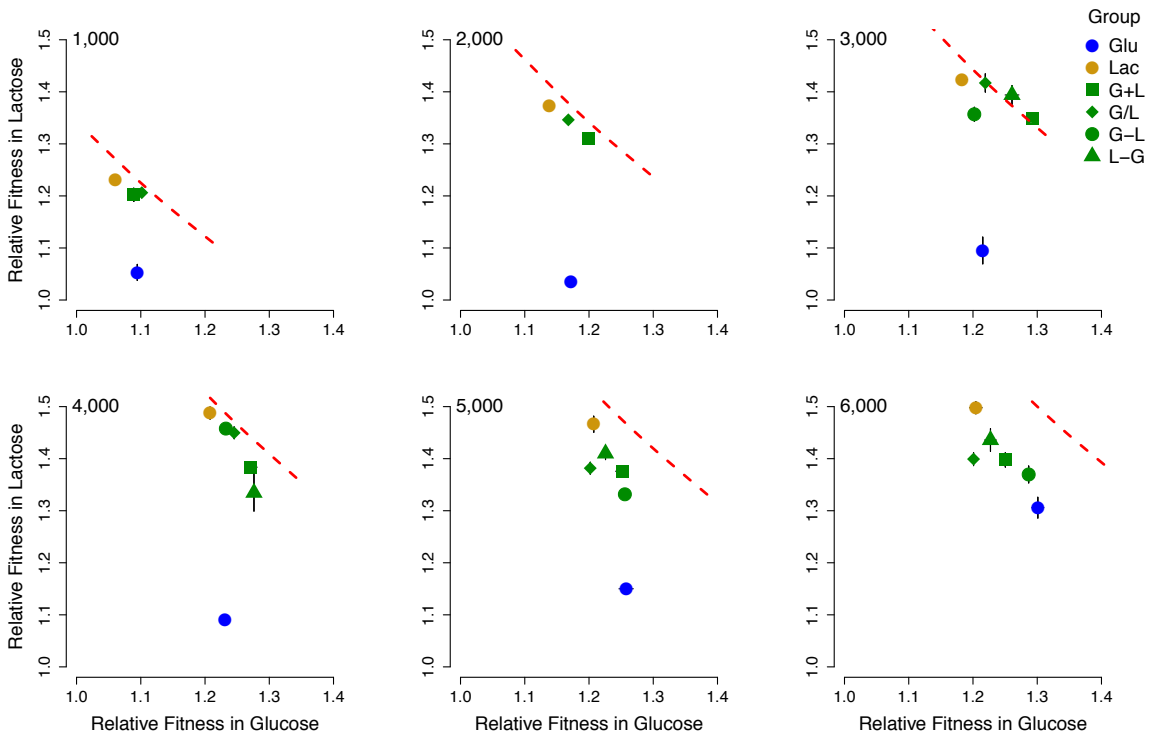


Figure 4. Mean relative fitness of all groups in glucose and lactose over the course of the experiment. Points and error bars represent the geometric mean relative fitness and standard error for the six replicate lines making up each group. Time in generations is indicated at the top left of each panel; symbols and colors are consistent throughout: blue circles, Glu group; yellow circles, Lac group; green squares, G+L group; green diamonds, G/L group; green circles, G-L group; and green triangles, L-G group.

The fact that the generalists eventually fell behind master of all trades fitness levels suggests that they were able to avoid or compensate for adaptive costs for the first several thousand generations, but this became increasingly difficult at later time points. If antagonistic pleiotropy became increasingly common as the generalist populations evolved, this could constrain the generalists' rates of adaptation through either of two possibilities. One is that if generalists fail to fix mutations with

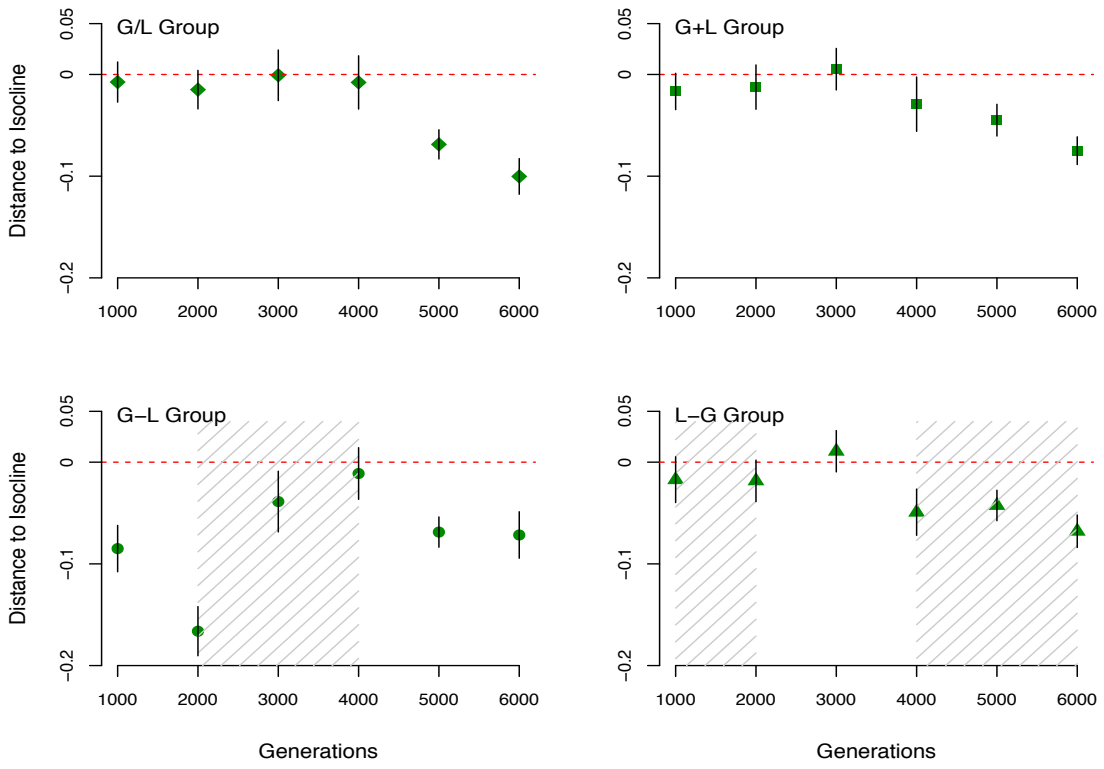


Figure 5. Euclidean distance between generalist groups and the MAT isocline (red dashed line at zero) over the course the experiment. The Y-axis indicates distance between the geometric mean direct response of specialist groups (MAT isocline) and the geometric mean correlated response of the generalist groups. Points represent the geometric mean relative fitness for the six replicate lines making up each group, and error bars are 95% confidence intervals. Both were calculated using a bootstrap analysis (see Methods). For the long-term fluctuating groups, shaded regions indicate periods of selection in lactose.

associated AP, they would be limited by the supply of mutations from increasing their fitness as much in two environments as specialists each evolved in those environments. A possible example of this is the L-G group, which had mostly positive correlations between fitness in the two environments, suggesting the avoidance of AP (Figure 3.F.), but which increased its mean fitness in lactose less than the Lac, G+L, and G/L groups (Table 1). Another possibility is that if generalists

adapted with AP, they would suffer a decreased rate of adaptation relative to the specialists as costs accumulated in each of the two environments. I found indications of AP in the daily and long-term fluctuating groups, and a previous experiment confirmed the presence of two mutations with AP in this system. Quan et al. (2012) identified mutations in the LacI and LacO genes, both of which were beneficial in lactose and deleterious in glucose, and had fixed in the majority of replicate lines from the Lac, G+L, and G/L group after 2,000 generations of evolution. This lends some confidence to my inferences, though the relative abundance of AP in evolved generalist genotypes cannot be determined based on my analyses alone. Still, the results of my long-term comparisons between specialists and generalists demonstrate increasing adaptive costs.

3.4 Variation in Response

The previous analyses focused on comparing adaptive patterns between groups, but comparing the direct response of replicate lines within a group allowed me to explore the possibility that populations were evolving toward different adaptive peaks in the same environment. I performed a series of ANOVAs to examine the significance of among-population variation in direct response after 6,000 generations of evolution (Table 2). A previous study performed the same analysis with these populations after 2,000 generations, and reported significant variation in the Lac and G/L groups, with only the G/L group remaining significant after sequential x correction (Cooper and Lenski 2010). At 6,000 generations, I found that

the Lac, G/L, and L-G groups each had significant among-population variation both before, and after correction for multiple comparisons. In addition to the G/L group, which showed signs of divergent evolution at both time points examined, both groups that experienced an initial period of selection in a lactose-limited environment showed signs of increasing divergence. A caveat to this analysis is that the presence of significant differences in direct response cannot be ruled out even if none are detected through this analysis; for example if 95% confidence intervals for a single replicate line overlap those of the others, no difference will be detected, even if the means are quite different.

Group	Mean direct response	F	p-value
Glu	1.302497	2.5661	0.051
Lac	1.495232	6.9556	**<0.001**
G+L	1.389957	1.4582	0.240
G/L	1.363739	7.0299	**<0.001**
G-L	1.285213	0.789	0.568
L-G	1.432832	9.9503	**<0.001**

Table 2. Results of one-way ANOVA for within group variation in direct response. Bolded p-values indicate a significant variation in direct response after correction for multiple comparisons.

3.5 Direct Competitions

In an effort to more thoroughly interpret the indirect comparisons between groups which made up the bulk of my experiment, I performed a series of competitions wherein fitness was measured directly, rather than relative to the ancestor. The results of all direct competitions were interpreted by comparison to the results of

indirect competitions; therefore, I plotted the mean and 95% confidence intervals for both direct and indirect fitness estimates side by side. In all cases, means and error bars were calculated with a bootstrap analysis (see Methods). I interpreted no significant difference if the confidence intervals from both sets of fitness measures overlapped the other's mean; otherwise, interpretations depended on the degree of overlap. First, I competed two lines from each specialist group against one line from each generalist group (Figure 6). These lines were selected randomly with respect to their performance in relative fitness competitions. I competed Glu and Lac specialists in their respective environments, in keeping with previous comparisons between generalist and specialist fitness estimates.

A majority (thirteen of sixteen) of direct fitness estimates showed little or no difference from their corresponding relative fitness estimates; notably, the two direct estimates most different from the corresponding indirect estimates were both in lactose.

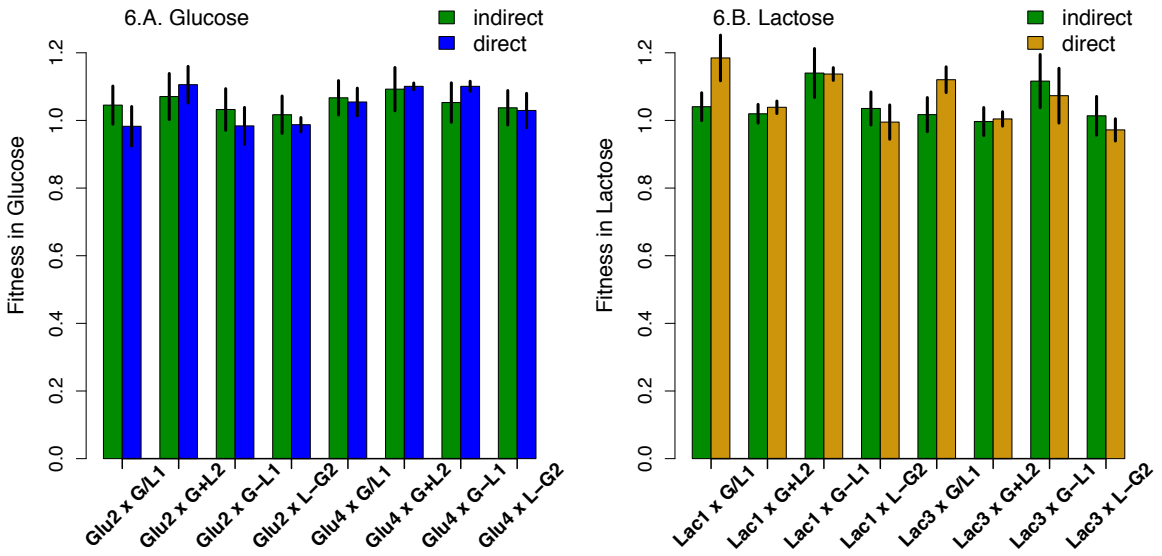


Figure 6. Fitness estimates from direct competitions between specialist and generalist populations. Competitions were performed at 6,000 generations in the evolution environment of the specialist used (i.e. competitions with glucose specialists were performed in glucose). Competed populations are indicated on the X-axis. Direct fitness estimates are shown in green. Relative fitness estimates are shown in blue for glucose specialists and yellow for lactose specialists. Points and error bars representing the mean and 95% confidence intervals were calculated from a bootstrap analysis (see Methods).

Next, I performed direct competitions between generalist lines, again selected randomly with respect to their performance in relative fitness competitions. I competed one population from the G+L group against two populations each from the G/L, G-L, and L-G groups. These competitions were performed in both glucose and lactose environments, as generalist fitness in both environments was considered in previous analyses. Figure 7 shows the mean and 95% confidence intervals for both indirect and direct fitness estimates measured in glucose and lactose. These results were more variable than the previous set. Only two of six

direct fitness estimates showed little or no difference from their corresponding relative fitness estimates in either environment, while four showed a significant difference in one of the two environments. Again, differences occurred more often in lactose (3 of 4 observed here). Differences in direct and indirect fitness estimates could reflect a number of within population dynamics, discussed at greater length below.

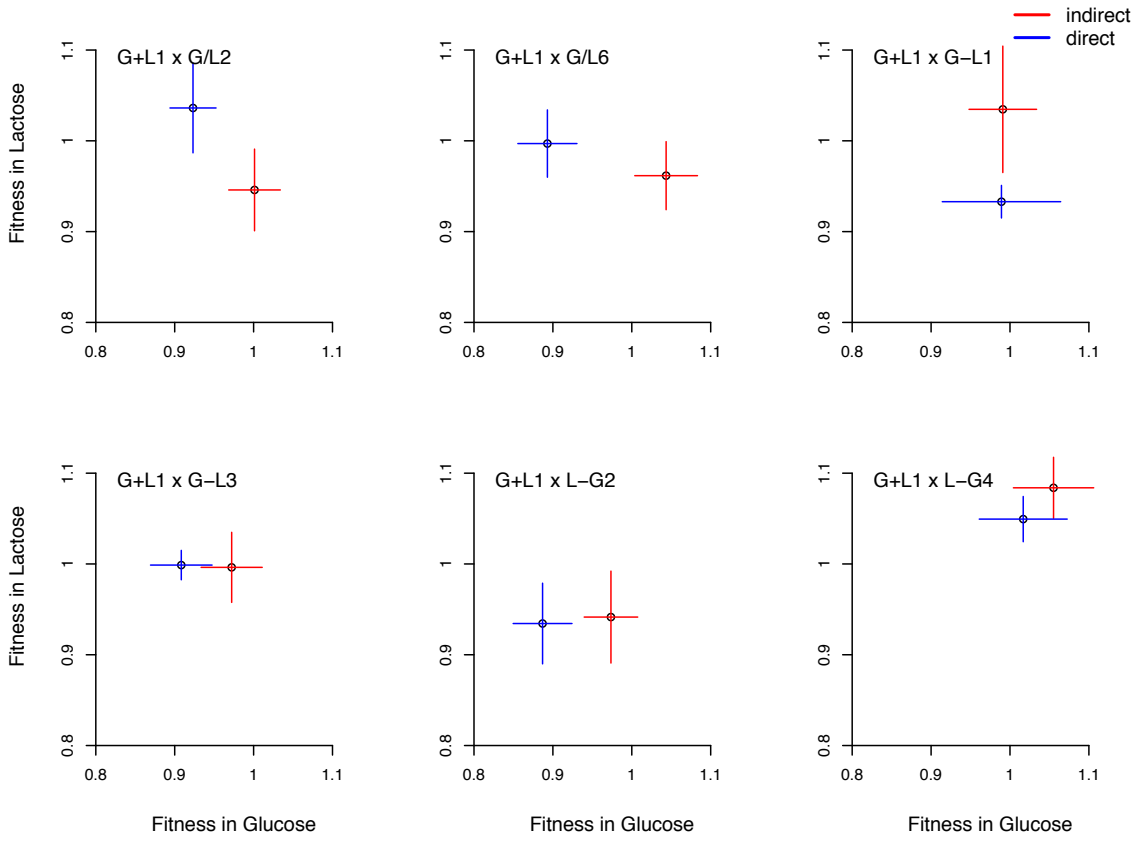


Figure 7. Fitness estimates from direct competitions between generalist populations. Competitions were performed at 6,000 generations in glucose and lactose. Competed populations are indicated at the top left of each panel. Direct fitness estimates are shown in blue. Relative fitness estimates are shown in red. Points and error bars representing the mean and 95% confidence intervals were calculated from a bootstrap analysis (see Methods).

3.6 Glucose Adaptation

The final series of direct competitions I performed was in response to a signal of unusual within-group dynamics. This signal was the accelerated rate at which the Glu group adapted to the lactose environment (despite never having experienced selection in lactose) during the final phases of the experiment (Figure 2.B.). Specifically, that group saw a mean relative fitness increase of 15% in lactose between 5,000 and 6,000 generations that was not an artifact of outlying populations with high individual increases; in fact, only one of six populations increased fitness in lactose less than 9%. Before considering which genetic mechanisms could best explain this increase, I needed to rule out the possibility that it was due to experimental error. The simplest way to do this was to plate each population on TA agar and check for inconsistencies in Ara markers. Such inconsistencies would indicate cross-contamination or a confusion of the lines that went unnoticed during propagation; however, I found evidence of none. As an added precaution, I sequenced genes from a subset of the replicate populations to identify mutations that were present at both early and late experimental time points. First, I selected five clones from each of three populations at two times points (2,000 and 6,000 generations) after plating on nutrient-rich agar. I then identified candidate genes containing mutations that occurred prior to 2,000 generations, based on previous whole genome sequencing of evolved clones (data not shown). I used standard PCR methods to amplify candidate genes and purify reactions for sequencing, and ultimately identified a total of five distinct point mutations that

were present in clones from both time points (Table 3). These results indicated that a confusion of the lines was statistically improbable.

Population	Generation	Number of Clones	Gene	Annotation
Glu4	2,000	1	*pykF*	D336N (GAC→AAC)
	6,000	5	*pykF*	D336N (GAC→AAC)
Glu4	2,000	1	*spoT*	F409V (TTC→GTC)
	6,000	5	*spoT*	F409V (TTC→GTC)
Glu5	2,000	4	*recD*	V10A (GTT→GCT)
	6,000	5	*recD*	V10A (GTT→GCT)
Glu5	2,000	5	*spoT*	R701Q (CGA→CAA)
	6,000	5	*spoT*	R701Q (CGA→CAA)
Glu6	2,000	2	*spoT*	(CGT→CTT)
	6,000	5	*spoT*	(CGT→CTT)

Table 3. Sequencing results from Glu group clones at 2,000 and 6,000 generations (to confirm the identity of the replicate populations). The number of individual clones from each time point in which the mutation was identified is indicated.

One hypothesis that could explain a dramatic fitness increase in a non-selective environment is the presence of ecological interactions within populations, e.g., cross-feeding between coexisting subpopulations. This could lead to accelerated coevolution between subpopulations, such that relative fitness estimates would no longer be representative of evolution in a stable environment (deVisser and Rozen 2005). If such interactions were present in the Glu group and had resulted in the detection of a non-transitive fitness increase in lactose, I would expect that fitness estimates derived from direct competitions would be significantly different from the previous relative fitness estimates. Therefore, I competed all populations from the Glu group at 5,000 and 6,000 generations directly against themselves (Figure 8); I

did this in the lactose environment, in which I expected direct and relative fitness estimates to be significantly different if non-transitivity had been an influence, and in the glucose environment to serve as a control.

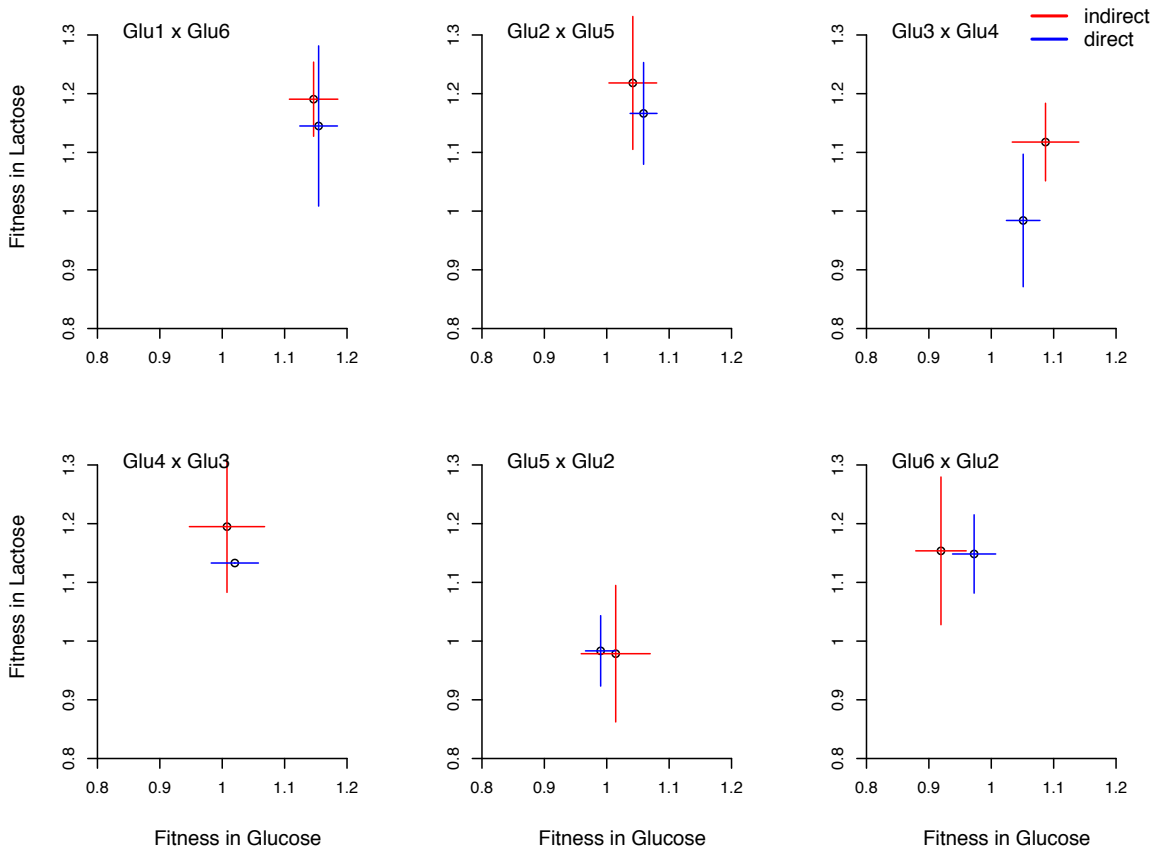


Figure 8. Fitness estimates from direct competitions between replicate lines of the Glu group. Competitions were performed at 5,000 and 6,000 generations in glucose and lactose environments. Competed populations are indicated at the top left of each panel (5,000 x 6,000). Direct fitness estimates are shown in blue. Relative fitness estimates are shown in red. Points and error bars representing the mean and 95% confidence intervals were calculated from a bootstrap analysis (see Methods).

The majority of direct fitness estimates showed little or no difference from their corresponding relative fitness estimates in either environment (Figure 8), and there was no tendency for a greater difference in either environment. Though these results do not rule out the existence of ecological interactions within these populations, they demonstrate that such interactions are unlikely to have impacted relative fitness competitions in a meaningful way.

An alternative hypothesis to explain how the Glu group was able to overcome its cost of adaptation and increase its fitness in the lactose environment is through the influence of epistatic (gene by gene) interactions. If the effects of mutations were dependent on the genetic background in which they occurred, mutations that would have been selectively deleterious at an early time point could be beneficial if they occurred at a later time point. I explored this possibility by screening for constitutive expression of the lactose metabolism machinery, a phenotype which has been linked to mutations in the LacI and LacO genes and shown to impose a cost in the glucose-limited environment (Quan et al. 2012). If either of these mutations was responsible for the large fitness increase in lactose, I expected that most populations would have the constitutive expression phenotype at 6,000, but not 5,000 generations. I screened ~250 colonies from each of the six Glu group populations, at both time points, on agar supplemented with X-gal (on which the wildtype and mutant phenotypes can be visibly distinguished). No populations displayed the mutant phenotype at 5,000 generations, but four of six had

frequencies of approximately 30% or higher at 6,000 generations. To confirm the results of my plating assay, I amplified and sequenced the LacI and LacO genes in clones from each of the four lines that expressed the mutant phenotype (at the 6,000 generation time point). I found mutations in *LacI* in three of these lines, and in *LacO* in the other (Table 4). As an added precaution against cross-contamination, I sequenced other genes in the same clones, in which mutations had been found previously (e.g., Table 3). My logic was that finding a single clone with both the new and old mutations supported the hypothesis that the new mutation had occurred in the same lineage and been selected for.

Population	Clone Name	Gene	Annotation
Glu3	3_1	*lacI*	(T→C), (T→G)
Glu4	4_1	*lacI*	G deletion
	4_1	*pykF*	D336N (GAC→AAC)
Glu5	5_1	*lacO*	(G→A)
	5_1	*lacI*	(T→G)
Glu5	5_2	*lacI*	(T→G), G insertion
	5_2	*spoT*	R701Q (CGA→CAA)
	5_2	*recD*	V10A (GTT→GCT)
Glu6	6_1	*lacI*	(T→G), (A→G)
	6_2	*lacI*	(T→C), G insertion
	6_2	*spoT*	(CGT→CTT)

Table 4. Sequencing results from Glu group clones at 6,000 generations (to confirm the presence of mutations causing constitutive expression of the lactose metabolism machinery). Clone name is included to allow multiple clones from the same population which had different combinations of mutations to be distinguished.

I found clones with mutations in *LacI*, and with the same point mutations identified previously in other genes, in single clones from the Glu4, Glu5, and Glu6 populations (Table 4). Therefore I have some confidence that the evolved genetic background acted epistatically with these (previously deleterious) mutations, allowing them to rise to high frequency between 5,000 and 6,000 generations.

4 DISCUSSION

I compared the fitness of specialist and generalist populations, measured in different environments, at regular intervals over 6,000 generations of evolution. My aim in doing this was to elucidate the extent to which costs of adaptation influence long-term evolutionary trajectories. To achieve this aim, I tested the fit of standard AP and MA models to the adaptive patterns I observed, and evaluated both between-group and within-group dynamics. My results indicate that costs of adaptation are ultimately unavoidable, though populations may not experience them for a substantial number of generations.

4.1 Specificity & Costs of Adaptation

After 6,000 generations, all groups had significantly increased their fitness in each of the two resources making up the experiment (Figure 2.). These results demonstrate that selection in the single-resource environments was not required for adaptation to them, which was not necessarily expected. However, it is not particularly surprising, given that there is overlap in the pathways by which *E. coli* metabolizes glucose and lactose, and a previous study linked the amount of beneficial pleiotropy in adaptation to the degree of overlap in the import and metabolic pathways of different resources (Travisano and Lenski 1996). Additionally, I was able to confirm that the majority of adaptation was specific to the resource-regimes present in the environments (Table 1), which gave me confidence that differences in the environments were large enough that my central question could be tested reliably

using this system. I expected that costs would be a prevalent adaptive mechanism, as ample evidence of AP has been found previously in a similar system (Cooper and Lenski 2000). I attempted to determine whether AP was the underlying genetic basis of adaptive specificity in the Glu and Lac groups by examining the relationship between their direct and correlated responses (Figure 3). Correlations were non-significant, so I was unable to conclude whether the observed specificity was due to beneficial or deleterious pleiotropic effects. However, considering each group's mean fitness in glucose and lactose, alongside the within-group relationships between fitness across environments, allowed me to detect patterns of cost in both specialist and generalist groups.

The specialists maintained distinct adaptive trajectories throughout the experiment; I found that in general, adaptation to glucose tended to impose costs in lactose, but not vice-versa. I offer three lines of evidence to support this interpretation. First, by 1,000 generations the Glu group had a significant positive direct response, but some replicate populations had decreased their fitness in lactose, revealing a pattern consistent with MA (Figure 2.A.) Second, for the first half of the experiment, populations evolving in glucose had substantial fluctuations in lactose fitness while steadily increasing fitness in glucose (Figure 2.A.), indicating the presence multiple adaptive mutations with different pleiotropic effects. Finally, the overall adaptive patterns of the Lac group suggest that it was able to adapt using (mostly) mutations with positive or no pleiotropic effects in glucose; the only suggestion of a cost was a

(nonsignificant) negative correlation at 1,000 generations. The specialists' overall rates of adaptation also sharply contrast; the Glu group increased its fitness in both glucose and lactose during the later stages of the experiment, which suggests that costs accrued early on were compensated by the fixation of additional mutations. The Lac group, however, had no significant fitness increase in either environment between 4,000 and 6,000 generations, suggesting that the average effect of mutations that fixed in that group was diminishing over time. Both patterns are consistent with increasing epistasis, such that gene by gene interactions became more common and modified the effects of mutations that occurred during the later stages of adaptation; interestingly, the interactions within the specialist groups seemed to be having opposite effects. Adaptive mutations in the Glu group seem to have larger benefits in lactose at later time points, whereas all mutations in the Lac group at later time points have diminished effects. I discuss the effects of epistasis in the Glu group in more detail below.

I was able to infer the presence of AP in the daily fluctuating generalist group based on its higher number of negative correlations between fitness in glucose and lactose, and slower rate of adaptation to glucose than the mixed resource regime. Bailey and Kassen (2012) found a similar result when comparing rates of adaptation in mixed and spatially fluctuating environments, such that populations evolved in mixed environments adapted significantly faster. In my case, the effect was not large

enough to cause a difference in the average correlated response of the G/L group, relative the G+L group, but the overall pattern suggests competing adaptive mutations having different pleiotropic effects, providing a strong signal of AP. Decreases in the mean fitness of the long-term fluctuating generalists, in non-selective environments following the switch to a new selective resource, were also consistent with the presence of costs. These costs were more likely due to AP than MA because they occurred at the same time as significant increases in direct response (for both groups). I did find some signal of MA in two replicate lines of the L-G group, which decreased their fitness in the non-selective environment, and simultaneously failed to increase their fitness in the selective environment. My results, coupled with previous identification of two mutations with AP that had fixed in most generalist groups by 2,000 generations (Quan et al. 2012), indicate that costs due to AP and MA were, to some extent, present in this system. Identifying costs themselves was not my central aim, and to determine the comprehensive genetic makeup of these populations would require whole-genome sequencing and a series of allele replacement experiments. However, I was able to quantify the influence of mechanisms underlying adaptation (and thus make inferences about their relative abundance) by comparing the fitness of specialist and generalist groups at regular intervals.

4.2 Specialists vs. Generalists

To test predictions about which genetic mechanisms produced the observed patterns of adaptation in the specialists, and thereby infer which mechanisms gave rise to the adaptation of the generalists, I compared the geometric mean relative fitness of all groups in glucose and lactose over the course of the experiment. Generalists were considered masters of all trades at any time point if they had geometric mean relative fitness not significantly different from the MAT isocline, calculated from the combined direct response of the specialists (Figure 5). If costs due to AP were prevalent, I expected that generalists would be constrained from reaching master of all trades fitness levels. I also considered whether beneficial pleiotropy was common, which would have lead to the generalists and specialists maintaining similar levels of fitness throughout the experiment.

The mix and daily fluctuating generalists were already masters of all trades at 1,000 and 2,000 generations, and in fact, most generalists were masters of all trades during the mid-points of the experiment. This suggests that mutations with deleterious effects in either environment largely failed to fix during the first 4,000 generations, though the generalists' patterns of correlated response indicate that such mutations were sometimes present at reasonably high frequencies . The effects of those that were accrued did not constrain the generalists' rate of adaptation, relative to the specialists', until 5,000 and 6,000 generations, when all generalists fell significantly behind the MAT isocline. This pattern of eventual decline provides a

strong signal of the effects of AP, as the constraining effects of MA are expected to decrease, rather than increase with the fixation of adaptive mutations. Though I previously described results indicating the presence of some costs, and a few mutations with AP have been identified in this system (Quan et al. 2012), I found more evidence that the avoidance of deleterious pleiotropic effects lead to decreased rates of adaptation in the generalists, rather than the accumulation of costs in both single-resource environments. One observation that supports this hypothesis is that the patterns of adaptation in the Lac group indicate the general avoidance of costs, and this group also had a decreased rate of adaptation during the later stages of the experiment. It is worth mentioning that if the experiment had ended after 3,000 or even 4,000 generations of evolution, conclusions about the role of costs in constraining long-term adaptive trajectories would have been completely different. In all, I found that costs are indeed an essential component of adaptation, though that they may not act to constrain evolution for a substantial amount of time.

4.3 Variation in Response

I performed additional tests to examine the dynamics of adaptation in my populations in greater detail; first, I performed a series of ANOVAs to test for within group (among population) variation in direct response. Significant differences in the direct response of replicate lines, founded from the same ancestor and evolved in the same selective environment, may indicate that populations are evolving to different adaptive peaks. After 2,000 generations of evolution, a single group of

replicate populations, evolved in the daily fluctuating environment, was found to have significant differences in direct response after correction for multiple comparisons (Cooper and Lenski 2010). I performed the same analysis after 6,000 generations of evolution, and found that the same group, as well as two others (Lac and L-G) had significant differences in direct response. The maintenance of significant variation in the G/L group is not surprising, and could in fact be aligned with my hypothesis about the increased presence of AP in this group. I found signal of multiple mutations with deleterious pleiotropic effects in this group, which are likely a result of the daily fluctuations in selective pressures; such mutations would be likely to direct populations toward different adaptive peaks, as they act to constrain simultaneous adaptation to different selective pressures. I should note that the Lac group had a high level of variation in direct response at 2,000 generations, but that this variation was not significant after correction for multiple comparisons. Still, my results indicate that this variation has increased, suggesting that mutations which fixed early in this group's adaptive history were the source of initial variation. This is supported by the fact that the L-G group also had significant variation in direct response by 6,000 generations, as these groups had identical adaptive trajectories for the first 2,000 generations.

4.4 Direct Competitions

In an effort to better interpret the relative fitness estimates which form the basis of my project, I performed a series of direct competitions between evolved

populations. This served as a check for non-transitivity in my system, though it was not exhaustive (had the results of the Glu group competitions been more variable, I may have had reason to perform a more comprehensive survey). As my central question involved a comparison between the response of specialist and generalist groups in the specialists' selective environments, I first performed direct competitions between a selection of specialist and generalist populations, in the specialists' environments (Figure 6). The fitness estimates produced by these direct competitions were, for the most part, not significantly different from those previously found in the equivalent indirect competitions. The results of direct competitions between generalist populations were more variable (Figure 7). I note, however, than even when differences were found, they tended to be small relative to the overall fitness improvement of each population. Additionally, my central question relied on comparisons between specialists and generalists, and I found no indication that non-transitivity had influenced those results.

4.5 Glucose Adaptation

An unexpected result was the accelerated rate with which the Glu group adapted to lactose during the later stages of the experiment. Selection for compensatory mutations can be ruled out the large fitness increase occurred in a non-selective environment. Additionally, for the effects of pleiotropy alone to explain the observed pattern requires that specific types of mutations would have had to occur preferentially at different stages of adaptation; for example, if mutations with

antagonistically pleiotropic effects had larger selective benefits than mutations with beneficial pleiotropic effects for the first 3,000 generations of evolution, after which time the pattern reversed. Having ruled out the likelihood of cross-contamination, another hypothesis was the presence of ecological interactions within populations making up the group, e.g., cross-feeding between coexisting subpopulations. Such interactions have been detected in evolving lines of *E. coli* (Turner et al. 1996; Rozen and Lenski 2000; Spencer et al. 2008), and have been shown to lead to non-transitivity between relative fitness estimates (deVisser and Rozen 2005). If such interactions were present in the Glu group and had spuriously inflated relative fitness estimates for this group measured in lactose at 6,000 generations, I would expect that fitness estimates derived from direct competitions at the same time point would be significantly different. However, the majority of comparisons showed no significant difference.

Positive epistasis is an alternative explanation for the Glu group's ability to overcome its initial constraint in lactose. I explored this possibility by confirming the presence of mutations in *LacI* and *LacO*, known to have antagonistically pleiotropic effects in this system; each are beneficial in lactose but deleterious in glucose (Quan et al 2012). My findings suggest that these mutations, having occurred in the evolved genetic background of four Glu group populations, no longer have deleterious effects in glucose but were instead selectively beneficial, and indeed rose to relatively high frequencies between 5,000 and 6,000 generations.

However, to confirm the presence of positive epistatic interactions between the evolved genetic background and each of these mutations requires an allele replacement experiment. For example, one could replace the mutant allele with the ancestor allele in the evolved genetic background, then measure the fitness of these new clones in both single-resource environments. If these mutations are in fact beneficial in the evolved genetic background, replacing them with the ancestral allele should cause fitness to decrease in glucose, as well as lactose. Further experiments, in which evolved alleles are combined in the ancestral background, could reveal specific interactions between mutations and potentially indicate the source of epistasis.

A final point of interest related to the presence of ecological interactions is the existence of slow-growing Ara- colonies in an historically Ara+ population. Figure 9 shows photographs of this population (Glu1) at 6,270 generations, plated on TA agar and incubated at 37^{o} C for 24 and 48 hours, respectively.

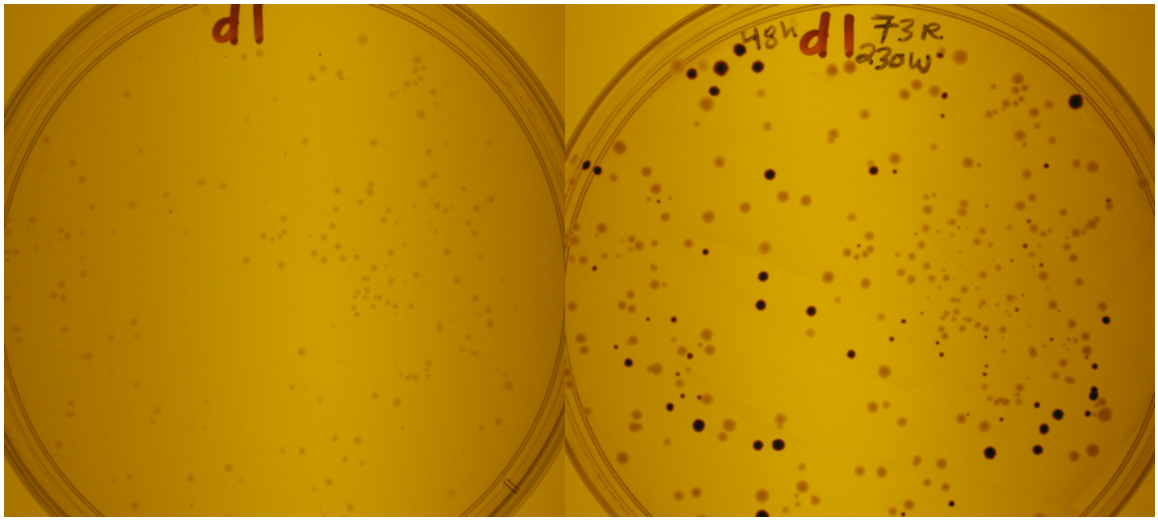


Figure 9. Photographs of the Glu1 population, plated on TA agar, after incubation for 24 hours (right) and 48 hours (left).

The coexistence of both morphs has continued for at least 270 generations, and is monitored during propagation by plating on TA every 12 transfers. Rozen and Lenski (2000) evaluated the dynamics of two coexisting morphs that arose in a single population of *E. coli* during a long term evolution experiment, and found that the relationship was maintained by frequency-dependent selection, such that each type had a selective benefit when it was rare. To confirm that this is the case in our experiment would require an additional experiment to explore the relationship between the two morphs (e.g., Elena and Lenski 1997). For example, clones of each type could be selected and competed at different starting frequencies, to determine the fitness of each type when rare. Note that if these subpopulations were both present during the final time point of the experiment and caused non-transitivity in relative fitness estimates, this would have been detected through direct competitions between the Glu group populations. These adaptive dynamics are an interesting point for follow-up, but have had no detectable influence on my results.

The novel aspect of my study was identifying the influence of costs on long-term evolution, i.e., their constraining effects on simultaneous adaptation to multiple resources. My interpretations of the traditional test for costs of adaptation (examining the relationship between fitness across environments, in search of a negative correlation), was aided by a set of comparisons between fitness measured in different environments at multiple time points. For several groups, fitness in non-selective environments fluctuated as a result of competing mutations, and the deleterious effects of those mutations would not have been evident in a 'snap-shot' of the relationship between fitness in the two environments at any one time point. Long-term observational studies are needed to inform the overall role of individual mutational mechanisms to adaptive evolution.

5 REFERENCES

Alizon S, van Baalen M 2005 Emergence of a convex trade-off between transmission and virulence. Am. Nat. 165: 155-167.

Bailey SF, Kassen R 2012 Spatial structure of ecological opportunity drives adaptation in a bacterium. Am. Nat. 180: 270–283.

Bataillon TT, Zhang TT, Kassen, RR 2011 Cost of adaptation and fitness effects of beneficial mutations in *Pseudomonas fluorescens*. Genetics 189: 939–949.

Bell G 1997 Experimental evolution in *Chlamydomonas*. I. Short-term selection in uniform and diverse environments. Heredity 78: 490-497.

Bell G, Reboud X 1997 Experimental evolution in *Chlamydomonas*. II. Genetic variation in strongly contrasted environments. Heredity 78: 498-506.

Bennett AB, Lenski RE, Mittler JE 1992 Evolutionary adaptation to temperature. I. Fitness responses of *Escherichia coli* to changes in its thermal environment. Evolution 46: 16-30.

Berngruber TW, Froissart R, Choisy M, Gandon S, Balloux F 2013 Evolution of virulence in emerging epidemics. PLoS Pathog. 9(3): e1003209.

Berlocher SH, Feder JL 2002 Sympatric speciation in phytophagous insects: moving beyond controversy? Annu. Rev. Entomol. 47: 773–815.

Birand A, Vose A, Gavrilets S 2012 Patterns of species ranges, speciation, and extinction. Am. Nat. 179: 1–21.

Björkman JJ, Samuelsson PP, Andersson DID, Hughes DD 1999 Novel ribosomal mutations affecting translational accuracy, antibiotic resistance and

virulence of *Salmonella typhimurium*. Mol. Microbiol. 31: 53–58.

Brown, SP, Cornforth DM, Mideo N 2012 Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. Trends Microbiol. 20: 336–342.

Buckling A, Maclean RC, Brockhurst MA, Colegrave N 2009 The beagle in a bottle. Nature 457: 824–829.

Bull JJ, Badgett MR, Wichman HA 2000 Big-benefit mutations in a bacteriophage inhibited with heat. Mol. Biol. Evol. 17: 942–950.

Burch CL, Chao L 1999 Evolution by small steps and rugged landscapes in the RNA virus phi6. Genetics 151: 921–927.

Cairns J, Foster PL 1991 Adaptive reversion of a frameshift mutation in *Escherichia coli*. Genetics 128: 695–701.

Clavel J, Julliard R, Devictor V 2011 Worldwide decline of specialist species: toward a global functional homogenization? Front. Ecol. Environ. 9: 222–228.

Colautti RI, Lee C-R, Mitchell-Olds T 2012 Origin, fate, and architecture of ecologically relevant genetic variation. Curr. Opin. Plant Biol. 15: 199–204.

Cooper T, Lenski RE 2010 Experimental evolution with *Escherichia coli* in diverse resource environments. I. Fluctuating environments promote divergence of replicate populations. BMC Evol. Biol. 10:11

Cooper VS, Lenski RE 2000 The population genetics of ecological specialization in evolving *Escherichia coli* populations. Nature 407: 736-739.

Cooper TF, Ostrowski EA, Travisano M 2007 A negative relationship between mutation pleiotropy and fitness effect in yeast. Evolution 61: 1495–1499.

Crill WDW, Wichman HAH, Bull JJJ 2000 Evolutionary reversals during viral adaptation to alternating hosts. Genetics 154: 27–37.

de Visser JAGM, Rozen DE. 2005 Limits to adaptation in asexual populations. J. Evol. Biol. 18: 779–788.

Draghi JA, Whitlock MC 2012 Phenotypic plasticity facilitates mutational variance, genetic variance, and evolvability along the major axis of environmental variation. Evolution 66: 2891–2902.

Duffy S 2005 Pleiotropic costs of niche expansion in the RNA bacteriophage 6. Genetics 172: 751–757.

Dykhuizen DE, Dean AM 2004 Evolution of specialists in an experimental microcosm. Genetics 167: 2015–2026.

Elena SF, Agudelo-Romero P, Lalić J 2009 The evolution of viruses in multi-host fitness landscapes. Open Virol. J. 3: 1–6.

Elena SF, Lenski RE 1997 Test of synergistic interactions among deleterious mutations in bacteria. Nature 390: 395–398.

Elena SF, Lenski RE 2003 Microbial genetics: Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. Nat. Rev. Genet. 4: 457–469.

Featherstone DE, Broadie K 2002 Wrestling with pleiotropy: genomic and topological analysis of the yeast gene expression network. BioEssays 24.3: 267-274.

Felsenstein J 1981 Scepticism towards Santa Rosalia, or why are there so many kinds of animals? Evolution 35: 124-138.

Fisher, RA The Genetical Theory of Natural Selection (Oxford Univ. Press, Oxford, 1930).

Foster KR, Shaulsky G, Strassmann JE, Queller DC, Thompson CRL 2004 Pleiotropy as a mechanism to stabilize cooperation. Nature 431: 693–696.

Frank SA 1996 Models of parasite virulence. Q. Rev. Biol. 71: 37-78.

Fry JD 1993 The "general vigor" problem: can antagonistic pleiotropy be detected when genetic covariances are positive? Evolution 47: 327-333.

Fry JD 2003 Detecting ecological trade-offs using selection experiments. Ecology 84: 1672-1678.

Funchain P, Yeung A, Stewart JL, Lin R, Slupska MM, Miller JH 2000 The consequences of growth of a mutator strain of *Escherichia coli* as measured by loss of function among multiple gene targets and loss of fitness. Genetics 154: 959-970.

Futuyma DJ, Moreno G 1988 The evolution of ecological specialization. Ann. Rev. Ecol. Syst. 19: 207-233.

Gallet R, Cooper TF, Elena SF, Lenormand T 2012 Measuring selection coefficients below 10^{-3}: Methods, questions, and prospects. Genetics 190: 175–186.

Gillespie J Population Genetics: a concise guide (Johns Hopkins Univ. Press, Baltimore, 2004)

Hawthorne DJ, Via S 2001 Genetic linkage of ecological specialization and reproductive isolation in pea aphids. Nature 412: 904-907.

Hedrick PW 1986 Genetic polymorphism in heterogeneous environments: a decade later. Ann. Rev. Ecol. Syst. 17: 535-566.

Jasmin J-NJ, Dillon MMM, Zeyl CC 2012 The yield of experimental yeast populations declines during selection. Proc. R. Soc. B. 279: 4382–4388.

Jasmin J-NJ, Kassen R 2007 On the experimental evolution of specialization and diversity in heterogeneous environments. Ecol. Lett. 10: 272-281.

Kassen R 2002 The experimental evolution of specialists, generalists, and the maintenance of diversity. J. Evol. Biol. 15: 173-190.

Kassen R, Bell G 1998 Experimental evolution in *Chlamydomonas*. IV. Selection in environments that vary through time at different scales. Heredity 80: 732–741.

Koskella B, Lin DM, Buckling A, Thompson JN 2012 The costs of evolving resistance in heterogeneous parasite environments. Proc. R. Soc. B. 279: 1896-1903.

Lennon JT, Khatana SAM, Marston MF, Martiny JBH 2007 Is there a cost of virus resistance in marine cyanobacteria? ISME Journal 1: 300–312.

Lenski, RE 1988 Experimental studies of pleiotropy and epistasis in *Escherichia coli*. I. Variation in competitive fitness among mutants resistant to virus T4. Evolution 42: 425–432. 1988a

Lenski RE 1988 Experimental studies of pleiotropy and epistasis in *Escherichia coli.* II. Compensation for maldaptive effects associated with resistance to virus T4. Evolution 42: 433-440. 1988b

Lenski RE, May RM 1994 The evolution of virulence in parasites and pathogens: reconciliation between two competing hypotheses. J. Theor. Biol. 169: 253-265.

Lenski RE, Rose MR, Simpson SC, Tadler SC 1991 Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. Am. Nat. 138: 1315-1341.

MacLean RC 2004 The evolution of a pleiotropic fitness tradeoff in *Pseudomonas fluorescens*. P. Natl. Acad. Sci. USA 101: 8072–8077.

MacLean RC, Bell G 2002 Experimental adaptive radiation in *Pseudomonas*. Am. Nat. 160: 569-581.

Ostrowski EA, Rozen DE, Lenski RE 2005 Pleiotropic effects of beneficial mutations in *Escherichia coli*. Evolution 59: 2343–2352.

Poon AFYA, Chao LL 2006 Functional origins of fitness effect-sizes of compensatory mutations in the DNA bacteriophage phiX174. Evolution 60: 2032–2043.

Postma PW, Lengeler JW, Jacobson GR 1993 Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. 57: 543-594.

Quan S, Ray JCJ, Kwota Z, Duong T, Balázsi G, Cooper TF, Monds RD 2012 Adaptive evolution of the lactose utilization network in experimentally evolved populations of *Escherichia coli.* PLoS Genetics 8: e1002444–e1002444.

Ravigné V, Dieckmann U, Olivieri I 2009 Live where you thrive: Joint evolution of habitat choice and local adaptation facilitates specialization and promotes diversity. Am. Nat. 174: 141–169.

Reboud X, Bell G 1997 Experimental evolution in *Chlamydomonas*. III. Evolution of specialist and generalist types in environments that vary in space and time. Heredity 78: 507-514.

Remold S 2012 Understanding specialism when the jack of all trades can be the master of all. Proc. R. Soc. B. 279: 4861-4869.

Remold SK, Rambaut A, Turner PE 2008 Evolutionary genomics of host adaptation in vesicular stomatitis virus. Mol. Biol. Evol. 25: 1138–1147.

Rice WR 1989 Analyzing tables of statistical tests. Evolution 43: 223–225.

Roff DA 1996 The evolution of genetic correlations: an analysis of patterns. Evolution: 1392–1403.

Roff DA, Fairburn DJ, 2007 The evolution of trade-offs: where are we? J. Evol. Biology. 20: 433-447.

Rose M, Charlesworth B 1980 A test of evolutionary theories of senescence. Nature 287: 141-142.

Rozen DE, Lenski RE 2000 Long-term experimental evolution in *Escherichia coli.* VIII. Dynamics of a balanced polymorphism. Am. Nat. 155: 24–35.

Schrag SJS, Perrot VV, Levin BRB 1997 Adaptation to the fitness costs of antibiotic resistance in *Escherichia coli*. Proc. R. Soc. B. 264: 1287–1291.

Selman C, Blount JD, Nussey DH, Speakman, JR 2012 Oxidative damage, ageing, and

life-history evolution: where now? Trends Ecol. Evol. 27: 570-577.

Service PM, Rose MR 1985 Genetic covariation among life-history components: the effect of novel environments. Evolution 39: 943–945.

Sgrò CM, Hoffmann AA 2004 Genetic correlations, tradeoffs and environmental variation. Heredity 93: 241–248.

Sniegowski PD, Gerrish PJ, Lenski RE 1997 Evolution of high mutation rates in experimental populations of *E. coli*. Nature 387: 703–705.

Spencer CC, Tyerman J, Bertrand M, Doebeli M 2008 Adaptation increases the likelihood of diversification in an experimental bacterial lineage. P. Natl. Acad. Sci. USA. 105: 1585–1589.

Travisano M 1997 Long-term experimental evolution in *Escherichia coli*. VI. Environmental constraints on adaptation and divergence. Genetics 147: 471-479.

Travisano M, Lenski RE 1996 Long-term experimental evolution in *Escherichia coli.* IV. Targets of selection and the specificity of adaptation. Genetics 143: 15-26.

Travisano M, Vasi F 1995 Long-term experimental evolution in *Escherichia coli*. III. Variation among replicate populations in correlated responses to novel environments. Evolution 49: 189-200.

Trindade SS, Sousa AA, Xavier KBK, Dionisio FF, Ferreira MGM, Gordo II 2009 Positive epistasis drives the acquisition of multidrug resistance. PLoS Genetics 5: e1000578–e1000578.

Turner PE, Elena SF 2000 Cost of host radiation in an RNA virus. Genetics 156: 1465–1470.

Turner P, Souza V, Lenski R 1996 Tests of ecological mechanisms promoting the stable coexistence of two bacterial genotypes. Ecology 77: 2119–2129.

Van Tienderen PH 1997 Generalists, specialists, and the evolution of phenotypic plasticity in sympatric populations of distinct species. Evolution 51: 1372–1380.

Velicer GJ, Lenski RE 1999 Evolutionary trade-offs under conditions of resource abundance and scarcity: Experiments with bacteria. Ecology 80: 1168-1179.

Wagner GP, Kenney-Hunt JP, Pavlicev M, Peck JR, Waxman D, Cheverud JM 2008 Pleiotropic scaling of gene effects and the "cost of complexity". Nature 452: 470–472.

Ward H, Perron GG, MacLean RC 2009 The cost of multiple drug resistance in *Pseudomonas aeruginosa*. J. Evol. Biol. 22: 997–1003.

Weaver SC, Brault AC, Kang W, Holland JJ 1999 Genetic and fitness changes accompanying adaptation of an arbovirus to vertebrate and invertebrate cells. J. Virol. 73: 4316-4326.

Whitlock MC 1996 The red queen beats the jack-of-all-trades: the limitations on the evolution of phenotypic plasticity and niche breadth. Am. Nat. 148: S65-S77.

Wilson DS, Yoshimura J 1994 On the coexistence of specialists and generalists. Am. Nat. 144: 692–707.

Zhang W, Sehgal V, Dinh DM, Azevedo RBR, Cooper TF, Azencott R 2012 Estimation of the rate and effect of new beneficial mutations in asexual populations. Theor. Popul. Biol. 81: 168–178.