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Decay of unused characters by selection and drift

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Abstract

The reduction and loss of redundant phenotypic characters is a common feature of evolution. However, the mechanisms that drive deterioration of unused characters remain unclear. Here, we outline a simple framework where the relative importance of selective and neutral processes varies with environmental factors, because of variation in the fitness costs associated with unused traits. We tested our hypotheses using experimental evolution of the bacterium *Pseudomonas fluorescens* in spatially uniform environments. Results show that an unused character, swimming motility, decayed over evolutionary time and the rate of this decay varied among selection environments with different levels of resource availability. This is explained in the context of an environment-specific genetic correlation between motility and fitness, which is negative when resources are limited but neutral at higher resource levels. Thus, selection against an unused character was most effective in environments where the fitness cost was the greatest. This suggests that the same character can decay by different mechanisms depending upon environmental factors and supports previous evidence to show that resource availability can critically affect the outcomes of evolution.

Introduction

The loss of redundant or disadvantageous characters over evolutionary time is widespread and well documented (Darwin, 1859; Fong *et al.*, 1995; Porter & Crandall, 2003). Familiar examples include the loss of functional eyes in cave-dwelling species (Jeffery, 2005; Romero & Green, 2005) and the evolution of flightlessness in birds and insects (McNab, 1994). The reduction in such unused characters may result in the evolution of ecological specialization (Futuyma & Moreno, 1988). However, the evolutionary mechanisms that drive character reduction remain unclear and the relative contributions of selective and neutral forces are generally ambiguous (Romero & Green, 2005; Maughan *et al.*, 2006).

Two principal mechanisms are proposed for the decay of unused characters that need not act exclusively. The first is selection, or antagonistic pleiotropy, where any previous benefit of a character is outweighed by a fitness cost in the present environment and its decay is adaptive (Cooper & Lenski, 2000). Here, reduction in unused traits is associated with increasing fitness (MacLean & Bell, 2002). The second is neutral mutation accumulation, where the character has no significant effect on fitness and decays due to the spread of mutations that are selectively neutral but have a deleterious effect on the unused character (Haldane, 1933; Fry, 1996; Kawecki *et al.*, 1997). Neutral mutation accumulation is expected to proceed stochastically at a rate determined by the genomic rate of mutation (Kimura, 1983) and independent of the rate of fitness increase. This is true even in an asexual population that is substituting beneficial mutations at other sites, as although this affects the effective population size and the dynamics of a particular neutral mutation, it will not affect the overall rate of neutral substitution, which is independent of effective population size (Kimura, 1983; Cooper *et al.*, 2001). Previous studies have successfully distinguished antagonistic pleiotropy and mutation accumulation in particular sets of conditions (Cooper & Lenski, 2000; Collins & Bell, 2004; Dorken *et al.*, 2004; Maughan *et al.*, 2006). However, the relative importance of selection and drift may often be determined by environmental factors.

Theoretical and experimental evidence show that trade-offs between different traits can vary across

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environments (Stearns, 1992; Perrin & Sibly, 1993; Sgro & Hoffmann, 2004), depending on variation among individuals for resource acquisition and allocation (van Noordwijk & de Jong, 1986). This is partly because the energetic cost of a given trait only translates to a fitness cost in certain environments. When resources are limited, expenditure on one trait reduces the amount that can be allocated to other traits. When resources are abundant, expenditure need not compromise allocation to other traits; so, it is possible to maintain fitness while allocating resources to both adaptive and unused characters. For example, Gebhardt & Stearns (1988) showed that genetic correlations among life-history traits in *Drosophila mercatorum* vary with the level of dietary yeast. In fact, studies from a range of taxa show that trade-offs are more likely to be detected when resources are limited than when they are abundant (Spitze, 1991; Brown, 2003; Blanckenhorn & Heyland, 2004; Ernande *et al.*, 2004). It follows that the fitness cost of an unused character, and therefore the mechanism driving its decay, is likely to depend upon resource supply.

The fitness cost imposed by an unused trait should be greatest in environments where resources are scarce, resulting in selection against it and rapid decay that is linked to increasing fitness. By contrast, the cost of an unused character may be independent of fitness when resources are abundant, so that decay occurs stochastically by mutation accumulation. It has not yet been demonstrated experimentally that the same character can decay by different mechanisms because of variation in environmental factors. Here, we use experimental evolution of a bacterium *Pseudomonas fluorescens* to test the hypothesis that the fitness cost of an unused function depends upon resource availability and in turn that the same trait can decay by different mechanisms in different environments. Microbes are ideal for this work as they are small and reproduce rapidly; hence, evolution can be observed in real time and in controlled laboratory environments (Dykhuizen, 1992; Elena & Lenski, 2003; Jessup *et al.*, 2004). *Pseudomonas fluorescens* in particular adapts rapidly to novel experimental environments and has been used in a wide range of evolutionary studies (Rainey & Travisano, 1998; MacLean & Bell, 2002; Buckling *et al.*, 2003; Brockhurst *et al.*, 2005).

We treat swimming motility in spatially homogeneous environments as an unused character, as we expect the advantage that flagella-driven motility confers in natural environments to be reduced or lost in spatially uniform conditions. Motility is important for *P. fluorescens* in competitive colonization and growth in the rhizosphere (Capdevilla *et al.*, 2004; Martinez-Granero *et al.*, 2006), and may be used in evasion of protozoan predators (Matz & Jurgens, 2005). However, flagella are highly complex organelles composed of several protein species with several more for assembly and regulation (Bardy *et al.*, 2003), and so the advantage of motility is weighed against an energetic cost. We expected the effects of this cost on fitness to vary with the availability of resources, and for this to affect the deterioration of motility during evolution. We tested this with a two-part experiment. First, we conducted a selection experiment where replicate lines evolved in spatially uniform environments at different resource supply rates. Secondly, to determine the fitness cost of motility at different levels of resource availability, we estimated the genetic correlation between motility and fitness, measured as growth rate, at each level of resource supply for a sample of individual genotypes isolated from a single evolved population.

We predicted, first, that motility relative to the ancestral clone would decrease following selection in spatially uniform environments as the fitness advantage conferred by motility would be reduced or lost. Secondly, we predicted that the genetic correlation between motility and fitness would vary with resource availability, as we expect motile individuals to incur a fitness cost when resources are scarce but not when resources are abundant. Thirdly, we predicted that loss of motility would occur more rapidly and to a greater extent in environments with low resource availability as selection against motility will be strongest here, whereas in environments where motility has no significant effect on fitness it should decay stochastically by neutral mutation accumulation. Fourthly, we expected that changes in motility would be coupled with changes in growth rate in environments where motility decayed because of selection.

Materials and methods

Selection experiment

A clonal isolate of *P. fluorescens* SBW25 was used to found all selection lines, so that they were initially isogenic. Storage of the ancestral strain and subsequent samples is in 50% v : v glycerol at −80 °C. Selection was carried out in 28 mL glass universals containing liquid media under shaken conditions at 28 °C. This constitutes a spatially homogeneous environment. Liquid media in each tube comprised 6 mL of M9 salt solution (NH_4Cl 1 g L^{-1}, Na_2HPO_4 6 g L^{-1}, KH_2PO_4 3 g L^{-1}, NaCl 0.5 g L^{-1}) plus an equal concentration (g L^{-1}) of each of four carbon substrates. Carbon substrates were selected that have previously been shown to support growth and adaptation of *P. fluorescens* SBW25 (MacLean *et al.*, 2004; Barrett *et al.*, 2005): acetic acid, glycerol, malic acid and succinic acid. Resource supply rate was manipulated by changing the total concentration of carbon substrates in liquid media at the start of each transfer, so that at different concentrations carbon substrates were supplied at different rates over the course of the experiment. Following pilot work to determine the effect of different concentrations on the growth of the ancestral clone, concentrations were chosen so that growth varied among selection environments and displayed a saturating relationship with resource supply.

Although this also generates considerable variation in population size among treatments, effective population sizes were large enough in all lines (N_e between $\sim 7.2 \times 10^6$ and $\sim 7.2 \times 10^7$, estimated as the harmonic mean of N following Lenski *et al.*, 1991; Hartl & Clark, 1997) to discount significant variation in population genetic processes such as the supply of beneficial mutations or the sampling effects of random drift. Population density was measured following growth over 48 h by optical density (OD) at 600 nm using a Jenway 6300 Spectrophotometer (Jenway, Essex, UK).

Eight replicate selection lines were initiated in each of six selection environments, each with a specific resource supply rate. Resource supply was increased twofold between selection environments from the lowest level, so that lines were selected at the following concentrations: 0.009375, 0.01875, 0.0375, 0.075, 0.15 and 0.3 g L^{-1}. Selection proceeded by transferring 60 μL of each 6-mL culture to new media every 2 days for 50 transfers, as described in Hall & Colegrave (2007). If populations grow to stationary phase between each transfer and then stop, a 100-fold dilution would allow approximately 6.7 generations per transfer. However, Gram-negative bacteria, such as *P. fluorescens*, do not stop growing completely (Zambrano *et al.*, 1993) or evolving (Finkel & Kolter, 1999) once they reach stationary phase and there is some cell turnover without increasing population size (Zambrano & Kolter, 1996). This makes the number of generations per transfer difficult to determine exactly. We estimate that our selection experiment involved a minimum of 350 generations of growth, with the actual number probably closer to 500. Populations were frozen every 10 transfers.

Motility and growth assays

Following selection, motility and growth scores were taken for all selection lines at all time points. Growth scores for each population were recorded in the same liquid media that had been used during selection. Prior to assay, all populations were reconditioned from frozen by growth in King's Medium B (KB: proteose peptone 20 g L^{-1}, glycerol 12 g L^{-1}, K_2HPO_4 1.5 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 1.5 g L^{-1}) for 2 days at 28 °C in shaken conditions. Nine replicate populations of the ancestral clone were also reconditioned. For each population, the same reconditioned culture was used to estimate growth and motility scores. All assays were performed three times.

In preparation for growth assays, each reconditioned population was starved for 2 h by dilution in M9 salt solution. Twenty microlitres of starved cells (approx. 10^5 viable cells) were transferred to individual wells of 96-well microplates containing 180 μL of the appropriate assay media (M9 salt solution plus 0.009375, 0.01875, 0.0375, 0.075, 0.15 or 0.3 g L^{-1} of each carbon substrate depending on the selection line). Microplates were then kept at 28 °C for 48 h. Cell number was then estimated by measuring OD at 600 nm using a SpectraMax M2 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) and subtracting control well scores (sterile media). ODs can be converted to cellular densities (CFU mL^{-1}) as follows: CFU mL^{-1} = $(1.997 \times 10^9$ OD) − 4.705×10^6. Thus, cell density ranged from approximately 4.0×10^7 to 4.4×10^8 CFU mL^{-1} among our experimental treatments. Ideally, growth rate would be measured under the same conditions as selection (i.e. in 28 mL glass tubes over 48 h). However, growth measured as described here strongly correlates with growth rate under the conditions of selection ($r = 0.93$, $N = 40$, $P < 0.001$), and allows a greater level of replication.

Motility was measured by swimming ability on soft agar plates containing 25 mL of M9 salts plus 0.15 g L^{-1} of each carbon substrate and 3 g L^{-1} agar. The same concentration of carbon sources was used for all motility assays to avoid motility measures being confounded by nutrient concentration. Five microlitres of reconditioned culture was spotted onto the plate and the diameter of swimming haloes was measured with callipers after incubation at 28 °C for 24 h. Pilot work showed that the duration of assays did not qualitatively affect results as scores after 24 and 40 h were strongly correlated ($r = 0.86$, $N = 54$, $P < 0.0001$) and that replicate measures for the same culture were highly repeatable (intraclass correlation coefficient between 0.9 and 0.98).

Estimating genetic correlations in different environments

To determine the effect of resource supply on the genetic correlation between growth and motility, we compared the regression of growth on motility at different resource concentrations for a sample of genotypes from a single evolved population. A single population was used so that variation among individuals for growth and motility was not confounded by differences between populations. We chose a population that had been selected at 0.3 g L^{-1} as this was where we expected variation in motility within populations to be the highest. Genotypes were isolated by first reconditioning the population overnight in KB at 28 °C in shaken conditions; then, the culture was diluted and spread onto a KB agar plate. Eighty colonies were then picked off at random, constituting distinct genotypes, and grown for 2 days as above in preparation for growth and motility assays. A single motility score was estimated for each genotype as well as growth at each of the six resource supply rates.

Statistical analysis

To test for changes in motility and growth after selection at different resource supply rates, mean motility and growth scores for evolved lines were compared with those for the ancestral clone by student's *t*-tests, with significance levels adjusted by sequential Bonferroni correction,

performed in JMP 5.1 (SAS Institute, Cary, NC, USA). Then, variation in the change in motility among selection environments was tested by a one-way ANOVA.

To test for variation in the fitness cost of motility among environments, we ran models testing for an interaction of motility with resource supply rate in determining the growth rates of a sample of genotypes isolated from a single population. As growth scores at different resource supply rates were repeated measures on the same genotypes, we used a repeated measures design in the MANOVA function of JMP. Motility scores were transformed to natural logarithms to account for a non-normal distribution and degrees of freedom were adjusted for within-subject factors (resource supply and the interaction with motility) using a Greenhouse–Geisser estimate of epsilon (ϵ) as an index of sphericity. To estimate the genetic correlation at each level of resource supply, linear regressions of growth by motility were fitted using the same parameters as in the overall model, with significance levels adjusted by sequential Bonferroni correction to account for family-wise type I error. We found some extreme values in the distribution of motility scores, which we accounted for by testing the leverage and influence of all data points. Although some points have high leverage scores [above $2(p/N)$ where p is the number of parameters and N is the sample size], they do not have high influence (measured using Cook's D) and plots of residuals confirmed that they did not bias or deviate from fitted models.

Finally, to test whether changes in motility occurred stochastically over time or were coupled with changes in growth rate, we compared the predictive power of models including either transfer number or growth score to determine which was more closely linked to changes in motility over the course of selection. Partly nested linear mixed models included motility and growth scores taken at 10-transfer (~100 generation) intervals for lines selected at different resource supply rates. Motility score was taken as the response variable, selection environment and transfer number as fixed factors, selection line nested within selection environment as a random factor (accounting for repeated measures on the same selection lines), and growth score as a covariate. Each model also included the interaction of either growth or transfer number with selection environment to allow for variation in the mechanism of decay with resource supply rate. Motility scores were Box–Cox transformed to account for a non-normal distribution and degrees of freedom for within subject factors were adjusted for nonsphericity.

Results

Changes in motility and growth over the course of selection

Following selection, growth increased in all treatments, whereas motility generally declined (Fig. 1; Table 1). Our experimental manipulation of the concentration of carbon substrates led to variation in population density among selection environments ($F_{5,12} = 79.57$, $P < 0.0001$; Fig. 1b at $t = 0$). The reduction in motility at the end of selection also varied considerably among selection environments ($F_{5,38} = 17.92$, $P < 0.0001$; Fig. 1a at $t = 50$), with the greatest response to selection at low resource supply rates and little or none at higher levels. The largest increases in growth rate occurred within the first 100 generations in all selection environments but one (0.3 g L^{-1}) and the response to selection for growth rate at the end of the experiment varied with resource concentration ($F_{5,42} = 29.85$, $P < 0.0001$), peaking at intermediate levels.

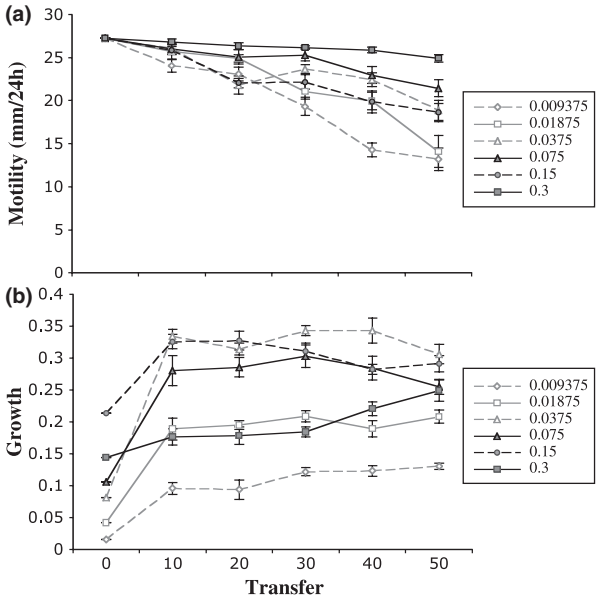

Fig. 1 (a) Motility and (b) growth scores over the course of selection in different environments (shown at right as resource supply rate in g L^{-1}). Scores at transfer 0 show growth of the ancestral clone, measured over a single 2-day transfer, in different selection environments. Points show mean ± 1 SE.

The genetic correlation between motility and fitness varies with resource supply

The relationship between motility and fitness depends upon resource availability, which was detected by a significant interaction between resource supply rate and motility in explaining the growth rate of a large sample of independently isolated genotypes from a single population (repeated measures ANOVA: $F_{2.86,217.53} = 25.42$, $P < 0.0001$). Specifically, high motility incurs reduced fitness at low resource supply, but is independent of fitness when resources are more abundant. This was shown by variation in the slope (b) of growth against motility among environments with different resource

Table 1 Student's *t*-tests of growth and motility scores of evolved populations compared with the ancestral clone.

	Motility			Growth		
Resource supply (g L^{-1})	*t*	*N*	*P*	*t*	*N*	*P*
0.009375	−10.97	4	0.002*	22.64	8	< 0.0001*
0.01875	−7.95	8	< 0.0001*	15.76	8	< 0.0001*
0.0375	−7.58	8	0.0001*	14.87	8	< 0.0001*
0.075	−7.15	8	0.0002*	12.49	8	< 0.0001*
0.15	−6.49	8	0.0003*	6.17	8	0.0005*
0.3	−1.81	8	0.11	6.17	8	0.0005*

Asterisks indicate significance after sequential Bonferroni correction.

Table 2 Regression analysis of growth against motility at different resource supply rates.

Resource supply (g L^{-1})	r^2 (N = 78)	*b*	$F_{1,76}$	*P*
0.009375	0.215	−0.043	20.79	< 0.0001*
0.01875	0.202	−0.072	19.21	< 0.0001*
0.0375	0.082	−0.052	6.79	0.011*
0.075	0.013	−0.020	0.98	0.326
0.15	0.071	0.046	5.77	0.019
0.3	0.0008	0.004	0.06	0.801

Asterisks indicate significance after sequential Bonferroni correction.

supply rates, which is negative at low resource supply but neutral at higher levels (Table 2; Fig. 2). Thus, high motility was associated with reduced growth rate in environments where we detected the greatest deterioration over the selection experiment.

Loss of motility over time and with changes in fitness

If changes in motility were associated with increasing fitness, we would expect growth score to have a significant predictive effect on motility scores in a linear model including data from each time point. By contrast, if changes in motility were because of the stochastic effects of genetic drift, they would occur at an approximately constant rate over the course of selection and the number of elapsed generations would be more informative than changes in growth rate.

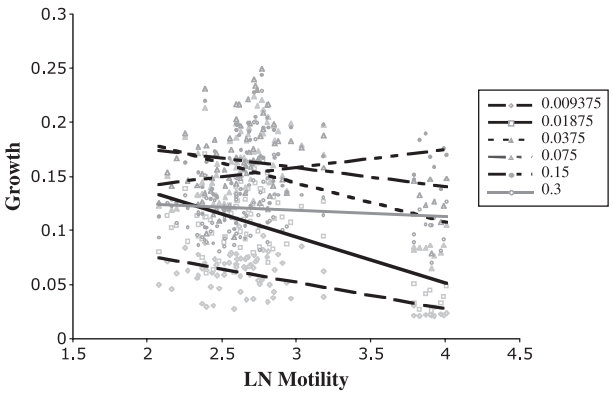

Fig. 2 Genetic correlation between growth and motility at different resource supply rates. Data show growth scores at each resource supply rate plotted against a single motility score for each of 78 independent genotypes isolated from a single evolved population. Further details are given in Table 2.

Changes in motility during our experiment were better explained by the number of elapsed generations than by changes in growth rate, as a model including transfer number was considerably better at explaining the loss of motility ($r^2 = 0.83$, $P < 0.0001$) than one including growth scores at each time point and excluding transfer number ($r^2 = 0.46$, $P < 0.0001$). This was also true in all selection environments when they were tested separately. The inclusion of a quadratic term (growth × growth) to test for a polynomial regression of motility by growth did not significantly increase the predictive power of the model ($r^2 = 0.49$, $P < 0.0001$). Thus, changes in motility and growth occurred over different timescales. Furthermore, the rate at which motility decreased varied among selection environments, shown by a significant interaction between selection environment and transfer number ($F_{12.38,86.63} = 5.68$, $P < 0.0001$) and illustrated in Fig. 1a by the nonparallel slopes of motility over time at different resource supply rates. Therefore, changes in motility were approximately linear over time, although changes in fitness were not, and the rate of decay varied among treatments.

Discussion

Swimming motility deteriorated over evolutionary time in spatially uniform conditions, as expected of a trait with

little or no function in the prevailing environment. Furthermore, we found that the rate and extent of this deterioration varied among selection environments, with the greatest deterioration in environments with low resource availability. We suggest that this is because the relative effects of selective and neutral forces on an unused character depend upon the availability of resources. Specifically, at low resource supply, motility has a negative relationship with fitness, resulting in selection against it and rapid decay over evolutionary time, whereas at high resource supply motility is selectively neutral and decays due to the stochastic spread of mutations that have deleterious effects on motility. Overall, our results support the notion that the importance of trade-offs varies with environmental factors, and that this can determine whether and how quickly phenotypic traits are lost or maintained.

The most compelling evidence that motility is under selection in some environments and not others is that it has a fitness cost at low resource supply but is independent of fitness at higher levels. This interpretation is supported by the relatively rapid deterioration in environments where we expected selection against motility to be the strongest. In contrast to previous studies (Cooper & Lenski, 2000; Maughan *et al.*, 2006), we found that the effects of selection against an unused character were not coupled to changes in fitness over time. These differences in timescale may be explained by variation in the rate at which different mutations are fixed depending upon their relative contributions to fitness. During adaptation to a novel environment, mutations of large effect tend to be fixed early on (Orr, 1998, 2005), so that fitness increases rapidly during the early stages of selection. In our experiment, the largest increases in fitness did indeed occur early on, which is concurrent with previous findings for bacteria in general (Elena & Lenski, 2003) and *P. fluorescens* in particular (Barrett & Bell, 2006; Barrett *et al.*, 2006). However, mutations that affect motility presumably have a smaller effect on fitness than those that increase growth rate directly, for instance, by enhancing metabolism of different carbon substrates. Thus, deterioration of motility was approximately linear over the course of our experiment, whereas fitness clearly increased at an inconsistent rate.

The different timescales of increasing fitness and decreasing motility suggest that the deterioration of unused characters occurs more slowly than adaptation to the experimental environment. It follows that changes in the rate of deterioration might occur over thousands as opposed to hundreds of generations. For example, Cooper & Lenski (2000) identified nonlinear decay of total catabolic function in *Escherichia coli* over 20 000 generations of evolution. This demonstrates that phenotypic evolution continues long after the initial stages of rapid adaptation in microbial selection experiments. The relative rates of adaptation and decay of unused functions might also be influenced by factors that arise over the course of evolution, such as changes in mutation rate, covariance with other phenotypic characters, or compensatory mutations at other loci.

One alternative explanation for the observed pattern is the evolution of mutator genotypes in some treatments. The evolution of higher mutation rates could accelerate neutral mutation accumulation, speeding up the decay of an unused trait. The evolution of mutator strains has been observed in other experimental evolution studies with similar population sizes to those used here (Sniegowski *et al.*, 1997). However, to explain the patterns observed here would require a negative relationship between the frequency of mutators and resource supply rate. Although mutators are expected to spread relatively rapidly in poorly adapted populations (Taddei *et al.*, 1997; Giraud *et al.*, 2001), the low growth rate in our low-resource treatments does not necessarily reflect a lower level of adaptation to the available carbon substrates. Indeed, the response to selection for fitness at the end of the experiment was greatest at intermediate resource supply rates. This suggests that low-resource treatments may not impose significantly novel selection pressures relative to intermediate and high levels when the available carbon substrates are the same in all treatments. Additionally, the evolution of mutators cannot explain variation in the genetic correlation between motility and growth across environments, as these were estimated for the same sample of genotypes isolated from a single population and any variation in slope is therefore because of environmental and not genetic effects. Unfortunately, we cannot rule out the appearance of mutators in some lines, but we do not expect this to explain the parallel phenotypic deterioration in replicate selection lines at a low resource supply rates.

The fitness effects of an unused trait could also be modified by covariance with other phenotypic characters during short-term responses to environmental variation. Environmental factors can affect the growth rate, cell size and swimming motility of microbes concurrently (Matz & Jurgens, 2005). In fact, there is evidence from *E. coli* that motility can increase over the short term in low-quality environments as part of a broad transcriptional programme that is interpreted as an increase in foraging effort in stressful conditions (Liu *et al.*, 2005). Therefore, selection against motility in our experiment might have been amplified by such a response at low resource concentrations. We accounted for this possibility by making observations of the ancestral clone at different resource concentrations under the microscope. We found no discernible differences among cultures other than variation in cell density, providing further evidence that low resource environments did not present unusually stressful conditions here. The discrepancy between this result and that of Liu *et al.* (2005) probably stems from the fact that we used the same carbon substrates in all environments, whereas they detected variation among

environments containing carbon substrates of varying quality.

Phenotypic deterioration may be particularly relevant for complex traits like motility as there are several different mutations that can result in loss of function. By contrast, the fitness costs associated with simple traits that can only be knocked out by one particular mutation may be ameliorated by compensatory mutations at other loci that decrease the cost without affecting the redundant character's function (Cohan *et al.*, 1994; Levin *et al.*, 2000; Maisnier-Patin & Andersson, 2004). For example, microbial resistance to antibiotics can be maintained in the short term even in the absence of antibiotics if the fitness cost associated with resistance is reduced (Schrag *et al.*, 1997). In other words, compensatory adaptation should be more important when the frequency of loss-of-function mutations is low relative to that of compensatory mutations. Thus, the cost of motility in our experiment was reduced by mutations affecting motility directly and not by compensatory mutations at other loci.

In summary, our results support existing evidence that the importance of trade-offs varies with resource availability (van Noordwijk & de Jong, 1986; Sgro & Hoffmann, 2004). Furthermore, we show how this can determine the strength of selection against redundant phenotypic characters. Although the deterioration of unused traits also depends upon a number of genetic factors, the debate over when and why different mechanisms lead to decay might only be resolved by considering how the fitness costs of unused characters vary with environmental factors.

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