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Spontaneous Mutational Correlations for Life-History, Morphological and Behavioral Characters in *Caenorhabditis elegans*

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ABSTRACT

The pattern of mutational covariance among traits plays a central, but largely untested, role in many theories in evolutionary genetics. Here we estimate the pattern of phenotypic, environmental, and mutational correlations for a set of life-history, behavioral, and morphological traits using 67 self-fertilizing lines of *Caenorhabditis elegans*, each having independently experienced an average of 370 generations of spontaneous mutation accumulation. Bivariate relationships of mutational effects indicate the existence of extensive pleiotropy. We find that mutations may tend to produce manifold effects on suites of functionally related traits; however, our data do not support the idea of completely parcelated pleiotropy, in which functional units are separately affected by mutations. Positive net phenotypic and mutational correlations are common for life-history traits, with environmental correlations being comparatively smaller and of the same sign for most pairs of traits. Observed mutational correlations are shown to be higher than those produced by the chance accumulation of nonpleiotropic mutations in the same lines.

GENETIC associations between characters are of special importance in evolutionary biology because, depending on their sign and magnitude and on features of the adaptive landscape, they can either facilitate or hinder the integrated evolution of the traits involved (Lande 1981; Via and Lande 1985; Houle 1991; Arnold 1992; Partridge and Barton 1993; Björklund 1996; Raff 1996, Chap. 9; Schluter 1996; Crespi 2000; Etterson and Shaw 2001; Roff 2002; Phillips and McGuigan 2005). The additive genetic covariance among traits is an essential element in many evolutionary genetic theories, including those concerning the amount of standing genetic variation and covariation that can be maintained in populations (*e.g.*, Lande 1975, 1980, 1984; Turelli 1985, 1988; Keightley and Hill 1990; Houle 1991; Charlesworth and Hughes 2000; Zhang and Hill 2003) and the direction and speed of multivariate divergence in response to selection or genetic drift (Lande 1979). Hence, considerable effort has gone toward identifying and measuring genetic correlations in both laboratory and natural populations (*e.g.*, Arnold 1981; Riska *et al.* 1989; Roff 2000). Although the tremendous growth in evolutionary quantitative genetics over the last two decades has provided a large number of estimates of genetic covariances, we still know very little about their underlying causes, especially from the standpoint of deciphering the forces that influence the long-term evolution of genetic covariance structure (Lande 1980; Cheverud 1984; Steppan *et al.* 2002). While there have been studies of the effects of selection (Wilkinson *et al.* 1990; Shaw *et al.* 1995) and genetic drift (Phillips *et al.* 2001; Whitlock *et al.* 2002), there is virtually no empirical information on what may turn out to be the most important determinant of genetic covariances, the pattern of pleiotropic mutation (Jones *et al.* 2003).

Due to the complexities and interrelatedness of biochemical pathways underlying complex trait development, pleiotropy—the manifold effects of a single gene or set of genes on traits—is almost certain to be a ubiquitous feature of genetic systems (*e.g.*, Wright 1968, 1980; Lande 1980) and is thought to be required for long-term genetic constraints. For an accurate null model against which to compare and explore the causes of natural patterns of genetic covariation, the contribution of new mutations to the genetic covariance structure (*i.e.*, the rate of input of new genetic covariance and the pattern of its effects) must be taken into account (Lande 1980). This issue has been generally neglected in studies of multivariate evolution. For example, models treating the long-term response of populations to selection assume that patterns of genetic variation and covariation (summarized by the matrix **G**) remain fairly constant or change only in a proportional manner through time (Lande 1975, 1979; Arnold *et al.* 2001). Yet the pleiotropic input of mutation would be expected to alter the structure of **G** unless mutation tends to precisely recreate the patterns of covariance maintained

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by selection (*e.g.*, Deng *et al.* 1999). Further, understanding the pleiotropic effects of new mutations is critical for testing hypotheses regarding phenotypic modularity and the evolution of pleiotropy itself (Cheverud 1996; Wagner 1996; Wagner and Altenberg 1996; Hansen 2003).

The inherent pleiotropic effects of spontaneous mutations manifest themselves in divergence or artificial selection experiments as mutational covariances and correlations. Despite their theoretical importance, empirical data on these parameters are quite scarce. This is due primarily to the requirement for a large number of accumulated mutations to achieve sufficient statistical power to detect mutational correlations. The few studies investigating the pleiotropic effects of spontaneously generated mutations are mainly restricted to a few life-history traits in a single species, *Drosophila melanogaster* (Yoshimaru and Mukai 1985; Houle *et al.* 1994; Fernández and López-Fanjul 1996; but see Lynch 1985). The estimates of genetic correlations were significantly different from +1 in only a few cases. Additional data from mutagenesis studies (Camara *et al.* 2000; Keightley *et al.* 2000) exist, but because chemical mutagenesis may produce a spectrum of mutations different from that arising as a result of natural processes, it is unclear how meaningful such results are for natural populations. Further, the extremely high rates of mutation induced in these experiments, coupled with the possibility of unequal mutagen dosage across lines, make it difficult to determine the extent to which the correlations detected reflect clustering of nonpleiotropic mutations within certain lines *vs.* actual pleiotropy. In any case, the general consensus from these studies seems to be that mutations affecting life-history traits tend to produce intermediate-to-high positive correlations (but see Fernández and López-Fanjul 1996) with little evidence of genetic trade-offs generated by antagonistic pleiotropy.

To increase our understanding of the consequences of the pleiotropic effects of mutation for populations, we surveyed the joint influence of spontaneous mutations on pairs of life-history, behavioral, and morphological characters in long-term mutation accumulation lines of the nematode *Caenorhabditis elegans*. This study constitutes the first rigorous investigation of the covariance among *spontaneous* mutations for a broad variety of complex characters.

MATERIALS AND METHODS

Mutation accumulation lines: The current experiment was conducted using 67 mutation accumulation (MA) lines of *C. elegans* (Vassilieva and Lynch 1999; Vassilieva *et al.* 2000; Denver *et al.* 2000, 2004; Ajie *et al.* 2005, accompanying article in this issue) that had undergone an average of 370 single-individual population bottlenecks. The MA experiment was initiated from offspring of a single, highly inbred Bristol-N2 hermaphrodite. As described in Vassilieva and Lynch (1999), offspring of that individual were used to establish 100 replicate lines that were then propagated independently by transfer of a single, randomly selected L4 hermaphrodite each generation. Such treatment minimizes selection against mildly detrimental mutations, ensuring accumulation at a rate nearly equal that of their occurrence. Because *C. elegans* reproduces by self-fertilization, this procedure also rapidly removes heterozygosity. During the initial subdivision of the MA lines, many thousands of worms were stored cryogenically for use as a control. To prevent accidental line loss, MA lines that went extinct were reinitiated from populations of the previous generation as many as five consecutive times prior to being considered extinct in the experiment. Even with this treatment, 33 of the original 100 lines went extinct at a fairly steady pace over the course of the MA experiment, suggesting the possibility that many of these extinctions were a direct consequence of deleterious mutation accumulation. In any event, this study disregards lethal and extremely detrimental mutations. Experimental lines were cultured at 20° on petri plates containing NGM agar seeded with a suspension of OP50 *Escherichia coli* as a food source (Sulston and Hodgkin 1988).

The MA lines had acquired significant mutation loads at the outset of this experiment (Vassilieva *et al.* 2000; Ajie *et al.* 2005, accompanying article). The mean phenotypes relative to control values had declined by ~46% for progeny production, 12% for survival to maturity, and 32% for intrinsic population growth rate (r) after an average of 214 single-individual bottlenecks (see Vassilieva *et al.* 2000, Figure 2). Average estimates of the per-generation genomic rate of mutation to deleterious alleles made using the rate of change in the means and among-line variances of traits are 0.033 for productivity, 0.003 for survival, and 0.025 for r. (These values represent the average of estimates made using the Bateman-Mukai [B-M] and maximum-likelihood techniques from Vassilieva *et al.* 2000.) This leads to the prediction that, after 370 generations of MA, an average of ~6.1, 0.6, and 4.6 mutations/line with measurable effects on each of these traits is expected. On the basis of the nuclear mutation rate estimated from direct sequencing of >4 Mb of DNA from a recent study of these lines (Denver *et al.* 2004), 2.1×10^{-8}/generation, we can approximate the expected mean number of mutations per line with potential effects on fitness. Coding DNA constitutes ~30% of the *C. elegans* genome, or $\sim 26.2 \times 10^6$ bp (*C. elegans* Sequencing Consortium 1998). Disregarding codon bias (making our estimate more conservative) and assuming all types of substitutions are equally probable, 75% of all possible nucleotide substitutions are expected to cause an amino acid change (Li 1997, Table 1.4). Using these parameters, we expect an average of ~153 nonsynonymous mutations/line after 370 generations (*i.e.*, $2.1 \times 10^{-8} \times 26.2 \times 10^6$ bp coding DNA × 370 generations × 0.75). The source of the discrepancy between the estimates of mutation rate based on phenotypic *vs.* molecular analyses is unlikely due to insufficient statistical power of divergence experiments to detect mutations; if mutations of small effect occurred frequently enough, their cumulative effects should be detectable. Rather, the difference is most likely indicative of a class of mutations with effects nearly or entirely neutral in the laboratory environment (Davies *et al.* 1999; Estes *et al.* 2004).

Body size and life-history assays: Prior to the 370-generation assay, all lines were expanded into five replicates by transferring single, randomly selected L4 individuals to fresh plates. These animals were allowed to self-reproduce and random L4 worms were again transferred to new plates. Immediately after these second-generation L4 individuals were transferred, a digital image was recorded for each animal using a Nikon digital camera mounted to a compound microscope. Individual body width at the vulva (easily identifiable at the L4 stage)

was measured manually using Image Pro Plus image analysis software.

The above animals were allowed to self-reproduce and fitness components were measured on randomly selected offspring from each replicate. The same general procedure of line subdivision was applied to 20 thawed control animals. Single individuals were transferred to fresh plates daily and progeny production was measured by directly counting the progeny produced over the first 4 days of life, covering the majority of the reproductive period. "Early productivity" is the number of offspring produced on the first 2 days of reproduction combined, whereas "late productivity" is the number of offspring produced on the third and fourth days of reproduction. Intrinsic population growth rate was also calculated for each line by solving $\sum e^{-rx}\, l(x)\, m(x) = 1$ for r, where $l(x)$ is the proportion of worms surviving to day x and $m(x)$ is the fecundity at day x.

Behavioral assays: As detailed in Ajie *et al.* (2005, accompanying article), we estimated the effects of spontaneous mutation on behavioral traits by measuring previously well-characterized and ecologically relevant aspects of individual behavioral response (Pierce-Shimomura *et al.* 1999) to the chemical repellant linoleic acid, a nematicidal fatty acid isolated from Bascidiomycetes (Stadler *et al.* 1994). Using a computer tracking system (Pierce-Shimomura *et al.* 1999; Ajie *et al.* 2005, accompanying article), we measured three behavioral characters related to chemotaxis on individual worms: (1) "directness," the ratio of the beeline distance to the total path length traversed by a worm; (2) turn frequency (>90° changes in direction per minute); and (3) average instantaneous velocity. To establish a baseline for locomotory response in the absence of an olfactory cue, a series of assays were also conducted for the control lines with no repellant (Ajie *et al.* 2005, accompanying article).

Due to the time involved in the behavioral measurements, these assays had to be carried out over a number of days. To control for possible variation under laboratory conditions across days, each MA line was assayed in parallel with a control line. We initiated a single base control line from our frozen ancestral N2 stock and propagated this line for four generations prior to the assay by single-progeny descent to avoid maternal environment effects and to ensure homozygosity. From this line, maintained by single-progeny descent for the remainder of the assay, control pseudolines were established as necessary. Assays were conducted on five randomly selected progeny descendant from the founding individual of each MA and N2 line. Assays were carried out in a temperature-controlled room at 21° for 26 days over the course of almost 2 months. This amount of time corresponds to ~14 generations in *C. elegans*, and as the per-character mutation rate is quite low (Vassilieva *et al.* 2000), it is unlikely that this was a sufficient period to cause significant mutational deterioration in the control.

A slight temporal trend across days in turn frequency and velocity data was corrected by regressing the control line scores on day and then using the residuals for subsequent analysis. Directness was arcsine square root transformed and velocity and turn frequency were square root transformed before analyses.

Effect of spontaneous mutation on trait means and variances: Insight into the mutability of each trait can be gained from the per-generation rate of decline in mean phenotype (R_m) and the mutational heritability (h^2_m), defined as the per-generation rate of increase in heritability due to mutation. H2boot (Phillips 2002; subroutine for a one-way ANOVA among recombinant inbred lines) was used to calculate R_m, the mutational variance (V_m), and h^2_m for each trait. The rates of change in the mean of each phenotype were calculated simply as the difference between the MA and the control mean scaled by the number of generations of MA. The per-generation percentage change in the mean of each phenotype was calculated as R_m divided by the control mean phenotype for each trait. Mutational variance, the per-generation increase in additive genetic variance caused by mutation, is taken to be half the rate of increase in the among-line variance, assumed to have begun at zero. Because these calculations are made on the basis of a single assay, they assume a linear decline in mean phenotype and a linear increase in among-line variance as mutations accumulate. Such a pattern has been shown for life-history traits in these lines (Vassilieva *et al.* 2000) and is a common feature of most MA studies (Lynch and Walsh 1998). Barring mutations that arose during the previous three to four generations, which thus might still be segregating (see discussion in Estes and Lynch 2003), replicates within each MA line will be genetically identical. Therefore, the within-line component of variance is taken to be a direct estimate of environmental sources of variation and the among-line variance a measure of total genetic variation. Significance levels were determined by generating 10,000 bootstrap estimates (with replacement) resampled at the level of line.

Estimation of covariances and correlations: Covariance estimates generated by H2boot (Phillips 2002) were poorly behaved due to extreme outliers; therefore, covariances and correlations at the phenotypic, genotypic (mutational), and environmental levels and their standard errors were estimated for the MA lines by least-squares estimation of the variance and covariance components with a delete-one-family jackknife procedure. The quantitative genetic parameters reported are thus the averages of the jackknifed estimates for the data set. Significance levels of these parameters were adjusted using the sequential Bonferroni method for 21 tests (Rice 1989). For the mutational correlations found to be significantly different from zero, we conducted *t*-tests using the jackknife estimates of mutational correlations as the sample mean and their standard errors as the standard error of the mean to test whether mutational correlations were also significantly different from +1.0 (Knapp *et al.* 1989).

For logistical reasons, behavioral and life-history traits were measured for different individuals from each line (see above), but both assays were conducted at approximately the same time by two experimenters and individuals in each assay shared the same incubator. Body width was measured on the parents of the individuals included in the life-history assay. Consequently, the correlations that we report between different character classes are technically the correlations between genetically identical pairs of lines that were not separated by more than 10 generations of single-individual bottlenecks. Because of our experimental design, we feel that it is reasonable to interpret these correlations as if the measurements were taken on the same individuals. This approach is conservative since in this case the within-family (environmental) variance is increased relative to the among-family (genetic) component of variance.

Variance among control lines: A caveat for all of the above analyses is that variation among control lines is not zero for all traits—not a new problem to the long-term MA experiment (*e.g.*, Vassilieva *et al.* 2000). For the current study, we estimate low (~0.1) but significant broad sense heritabilities for late productivity, body width, and turn rate in the control (data not shown). Although the order and orientation of trays on which we kept the petri plates containing our lines were randomized on each day of the assay, replicate lines were kept in a nonrandom order on the trays. This likely indicates that a portion of the (co)variation of traits measured on MA lines is not due to new genetic variability, but is rather partially

TABLE 1

Mutational variances, covariances, and correlations

	Early productivity	Late productivity	*r*	Body width	Directness	Velocity	Turn rate
Early productivity	102.6***	0.849***	0.851***	0.492***	−0.062	0.398**	−0.076
Late productivity	290.0***	1141***	0.923***	0.513***	0.382	*0.470**	0.048
r	2.316***	8.331***	0.071***	0.583***	−0.053	*0.311**	−0.076
Body width	0.020***	*0.067**	*0.001**	1.497×10^{-5}*	−0.309	*0.243**	0.019
Directness	−0.028	1.329	−0.001	-1.056×10^{-4}	0.007*	−0.017	−0.549***
Velocity	*1.162**	4.610	0.023	2.779×10^{-4}	−0.001	0.080*	*−0.721**
Turn rate	−0.148	1.352	−0.006	3.261×10^{-5}	*−0.019***	−0.063	0.141*

Mutational correlations for each pair of traits are above the diagonal; mutational variances are on the diagonal; mutational covariances are below the diagonal. *, **, and *** denote significant differences from zero at the 0.05, 0.01, and 0.001 levels, respectively. Italics indicate estimates that were nonsignificant after correction for multiple comparisons (sequential Bonferroni; Rice 1989).

a result of shared environmental effects due to geographic structure in the experimental lines. We repeated the analyses after subtracting the control among-line variance. This correction had no effect on estimates of mutational correlations and minor effects on other parameter estimates (data not shown), so we present the estimates without this correction to facilitate statistical analysis.

In addition, there was a significant effect of the order of transfer on mean late productivity, *r*, and body width in the control lines such that all three traits declined as the assay progressed. As described in detail in Vassilieva and Lynch (1999), great care was taken to ensure that hermaphrodites entering the fitness assays were transferred to fresh plates at the same time (±30 min) during each day of the assay. We are therefore uncertain as to the source of this trend, but corrected for it by first regressing the control line means on elapsed time during the first transfer of the assay for each of the three characters. Scores for each MA line were then corrected using $Y = b$(control grand mean − control block mean), where Y is the corrected MA line score, b is the coefficient from the regression of control line means on elapsed time, and block is the particular tray on which the group of control and MA lines was placed during the assay. The correction had no substantial effect on the main results and, since no such temporal trend was present in the MA data, it is possible that the correction was overly conservative.

RESULTS

Mutational covariances and correlations: As confirmed previously for life-history characters (Vassilieva and Lynch 1999; Vassilieva *et al.* 2000), all traits showed significant values of V_m (Table 1), as well as a significant decline in mean phenotype (R_m) compared to the control (accompanying article by Ajie *et al.* 2005). The mutational covariances are reflected in the patterns observed for the mutational correlations for each of the 21 trait pairs (Table 1). Mutational correlations are large, positive, and highly significant between all pairs of life-history traits. Positive intermediate mutational correlations are also observed between body width and the three life-history traits related to reproduction (*e.g.*, Figure 1). Two pairs of behavioral traits showed significant, negative mutational correlations: turn rate directness (Figure 1) and turn rate velocity, although the latter was not significant after correcting for multiple comparisons. Turn rate and directness are expected to be intrinsically correlated to some degree as a worm that turns more frequently is necessarily less direct in its trajectory away from a chemical repellant. However, these are not simply different measurements of the same trait since the opposite scenario is not always true (*i.e.*, worms with a low turn rate do not always exhibit high directness). In fact, data from a natural population of a different Caenorhabditis species show no correlation between these traits (P. C. Phillips and B. C. Ajie, unpublished data). Finally, there are significant mutational correlations between velocity and the three fertility-related traits and between velocity and body width, although none were significant after the correction for multiple comparisons was performed (Table 1).

Phenotypic and environmental correlations: The phenotypic correlations (Table 2), composite functions of the genetic and environmental correlations, mirror quite closely the mutational correlations in both sign and relative magnitude ($r = 0.90$, $P < 0.001$). Environmental correlations, the correlation of environmental deviations including nonadditive genetic deviations (Falconer and Mackay 1996), are positive and significant for all pairs of life-history traits. All statistically significant environmental correlations are of the same sign as the corresponding estimates for phenotypic correlations.

DISCUSSION

We have studied the effects of spontaneous mutations on life-history, body size, and behavioral characters in a set of lines independently derived from a homozygous base population. Analyses reveal significant changes in the mean of each trait as well as significant levels of accumulated mutational variance in accordance with

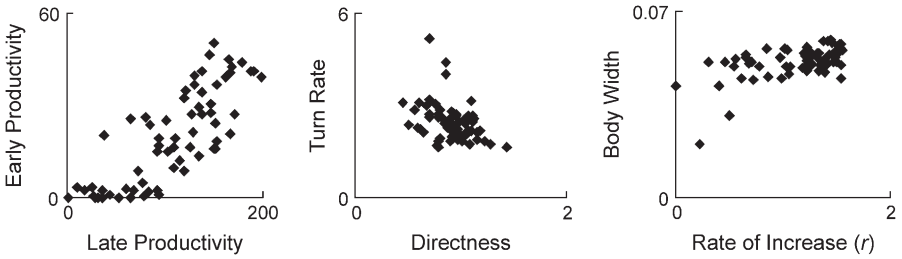

FIGURE 1.—Examples of bivariate relationships of MA line means for life-history, behavioral, and morphological characters. MA line-mean correlations provide a rough estimate of mutational correlations.

previous results for life-history traits (VASSILIEVA *et al.* 2000). The behavioral traits that we measured are each related to chemotaxis, the primary means whereby *C. elegans* perceives and responds to its environment (TROEMEL 1999; JOVELIN *et al.* 2003). Consequently, these traits have great potential to affect total fitness in nature. Putatively, 10% of the *C. elegans* genome is devoted to chemosensory function (BARGMANN 1998). Combined with genes controlling locomotion (*e.g.*, toward or away from a chemical stimulus), these genomic regions are expected to compose a fairly large mutational target, although likely not equivalent to those underlying life-history characters (AJIE *et al.* 2005, accompanying article).

Our findings for body width are qualitatively similar to those from a recent analysis of total body volume for the same lines after 152 generations of MA (AZEVEDO *et al.* 2002). Their study, also consistent with mutagenesis experiments in Drosophila (KEIGHTLEY and OHNISHI 1998), found that mutation leads to reduced body size far more often than to increased body size. However, AZEVEDO *et al.*'s (2002) estimate of h^2_m for their measurement of body size—total body volume—is more than twice what we estimate in the current study for body width (0.4 *vs.* 0.1%/generation). This, not surprisingly, indicates that total body volume is a more mutable trait than body width.

We find that newly arising mutations affecting life history tend to act pleiotropically, decreasing bivariate phenotypes. All life-history characters are significantly, positively correlated at the phenotypic, mutational, and environmental levels, suggesting that the majority of new mutations will have deleterious pleiotropic effects on all components of fitness (*e.g.*, Figure 1). We find no evidence at the phenotypic or genetic level for life-history trade-offs being generated by antagonistic pleiotropy, in agreement with findings from EMS-mutagenized lines of *C. elegans* (KEIGHTLEY *et al.* 2000). Although the mutational correlations between certain life-history traits are extremely high (*e.g.*, early productivity and *r*) and to some extent must reflect overlapping measurement, there is little evidence from the bivariate analyses for the existence of any absolute genetic constraints among the traits we measured. All mutational correlations that showed a significant difference from zero were also statistically different from +1.0 (*t*-tests, $P < 0.05$). Thus, there is unlikely to be complete genetic overlap in the control of any trait pairs that we studied.

Statistically significant environmental correlations were found to be of the same sign as mutational correlations in every case. This indicates that genetic and environmental sources of variance operate along similar pathways and that, insofar as the environmental correlations detected here reflect those likely to be present in nature, residual environmental effects would not reduce the efficiency of natural selection by diminishing the correlation between genotype and phenotype. In a few cases (*e.g.*, between directness and velocity) highly significant phenotypic correlations were solely a result of environmental correlations. This exemplifies the well-known hazard involved in inferring genetic correlations from phenotypic data (*e.g.*, WILLIS *et al.* 1991).

TABLE 2

Phenotypic and environmental correlations

	Early	Late	*r*	Width	Direct	Velocity	Turn
Early productivity		0.651***	0.705***	0.179***	0.120	0.233**	0.075
Late productivity	0.530***		0.832***	0.257***	0.396	*0.380**	0.167
r	0.619***	0.773***		0.274***	0.045	*0.139**	0.003
Width	0.015	*0.125**	0.109		−0.068	0.013	0.149**
Directness	0.165	0.427	0.427	−0.029		−0.296***	−0.172**
Velocity	0.154	0.337	0.337	−0.081	−0.354***		−0.374***
Turn rate	0.129	0.201	0.201	0.194***	−0.116	−0.291***	

Phenotypic (environmental) correlations for each pair of traits in the MA lines above (below) the diagonal. *, **, and *** denote significant differences from 0 at the 0.05, 0.01, and 0.001 levels, respectively. Italics indicate estimates that were nonsignificant after correction for multiple comparisons (sequential Bonferroni; RICE 1989).

Underlying causes of detected mutational correlations: The necessity of large sample sizes, in addition to the time involved in measuring a number of different characters, makes the estimation of mutational correlations a challenging endeavor. And even when statistically significant mutational correlations are detected in a quantitative genetic experiment, there is generally not one indisputable interpretation of the result. As outlined below, several nonmutually exclusive possibilities exist when such correlations are detected.

Pleiotropic mutations with correlated effects: This is the case as modeled by Lande (1980) in which single mutations produce correlated effects on the traits that they influence; *i.e.*, the effects of a mutation on each of the characters that it influences are drawn from the same (multivariate Gaussian) distribution. Although different researchers have employed a variety of distributions for mutational effects, this is the general model of pleiotropic mutation assumed in most quantitative genetic theoretical studies (*e.g.*, Jones *et al.* 2003; Zhang and Hill 2003).

Pleiotropic mutations with uncorrelated effects: For their maximum-likelihood inferences of bivariate mutational effects, Keightley *et al.* (2000) employed a model in which every mutation produces some effect on each of two traits (*i.e.*, universal pleiotropy), but these effects can be uncorrelated. Specifically, the effects of a single mutation on the two traits were independently drawn from bivariate gamma distributions having different scale parameters, depending on the mutation rate and the reduction in mean phenotype observed in their MA lines. This model simply highlights the point that, although mutations generating genetic correlations may be pleiotropic on both traits, the distribution of mutational effects need not be correlated.

This situation can lead to the over- or underestimation of mutational correlations from mutation accumulation experiments, particularly when experimental lines contain different numbers of pleiotropic mutations. This is because lines that harbor the most mutations (pleiotropic or not) will tend to be extreme for both traits, even if the effects of the pleiotropic mutations are uncorrelated. The results of Keightley *et al.* (2000) suggest that MA experiments may be inadequate for describing the fine-scale distribution of pleiotropic effects, but do not imply that detection of evolutionarily meaningful mutational correlations is beyond the reach of this experimental approach. An extreme example of this would be lethal mutations, as these lines display generalized deleterious effects. Inclusion of these lines in the current analysis would have the predictable effect of enhancing the overall pattern of genetic correlation, although the usefulness of those correlations would be less because this effect would be due to "diffuse" pleiotropy.

Statistical association among traits due to sampling: A far more insidious problem could arise when *apparent* mutational correlations are generated by sampling the "wrong" experimental lines by chance. The worst-case scenario here would occur when mutations of large effect at independent loci—each independently affecting two or more traits—happen to arise in the same line. In this case there would be mutational correlation without pleiotropy. If this occurs frequently, then MA experiments will be practically useless for studying any properties of mutational covariance. We performed calculations to test the likelihood that such sampling could produce spurious mutational correlations of the magnitude that we estimate from our data. On the basis of B-M estimates of mutation rate and average effect size for each character, we calculated the expected mutational correlation due to the accumulation of nonpleiotropic mutations in our lines. We found that the expected correlations due to sampling fell between $+0.262$ and -0.257 (data not shown). Since all of our statistically significant correlation estimates fall outside of this range (Table 1), the effects of sampling appear insufficient to explain our findings. Additionally, these analyses confirmed that neither the mutational effect size nor the number of mutations per line substantially affects the expected correlation due to sampling.

Although we cannot formally distinguish between the effect of lines having accumulated multiple, nonpleiotropic mutations and actual pleiotropy in our study, the likelihood of multiple nonpleiotropic mutations arising in the same MA lines is less likely in our study than in studies utilizing chemical mutagenesis (*e.g.*, Camara *et al.* 2000; Keightley *et al.* 2000). The high mutation rates induced in these studies along with the chance of unequal mutagen doses across lines enhance the likelihood of correlations among mutational probabilities being generated. In any case, some of our most convincing evidence for the existence of true pleiotropy is the fact that we observe no generalized mutational correlations across all traits. This pattern would be expected if the apparent correlations were driven purely by the existence of multiple nonpleiotropic mutations in the experimental lines.

Hidden pleiotropy: Finally, only the net pleiotropic effects are likely to be captured by MA experiments. If the pleiotropic effects of different loci tend to cancel each other, there could be some degree of "hidden pleiotropy" (Baatz and Wagner 1997). Strong pleiotropy can therefore exist without resulting in strong mutational correlations.

Significance for evolutionary and conservation genetics: Genetic variation observed for fitness correlates is thought to be at least partly explained by a balance between recurrent mutation, genetic drift, and selection (Barton and Turelli 1989; Houle *et al.* 1996; Falconer and Mackay 1996, Chap. 20; Lynch *et al.* 1998; Charlesworth and Hughes 2000). Our study indicates that polygenic covariance will be continually augmented by new, heritable covariance as well. In agree-

ment with most studies of mutation, the majority of the mutational variation (and covariation) that accumulated in our lines is clearly detrimental in the laboratory environment. Such observations have led to the proposal that a large portion of the standing genetic variance in populations may simply reflect transient deleterious variation ineffectual for adaptive evolution (Houle *et al.* 1996), a view supported by a number of empirical studies (Houle *et al.* 1996; Lynch and Walsh 1998, Chap. 12). Under a scenario of deleterious mutation-selection balance, depending on the bivariate distribution of mutational effects, the synergistic pleiotropy for fitness correlates detected in this study would likely be beneficial as selection could more effectively eradicate such mutations from a population.

Similarly, positive pleiotropy may have greatly facilitated the fitness restoration observed in a previous study of these lines (Estes and Lynch 2003). Under a regime of large population size exposed to selection, MA lines were shown on average to rapidly recover original levels of mean fitness, most likely as a result of selection for compensatory mutations. If a compensatory mutation had positive pleiotropic effects on multiple components of fitness, the selective advantage of this allele would be considerably larger than for mutations that acted to compensate single traits (*e.g.*, Poon and Otto 2000).

While positive pleiotropic effects of deleterious mutations could promote the eradication of such variants in nature, in populations where natural selection is relaxed (*e.g.*, experimental lines, small captive populations of endangered species) if a mutation negatively influences multiple fitness components, the total effect of mutation for populations will obviously be magnified. This mutational load will be underestimated if all such components are not measured. Alternatively, negative pleiotropic effects could result in overestimates of the total mutational load on fitness. Our results indicate that the former could be a significant problem, particularly since many studies of spontaneous mutation have focused on a single component of fitness such as juvenile viability or adult productivity. Additionally, if pleiotropic gene action is environmentally dependent, these biases could be even more extreme.

Implications for genetic modularity and evolution of genetic correlations: Understanding the mechanisms that bring about correlations between different characters will be essential to understanding phenotypic and genetic integration (*e.g.*, Wright 1918, 1935; Berg 1960; Cheverud 1996; Wagner 1996; Wagner and Altenberg 1996; Hansen 2003) and its role in promoting or constraining evolution (*e.g.*, Steppan *et al.* 2002; Jones *et al.* 2003). Wagner's (1996) model addressing the evolution of pleiotropy by differential epistasis predicts that only loci selected for a common function will evolve or maintain pleiotropic effects. This is taken to mean that natural patterns of covariance should evolve to match the pattern of stabilizing selection and the pattern of mutational effects, thereby permitting the integrated evolution of functionally related characters (Cheverud 1984, 1996; Bürger 2000).

Recent quantitative genetic and QTL studies have yielded some support for these ideas as genomic regions found to produce manifold effects have been generally restricted to suites of functionally and developmentally related traits (Cheverud *et al.* 1997, 2004; Leamy *et al.* 1999; Shook and Johnson 1999; Klingenberg *et al.* 2004, but see Knight *et al.* 2001). For newly arising variants, we too find little evidence for widespread pleiotropy between traits from different functional classes—at least as we have defined such classes. There were, however, extensive correlations between width of L4 individuals and fertility traits (*e.g.*, Figure 1). Yet, these traits are likely to be at least partially functionally correlated, as body width would place an upper bound on gonad size and, presumably by default, on fertility. Although we did not measure a large of number traits from "unrelated" character classes, our results lend provisional support for the hypothesis of genetic independence of functionally unrelated traits. However, in the absence of data on the action of natural selection on patterns of covariance, the hypothesis of the evolution of conformity between mutational and natural covariance patterns remains untested.

If the G-matrix were stable over the course of evolutionary time, extrapolation of the multivariate breeders' equation over multiple generations would be useful for predicting long-term evolutionary potential and revealing genetic constraints (Lande 1979). However, since the components of **G**, the genetic (co)variances, are functions of underlying gene frequencies (Falconer and Mackay 1996), the G-matrix is subject to evolutionary modification by all of the same forces that affect the genetic variation. Theoretical and empirical evidence clearly demonstrate that **G**, while it may in some cases remain stable at the within-species level, can change in variety of ways over long periods of time (reviewed in Steppan *et al.* 2002). As such, predictive theories of character evolution must eventually be reconciled with a mechanistic understanding of precisely how G-matrix components evolve (Arnold *et al.* 2001; Phillips and McGuigan 2005). Our study indicates that the pleiotropic input of mutation cannot be ignored as a potential evolutionary force for **G**. However, in this context, data on the mutational integration of traits are of limited meaning in the absence of knowledge of patterns of covariance among characters in natural populations. If the majority of mutations have large negative impacts on fitness, pleiotropism will act to increase their selective effects in nature. This will cause such mutations to be more effectively purged from populations, thereby prohibiting their involvement in the evolution of G-matrices.

Comparative studies of mutational and standing genetic covariance to determine how natural selection

responds to the genetic (co)variance generated by pleiotropic mutation and whether mutation tends to recreate patterns of covariation maintained by selection (Lande 1980) are badly needed. Such studies are crucial for generating accurate null models to test alternative hypotheses regarding the underlying causes of patterns of divergence between taxa (Lande 1975, 1979; Lovsfold 1986; Lynch 1990; Arnold *et al.* 2001; Steppan *et al.* 2002) for inferring evolutionary constraints or limits to artificial selection (Arnold 1992; Falconer and Mackay 1996), as well as for assessing the adequacy of models regarding the mechanisms maintaining genetic variation (Barton and Turelli 1989; Houle *et al.* 1994) and the evolution of pleiotropism (Cheverud 1996).

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