An Experimental Evolutionary Study on Adaptation to Temporally Fluctuating pH in *Escherichia coli*

Bradley S. Hughes*
Alistair J. Cullum
Albert F. Bennett
Department of Ecology and Evolutionary Biology, University of California, Irvine, California 92697-2525

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ABSTRACT

In this study, we use the bacterium *Escherichia coli* to examine evolutionary responses to environmental acidity fluctuating temporally among pH 5.3, 6.3, 7.0, and 7.8 (5,000–15 nM $[H^+]$). Two experimental protocols of temporal variation were used. One group (six replicate lines) of populations evolved for 2,000 generations during exposure to a cycled regime fluctuating daily between pH 5.3 and 7.8. The other group (also in six replicate lines) evolved during exposure for 2,000 generations to a randomly shifting regime fluctuating stochastically each day among pH 5.3, 6.3, 7.0, and 7.8. Adaptation to these fluctuating acidity regimes was measured as a change in fitness relative to the common ancestor by direct competition experiments in both constant and fluctuating pH regimes. For comparisons with constant pH evolution, a group evolved at a constant pH of 5.3 and another group evolved at pH 7.8 were also tested. This study initiated the first long-term laboratory natural selection experiment on adaptation to variable acidity and addressed key questions concerning patterns of adaptation (trade-offs, specialists, generalists, plasticity, transitions, and acclimation) in temporally fluctuating environments.

Introduction

In our ever-changing world, evolutionary physiologists are interested in understanding how organisms function in variable environments and how populations of organisms adapt evolutionarily to environmental fluctuation. Although there is strong empirical support for the argument that environmental heterogeneity does indeed represent a general explanation for diversity in natural systems, there are comparatively few data on the response of natural selection to environmental variability (Kassen 2002). A common hypothesis is that natural selection within variable environments will create generalists, while specialists will evolve in environments that stay more constant in space and time (e.g., Levins 1968; Huey and Slatkin 1976; Huey and Hertz 1984; Lynch and Gabriel 1987). The use of temporal variation in serial transfer regimes used to propagate bacterial populations in batch culture is analogous to a type of seasonal environment and allows for the empirical study of the environmental conditions promoting the evolution of specialists versus generalists (e.g., Rainey et al. 2000).

Experimental evolution is an effective laboratory technique for examining adaptation to abiotic environments, since it affords rigorous regulation and control of environmental conditions. Many replicate populations can be exposed simultaneously to novel stressful environments, and evolutionary changes can be observed subsequently over a number of generations (Rose et al. 1996; Bennett and Lenski 1999; Bennett 2003). Experimental evolutionary methods have previously been applied to studying adaptation to temperature (e.g., Huey 1991; Huey et al. 1991; Bennett et al. 1992), while here we expand the scope of experimental evolution to include acidity as an environmental variable.

The pH was selected as an experimental variable because of the importance of this environmental stressor to enteric bacteria such as *Escherichia coli*. While many organisms may not experience changing environmental acidity and alkalinity at stressful levels, variation in pH is a biologically significant environmental stress for enteric bacteria. The natural history of these bacteria suggests that they may spend hundreds or thousands of generations in the relatively neutral environment of the colon, punctuated by brief exposures to extreme acidity during passage through the stomach, followed by modest alkalinity in the small intestine during colonization of a new host. With modern sewage handling (or mishandling), enteric bacteria may also experience periods of exposure to the ocean, with a pH near 8.0, before infecting a new host. Thus, over macroevolutionary timescales, *E. coli* have experienced countless iterations of pH fluctuation between acidic and basic environments, and tolerance to high- and low-pH environments as well as to rapid transitions between these conditions is fundamental to the survival of this species.

Known mechanisms of acid and alkaline resistance in *E. coli* were previously described by Hughes et al. (2007). Physiologically, *E. coli* are often considered neutrophiles that grow best

* Corresponding author; e-mail: bhughes@uci.edu.

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at neutral pH, although they can also grow in moderate acid or base. The growth of *E. coli* at pH 5.0 lowers cytoplasmic pH by 0.6 units (Hickey and Hirshfield 1990), which can alter catalytic properties of regulatory enzymes and membrane functions (Somero 1986). Different physiological patterns in growth in the presence of glutamate or arginine reveal that *E. coli* can reverse the electrical membrane potential to make the inside of the cell positively charged, which is the same strategy that is used by various acidophiles to avoid cellular damage through protein denaturing, depurination of DNA, and damage to membranes (Richard and Foster 2004). During alkaline stress, the peptidoglycan layer of gram-negative bacteria, weakened by high pH, may be less capable of preventing the cytoplasmic membrane from bursting in *E. coli* at pH 10 (Mendonca et al. 1994). Although much less is known about alkaline stress resistance, it may involve protein repair of L-isoaspartyl protein carboxyl methyltransferase (Hicks et al. 2005). While *E. coli* may be capable of survival through brief exposure to extreme acidity or alkalinity, it is incapable of growing during such conditions. The pH limits for growth of *E. coli* K-12 are between 5.0 and 9.0 (Zilberstein et al. 1984) and between 4.8 and 8.4 for our ancestral strain of *E. coli* B (A. J. Cullum, unpublished data). In the stationary phase, however, *E. coli* can survive environments below pH 2, that is, more than 1,000 times $[H^+]$ beyond its lower growth limit, while lethal base stress occurs at pH 10, only a single pH unit beyond the upper growth limit. In this evolution experiment, we confined our selective environments within the growth limits of these bacteria, between pH 5.3 and 7.8, providing a significant range of acidic exposures (more than 300 times $[H^+]$) that still permit adequate growth for regeneration in serial dilution culture. This study initiated the first long-term laboratory natural selection experiment on adaptation to variable pH and addressed several questions concerning patterns of adaptation in temporally fluctuating environments.

Temporal variation of pH in this evolutionary system allowed us to address multiple hypotheses relating to evolution in variable environments. The assertion that the breadth of adaptation evolves to match the degree of environmental variation (Via and Lande 1985; Futuyma and Moreno 1988; Scheiner 1993) was examined through assessment of the differential patterns of fitness trade-offs in adaptation to fluctuating versus constant acid environments. Trade-offs are often assumed to be characteristic of niche specialists, while a lack of trade-offs is thought to be a hallmark of niche generalists. It has been shown empirically, however, that the common theoretical expectation of the general necessity of trade-offs is not a universal adjunct of adaptation to temperature (e.g., Bennett et al. 1992; Bennett and Lenski 1993; Mongold et al. 1996; Portner et al. 2006), so an investigation of the presence of trade-offs during adaptation to constant and variable acidity is of interest and provides an additional test of this concept. Evolution of generalists in variable environments is sometimes hypothesized to involve an adaptive cost ("jack-of-all-trades, master of none"; see Huey and Hertz 1984; Joshi and Thompson 1995; Via et al. 1995; Fry 1996; Whitlock 1996; DeWitt et al. 1998). This potential cost was directly addressed in our system by comparing the average fitness of pH specialists with that of groups evolved in environments of variable pH. The hypothesis that evolution in a temporally varying environment increases phenotypic flexibility for making transitions (e.g., Levins 1968, 1969; Feder 1978; Tsuji 1988) was not supported in thermal evolution experiments (e.g., Leroi et al. 1994*b*). This hypothesis was reexamined here in the same bacterial system but with pH as the environmental variable. Finally, in regard to acclimation, numerous thermal studies of beneficial acclimation (Huey and Kingsolver 1989; Rice and Bazzaz 1989; Krebs and Loeschcke 1994; Leroi et al. 1994*a*; Kingsolver 1995; Schmitt et al. 1995; Zamudio et al. 1995; Dudley and Schmitt 1996; Huey and Berrigan 1996; Bennett and Lenski 1997; Woods 1999; Gibert et al. 2001; Gilchrist and Huey 2001; Huey et al. 2002; Garland and Kelly 2006; Terblanche and Chown 2006) have demonstrated a mix of results, indicating a lack of a generality for the benefit of acclimation. The seemingly paradoxical result of Leroi et al. (1994*b*) that acclimation benefit declines during evolution in a variable environment may be less counterintuitive if one considers how bacteria in a fluctuating environment must often compete without the benefit of an acclimation period. Such relentless selection for the ability to transition to new environments without the aid of acclimation might eclipse the adaptive importance of maintaining an ability to benefit from acclimation. We tested the hypothesis that propagation in variable environments actually results in the evolution of acclimation insensitivity when compared with evolution in constant environments. In contrast, beneficial acclimation under historical conditions plays a consistent role in thermal environments in *E. coli* (Bennett and Lenski 1997), and we also examined this feature in pH evolution.

Here we used measurements of relative fitness from four groups, each consisting of six independent lines, with each evolved in one of the following conditions: constant acid (A), constant base (B), randomly fluctuating (R), or cycling (C) pH. After either 1,000 generations of evolution in constant pH or 2,000 generations of evolution in fluctuating pH, these groups were assessed for their evolved performance in four different environments to (1) determine the extent of adaptation of the various groups in each pH regime condition, (2) characterize trade-off patterns associated with the evolution of generalists or specialists in temporally fluctuating and constant pH regimes, (3) compare evolutionary fitness responses in randomly versus regularly cycled pH regimes, (4) assess the extent to which there was adaptation to transitions between the acid and alkaline conditions rather than only adaptation to static pH conditions, and (5) test the benefit of acclimation preceding fitness competitions.

Material and Methods

Bacteria

The bacterial study system in this experiment has been previously described (Bennett et al. 1992; Hughes et al. 2007). Briefly, the ancestral strain of *Escherichia coli* used to found the 24 lines of this study is asexual, prototrophic (but originally incapable of growing on L-arabinose [Ara−]), and absent of plasmids or functional bacteriophages, and it was isolated as a clone from one of 12 populations that were part of an earlier experimental evolutionary study (Lenski et al. 1991). During this earlier study, our ancestral lineage was allowed to evolve for 2,000 generations (300 d) in a standard laboratory environment of pH 7.0 and a temperature of 37°C in a regimen of daily serial dilution of 1 : 100 (0.1 mL in 9.9 mL) in a standard Davis minimal medium (DM; Carlton and Brown 1981). Most of the fitness increase during this laboratory propagation happened within the first 1,000 generations, so it was inferred that after 2,000 generations in this environment our ancestor was already relatively well adapted to the basic culture conditions of the evolutionary regimes, except for the differences in pH levels we would manipulate in this experiment. This assumption was verified in subsequent experiments, in which fitness increased minimally during a subsequent 2,000 generations of continued exposure to this environment (Bennett et al. 1992; Hughes et al. 2007). Two marker states differing in ability (+) to utilize the sugar arabinose allow visual differentiation between two lineages competing directly against each other in the same flask, when they are oppositely marked (Lenski et al. 1991). Hughes et al. (2007) verified the neutrality of this marker gene over the range of acidities used in this study. The two distinct (+, −) ancestral genotypes as well as the lineages we evolved were stored at −80°C and could be revived at any time for use in future experiments.

Culture Conditions

Culture conditions of our evolution experiments were identical to those previously described (Bennett et al. 1992; Hughes et al. 2007), except for the modification of pH in the media. Temperature was maintained at a constant 37°C. Lines were revived from the freezer by initial inoculation into Luria broth, but otherwise propagation was in DM, modified by varying proportions of the mono- and dibasic potassium phosphate present in the buffer system. The DM mixtures used for our experimental regimes had H^+ concentration levels of 5,000 nM [H^+] (pH 5.3), 500 nM [H^+] (pH 6.3), 100 nM [H^+] (pH 7.0), and 15 nM [H^+] (7.8 pH), all ± 0.1 pH units. Although in nonbuffered media at high cell densities bacterial metabolism will shift pH significantly, the low densities and buffered media of our experiments restricted such pH shifts to no more than 0.1 pH unit. Propagation proceeded through daily serial transfer of 0.1 mL of each culture into 9.9 mL of fresh medium, buffered to the appropriate pH levels, and these daily transfers effectively diluted population counts by 100-fold, so that the bacterial population grew by 100-fold (~6.64 generations) to regain its stationary phase density. Competition assays were performed through differential enumeration of colonies grown on TA agar plates, containing triphenyltetrazolium chloride and arabinose, with incubation at 37°C.

Evolving Lines

Six lines were created within each of the four evolutionary pH regime groups for a total of 24 experimental lines. The C group was alternated daily for 300 d (~2,000 generations) between pH 5.3 and 7.8, spending 24 h at 5,000 nM [H^+] (pH 5.3) and the next 24 h at 15 nM [H^+] (pH 7.8) for an abrupt shift of more than 300 times [H^+]. The R group was stochastically changed daily for 300 d (~2,000 generations) among four levels of pH (5.3, 6.3, 7.0, and 7.8). Thus, the C group evolved for a total of 150 d in pH 5.3 and 150 d in pH 7.8, and the R group spent a random amount of time, stochastically approaching a total of ~150 d in acidic conditions (pH 5.3 and 6.3) and ~150 d in neutral or basic conditions (pH 7.0 and 7.8). The A and B groups used in these experiments were each propagated in constant pH conditions for 150 d (~1,000 generations), with pH 5.3 for the A group and pH 7.8 for the B group. We chose to use these 1,000 generation lines for the A and B groups because we wanted to be able to address the question of whether the C and R groups could adapt as quickly (change in fitness per generation) to each pH as the A and B groups could to their respective pH's, despite the potential pleiotropic challenges the C and R groups faced in having to adapt to additional pH environments at the same time. Thus, although the C and R groups have spent more total generations in the general experimental environment than the A and B lines, the generations spent in any given environment (either acid or base) are the same for all groups. (The A and B groups were also allowed to evolve for an additional 1,000 generations in their respective environments, and adaptation of these groups after 2,000 generations has been reported previously [Hughes et al. 2007].) In order to determine whether our choice of the 1,000- versus 2,000-generation samples might have influenced the outcome of the comparisons in this study, we compared the 1,000- and 2,000-generation relative fitness values for each of the two groups in both pH 5.3 and 7.8 constant conditions. In no case did the two generations differ significantly, and so we believe that the analyses reported here would have had similar outcomes regardless of the generation of the A and B groups used.

Half of the lines in each group originated from the Ara− form of the ancestor, which is incapable of arabinose use, and the other half from the arabinose-utilizing Ara+ form isolated as a spontaneous mutation via high-volume TA plating (Lenski et al. 1991); these two forms of the ancestor are otherwise

Table 1: Evolved fitness of groups tested in different pH regimes

Group and Test Regime	Mean ± SE Relative Fitness[a]	*P* (Two Tailed)[b]
C:		
Constant pH 7.8	1.147 ± .022	.001
Constant pH 5.3	1.188 ± .009	<.001
pH 5.3 → 7.8	**1.143 ± .028**	**.004**
pH 7.8 → 5.3	**1.108 ± .013**	**<.001**
pH 5.3 → 7.8 → 5.3	**1.120 ± .008**	**<.001**
R:		
Constant pH 7.8	1.035 ± .021	.156
Constant pH 5.3	1.142 ± .014	<.001
pH 5.3 → 7.8	1.073 ± .030	.060
pH 7.8 → 5.3	1.103 ± .016	.001
pH 5.3 → 7.8 → 5.3	1.088 ± .022	.011
B:		
Constant pH 7.8	**1.010 ± .033**	**.782**
Constant pH 5.3	.809 ± .062	.027
pH 5.3 → 7.8	1.175 ± .033	.003
pH 7.8 → 5.3	.824 ± .065	.042
pH 5.3 → 7.8 → 5.3	.972 ± .040	.518
A:		
Constant pH 7.8	.708 ± .144	.098
Constant pH 5.3	**1.276 ± .030**	**<.001**
pH 5.3 → 7.8	.098 ± .233	.012
pH 7.8 → 5.3	.939 ± .090	.527
pH 5.3 → 7.8 → 5.3	.704 ± .125	.063

Note. Values shown in bold represent the condition(s) in which the group evolved.

[a] Mean ± SE values of fitness relative to the ancestor based on six replicate lines in each experimental group.

[b] Two-tailed probabilities were calculated using the t distribution with $n - 1 = 5$ df; the null hypothesis is that the mean fitness equals 1.

identical and show no fitness differences in DM. Individual lines were identified by the abbreviated group name, a "+" or "−" to identify the marker state, and a replicate number. As an example, the C group is comprised of the C+1, C+2, C+3, C−1, C−2, and C−3 lines. Samples of each evolving population were plated every 100 or 200 generations, and a single colony was randomly selected. These sampled clonal isolates were then grown to a high density in a glycerol-containing suspension medium and frozen at −80°C for storage to allow later revival for experimental analysis. (Mixed population samples containing the entire genetic diversity within a line were also frozen and stored for future experimentation with the full diversity of the population but were not assessed in this study.) Given the initial homogeneity of the lines and the asexuality of the bacterium, the evolutionary responses to our pH regimes must have been the result of mutations that occurred de novo within the lines.

Measurements of Relative Fitness

Using the A, B, C, and R groups isolated after their evolutionary regimes were completed, measurements of relative fitness for each of the 24 evolved lines were made in various pH regimes following the methods of Bennett et al. (1992). Fitness was determined through direct competition between each evolved line and the reciprocally marked (+, −) ancestral genotype in a single flask. Different competitions were conducted in regimes of constant pH 5.3 and constant pH 7.8 and through a pH 5.3 → 7.8 → 5.3 cycle reflecting the evolutionary conditions of the C group. In all of the competitions, the two competing strains were revived from −80°C storage and separately inoculated into a standard nutrient-rich Luria broth culture medium, with a pH of 7.0 and temperature of 37°C, for 24 h of growth. The lines were then each transferred into the ancestral pH 7.0 Davis minimal media for the second day's growth. For the constant regimes of pH 5.3 and 7.8, lines were separately exposed on the third day to the pH environment in which they would eventually compete to provide opportunity for phenotypic acclimation to the test regime before competition. On the fourth day of the constant regime assays, competitions were initiated by a transfer mixing together 50 μL of the competing evolved line and ancestral line into 9.9 mL of the same test pH media and were then incubated under standard conditions for 24 h. A census of initial and final population density of each competitor was taken by plating diluted samples on TA agar, on which the two competitors could be distinguished and enumerated through the differential colony color afforded by the reciprocal neutral marker. The relative fitness of the evolved line was calculated as the ratio of the number of its doublings compared with the doublings of the ancestor during the 100-fold combined population growth of the competition period (Lenski 1988*a*, 1988*b*; Bennett et al. 1990; Lenski et al. 1991). For the cycled competition regimes, the pH of the third-day media was set at 5.3. The fourth-day transfer was made by combining both ancestor and evolved line into pH 7.8 buffered media to begin the competition, with initial counts obtained by immediate plating. On the fifth day, a second census was taken, and the competitors were transferred back into pH 5.3 media, and 24 h later, on the sixth day, a final plating was done to complete the competition measurements. The intermediate plating permitted the assessment of acclimation state on fitness. Six simultaneous replicates were run for each line in each competition regime.

Statistical Analysis

We compared the fitness measurements produced in various conditions between and among both evolved individual lines and groups using conservative approaches to test our a priori hypotheses, and while having examined only a small subset of the many comparisons possible, we have presented the results

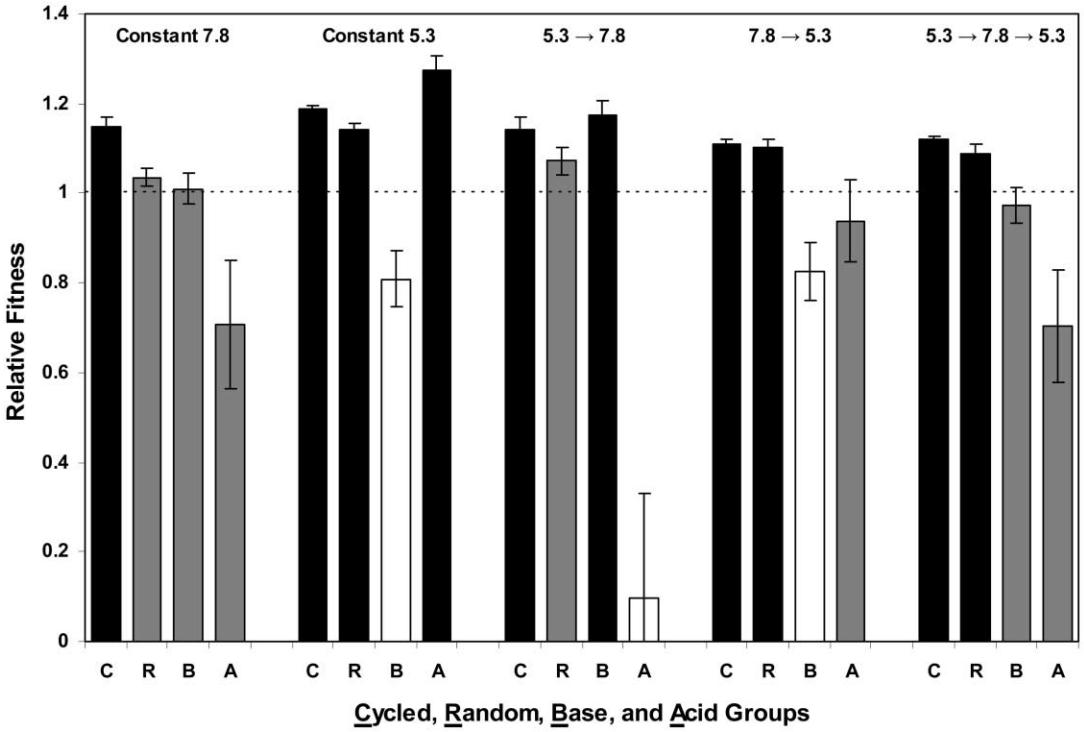

Figure 1. Mean fitness responses (relative to the common ancestor) of the experimentally evolved groups are shown with SE bars. A value of 1.0 (*dashed line*) represents no change relative to the ancestor. Groups with significantly increased relative fitness are shown as black bars, groups with significantly decreased fitness are shown as white bars, and groups with no significant change are shown as gray bars. The C group evolved for 2,000 generations in pH cycled between 5.3 and 7.8; the R group evolved for 2,000 generations in pH fluctuating randomly among 5.3, 6.3, 7.0, and 7.8; the B group evolved for 1,000 generations in a constant basic pH of 7.8; and the A group evolved for 1,000 generations in a constant acidic pH of 5.3. After evolution was completed, each of the four groups was tested under five pH regimes, as designated by the labels above the bars.

of all comparisons made. Significance of evolved relative fitness means were analyzed by t distributions compared with a null hypothesis value of 1, representing fitness equal to the ancestor, and were assessed for significance using two-tailed probabilities and $n - 1 = 5$ df. The statistical inferences for testing our hypotheses were based on the number of independent lines or replications to establish the degrees of freedom, which were $df = n - 1 = 5$, and adjustments to the degrees of freedom were made for unequal variance and comparisons of larger sets of groups. Comparisons of different groups were analyzed to report differences and standard error of the differences using the unpaired two-tailed t-test, with a null hypothesis that the difference is 0, assuming unequal variance and the specific degrees of freedom listed in each table. Probability analysis of transitional adaptation, with standard errors for the means, was compared within groups in different conditions, using t distributions with the null hypothesis of 1 for fitness and 0 for difference in fitness. Acclimation benefit was analyzed using paired comparisons of the same group measured in different conditions with the t-test for matched pairs, with a null hypothesis value of 0 and $df = n - 1 = 5$. The Shapiro-Wilk W-test was employed to detect possible nonnormality of matched pair differences, but no problematic distributions were found. The statistical inferences for testing our hypotheses all used the number of independent lines or replications to establish the degrees of freedom, which were $df = n - 1 = 5$ in all cases.

Results

Patterns of Adaptation

Patterns of adaptation, as measured by relative fitness, are summarized statistically in Table 1 and graphically in Figure 1, with reference to the specific pH regimes of each group's evolution and the conditions under which fitness was assayed. Black bars (Fig. 1) indicate significant fitness gains relative to the common ancestor, while open bars indicate significant fitness losses. Note that the fitness of a group in a particular pH can change drastically depending on the pH the group experienced the previous day, that is, whether the fitness was measured in a constant or transitional regime.

The C group had significantly improved fitness in all regimes, measured in constant pH 5.3 and 7.8, in transitioning pH

Table 2: Cost of plasticity in generalists versus specialists

Comparison	Test Regime	Difference ± SE[a]	df	P[b]	Cost[c]
C − (A and B)[d]	pH 5.3 and 7.8	+.025 ± .047	12.50	.608[A]	No
R − (A and B)[d]	pH 5.3 and 7.8	−.054 ± .046	11.96	.300[A]	No
C − (A and B)[d]	pH 5.3 and 5.3 → 7.8	*−.060 ± .032*	16.00	.077[A]	No
R − (A and B)[d]	pH 5.3 and 5.3 → 7.8	*−.118 ± .030*	15.59	.001[A]	Yes
C − R[e]	pH 5.3 and 7.8	+.078 ± .016	16	.001[B]	…

[a] Difference ± SE between the sample means of the generalist and specialist fitness values.

[b] Two-tailed probabilities for differences were calculated using the *t*-test, assuming equal (superscript B) or unequal (superscript A) variances; $n = 6$ for C or R, and $n = 12$ for the combined A and B. Degrees of freedom as shown.

[c] Indicates whether there is a fitness cost to being a generalist.

[d] Comparisons are between the relative fitness values of the six lines of the generalist group shown (C or R) and the relative fitness values of the 12 lines making up the two specialist groups (A and B). The single value used for each generalist line is the mean of its fitness scores in the two test regimes shown. The value used for each specialist line is the single value measured in constant pH 5.3 (A group), constant pH 7.8, or transitional pH 5.3 → 7.8 (B group), as shown.

[e] Comparison is between the relative fitness values of the six lines of the C group and the six lines of the R group. The single value used for each line is the mean of its fitness scores in the two test regimes shown.

5.3 → 7.8 (fitness measured in pH 7.8 after transfer from pH 5.3) and pH 7.8 → 5.3, and in the overall pH 5.3 → 7.8 → 5.3 transitional cycle (fitness measured over 2 d). Adaptation of the R group produced significant fitness gains only in pH 5.3, pH 7.8 → 5.3, and pH 5.3 → 7.8 → 5.3 but not in constant pH 7.8 or transitional pH 5.3 → 7.8 (Fig. 1, *gray bars*). The B group demonstrated a surprising pattern, with no change in fitness in the constant pH 7.8 conditions but a significant fitness gain in the transition from acid to base, pH 5.3 → 7.8 condition. (Note that the constant base group had not significantly increased fitness in its selection environment of pH 7.8 even after 2,000 generations of selection [Hughes et al. 2007].) Fitness also did not change for the B group in the pH 5.3 → 7.8 → 5.3 cycle. There was significant loss in fitness for the group when tested in both constant acid pH 5.3 and transition from base to acid, pH 7.8 → 5.3. The A evolved group developed a highly significant fitness gain of 28% in constant pH 5.3 but showed no significant gain in fitness when measured in the acidic conditions after transitioning from base, pH 7.8 → 5.3, demonstrating the potential difference between constant and transitional regimes. The A group showed no change in relative fitness in the cycling pH 5.3 → 7.8 → 5.3 conditions. Also, although the A group's 30% loss of fitness in constant pH 7.8 was not statistically significant, because of high variance among the A lines, the A group displayed a significant and very substantial 90% loss of fitness in the transitional pH 5.3 → 7.8 test.

Generalists, Specialists, and Trade-Offs in Evolution to Fluctuating versus Constant pH

Further analysis of the various evolved fitness measurements (Table 1; Fig. 1) revealed interesting patterns involving trade-offs, which are defined here as a significant loss of fitness in one or more pH conditions accompanying a significant gain of fitness in one or more selective pH conditions. These trade-off patterns indicate the evolution of specialists, defined here as a group exhibiting a significant increase in fitness with accompanied trade-offs, or generalists, which are defined here as

Table 3: Comparisons of fitness in C versus R groups

	Means		Relative Fitness		P
Test Regime	C Group	R Group	Difference ± SE[a]	df	(Two Tailed)[b]
Constant pH 7.8	1.147	1.035	.112 ± .031	9.983	.005
Constant pH 5.3	1.188	1.142	.045 ± .017	8.678	.028
pH 5.3 → 7.8	1.143	1.073	.070 ± .041	9.969	.120
pH 7.8 → 5.3	1.108	1.103	.004 ± .020	9.680	.834
pH 5.3 → 7.8 → 5.3	1.120	1.087	.033 ± .024	6.434	.209

[a] Difference ± SE in relative fitness between the experimental groups, based on six replicate lines in each experimental group compared between the two regimes.

[b] Two-tailed probabilities were calculated using *t*-tests, assuming unequal variance, with the null hypothesis that the mean difference equals 0. Degrees of freedom as shown.

Table 4: Constant pH fitness versus transitional fitness of the A group lines

Line	Line Means: Constant pH 7.8 → 7.8 (a)	Constant pH 5.3 → 5.3 (b)	Transition pH 5.3 → 7.8 (c)	Transition pH 7.8 → 5.3 (d)	Transitional Fitness Differential (c + d) − (a + b)
A−1	.781	1.313	−.250	.808	−1.536
A−2	.918	1.271	−.316	.849	−1.656
A−3	.047	1.297	−.240	.704	−.880
A+1	.632	1.383	−.275	.869	−1.420
A+2	1.042	1.206	.805	1.307	−.136
A+3	.828	1.182	.862	1.094	−.054
Group means	**.708**	**1.276**	**.098**	**.938**	**−.947**
SE[a]	.144	.030	.233	.090	.290
P[b]	.097	<.001	.012	.526	.022

Note. Values shown in bold represent the group means. The A group evolved in a regime of constant pH 5.3. Shown for each of the four pH regimes tested is the mean relative fitness of each line, based on six replicates, plus the group mean calculated from the six line means.

[a] SE of the group mean (columns a–d) and SE of the mean difference (right column).

[b] Two-tailed probabilities based on t distributions, with $n - 1 = 5$ df and the null hypothesis that mean fitness equals 1 (columns a–d) or difference equals 0 (right column).

a group exhibiting higher average fitness than the ancestor in at least one regime without significant trade-offs in any of the other regimes tested. By these criteria, the B group consists of specialists, with significant fitness gains from transitioning into alkalinity, pH 5.3 → 7.8, accompanied by significant fitness losses in both constant pH 5.3 and transition into acid, pH 7.8 → 5.3. Likewise, the A group also consists of specialists, with a fitness loss in the transition to alkalinity, pH 5.3 → 7.8. In contrast, the two groups evolved in variable pH regimes, R and C, both exhibited generalist fitness patterns, with neither group having any significant loss in fitness in any of the regimes assayed. Thus, evolution in both of the fluctuating pH regimes produced generalists, while both constant pH regimes produced specialists.

It has been a common assumption that the plasticity of a generalist must come with a cost in performance when directly compared with a specialist in the specialist's native environment. In assessing whether the jack-of-all-trades was actually

Table 5: Constant pH fitness versus transitional fitness of the B group lines

Line	Line Means: Constant pH 7.8 → 7.8 (a)	Constant pH 5.3 → 5.3 (b)	Transition pH 5.3 → 7.8 (c)	Transition pH 7.8 → 5.3 (d)	Transitional Fitness Differential (c + d) − (a + b)
B−1	.971	.951	1.184	.966	.228
B−2	1.144	.778	1.284	.628	−.010
B−3	1.007	.959	1.190	.959	.183
B+1	.974	.819	1.129	.921	.257
B+2	1.056	.543	1.044	.627	.072
B+3	.906	.805	1.217	.844	.350
Group means	**1.010**	**.809**	**1.175**	**.824**	**.179**
SE[a]	.033	.062	.033	.065	.053
P[b]	.782	.027	.003	.042	.020

Note. Values shown in bold represent the group means. The B group evolved in a regime of constant pH 7.8. Shown for each of the four pH regimes tested is the mean relative fitness of each line, based on six replicates, plus the group mean calculated from the six line means.

[a] SE of the group mean (columns a–d) and SE of the mean difference (right column).

[b] Two-tailed probabilities based on t distributions, with $n - 1 = 5$ df and the null hypothesis that mean fitness equals 1 (columns a–d) or difference equals 0 (right column).

Table 6: Constant pH fitness versus transitional fitness of the R group lines

Line	Line Means: Constant pH 7.8 → 7.8 (a)	Constant pH 5.3 → 5.3 (b)	Transition pH 5.3 → 7.8 (c)	Transition pH 7.8 → 5.3 (d)	Transitional Fitness Differential (c + d) − (a + b)
R−1	1.033	1.148	1.048	1.068	−.065
R−2	1.017	1.146	1.068	1.124	.029
R−3	1.028	1.162	1.187	1.164	.161
R+1	.966	1.184	.960	1.067	−.124
R+2	1.042	1.080	1.087	1.080	.046
R+3	1.126	1.134	1.085	1.118	−.058
Group means	**1.035**	**1.142**	**1.073**	**1.103**	**−.002**
SE[a]	.021	.014	.030	.016	.041
P[b]	.156	<.001	.060	<.001	.966

Note. Values shown in bold represent the group means. The R group evolved in a regime of randomly fluctuating pH environments. Shown for each of the four pH regimes tested is the mean relative fitness of each line, based on six replicates, plus the group mean calculated from the six line means.

[a] SE of the group mean (columns a–d) and SE of the mean difference (right column).

[b] Two-tailed probabilities based on t distributions, with $n - 1 = 5$ df and the null hypothesis that mean fitness equals 1 (columns a–d) or difference equals 0 (right column).

a master of none, we measured the cost to being a generalist when competing in the specialists' regimes. The fitness values of the specialist lines, each measured in the regime in which it evolved (i.e., the fitness of the A group in constant pH 5.3 and the B group in constant pH 7.8), were pooled and then compared with the fitness of the generalists, measured in each of the two regimes and then averaged for each line, using a t-test. The C and R groups were each compared with the specialists separately, as shown in the first and second rows of Table 2, and in neither case was there a significant cost to being a generalist.

A slightly different set of values could be used in these comparisons if one considered it important that the B group did not actually evolve improved fitness in the constant 7.8 pH regime in which it evolved but instead showed a fitness gain only in the transitional pH 5.3 → 7.8 regime. We therefore also compared the specialists and generalists for the combination of constant pH 5.3 and transitional pH 5.3 → 7.8 environments,

Table 7: Constant pH fitness versus transitional fitness of the C group lines

Line	Line Means: Constant pH 7.8 → 7.8 (a)	Constant pH 5.3 → 5.3 (b)	Transition pH 5.3 → 7.8 (c)	Transition pH 7.8 → 5.3 (d)	Transitional Fitness Differential (c + d) − (a + b)
C−1	1.208	1.187	1.173	1.089	−.134
C−2	1.177	1.177	1.155	1.120	−.079
C−3	1.066	1.213	1.252	1.056	.030
C+1	1.097	1.148	1.045	1.126	−.073
C+2	1.163	1.196	1.119	1.149	−.093
C+3	1.173	1.204	1.112	1.107	−.160
Group means	**1.147**	**1.188**	**1.143**	**1.108**	**−.084**
SE[a]	.022	.009	.028	.013	.010
P[b]	.001	<.001	.004	<.001	.025

Note. Values shown in bold represent the group means. The C group evolved in a regime cycling between pH 5.3 and 7.8. Shown for each of the four pH regimes tested is the mean relative fitness of each line, based on six replicates, plus the group mean calculated from the six line means.

[a] SE of the group mean (columns a–d) and SE of the mean difference (right column).

[b] Two-tailed probabilities based on t distributions, with $n - 1 = 5$ df and the null hypothesis that mean fitness equals 1 (columns a–d) or difference equals 0 (right column).

Table 8: Adaptation to complete cycled regime in variable versus constant groups

Groups Compared	Means: Variable Groups Tested in pH 5.3 → 7.8 → 5.3	Means: Constant Groups Tested in pH 5.3 → 7.8 → 5.3	Transitional Advantage of Variable Evolution Percentage Change (Difference ± SE)[a]	*P* (Two Tailed)[b]
C − A	1.120	.704	+59 (.417 ± .125)	.020
C − B	1.120	.972	+15 (.148 ± .041)	.013
R − A	1.087	.704	+54 (.384 ± .027)	.027
R − B	1.087	.972	+12 (.115 ± .046)	.036

[a] Percentage difference calculated by dividing variable by constant group relative fitness (and absolute difference with SE of the difference) tested in the same overall regime, based on six replicate lines in each experimental group.

[b] Two-tailed probabilities were calculated using a *t*-test, assuming unequal variance, with the null hypothesis that the mean difference equals 0, and $n_1 + n_2 = 12$ with 5.046 df (first row), 5.347 df (second row), 5.315 df (third row), and 7.795 df (fourth row), when adjusted for unequal variance.

again using the fitness of the A lines in the former and the B lines in the latter as the pooled specialist values, and tested against the fitness of each set of generalist lines averaged for the two environments. As shown in rows three and four of Table 2, the C group still showed no significant cost, but the R group did reveal a significant cost compared with the specialist in this particular condition. Overall, however, these comparisons suggest that the jack-of-all-trades may be a master of at least some as well.

Evolution in Cycled versus Random pH Fluctuation Regimes

Because the selective regimes of the C and R groups did differ, despite both being classified as generalists, we also compared the overall fitness of the two groups directly in a manner similar to the generalist-specialist comparisons above. Again we calculated for each line the mean of its fitness values in the constant pH 5.3 and 7.8 regimes and then compared the six values for each group by a *t*-test. The R group had a significantly greater cost of plasticity, with a relative fitness 0.078 lower than the C group.

The generalist patterns of the C and R groups were also not synonymous in other respects (Table 1; Fig. 1). The R group showed no significant gains under some regimes (i.e., constant pH 7.8 and pH 5.3 → 7.8). However, the C group had significantly higher fitness than the ancestor in every regime tested and therefore could be classified as a superior generalist. The C group fitness was also higher than the R group in every regime tested (Table 3), but only the constant pH 5.3 and 7.8 regimes produced statistically significant differences. At constant pH 7.8, there was a highly significant fitness advantage of 0.011 for the C group over the R group, while at constant pH 5.3, the advantage was only 0.050. In all of the fluctuating pH assays, the cycled and random groups were statistically indistinguishable.

Adaptation to Transitions versus Constant Components in the pH Environment

To assess the extent to which there was adaptation by the C and R lines to the abrupt transitions between acid and alkaline conditions rather than only adaptation to the two pH environments per se, comparisons of relative fitness performance between transitional (cycled) and constant regimes were made for each line in all four groups (Tables 4–7). These comparisons thus allowed us to examine the role that periods of fluctuation themselves play in evolution to changing environments. The transitional fitness differentials reported in the last columns of Tables 4–7 were calculated for each line by subtracting relative fitness performance measurements in the two constant regimes from those measurements in the two transitional regimes. The A group, evolved in constant acid conditions, exhibited a sig-

Table 9: Benefit of acclimation to basic pH for group A

Line	Line Means: Acclimated pH 7.8 → 7.8	Line Means: Nonacclimated pH 5.3 → 7.8	Acclimation Benefit (Difference)
A−1	.781	−.250	1.031
A−2	.918	−.316	1.234
A−3	.047	−.240	.287
A+1	.632	−.275	.907
A+2	1.041	.804	.236
A+3	.828	.862	−.034
Group means	**.708**	**.098**	**.610**
SE[a]			.209
95% CI[b]			.538
P[c]			.033

Note. Values shown in bold represent the group means.

[a] SE of the mean difference.

[b] Confidence interval (CI) for the group mean difference.

[c] Two-tailed probabilities based on *t* distributions, with $n - 1 = 5$ df and the null hypothesis that the mean difference equals 0.

Table 10: Benefit of acclimation to acid pH for group A

Line	Line Means: Acclimated pH 5.3 → 5.3	Line Means: Nonacclimated pH 7.8 → 5.3	Acclimation Benefit (Difference)
A−1	1.313	.808	.505
A−2	1.271	.849	.422
A−3	1.298	.704	.593
A+1	1.383	.869	.514
A+2	1.206	1.307	−.101
A+3	1.182	1.094	.088
Group means	**1.276**	**.939**	**.337**
SE[a]			.113
95% CI[b]			±.292
P[c]			.031

Note. Values shown in bold represent the group means.

[a] SE of the mean difference.

[b] Confidence interval (CI) for the group mean difference.

[c] Two-tailed probabilities based on *t* distributions, with $n - 1 = 5$ df and the null hypothesis that the mean difference equals 0.

nificant and detrimental transitional fitness differential of −0.95, demonstrating a very substantial loss of fitness in transitions compared with its fitness in constant regimes (Table 4). In contrast, the B group, evolved in constant basic conditions, showed a significant +0.18 transitional fitness differential, indicating its substantial gain of fitness in transitions compared with its fitness in constant regimes (Table 5). This marked improvement at transitioning was due almost exclusively to an improvement at transitioning from pH 5.3 to 7.8, while the A group's loss of fitness was more evenly shared between both transitions.

The R group, evolved in a randomly fluctuating pH, exhibited no significant difference between its performance in constant and fluctuating conditions and thus demonstrated no transitional fitness differential (Table 6). However, the C group, evolved in a cycling of the extreme pH values, showed a significantly lower fitness in transitions compared with its fitness in constant conditions (Table 7), with a fitness differential of −0.08. It should be noted, however, that this negative transitional fitness differential in the C group is not due to any loss of fitness in the transitional conditions; in fact, the C group showed significant fitness gains in both transitions, but the gains in the constant regimes were even greater and hence resulted in the negative differential. In the R group, on the other hand, there was no significant adaptation to the constant pH 7.8 regime, and this reduced the differential.

A further analysis of comparative adaptation to overall transitions, shown in Table 8, determined the overall fitness gained in the combined transitions by the groups evolved in fluctuating regimes compared with those evolved in constant pH. This overall transitional advantage was calculated as the difference between groups in their overall performance in the pH 5.3 → 7.8 → 5.3 cycled regime. While this wider view of the adaptation pattern could not discern the more specific factors causing the evolved fitness, it provided characterization of performance in long-term transitioning environments, which we refer to as transitional advantage. Both fluctuating groups evolved substantial and similar performance advantages in the cycled regime when compared with the constant pH evolved lines. The C group had the largest transitional advantage of 59% over the A group and a significant advantage of 15% over the B group. The R group had a significant advantage of 54% over the A group and a significant advantage of 12% over the B group. Evidently, selection in variable environments did in fact produce significant improvements in the overall ability to transition among the combination of environments.

The Beneficial Acclimation Hypothesis

To test the potential benefit of acclimation preceding competition, the relative fitness of each line within each group was compared between conditions having a day of acclimation to the test regime and not having acclimation to the test pH (Tables 9–16). In the test with acclimation, on the day before competition, the pH was the same as it was on the competition day, designated by acid acclimation of pH 5.3 → 5.3 or base acclimation of pH 7.8 → 7.8. The nonacclimated test placed the competitors in a different pH condition during the day before the competition, such as pH 7.8 → 5.3 or pH 5.3 → 7.8. This study found no general statistical support for the universality of benefit from acclimation when examined broadly by combining all of the tested groups, although individual cases of benefit from acclimation were clearly evidenced. Finer-scale analysis of the benefit of acclimation, calculated as the differ-

Table 11: Benefit of acclimation to basic pH for group B

Line	Line Means: Acclimated pH 7.8 → 7.8	Line Means: Nonacclimated pH 5.3 → 7.8	Acclimation Benefit (Difference)
B−1	.971	1.184	−.213
B−2	1.143	1.284	−.140
B−3	1.007	1.190	−.183
B+1	.974	1.129	−.155
B+2	1.056	1.044	.012
B+3	.906	1.217	−.311
Group means	**1.010**	**1.175**	**−.165**
SE[a]			.043
95% CI[b]			±.111
P[c]			.012

Note. Values shown in bold represent the group means.

[a] SE of the mean difference.

[b] Confidence interval (CI) for the group mean difference.

[c] Two-tailed probabilities based on *t* distributions, with $n - 1 = 5$ df and the null hypothesis that the mean difference equals 0.

Table 12: Benefit of acclimation to acid pH for group B

Line	Line Means: Acclimated pH 5.3 → 5.3	Line Means: Nonacclimated pH 7.8 → 5.3	Acclimation Benefit (Difference)
B−1	.951	.966	−.015
B−2	.778	.628	.150
B−3	.959	.959	<.001
B+1	.819	.921	−.102
B+2	.543	.627	−.085
B+3	.805	.844	−.039
Group means	**.809**	**.824**	**−.015**
SE[a]			.037
95% CI[b]			±.094
P[c]			.700

Note. Values shown in bold represent the group means.

[a] SE of the mean difference.

[b] Confidence interval (CI) for the group mean difference.

[c] Two-tailed probabilities based on t distributions, with $n - 1 = 5$ df and the null hypothesis that the mean difference equals 0.

ence of the acclimation minus the nonacclimation fitness responses, revealed interesting patterns among all four groups. The constant acid–evolved group A had a very substantial and significant improvement in relative fitness of 0.61 (Table 9) from acclimation to alkalinity and also a substantial and significant fitness improvement of 0.34 (Table 10) from acclimation to acid. In contrast, the constant base–evolved group B had a significant detriment to fitness of 0.16 (Table 11) from acclimation to alkalinity and no significant benefit from acclimation to acid (Table 12). The random fluctuating pH–evolved group R had no significant improvement due to acclimation in either acid or base (Tables 13, 14). In the cycled pH–evolved group C, there was no significant effect of acclimation to basic conditions (Table 15) but a significant benefit to fitness of 0.08 resulting from acclimation to acid (Table 16). Evidently, the fluctuating selective environments produced little or no acclimation benefit compared with the constant evolutionary conditions.

The hypothesis for expecting a benefit from acclimation to the historical evolutionary regime had mixed support by results for the A group and was unsupported by our B group. The A group, which was historically adapted to pH 5.3, had a large loss to fitness of 0.61 after acclimation at the historical pH (pH 5.3 → 7.8) compared with the nonhistorical acclimation (pH 7.8 → 7.8; Table 9). It also experienced a fitness gain of 0.34 after historical acclimation (pH 5.3 → 5.3) compared with the nonhistorical acclimation (pH 7.8 → 5.3; Table 10). The B group, which was historically adapted to pH 7.8, had a fitness gain of 0.16 following acclimation at the historical pH (pH 7.8 → 7.8) compared with the nonhistorical acclimation (pH 5.3 → 7.8; Table 11), while it had no significant difference between the historical (pH 7.8 → 5.3) and nonhistorical (pH 5.3 → 5.3) acclimation condition (Table 12). While we had no historical test for the random group, with certain caveats, the cycled group could potentially be considered, if it were assumed that the historic environment for the cycled group could appropriately be characterized as the transitional pH 7.8 → 5.3 test, since this test occurred at the end component of a pH 5.3 → 7.8 → 5.3 cycle. If this cycling treatment was an appropriate proxy for cycled history, while the nonhistorical component was pH 5.3 → 5.3 (Table 16), then the C group's significant loss of fitness of 0.08 would also be discordant with the historical benefit hypothesis.

Discussion

Generally, our study showed that (1) distinct patterns of adaptation occurred in cycling pH, randomly fluctuating pH, constant acid, and constant base environments; (2) generalists evolved in variable pH environments, and specialists evolved in constant pH environments, although there were not necessarily costs to the generalist when tested in specialized environments; (3) with variable environments, the cycled group had a higher fitness compared with the randomly fluctuated group; (4) adaptation to transitions occurred in the constant base regime, yet it was substantially reduced in the constant acid regime, and, in comparison to both constant regimes, the variable regimes displayed a significant advantage in adaptation to transitions; and (5) the benefit of acclimation was not universally supported, even when analyzed by historical context. Interpretation of some results are also considered in light of the natural history of *Escherichia coli*, although assertions regarding macroevolutionary implications cannot be conclusive from this laboratory-based microevolution study alone, since

Table 13: Benefit of acclimation to basic pH for group R

Line	Line Means: Acclimated pH 7.8 → 7.8	Line Means: Nonacclimated pH 5.3 → 7.8	Acclimation Benefit (Difference)
R−1	1.033	1.048	−.015
R−2	1.017	1.068	−.051
R−3	1.028	1.187	−.159
R+1	.966	.960	−.006
R+2	1.042	1.087	−.045
R+3	1.126	1.085	.041
Group means	**1.035**	**1.073**	**−.037**
SE[a]			.028
95% CI[b]			±.072
P[c]			.243

Note. Values shown in bold represent the group means.

[a] SE of the mean difference.

[b] Confidence interval (CI) for the group mean difference.

[c] Two-tailed probabilities based on t distributions, with $n - 1 = 5$ df and the null hypothesis that the mean difference equals 0.

Table 14: Benefit of acclimation to acid pH for group R

Line	Line Means: Acclimated pH 5.3 → 5.3	Line Means: Nonacclimated pH 7.8 → 5.3	Acclimation Benefit (Difference)
R−1	1.148	1.068	.080
R−2	1.146	1.124	.022
R−3	1.162	1.164	−.002
R+1	1.184	1.067	.118
R+2	1.080	1.080	.000
R+3	1.134	1.118	.017
Group means	**1.142**	**1.103**	**.039**
SE[a]			.020
95% CI[b]			± .051
P[c]			.107

Note. Values shown in bold represent the group means.

[a] SE of the mean difference.

[b] Confidence interval (CI) for the group mean difference.

[c] Two-tailed probabilities based on t distributions, with $n - 1 = 5$ df and the null hypothesis that the mean difference equals 0.

real-world conditions involve vastly greater environmental complexity and evolutionary time lines.

Patterns of Adaptation

The mere occurrence of fitness increments due to evolutionary adaptation is a fundamental expectation of evolutionary biology and is not particularly interesting in and of itself. What is interesting here is that the complex patterns of adaptation in the various pH regimes were so different among the groups and revealed the first empirical characterization of the intricacies of evolution in response to variable pH. One of the more surprising patterns was the contrast between acid and base adaptation. In acid, the A, C, and R groups each had significant fitness gains, with that of A being greatest, while the generally opposite pattern occurred in base, with only the C group producing any significant fitness gains. Interestingly, even after 300 d (2,000 generations) of constant exposure to alkaline conditions, there was no significant improvement in fitness by the B lines (Hughes et al. 2007), while in this study, only 1,000 generations of exposure to alkaline conditions produced highly significant 15% fitness gains in the C group. Apparently, the cycling pH produced a selective pressure for improvement in constant alkaline environments that the constant environment itself did not provide, which may indicate further complexity or constraints on evolution of the underlying mechanisms of alkaline resistance. Interestingly, the random fluctuation was not adequate selection for such base fitness improvements but only the extreme and regular cycling of the C group.

Generalists, Specialists, and Trade-Offs in Evolution to Fluctuating versus Constant pH

Both the A and B constant pH groups in this study had correlated fitness decrements in nonselection environments, classifying them as specialists on their respective niches of acid and base and suggesting trade-offs in fitness during evolution. Both C and R variable pH groups evolved to be generalists, as in a previous variable thermal evolution experiment (Bennett and Lenski 1993) that showed that the same ancestral line as used here evolved into thermal generalists in a variable thermal environment.

Perhaps of more interest than the mere appearance of generalists was the relative cost of being a jack-of-all-trades generalist versus a specialist, and particularly whether there was necessarily a cost to the generalist genotype when tested in unvarying environments. Our experiments were able to address this question directly. Assuming that the C and R group generalists displayed increased phenotypic plasticity, as evidenced by their significant increases of fitness in the variable test environments, we were able to measure the cost to being a generalist and thereby the cost of plasticity by comparing the average fitness of generalists to that of specialists in the pH regimes in which the specialists evolved. Neither the C nor R group showed significant costs (i.e., reduced fitness) in these constant pH conditions when compared with the specialists. Curiously, the B group did not improve fitness in its evolutionary regime of constant pH 7.8, yet it did improve in the novel regime of pH 5.3 → 7.8. Because of this occurrence of exaptation (i.e., preadaptation), in which the B group evolved increased fitness to a condition (pH 5.3 → 7.8) other than the condition for which it was selected (constant pH 7.8), we also

Table 15: Benefit of acclimation to basic pH for group C

Line	Line Means: Acclimated pH 7.8 → 7.8	Line Means: Nonacclimated pH 5.3 → 7.8	Acclimation Benefit (Difference)
C−1	1.208	1.173	.035
C−2	1.177	1.156	.021
C−3	1.066	1.252	−.187
C+1	1.097	1.046	.051
C+2	1.163	1.119	.045
C+3	1.173	1.112	.061
Group means	**1.147**	**1.143**	**.004**
SE[a]			.039
95% CI[b]			± .099
P[c]			.914

Note. Values shown in bold represent the group means.

[a] SE of the mean difference.

[b] Confidence interval (CI) for the group mean difference.

[c] Two-tailed probabilities based on t distributions, with $n - 1 = 5$ df and the null hypothesis that the mean difference equals 0.

Table 16: Benefit of acclimation to acid pH for group C

Line	Line Means: Acclimated pH 5.3 → 5.3	Line Means: Nonacclimated pH 7.8 → 5.3	Acclimation Benefit (Difference)
C−1	1.188	1.089	.099
C−2	1.178	1.120	.057
C−3	1.213	1.056	.156
C+1	1.148	1.126	.021
C+2	1.196	1.149	.048
C+3	1.204	1.107	.097
Group means	**1.188**	**1.108**	**.080**
SE[a]			.020
95% CI[b]			± .050
P[c]			.010

Note. Values shown in bold represent the group means.

[a] SE of the mean difference.

[b] Confidence interval (CI) for the group mean difference.

[c] Two-tailed probabilities based on t distributions, with $n - 1 = 5$ df and the null hypothesis that the mean difference equals 0.

compared the two generalist groups with the B group in this transitional environment. Although we found a significant cost to being a generalist for the R group under these conditions, we still found no significant cost for the C. The general outcome for these experiments, then, is that a jack-of-all-trades may still be a master of many.

Evolution in Cycled versus Random pH Fluctuation Regimes

The higher fitness gains of the cycled C group, a superior generalist, compared with the random R group, a generalist with fewer significant gains, may support the predictions that plasticity is most favored when selection acts equally strongly across habitats, all habitats are equally regular, and interhabitat variability is high (Scheiner 1993; Garland and Kelly 2006). The R group's regime, which fluctuated stochastically among pH 5.3, 6.3, 7.0, and 7.8, included environments with relatively weaker selection and had lower average environmental variability when compared with the cycled regime, which fluctuated regularly between the two extremes of pH 5.3 and 7.8. However, such a conclusion would have to ignore the distinction between the types of environments that produced significantly different fitness between the C and R groups. Since only the constant pH regimes (pH 5.3 and 7.8) revealed statistically significant differences between the cycled and random groups, while all comparisons in the transitional test regimes (pH 5.3 → 7.8, pH 7.8 → 5.3, pH 5.3 → 7.8 → 5.3) were not significant, it could be inferred that plasticity was not enhanced by the increased regularity and intensity of the cycled regime versus the random one. However, the R group had a significantly greater cost of plasticity than the C group, offering some support for efficiency aspects of the evolved plasticity hypothesis. It may also suggest that this area of theory could be verified further by comparative analysis of adaptation to transitional and constant pH conditions.

Adaptation to Transitions versus Constant Components in the pH Environment

It has been repeatedly argued that temporally variable environments should select for increased phenotypic flexibility (Levins 1968, 1969; Feder 1978; Tsuji 1988), although this question has received very little empirical attention. Typically, environmental heterogeneity or environmental stress does induce phenotypic plasticity (Harshman et al. 1999; Wilson and Franklin 2002; Berrigan and Scheiner 2004; Gabriel 2005). The formal theoretical approaches of quantitative genetic models, optimality models, and gametic models all suggest that adaptive plasticity will evolve with environmental heterogeneity (Scheiner 1993). More specifically, the evolution of plasticity is suspected to depend on the speed of temporal environmental changes, the predictability of the temporal heterogeneity, and the duration of the heterogeneity (Garland and Kelly 2006). The hypothesis that evolution in a temporally varying environment would increase phenotypic flexibility for making transitions was not supported by variable thermal evolution (Leroi et al. 1994*b*), yet when it was reexamined here in the same bacterial system, adapted instead to environmental pH, highly significant support was found. Our investigation used two approaches to measure whether temporally varying environments evolved higher phenotypic plasticity to transitions or whether the adaptation merely improved performance in the constant components. The first approach involved within-group comparisons between constant and transitional fitness to arrive at a transitional adaptation for each group, which was markedly different for each group. Surprisingly, transitional adaptation showed the most changes in the constant evolution groups, with the greatest gain of 0.18 with the B group and the greatest loss of 0.95 in the A group, while the variable R group had no significant change, and the C group had only a modest 0.08 decrement (Tables 4–7). By this measure, the constant base evolution of the B group had the only demonstrated transitional adaptation that was not eclipsed by adaptation to the constant components. The constant acid evolution (A) produced huge reductions in transitional fitness compared with the adaptive gains it produced in the constant component. From this comparison, it could be concluded that differences between transitional and constant evolution were of greater magnitude in evolution in constant environments than in variable environments. However, this approach alone does not include the relative overall transitional adaptation differences between these two types of evolution environments. So, our second approach made a broader among-group comparison of fitness in the combination of transitions between the variable and constant groups' performances in the overall transitional test cycle pH

5.3 → 7.8 → 5.3 to arrive at the transitional advantage of variable evolution. Noting that this level of analysis did not attempt to discern the specific components underlying the fitness, this measurement did reveal that R and C groups had very similar and significant transitional advantages of 54% and 59%, respectively, over the A group and also 12% and 15% advantages, respectively, over the B group. By this analysis, we concluded that the variable groups were better overall at making the combination of transitions than were the constant evolved groups, even if the variable groups made similar or greater gains in the constant components. From this perspective, we also concluded that even in the dramatically different adaptive scenarios of acid and base evolution (seen by the first approach in Tables 4–7), the hypothesis that evolution in a temporally varying environment should increase phenotypic flexibility for making transitions was consistently supported (shown by the second approach in Table 8). That the greater plasticity evolved in variable lines was matched by adaptations to the constant components of the regime may relate to the capacity for the jack-of-all-trades to still be a master of many. It may have evolved by selecting against trade-offs, such that the variable lines simply did not lose as much plasticity as those in constant environments, or it may be related to other aspects of evolution such as acclimation.

The Beneficial Acclimation Hypothesis

The evolutionary study of acclimation using multicellular organisms is often complicated by behavioral effects (Huey 2003) and variant life stages (Terblanche and Chown 2006), while the use of large bacterial populations allows more control of these factors. In addition, acclimation studies often encompass only a small portion of an organism's life span, yet multigenerational exposure to acclimation conditions is desirable (Bennett and Lenski 1997), since acclimation effects may span three generations (Watson and Hoffman 1995). To mediate such limitations with bacteria, a six- to seven-generation span of the acclimation day of our experiment was used, without permitting the introduction of further genetic adaptations that might confound results (Leroi et al. 1994*a*, 1994*b*; Bennett and Lenski 1997).

Physiologists have long assumed that acclimation in an environment would beneficially enhance performance in that same environment (Prosser 1986; Hochachka and Somero 2002), yet tests of a universal beneficial acclimation hypothesis (Leroi et al. 1994*a*) and numerous other studies have discredited the universality of this notion. Consistent with other experimenters' mixed results, this study found substantial heterogeneity of responses to acclimation among the four groups tested. For example, although the A group evolved substantial benefits to both base acclimation and acid acclimation, the constant base–evolved B group had significant detriments from base acclimation. This single negative effect of acclimation demonstrated by the B group, with a base acclimation detriment of 0.16, contradicts the universality of a beneficial acclimation hypothesis.

Perhaps even more interesting was the contrast between the small or insignificant acclimation effects on the variable groups C and R compared with the extensive heterogeneous effects of acclimation on the constant A and B lines. This result supported the counterintuitive hypothesis that temporal variation will evolve reduced sensitivity to acclimation. While the decline of acclimation benefit during evolution in a variable environment was initially surprising (Leroi et al. 1994*b*), perhaps it could be expected, since the bacteria evolved in fluctuating environments must continually compete without acclimation.

In disparity with the strong support for historical effects on beneficial acclimation shown by Bennett and Lenski (1997) in thermal evolution experimentation, our pH evolution study offered little support for historical effects on beneficial acclimation. For example, when thermal evolved lines of *E. coli* are acclimated at their historical evolutionary temperature, they are frequently superior even at other temperatures (Bennett and Lenski 1997), but none of the pH groups produced any such benefits when tested likewise. Actually, only the A group had a significantly positive historical effect on acclimation, which was only positive in the selective pH, while this same historical acclimation produced a highly significant detriment of 0.61 when tested in a different pH environment of 7.8 (Table 9). In resonance with the broad examination of temporally variable pH evolution patterns, this study extended the test of the historical acclimation hypothesis to include the cycling group and found the results to also be incongruent with the hypothesis that historical evolutionary acclimation is beneficial.

Future Directions

We are currently examining the maximum tolerance range of all the groups discussed in this study and in that by Hughes et al. (2007) to determine whether and how upper and lower tolerance limits shift as environmental conditions change. Plans for future studies include the extension of this experimental evolution system applied to environmental questions regarding ways in which *E. coli* may be evolving fitness to survive within the coastal ecosystem or the human host. This work will also include gene expression array analysis comparing natural isolates and laboratory evolved lines of *E. coli*.

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