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EVOLUTIONARY ADAPTATION TO TEMPERATURE. I. FITNESS RESPONSES OF *ESCHERICHIA COLI* TO CHANGES IN ITS THERMAL ENVIRONMENT

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Abstract.—We used bacteria to study experimentally the process of genetic adaptation to environmental temperature. Replicate lines of *Escherichia coli*, founded from a common ancestor, were propagated for 2,000 generations in 4 different thermal regimes as 4 experimental groups: constant 32, 37, or 42°C (thermal specialists), or a daily alternation between 32 and 42°C (32/42°C: thermal generalists). The ancestor had previously been propagated at 37°C for 2,000 generations. Adaptation of the groups to temperature was measured by improvement in fitness relative to the ancestor, as estimated by competition experiments. All four experimental groups showed improved relative fitness in their own thermal environment (direct response of fitness). However, rates of fitness improvement varied greatly among temperature groups. The 42°C group responded most rapidly and extensively, followed by the 32 and 32/42°C groups, whose fitness improvements were indistinguishable. The 37°C group, which experienced the ancestral temperature, had the slowest and least extensive fitness improvement. The correlated fitness responses of each group, again relative to the common ancestor, were measured over the entire experimental range of temperatures. No necessary tradeoff between direct and correlated responses of fitness was apparent: for example, the improved fitness of the 42°C group at 42°C was not accompanied by a loss of fitness at 37°C or 32°C. However, the direct fitness responses were usually greater than the correlated responses, judged both by comparing direct and correlated responses of a single group at different temperatures and by comparing direct and correlated responses of different groups at a single temperature. These comparisons indicate that the observed adaptation was, in fact, largely temperature specific. Also, the fitness responses of the generalist group across a range of temperatures were less variable than those of the thermal specialist groups considered as whole.

Key words.—Bacteria, environment, *Escherichia coli*, evolution, fitness, temperature, thermal adaptation.

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Adaptation of living systems to their environments is central to our understanding of organismal diversity and has been a focal concern of biologists for hundreds of years. Aspects of the adaptive process, such as the rapidity, perfection, and specificity of its adjustments continue to be debated actively (e.g., Williams, 1966; Lewontin, 1978; Taylor and Weibel, 1981; Brandon, 1990). Most analyses of adaptation rely on comparative data, correlating organismal and environmental factors, and inferring pattern (e.g., Dill et al., 1964; Gordon et al., 1982; Prosser, 1986). Such approaches have been criticized for several shortcomings, including their inability to consider and test alterna-

tives to adaptation (e.g., Gould and Lewontin, 1979; Felsenstein, 1985). We believe that analyses of natural patterns, as exemplified by comparative studies, can be valuably supplemented by direct experimental studies on the process of adaptation to environmentally important variables.

Temperature is an environmental factor of great biological significance. The vast majority of organisms are ectothermic, and environmental temperature directly establishes organismal temperature and thereby exerts control on biological rate processes. These processes are typically increased by 7–10% for each increment of one degree Celsius (Prosser, 1973; Cossins and Bowler, 1987; Ingraham, 1987). Vital living processes, such as energy transduction, reproduction, and growth, are all similarly affected by temperature change. As an environmental factor, temperature is vari-

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able both in space and time. Therefore, we expect organisms to acclimate phenotypically to temperature change, and populations to adapt genetically to changing thermal conditions (Holt, 1990). The vast literature on the comparative thermal biology of natural organisms (e.g., Prosser, 1973; Hochachka and Somero, 1984) supports these expectations. Such comparative analyses, however, do not permit an assessment of the rate of thermal adaptation, its specificity, or the magnitude of any improvement in fitness. We believe that only direct experimental studies permit analysis of these crucial aspects of the adaptive process, and such studies are feasible using microorganisms.

Bacteria offer significant advantages for experimental studies of adaptive evolution. They can be maintained under defined culture conditions with enormous population sizes for thousands of generations. They can be frozen so that all stages of the experiment, including the founding ancestors of the evolving lines, can be preserved and compared, thereby permitting direct competition experiments to determine fitness consequences. Experimental lines may be replicated so that rigorous statistical analyses of the adaptive process are possible. Finally, tight control over experimental conditions permits comparison of evolution in constant or variable environments at the discretion of the investigator. For these reasons, we have undertaken experiments to analyze evolutionary adaptation of *Escherichia coli* to different thermal environments. This bacterial species has proved very useful in other experimental evolutionary studies (see Levin and Lenski, 1983; Dykhuizen, 1990a; Dykhuizen and Dean, 1990 for reviews) and was also well-suited to our needs.

Our general experimental design and the major questions addressed by this study are discussed in the next section; a more detailed description of methods is given subsequently. This paper reports changes in fitness observed during 2,000 generations of adaptation to different experimental temperature regimes. Later studies in the series will investigate such topics as thermal niche breadth and functional capacities of the evolved lines. A separate analysis of the fit-

ness responses of the high temperature (42°C) group during the first 400 generations of the experiment has been reported previously (Bennett et al., 1990).

EXPERIMENTAL OVERVIEW

Our experimental lines were founded from a common ancestral bacterium. This ancestor was taken from a line previously propagated for 2,000 generations under defined culture conditions at 37°C. This bacterium was used to found six replicate *lines* in each of four temperature treatment *groups*: constant 32, 37, and 42°C (thermal specialists) and alternation between 32 and 42°C (designated 32/42°C: thermal generalists). All experimental groups were propagated for another 2,000 generations in the same defined conditions, excepting temperature, as the common ancestor had been propagated. The 37°C group thus continued to be subject to the *ancestral* environmental condition, whereas the other groups experienced *novel* environmental conditions. An easily scored marker enabled us to estimate directly the fitness of a derived line relative to the common ancestor during the course of the experiment. We could thereby measure both rate and extent of fitness improvement (that is, adaptation) to different temperature conditions. When fitness of an experimental group relative to the common ancestor was measured under its own experimental thermal regime, we refer to this as a *direct* fitness response. When its relative fitness was measured under any other temperature regime, we refer to this as a *correlated* response (Falconer, 1952, 1981).

The analysis of this experiment was intended to answer the following questions:

1) *Is the direct fitness response positive in all environments?* That is, does adaptation occur under all thermal regimes?

2) *Is the direct fitness response greater under novel environmental conditions than under the ancestral condition?* The population from which the ancestor was taken had previously been maintained at 37°C for 2,000 generations and its mean fitness during that time increased ~35% (Lenski et al., 1991). In a sense, the 37°C group can be considered a control against which adaptation to temperature per se (rather than to other aspects

of the culture conditions) may be judged in the other groups.

3) *Is the direct fitness response similar across all novel environments?* Are the rates of adaptation equal in both warmer (42°C) and cooler (32°C) environments, and in the variable (32/42°C) environment in comparison with the thermally constant environments?

4) *Are correlated fitness responses necessarily negative?* How general are thermal tradeoffs (Huey and Hertz, 1984), whereby adaptation to one temperature entails a loss of fitness at other temperatures?

5) *Are direct fitness responses generally greater than correlated responses?* If so, adaptation may be specific to the temperature regime imposed during experimental evolution.

6) *How do the adaptive responses of generalists compare with those of specialists?* The 32/42°C lines are exposed to a much broader range of temperatures than the other experimental groups. How does this thermal variation affect their evolution?

Although we examine these questions within the context of one particular experiment in which temperature is the key environmental variable, they are in fact universally important for understanding adaptive evolution.

MATERIALS AND METHODS

Bacterial Strains.—The ancestral strain that we use in this study was isolated as a clone from one of 12 populations of *Escherichia coli* B that were maintained in the laboratory as part of an earlier study of evolution (Lenski et al., 1991). Each of the 12 populations from this earlier study had been propagated for 2,000 generations (300 days) at 37°C using the same medium and other culture conditions that we employ in the present study, excepting temperature where indicated. During these 2,000 earlier generations, the mean fitness of these 12 populations had increased by ~35% relative to their common progenitor. The rate of increase in mean fitness declined significantly with time, such that approximately two-thirds of this increase occurred within the first 1,000 generations. Therefore, with respect to the present study, one can infer that our ancestral strain is already well-adapted

to the basic culture conditions under which it has been propagated. Hence, any increase in mean fitness observed for groups maintained under novel temperature regimes is more likely to occur as the result of adaptation to the specific thermal regime, rather than adaptation to the general laboratory culture conditions.

E. coli B has no plasmids or functional bacteriophages, and is strictly asexual. It is also prototrophic. The particular ancestral strain that we isolated for this study cannot utilize the sugar L(+)-arabinose; its phenotype is therefore Ara⁻. A spontaneous Ara⁺ mutant of this ancestral strain was isolated by plating >10⁹ cells on minimal arabinose agar. Ara⁻ and Ara⁺ clones form red and white colonies, respectively, when plated on tetrazolium arabinose (TA) indicator agar (Levin et al., 1977). The Ara marker is effectively neutral under all of the culture regimes employed in this study, as will be shown in the Results section of this paper. The Ara⁻ and Ara⁺ ancestral strains were stored in a glycerol-based suspension at -80°C, so that it is possible to compare them directly with their experimental derivatives at any time.

Culture Conditions.—The culture medium employed in all experiments was Davis minimal broth supplemented with 25 μg ml⁻¹ glucose (Carlton and Brown, 1981; Lenski, 1988a). At 37°C, this glucose concentration supports a stationary phase density of the ancestral strain of ~4 × 10⁷ cells per ml; at 32°C this density is slightly higher, whereas at 42°C it is somewhat lower. Culture volume was 10 ml, and cultures were maintained in 50 ml Erlenmeyer flasks placed in shaking incubators set to 120 rpm. Temperatures of three shaking incubators were set to 32, 37, and 42°C and these were accurate to ±1°C including both temporal and spatial variation within the incubator.

Evolving populations (see below) were propagated daily by transferring 0.1 ml of each culture into 9.9 ml of fresh medium. These transfers were performed quickly at room temperature; the propagated cultures were returned to the appropriate shaking incubator within a few minutes. During each of these daily cycles, a bacterial population must grow ~100-fold to reattain its stationary phase density, requiring log₂ 100 =

6.64 generations of binary fission per day. Failure to undergo this amount of growth would result in rapid extinction of an experimental line.

Bacterial populations were enumerated by spreading diluted cultures onto TA indicator agar plates, which were incubated at 37°C for one day. The plating medium and temperature represent an arbitrary environment in which to enumerate the abundance of some pure or mixed population. Our estimates of relative fitness (see below) are unaffected by any differences in plating efficiency between two competitors.

Evolving Lines.—Twenty-four lines were founded using the two ancestral strains, 12 Ara⁻ and 12 Ara⁺. Each line was founded from a single colony, and hence from a single cell. Consequently, there was no genetic variation either within or between the founding populations, excepting the Ara marker.

Six lines (three Ara⁻ and three Ara⁺) were propagated for 300 days (=2,000 generations), under each of four different temperature regimes (=treatment groups), designated 32, 37, 42, and 32/42°C. The first three groups indicate that the lines were maintained at constant temperatures (within the limits described above), while the regime designated 32/42°C indicates that the lines were alternated daily between 32 and 42°C (~6.64 generations sequentially in each). Other culture conditions were identical for all four groups, as described above.

Evolving lines were plated on TA agar after 100, 200, and every succeeding 200 generations, through 2,000 generations total. For each line, a single colony was chosen randomly. These clonal isolates were re-grown in a glycerol-containing medium, and the resulting suspensions were stored at -80°C. Mixed population samples, which contained the entire genetic diversity within any line, were obtained at less frequent intervals, by adding glycerol to the entire population (less that part used to propagate the culture) and storing the suspension at -80°C. These mixed population samples provided a back-up from which a line could be re-started in the event of a laboratory mishap (see below).

Lines derived from the Ara⁻ and Ara⁺ ancestral strains were physically alternated

during their daily transfer to fresh medium. Thus, any cross-contamination (equivalent to "migration" between lines in a population genetics sense) would produce a readily detected change in the Ara marker state. No serious cross-contamination events occurred during this study, although colonies of inappropriate color were occasionally observed at very low frequency. In no case did cross-contaminants, once detected, appreciably increase in frequency; and none of the clones randomly chosen for further analysis had the inappropriate Ara phenotype. The initial genetic homogeneity of these lines, coupled with their asexuality and the lack of migration between them, indicates that adaptive evolution must result solely from changes in the frequencies of mutants that have appeared de novo within any particular line.

The evolving populations were also monitored for contamination from external sources whenever samples were obtained, using the procedures described in Lenski et al. (1991). Contamination of three lines occurred between 600 and 800 generations. These three lines were subsequently restarted from their corresponding mixed population samples that had been stored after 400 generations, and each was then propagated for a full 2,000 generations. None of the data indicated that the three lines that had been restarted differed from the 21 lines that were maintained continuously. Thus, we retained the full complement of six lines for each of the four experimental groups.

After each daily transfer, the previous day's cultures were placed in a refrigerator at 4°C, where they were held for one day. In the event of a mishap, such as a broken flask, these refrigerated cultures could be used to restart any or all of the lines with the loss of only a single day. On three occasions, the entire set of lines fell behind by one day, while on four other occasions, individual lines lost one day.

Measurements of Fitness in Competition Experiments.—Relative fitnesses of lines were assayed in competition experiments, using the same culture conditions described above, under one of the four specified temperature regimes. In all cases, one of the competitors was Ara⁺ and the other Ara⁻.

The basic procedures for estimating rel-

ative fitness at 32, 37, and 42°C were as follows. The two competing strains were both taken from the -80°C freezer storage, and then conditioned by growing each separately in the standard culture medium for one daily cycle at the temperature regime in which they would compete. Hence, the two competitors had comparable opportunity to acclimate physiologically to the conditions under which they would be competed. The two competitors were then mixed at a 1:1 volumetric ratio and diluted 100-fold into the standard culture medium, where they were allowed to grow and compete during a standard daily growth cycle. Initial and final densities of each competitor were estimated by plating dilutions onto TA agar, on which the two competitors could be distinguished by colony color. Relative fitness was calculated from the ratio of the number of doublings achieved by each of the two competitors during the resulting 100-fold net population growth (Lenski, 1988a, 1988b; Bennett et al., 1990; Lenski et al., 1991). This calculation is unaffected by any difference in the plating efficiencies of the two competitors, because any such difference influences relative abundances in the initial and final samples equally (Lenski, 1991).

These procedures were modified as follows for the variable 32/42°C temperature regime. Two competitors were conditioned separately at 32°C. They were then mixed as above, an initial sample was obtained, and the mixed competition culture was incubated at 42°C for one day. The mixed culture was then transferred by 100-fold dilution into fresh medium, and incubated for a second day at 32°C. At the end of the second day, the final sample was taken. Relative fitness was calculated as before based on the number of doublings achieved during this $100^2 = 10,000$ -fold net population growth. Note that each competitor experiences one sudden transition from 32 to 42°C, one day at 42°C, one sudden transition from 42 to 32°C, and one day at 32°C, which is identical to the cycle experienced by the lines of the 32/42° group.

Experimental Designs and Statistical Analyses.—To test the selective neutrality of the arabinose-utilization marker, 10 replicate competition experiments were per-

formed between the Ara⁻ and Ara⁺ ancestral strains under each of the four temperature regimes.

To examine the *direct response to selection* under each of the four temperature regimes, an experimental line was competed against the ancestor at the temperature regime of the experimental group. A single clone from each line was competed against the ancestral strain possessing the opposite Ara marker after 100, 200, and every succeeding 200 generations, through 2,000 generations. The only replication was provided by the six independently evolving lines in each group. The statistical uncertainty for a particular group at any given time may thus reflect the combined sources of variation due to measurement error, genetic heterogeneity among clones within a line, and divergence between replicate lines.

In addition, for each line, we used least-squares linear regression to estimate the *rate of change in fitness* (=slope) at its experimental temperature; the initial relative fitness (=intercept) was fixed at 1 in these regressions. These rates were then used to test for significant trends in fitness for each group, as well as to compare rates of fitness change among the four groups. We also performed nonlinear regressions to describe more precisely these direct responses; the form of these regressions will be discussed later.

To examine further the specificity of adaptation with respect to temperature, the *correlated responses of fitness* to the thermal regimes of other experimental groups were estimated for each line, using the isolates obtained at 2,000 generations. In particular, we estimated the fitness responses of (i) the 32°C group at 37°C and at 42°C; (ii) the 37°C group at 32°C and at 42°C; (iii) the 42°C group at 32°C and at 37°C; and (iv) the 32/42°C group at 32°C, at 37°C, and at 42°C. Pairwise comparisons between direct responses and corresponding correlated responses are of two distinct types: (1) the direct response of a group is contrasted with a correlated response of that same group at a different temperature; and (2) the direct response of a group is contrasted with the correlated response of another group at the same temperature.

All of our statistical inferences use the

number of independent lines to establish the degrees of freedom for hypothesis testing. In this respect, our inferences are conservative. At times, however, we test a series of related hypotheses that are not orthogonal (i.e., independent of one another) because they use the same experimental data. This situation can lead to spurious claims of significance, especially when either (i) the hypotheses are generated a posteriori, or (ii) a large number of hypotheses are tested, of which only a few are individually significant (Miller, 1981; Rice, 1989). We have sought to limit these potential problems in several ways. First, we have formulated our hypotheses a priori, and we have generally examined hypotheses in which the expected direction of an effect is specified in advance. Second, we have not performed all possible comparisons and contrasts, but instead restricted ourselves to the subset of comparisons that are most interesting to us. For example, we compare certain direct and correlated responses to establish the temperature-specificity of adaptations (e.g., the direct response of the 32°C group versus the correlated response of the 32°C group at 37°C); however, we do not make numerous other possible comparisons of direct and correlated responses that do not bear directly on the issue of the temperature-specificity of adaptations (e.g., the direct response of the 32°C group versus the correlated response of the 37°C group at 42°C). Third, we employ Holm's sequential Bonferroni criterion (Rice, 1989) to compute significance levels whenever the same data are used in multiple comparisons. We report these values in addition to significance levels calculated for each individual test.

RESULTS

Selective Neutrality of the Genetic Marker.—The effect of the arabinose-utilization genetic marker state on the fitness of the common ancestor was examined under all four experimental temperature regimes (Table 1). The marker state (Ara⁺ or Ara⁻) had no significant effect on fitness under any of the temperature regimes. Given the small standard errors of the estimates, any fitness effect of the marker that could have gone undetected is also small relative to the fit-

TABLE 1. Effective neutrality of the arabinose-utilization marker in the ancestral strain under each experimental temperature regime.

Regime	Fitness of Ara ⁺ Relative to Ara ⁻	
	Mean (\pm SE) ¹	Significance ²
32°C	1.006 (\pm 0.013)	0.629
37°C	1.001 (\pm 0.019)	0.961
42°C	0.993 (\pm 0.012)	0.588
32/42°C	0.993 (\pm 0.008)	0.436
Pooled	0.998 (\pm 0.007)	0.818

¹ Mean (and standard error of the mean) based on 10 assays for each experimental temperature regime.

² Two-tailed probability, using the *t*-distribution with $n - 1 = 9$ degrees of freedom ($4n - 1 = 39$ degrees of freedom for the pooled data); the null hypothesis is that the mean fitness equals 1.

ness changes that occurred during experimental evolution, which are described below. Moreover, the evolution experiment was balanced with respect to the marker state, so that any slight fitness effect of the marker in one line would tend to be offset by the opposite effect in another line within the same group. Therefore, the arabinose-utilization genotype can be used as an effectively neutral marker to distinguish the ancestor from experimental lines in competition experiments.

Direct Fitness Responses.—The direct fitness responses for each group are shown in Figure 1. These responses reflect the mean fitness of the lines within an experimental group relative to their common ancestor, under the same temperature regime at which they were propagated. Linear regressions were performed to estimate the average rate of change in fitness for each line during either the first 1,000 generations only or during the entire 2,000 generations. The initial fitness, or intercept, was constrained to equal 1 in these regressions. The means and standard errors of these rates (based on the replicate lines within each group) are summarized in Table 2. At 1,000 generations, statistically significant increments in fitness had occurred in all three novel temperature groups (32, 42, and 32/42°C); however, any fitness increase under the ancestral regime (37°C) was very small and not statistically significant. By 2,000 generations, fitnesses had increased significantly in all four temperature groups.

The fitnesses for each group predicted from the linear model (calculated from the

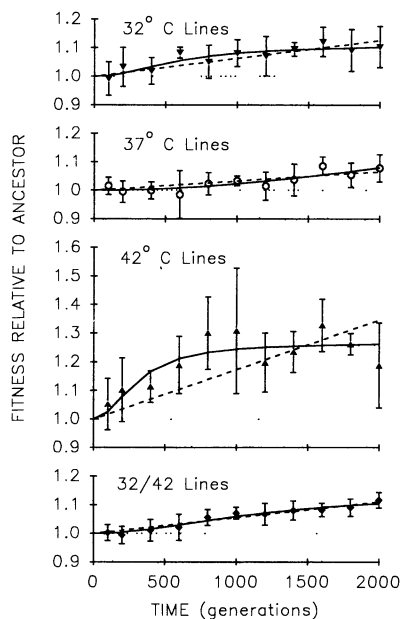


FIG. 1. Direct fitness response of each group over 2,000 generations of experimental evolution at constant 32, 37, or 42°C or alternating 32/42°C. Fitness is expressed relative to the common ancestor, and was assayed for each group under its own experimental temperature regime. Each point is the mean fitness of six replicate lines; the error bars show the 95% confidence interval calculated from the t -distribution with $n - 1 = 5$ degrees of freedom. For each group: The dotted line (\cdots) shows the ancestral fitness (=1). The dashed line ($---$) gives the average of the linear regressions for the six replicate lines, with the intercept constrained to 1 (Table 2). The solid curve ($---$) represents a fit of the mean fitness trajectory to a sigmoidal model (see text for further details).

mean of the slopes for the replicate lines during the entire 2,000 generations) are shown as dashed lines in Figure 1. The linear model does not provide a good description of the fitness trajectory for the 42°C group. A priori, we expect the mean fitness trajectory for a clonal population to increase very little at first, because the genetic variation necessary for any response to selection must arise by mutation and even very favorable alleles will take many generations to reach detectable frequencies. We further expect that, following a period of rapid increase, the fitness trajectory will become progressively more shallow as potentially advantageous mutations are exhausted

TABLE 2. Rates of change in fitness (relative to the common ancestor and assayed at the experimental temperature) for each group. The rate of change was obtained for each bacterial line by least-squares linear regression of fitness against time, with the intercept fixed at 1. Rates are expressed per generation.

Group	Mean (\pm SE) ¹	Significance ²
Rate over first 1,000 generations only		
32°C	$8.35 (\pm 1.49) \times 10^{-5}$	0.001
37°C	$1.96 (\pm 1.10) \times 10^{-5}$	0.068
42°C	$33.18 (\pm 6.01) \times 10^{-5}$	0.001
32/42°C	$5.94 (\pm 1.14) \times 10^{-5}$	0.002
Rate over entire 2,000 generations		
32°C	$6.27 (\pm 0.75) \times 10^{-5}$	<0.001
37°C	$3.25 (\pm 0.46) \times 10^{-5}$	<0.001
42°C	$17.32 (\pm 1.51) \times 10^{-5}$	<0.001
32/42°C	$5.51 (\pm 0.40) \times 10^{-5}$	<0.001

¹ Mean (and standard error of the mean) based on 6 replicate lines for each experimental group.

² One-tailed probability, using the t -distribution with $n - 1 = 5$ degrees of freedom; the null hypothesis is that the mean rate of change equals 0.

(Lenski et al., 1991). In other words, such fitness trajectories may be better described by an equation that generates a sigmoidal response than one that describes a straight line. Perhaps the most familiar equation that describes a sigmoidal response is the logistic, but this equation has the property that the initial slope is proportional to the intercept, which is inappropriate for fitness trajectories. We therefore used instead a sigmoidal equation of the following form:

$$W(t) = W(0) + B_1 t^2 / (B_2 + t^2),$$

where W is fitness, t is time, and B_1 and B_2 are regression coefficients. This equation was fit to the mean fitness trajectory for each group using least-squares nonlinear regression. The initial fitness, $W(0)$, was again constrained to equal 1 in each of these regressions. The predicted regression equations for the four groups are shown as solid lines in Figure 1. To determine whether the sigmoidal model (with two parameters) provided a significantly better fit to the mean fitness values than the linear model (with only one parameter), we calculated partial F -statistics (Kleinbaum and Kupper, 1978) as follows. First, the sum of squares representing the difference between the linear and sigmoidal models was obtained as the difference in the sums of squares explained by

TABLE 3. Pairwise comparisons of the rates of fitness improvement among the experimental groups. Rates were obtained over the first 1,000 generations only or over the entire 2,000 generations of experimental evolution (see Table 2).

Comparison	Mann-Whitney test statistic ¹	Significance ²	
		<i>t</i>	SB
First 1,000 generations only			
Novel versus ancestral			
32°C vs. 37°C	34	0.004	*
42°C vs. 37°C	36	0.001	*
32/42°C vs. 37°C	32	0.013	*
Novel versus novel			
42°C vs. 32°C	36	0.002	*
42°C vs. 32/42°C	36	0.002	*
32°C vs. 32/42°C	27	0.180	
Entire 2,000 generations			
Novel versus ancestral			
32°C vs. 37°C	33	0.008	*
42°C vs. 37°C	36	0.001	*
32/42°C vs. 37°C	32	0.013	*
Novel versus novel			
42°C vs. 32°C	36	0.002	*
42°C vs. 32/42°C	36	0.002	*
32°C vs. 32/42°C	22	0.592	

¹ Based on six replicate lines per experimental group.

² One-tailed probabilities were used for comparing a group propagated under a novel temperature regime (32, 42, or 32/42°C) with the group propagated at the ancestral temperature regime (37°C); two-tailed probabilities were used for comparing any two groups propagated under novel temperature regimes. Probabilities were calculated for each *individual* comparison (*t*) and by applying the *sequential Bonferroni* technique to each set of 3 comparisons that involves the same experimental group (SB). For the latter method, an asterisk (*) indicates that the null hypothesis of no difference in rates is rejected at $P < 0.05$, even after adjusting for the nonorthogonal comparisons.

the respective models. Then, we computed an *F*-ratio using the corresponding difference mean square (with 1 degree of freedom) and the error mean square (with 9 degrees of freedom) derived from the sum of squares unexplained by the full model. For the 42°C group, the sigmoidal model provided a significantly better fit than did the linear model ($F_s = 20.9$, $P < 0.005$). For the three other groups, the sigmoidal model provided a marginally nonsignificant improvement in fit (32°C, $F_s = 4.71$, $0.05 < P < 0.1$; 37°C, $F_s = 4.12$, $0.05 < P < 0.1$; 32/42°C, $F_s = 4.14$, $0.05 < P < 0.1$).

To discern whether the rate of adaptation varied among the groups, we performed pairwise comparisons of the rates of fitness increase that were obtained by linear regression (Table 3). Using the rates obtained

TABLE 4. Direct and correlated fitness responses for each group after 2,000 generations.

Group evolved at	Fitness assayed at	Mean (\pm SE) ¹	Significance ²
Direct responses			
32°C	32°C	1.104 (\pm 0.028)	0.007
37°C	37°C	1.078 (\pm 0.019)	0.004
42°C	42°C	1.188 (\pm 0.058)	0.012
32/42°C	32/42°C	1.116 (\pm 0.011)	<0.001
Correlated responses			
32°C	37°C	1.041 (\pm 0.035)	0.300
32°C	42°C	0.881 (\pm 0.103)	0.300
37°C	32°C	1.002 (\pm 0.015)	0.918
37°C	42°C	0.892 (\pm 0.083)	0.249
42°C	32°C	1.034 (\pm 0.028)	0.281
42°C	37°C	1.030 (\pm 0.023)	0.248
32/42°C	32°C	1.010 (\pm 0.017)	0.581
32/42°C	37°C	1.064 (\pm 0.014)	0.005
32/42°C	42°C	1.021 (\pm 0.023)	0.401

¹ Mean (and standard error of the mean) based on six replicate lines in each experimental group.

² One-tailed probabilities were used for direct responses; two-tailed probabilities were used for correlated responses. All probabilities were calculated using the *t*-distribution with $n - 1 = 5$ degrees of freedom; the null hypothesis is that the mean fitness equals 1.

over the first 1,000 generations, five of the six pairwise comparisons are statistically significant (based on either the individual comparison or the application of the sequential Bonferroni technique to all non-orthogonal comparisons). Despite the pronounced "plateau" in the fitness trajectory for the 42°C group, the same five comparisons are significant when the linearized rates of fitness improvement were calculated over the entire 2,000 generations. Therefore, we can rank the four experimental temperature regimes with respect to the average rate of adaptation of the groups, as follows:

$$42^\circ\text{C} > 32^\circ\text{C} = 32/42^\circ\text{C} > 37^\circ\text{C}.$$

In other words, (i) the rate of further adaptation is slower in the ancestral environment than in any of the novel environments, and (ii) among the novel environments, adaptation is more rapid at the higher temperature than at the lower temperature or in the fluctuating regime.

Correlated Fitness Responses.—A correlated fitness response is here defined as a change in fitness of a line (or group) that has been propagated under one temperature regime when fitness is now assayed under some other temperature regime. Correlated

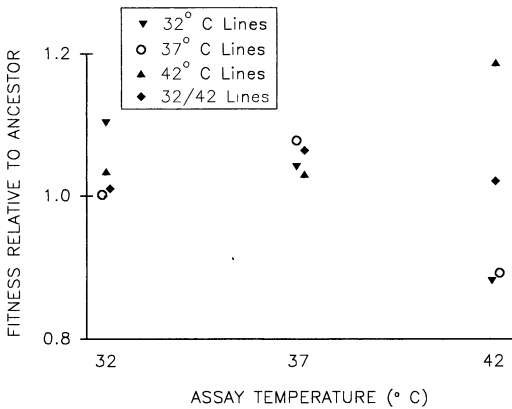


FIG. 2. Direct and correlated fitness responses of the experimental groups after 2,000 generations of evolution at constant 32, 37, or 42°C or alternating 32/42°C. Fitness is expressed relative to the common ancestor, and was assayed for each experimental group at 32, 37, and 42°C. The direct response is given when fitness was assayed at the same temperature as a group had been propagated during the experimental evolution. A correlated response is given when fitness was assayed at any other temperature. Each point is the mean fitness of six replicate lines; statistical analyses of these data are reported in Tables 4 and 5.

responses were measured at 2,000 generations; these correlated responses, along with the direct responses, are summarized statistically in Table 4 and graphically in Figure 2. As indicated above, all four of the direct fitness responses are positive; all four are statistically significant, even using only the 2,000-generation sample point.

Of the nine correlated responses that were measured, seven suggest an increase in fitness (relative to the ancestor), whereas two indicate a decrease in fitness. However, only one of these correlated responses is statistically significant. In particular, the 32/42°C group is more fit at 37°C than is the common ancestor. The large apparent decrements in mean fitness at 42°C for both the 32 and 37°C groups are not statistically significant, owing to the correspondingly large variances in these correlated responses. This variation reflects the fact that some lines in both the 32 and 37°C groups have fitnesses at 42°C that are $\ll 1$, whereas other lines have corresponding fitnesses of ~ 1 . Evidently, there is substantial heterogeneity among replicate lines in certain correlated responses.

The rate of further adaptation to the an-

cestral environment (37°C) was slower than the rate of adaptation under any of the novel thermal regimes (32, 42, and 32/42°C) (Table 3). This result suggests that much of the observed adaptation was temperature specific. However, the tendency towards correlated fitness gains at other temperatures (Table 4) seems to suggest adaptation to aspects of the culture conditions other than thermal regime (e.g., types and concentrations of nutrients in the media). In fact, these contrasting points of view can be reconciled by comparing the relative magnitudes of the direct and correlated responses, as summarized in Table 5. Two types of comparisons are relevant. First, one can compare the direct response of each group with the correlated fitness responses of that *same group* under *other temperature regimes*. Second, one can compare the direct response of each group with the correlated fitness responses of *other groups* under the *same temperature regime*. Using the data reported in Table 4, a total of 18 comparisons (9 of each type) can be made between direct and correlated responses. In all 18 cases, the direct response is greater than the correlated response; in 12 cases, these differences are significant at $P < 0.05$, based on individual significance tests. Many of these comparisons are not orthogonal, however, because each direct response is compared with three or five different correlated responses. The sequential Bonferroni technique was used to correct for this violation of statistical independence. Even with this correction, the difference between direct and correlated responses was significant at $P < 0.05$ in 10 of 18 cases. Evidently, much (but not all) of the observed fitness gains *are* temperature specific.

Comparison of Thermal Generalists and Specialists.—One may hypothesize that the 32/42°C group (having been propagated in a thermally varying environment) should be less variable in its fitness across a range of temperatures than the groups propagated in thermally constant environments (32, 37, and 42°C). To test this hypothesis, we computed for each line its variance in relative fitness, as measured at each of three assay temperatures: 32, 37, and 42°C (Fig. 2). The 6 generalist lines (32/42°C) had significantly smaller “reaction variances” than the 18

TABLE 5. Temperature specificity of adaptation during 2,000 generations of experimental evolution. Two types of statistical comparisons were made between direct and correlated fitness responses: (1) the direct response of a group was compared with a correlated response of that same group under a different thermal regime; and (2) the direct response of a group was compared with the correlated response of a different group under the same temperature regime. For both types of comparisons, temperature specificity of adaptation is indicated if direct responses are greater than corresponding correlated responses.

	Difference (\pm SED) ¹	Significance ²	
		I	SB
A. Direct response of the 32°C lines <i>minus</i>			
1. correlated response of 32°C lines at 37°C ³	0.063 (\pm 0.039)	0.085	
2. correlated response of 32°C lines at 42°C ³	0.223 (\pm 0.099)	0.037	
3. correlated response of 37°C lines at 32°C ⁴	0.102 (\pm 0.032)	0.004	*
4. correlated response of 42°C lines at 32°C ⁴	0.069 (\pm 0.040)	0.056	
5. correlated response of 32/42°C lines at 32°C ⁴	0.093 (\pm 0.033)	0.009	*
B. Direct response of the 37°C lines <i>minus</i>			
1. correlated response of 37°C lines at 32°C ³	0.077 (\pm 0.014)	0.002	*
2. correlated response of 37°C lines at 42°C ³	0.186 (\pm 0.091)	0.048	
3. correlated response of 32°C lines at 37°C ⁴	0.038 (\pm 0.040)	0.184	
4. correlated response of 42°C lines at 37°C ⁴	0.048 (\pm 0.030)	0.069	
5. correlated response of 32/42°C lines at 37°C ⁴	0.014 (\pm 0.023)	0.276	
C. Direct response of the 42°C lines <i>minus</i>			
1. correlated response of 42°C lines at 32°C ³	0.153 (\pm 0.082)	0.060	
2. correlated response of 42°C lines at 37°C ³	0.157 (\pm 0.058)	0.021	*
3. correlated response of 32°C lines at 42°C ⁴	0.307 (\pm 0.118)	0.013	*
4. correlated response of 37°C lines at 42°C ⁴	0.295 (\pm 0.101)	0.008	*
5. correlated response of 32/42°C lines at 42°C ⁴	0.167 (\pm 0.062)	0.012	*
D. Direct response of the 32/42°C lines <i>minus</i>			
1. correlated response of 32/42°C lines at 32°C ³	0.106 (\pm 0.024)	0.003	*
2. correlated response of 32/42°C lines at 37°C ³	0.052 (\pm 0.016)	0.011	*
3. correlated response of 32/42°C lines at 42°C ³	0.095 (\pm 0.022)	0.004	*

¹ Difference (and standard error of the difference) between indicated direct and correlated responses (Table 4).

² One-tailed probabilities were obtained for each *individual* comparison (I) and by applying the *sequential Bonferroni* technique to each set of three or five comparisons that uses the same direct response (SB). For the latter method, an asterisk (*) indicates that the null hypothesis of no difference between direct and correlated responses is rejected at $P < 0.05$, even after adjusting for the nonorthogonal comparisons.

³ Based on *t*-test for paired comparisons, with $n - 1 = 5$ degrees of freedom.

⁴ Based on *t*-test for two sample means, with $n_1 + n_2 - 2 = 10$ degrees of freedom.

specialist lines, taken as whole (Mann-Whitney $U_s = 87$, $0.01 < P < 0.025$, one-tailed). However, when the sequential Bonferroni technique is applied, none of the pairwise comparisons is significant. Thus, while the thermal generalist lines (32/42°C) are indeed less variable in their fitnesses across a range of temperatures than the thermal specialists, taken as a whole, the data do not clearly indicate whether this conclusion holds with respect to each particular group of thermal specialists (32, 37, or 42°C).

DISCUSSION

Temperature is a factor that is simultaneously biologically important, environmentally significant, and experimentally tractable. It has pervasive effects on all biological rate processes and on organismal and population-level traits that reflect those

processes. Temperature varies naturally both spatially and temporally, and comparative studies provide ample evidence that organisms have adapted, at least partially, to temperature differences. But the effects of these adaptations to temperature on fitness itself, the tradeoffs engendered by such adaptations, and the rapidity of the adaptive evolution are virtually unknown. Given the difficulty of observing the process (as opposed to the results) of adaptation by natural selection (Endler, 1986), it is reasonable to attempt to examine these issues by means of experimental evolution in the laboratory (Huey and Kingsolver, 1989; Bennett and Huey, 1990). Our results demonstrate that experimental studies of evolutionary adaptation to temperature are feasible using bacteria. This bacterial system has many advantages, among them the ability to de-

fine and test adaptation, to replicate lines and assays, and to propagate populations of organisms under a variety of different thermal regimes.

We now address each of the questions that we posed in the Experimental Overview:

1) *Fitness relative to the common ancestor increased in all four experimental groups.* Many previous studies with bacteria and other microorganisms have demonstrated increases in fitness of evolving lines, when fitness is measured relative to the ancestor and in the same environment in which the evolution occurred (see Dykhuizen, 1990a, for a recent review). There are, however, some circumstances in which fitness relative to an ancestor may decrease even when assayed in the same environment. Declining fitnesses can occur due to the accumulation of deleterious mutations (Chao, 1990), or as the result of multiple substitutions with nontransitive effects on fitness (Paquin and Adams, 1983). Evidently, such processes were not important in this study, because all four experimental groups had strongly positive direct responses to selection (Fig. 1, Table 2). This study demonstrates that *E. coli* can adapt genetically during propagation under a variety of thermal regimes, and that this adaptation can occur quite rapidly (between one and several months). This system is therefore amenable to evolutionary studies of thermal adaptation.

2) *The rates of increase in fitness were greater under all novel temperature regimes than under the ancestral regime.* This result (Table 3) suggests that much of the adaptation under the novel regimes was temperature specific, because adaptation to aspects of the environment other than thermal regime (e.g., composition of the culture medium) would be expected to be similar in all groups. Temperature specificity does not imply, however, that the observed fitness increases would still be manifest if other aspects of the culture conditions were changed. For example, the genetic changes that enhance growth rate at high temperatures in a minimal-salts medium may or may not enhance growth rate at high temperatures in a nutrient-rich medium.

If a population is maintained indefinitely in a constant environment, then eventually

that population may be depleted of the standing genetic variation that is necessary to sustain adaptation (Fisher, 1930). The depletion of genetic variation due to selection is exacerbated in asexual organisms, because the substitution of a favorable allele at one locus creates a population "bottleneck," which tends to purge genetic variation at other loci (Crow and Kimura, 1965; Kubitschek, 1974; Levin, 1981). To sustain adaptation in an asexual population, genetic variation must be generated *de novo* by mutation. But in a constant environment, the frequency of beneficial mutations and their average fitness effect may become progressively smaller as a population approaches an "adaptive peak" (Lenski et al., 1991). Hence, just as *standing* genetic variation for fitness may be depleted in a constant environment, so the *potential* for further beneficial mutations may be exhausted. If the environment changes, however, then some genetic variants that were neutral or even deleterious in the former environment may be advantageous, thereby promoting adaptation to the novel environment (Service and Rose, 1985). The ancestral strain used in this study was derived from another long-term evolution experiment, which ran for 2,000 generations (Lenski et al., 1991). In that earlier experiment, mean fitness steadily rose, while the frequency of further beneficial mutations (or their average fitness effect) declined with time. These earlier results suggest, therefore, that the potential for further adaptation to the ancestral environment was diminished. Hence, for the present study, we were able to increase the likelihood of adaptation by the bacteria to the novel aspect of their environment—temperature—by keeping all other aspects of their culture conditions unchanged from the earlier study.

Even so, the significant direct response of the 37°C lines (Table 2) indicates that some portion of the fitness gains observed in this study—under both ancestral and novel regimes—may reflect adaptation to aspects of the environment other than temperature.

3) *The rates of fitness improvement were not equal across the novel temperature regimes.* In particular, the 42°C group evolved much faster than did the other experimental groups (Table 3). In fact, a significant fitness

gain in these lines was observed within only 200 generations, about one month (Bennett et al., 1990). The magnitude of the temperature shift from the ancestral state was comparable for the 32 and 42°C regimes, so that an asymmetry exists in the rates of genetic adaptation by these bacteria to increases and decreases in temperature. This asymmetry is an intriguing result, and its generality may be important evolutionarily. Do populations of other organisms, closely or distantly related to *E. coli*, adapt to higher temperatures more rapidly than to colder temperatures? If so, this observation could have implications for the ability of organisms to respond evolutionarily to climatic change. Therefore, further investigation of the generality of this asymmetry may be quite interesting.

The basis of the more rapid genetic adaptation to high temperature is unknown. However, it is *not* due to more generations at higher temperatures, as might result from a general thermal stimulation of all biological reaction rates, including growth. The number of generations per day for each group, regardless of experimental temperature, was externally imposed by dilution and then subsequent growth until nutrient limitation prevented further multiplication. Also, as noted in the Materials and Methods section, population densities actually decreased slightly at the higher temperatures. The rate of mutation (presumably including advantageous as well as neutral and deleterious mutations) may have been affected by temperature. Some earlier studies (Novick and Szilard, 1950; Ryan and Kiritani, 1959) have suggested that rates of certain mutations are elevated at higher temperatures. To the extent that mutation rates are elevated at high temperature, the anticipated ordering of rates of adaptation to constant temperatures would be 42°C > 37°C > 32°C. This phenomenon could have contributed to the observed asymmetry in adaptation to high and low temperature, although it certainly is not sufficient to explain all aspects of our data, since the observed ordering of rates of adaptation to constant temperatures was 42°C > 32°C > 37°C. We plan to explore the effect of temperature on the rates of certain easily identifiable mutations in the future. A qualitatively differ-

ent kind of explanation supposes that, irrespective of the mutation rate per se, there is a broader spectrum of mutations that affect performance at higher temperatures than at lower temperatures. This condition may exist if, for example, the "prehistorical" (i.e., prelaboratory) ancestors of the bacteria used in this study had been more often selected at lower temperatures (e.g., outside the mammalian gut) than at higher temperatures (e.g., in febrile mammals).

4) *The correlated responses of fitness at other temperatures were not usually negative.* We observed no necessary decline in fitness under one temperature regime as a consequence of adaptation to another regime (Table 4). Most correlated fitness responses were not statistically significant, and in fact tended to be slightly positive rather than negative. A few lines propagated at 32 and 37°C apparently did lose fitness at high temperature (42°C), but this response was not general: most lines propagated at 32 and 37°C remained as fit as the ancestor at 42°C. Furthermore, 32°C lines did not lose fitness at 37°C, nor did 37°C lines lose fitness at 32°C, nor did 42°C lines lose fitness at 32 or 37°C. It remains to be determined how the reaction norm of fitness over a broader range of temperatures (i.e., the "thermal niche") has been shifted in these lines as the result of their adaptation under the different regimes.

Negative genetic correlations between fitness components or between measures of fitness in different environments may contribute to the stable maintenance of intraspecific (and interspecific) diversity for ecologically important traits. These genetically determined "tradeoffs" are widely believed to be quite common in nature. It is easily forgotten, however, that the expectation of such tradeoffs is based on the assumption that populations are at or near a selective equilibrium (Service and Rose, 1985; see also Lenski 1988b). We sought to achieve a quasi-equilibrium starting point by using an ancestral strain that had been propagated for 2,000 generations in a constant environment (37°C). However, as indicated above, we observed continuing adaptation even in the control group that was maintained at 37°C (Table 2), albeit at a slower rate than those in novel thermal environ-

ments (Table 3). The tendency towards positive correlated responses (Table 4) may similarly indicate that some of the fitness gains reflect adaptation to aspects of the environment other than temperature. We can use the average direct fitness gain of the 37°C control group ($1.078 - 1 = 0.078$; Table 4) as a plausible index of the extent of nontemperature-specific adaptation in the other experimental groups. Subtracting this index from each of the nine correlated responses in Table 4, we find that all of the resulting values are < 1 . These values suggest that tradeoffs in performance across temperature regimes may indeed exist, provided that these tradeoffs are expressed relative to an evolving control rather than relative to a static ancestor. To examine this issue more fully, we have systematically compared all corresponding direct and correlated fitness responses (Table 5).

5) *The direct fitness responses are consistently greater than the correlated responses.* We made two types of comparisons between direct and correlated fitness responses. In one type, the direct response of a group was compared with a correlated fitness response of that *same group* under a *different temperature regime*. For the other type, the direct response of a group was compared with the correlated fitness response of a *different group* under the *same temperature regime*. In all 18 such comparisons, the direct response was greater than the corresponding correlated response (Table 5). In 10 cases, this difference was significant at $P < 0.05$, even when using a sequential Bonferroni technique to adjust for nonorthogonal comparisons. These results provide the strongest indication that much (but not all) of the adaptation during this evolution experiment was indeed temperature specific.

The admixture of adaptation to temperature and to other aspects of the environment can, in principle, give rise to situations in which certain correlated fitness responses may actually be greater than corresponding direct responses. Consider a population living in a constant environment for a very long time, so that it is near—but not at—evolutionary stasis. Occasional beneficial mutations may arise from time to time in this population, but the fitness advantages are so small that many are lost to drift; and

for those that are not, it takes a very long time for the mutants to reach a frequency at which they have an appreciable effect on mean fitness (Lenski et al., 1991). Now consider that the population becomes divided: one half is subject to the same constant environment, while the other half is subject to an environment that is similar in most respects but differs in one important feature (such as temperature). A mutation that was only slightly beneficial in the ancestral environment may be very advantageous in the novel environment, and may therefore be rapidly swept to fixation there. If the two halves of the population are then brought back together in the ancestral environment, then the half that was selected in the novel environment will prevail in competition. That is, adaptation in the novel environment will have produced a correlated fitness increase in the ancestral environment that is greater than the direct response in the ancestral environment itself. In fact, we observed this situation early in our study (Bennett et al., 1990). By 400 generations, the 42°C group had improved in fitness not only at 42°C, but also—to a lesser extent—at 37°C as well. Yet in this time, no improvement in the group maintained at the ancestral 37°C was observed, so that the correlated fitness response of the 42°C group at 37°C was significantly greater than the direct response of the 37°C group. Other factors may have contributed to this unexpected difference, such as an increase in mutation rate at the higher temperature (Bennett et al., 1990) or frequency-dependent selection (Dykhuizen, 1990b). But in any case, with the passage of a few hundred generations more (this study), a direct response of the 37°C group did occur (Fig. 1), while the correlated response of the 42°C group to 37°C remained positive but small (Fig. 2). As a consequence of this further evolution, the advantage of the 42°C group at 37°C was eliminated (Table 5).

6) *The fitness responses of the thermal generalists are less variable across temperatures than are those of the thermal specialists.* The groups propagated at constant temperatures (32, 37, or 42°C) may be regarded as thermal specialists. That is, these groups have been selected for enhanced performance at only a single temperature, and there has been no direct selection to oppose

loss of performance (due to drift or antagonistic pleiotropy) at other temperatures. By contrast, the lines maintained under the 32/42°C variable regime may be regarded as thermal generalists. These lines have been selected for enhanced performance at two disparate temperatures, but for only half as much time at each; the only assay temperature (among those employed in this study) for which there was no direct selection to maintain performance was intermediate to the extremes. Therefore, one can hypothesize that the 32/42°C group should be less variable in its fitness across this range of temperatures than the other groups. To test this hypothesis, we estimated the “reaction variance” for each line, by calculating its variance in fitness across three assay temperatures (32, 37, and 42°C). The generalist lines have significantly smaller reaction variances than the specialist lines, taken as a whole. However, the data do not clearly indicate whether this conclusion holds with respect to each particular group of thermal specialists. Nor do we know whether the reduced reaction variance of the generalist lines would extend to more extreme temperatures. Nevertheless, these results are consistent with the view that evolution in a thermally varying environment produces an organism that is a “jack-of-all-temperatures” but a “master of none” (see Huey and Hertz, 1984).

The responses of the thermal generalist group are intriguing in other respects, as well. This group exhibited a significant direct response, as indicated by its fitness increase under the variable 32/42°C regime (Fig. 1, Table 2). It also improved in fitness at the midpoint temperature, 37°C, but did not show any significant fitness gain at either of the two component temperatures of its propagation regime, 32 and 42°C (Table 4). Moreover, the adaptation of the generalist group under the variable regime was significantly greater than its adaptation to either component temperature or to the midpoint temperature (Table 5). What is the explanation for these results? When fitnesses were assayed at either 32 or 42°C, the competitors were allowed to acclimate for one day to the assay temperature. By contrast, the group propagated under the 32/42°C regime always experienced a given

temperature on one day having been exposed the previous day to the alternate temperature. This alternation of temperatures is also reflected in the fitness assay procedures for the 32/42°C group, as described in the Materials and Methods section. Therefore, the adaptation of the 32/42°C group may reflect improved responsiveness to the *transient* aspects of the thermal environment, in addition to (or rather than) adaptation to the component temperatures per se. We intend to explore this possibility in our future work. More generally, a population subjected to a temporally varying environment may become better adapted to (i) some or all of the component environmental states, (ii) the “average” environmental state, and/or (iii) the serial correlations among environmental states. The generality and relative importance of these alternative evolutionary solutions to living in temporally varying environments are of considerable interest.

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