Spontaneous Mutation Rate of Modifiers of Metabolism in Drosophila

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ABSTRACT

A rigorous test of our understanding of evolutionary quantitative genetics would be to predict accurately the equilibrium distribution of a character from empirical estimates of the relevant parameters in a mutation-selection-drift balance model. An aspect of this problem that is amenable to experimental analysis is the distribution of the effects of new mutations. This study quantifies the divergence among 200 lines of *Drosophila melanogaster* as they accumulated mutations on the second chromosome and estimates the rate of increase of variation and covariation in metabolic characters. Amounts of stored triacylglycerol and glycogen and the activities of a series of 12 metabolic enzymes were assayed in a subset of lines at generations 0, 11, 22, 33 and 44. Analyses of the rate of increase in the among-line variance in each trait allowed estimation of V_m/V_e, the ratio of among-line variance added per generation to the environmental variance. Values of $\hat{V}_m/\hat{V}_e$ for the second chromosome ranged from 0.0004 to 0.0289 per generation. Six of the 16 characters showed significant departure from a normal distribution, and several lines exhibited large changes in more than one character. The covariance of pairs of traits also was partitioned into a within-line component (environmental covariance, Cov_e) and an among-line component (mutational covariance, Cov_m). Both variances and covariance among lines increased over time, as assessed by linear regression, whereas environmental covariance showed no such trend. Results indicate that the quantitative genetic parameters describing the variation in metabolic traits are similar to those of other continuous characters.

BEGINNING with the papers of KIMURA (1965), LATTER (1970), BULMER (1972) and LANDE (1975), extensive literature has accumulated on the theory of the evolution of a quantitative character in the face of mutation and selection. Initially the emphasis was on a single quantitative character determined by many loci with small additive effects. Most of the theory has focused on stabilizing selection (BARTON 1986; BARTON and TURELLI 1987; TURELLI 1988; NAGYLAKI 1989), where an important issue is the validity of assumptions that must be made to derive a Gaussian equilibrium distribution of allelic effects. A split arose between models that had a continuum of alleles having small mutational effects (also called "infinitesimal" models) and models that allowed mutations of large effect (also called the "rare alleles" or "house-of-cards" model). The parameters of these models include the mutation rate, the magnitude of mutational effects and the strength of selection. TURELLI (1984, 1986) pointed out that the continuum-of-alleles models require excessively high per-locus mutation rates to produce observed genetic variances with reasonable choices of other parameters. Theoreticians have called for more and better empirical estimates of relevant parameters, including the number of loci involved in quantitative traits, the rate of polygenic mutation and the magnitude of mutational effects.

Two classes of experiments have been done to estimate rates of polygenic mutation: quantification of divergence among initially identical lines and artificial selection on an inbred line (reviewed by LYNCH 1988). Consider a population where μ_i and $\sigma^2_{m,i}$ are the mutation rate and mutational variance (the variance in effects of new mutations) for the ith locus. When effects are assumed to be additive and there is no epistasis, these can be collapsed to one parameter, $V_m = 2\Sigma\mu_i\sigma^2_{m,i}$. LYNCH (1988) calculated the ratio of variance of mutational effects to the environmental variance ($\hat{V}_m/\hat{V}_e$) to scale the results among disparate experiments, and, in studies of Daphnia, Drosophila and crop plants, this ratio averaged $\sim 10^{-3}$. In three recent studies of polygenic mutation rates for loci affecting sternopleural and abdominal bristles in *Drosophila melanogaster,* estimates of $\hat{V}_m/\hat{V}_e$ ranged from 0.5 to 3.4×10^{-3} (MACKAY *et al.* 1992; SANTIAGO *et al.* 1992; LÓPEZ and LÓPEZ-FANJUL 1993a,b). In an experiment initiated with an inbred line, artificial selection on the weight of 6-week-old mice produced a response, which KEIGHTLEY and HILL (1992) used to estimate the rate of mutation that must have occurred during the experiment to yield the observed response. Their model produced an esti-

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mate of $\hat{V}_m/\hat{V}_e$ (which they call "mutational heritability") of 0.006–0.016, or nearly 10 times that of Drosophila bristles. Experiments of this sort consistently reveal a surprisingly large effect of new mutations. Even more surprising, experiments that estimate $\Sigma\mu_i$ separate from the magnitude of mutational effects consistently yield $\Sigma\mu_i \geq 10^{-2}$ (TURELLI 1984), and in the case of fitness, the mutation rate is nearly one (HOULE *et al.* 1992). This raises a paradox in explaining how the per-character mutation rate could be so high, when the per-locus mutation rates are on the order of 10^{-6}.

The discrepancy between per-locus and per-character mutation rates suggests that thousands of genes are mutable in ways that influence each character. This "mutation paradox" persists even when mechanisms other than mutation-selection balance are considered in models that maintain additive variation. Stabilizing selection acting on a character that exhibits genotype by environment interactions in varying environments can result in induced overdominance on the underlying loci (GILLESPIE and TURELLI 1989). Another possibility is that many loci do affect each character, but each mutation in turn affects many traits. The argument for this widespread pleiotropy is compelling (LANDE 1980; TURELLI 1985; BÜRGER 1986, 1989; BARTON 1990), and the models demonstrate how pleiotropy can result in apparent stabilizing selection, which reduces polygenic variation compared to single-character models. Widespread pleiotropic effects of mutations are plausible, but the amount of genetic variation maintained in mutation-selection balance depends heavily on the details of the genetic basis for the pleiotropy (BARTON and TURELLI 1989).

Enzyme activities are characters with some desirable properties for studies of evolutionary quantitative genetics, including the fact that we have some understanding of regulation at a molecular level and some knowledge of how they might relate to one another in metabolic pathways. Three published studies have examined the increase in variance in enzyme activities among mutation-accumulation lines of *D. melanogaster*. These studies were performed on MUKAI's mutation-accumulation lines, measuring the increase in among-line variance in enzyme activity. In the first such study, MUKAI *et al.* (1984) quantified the rate of mutation of polygenes affecting alcohol dehydrogenase activity. For this trait the estimate of $\hat{V}_m/\hat{V}_e$ was 0.0006, which is 15 times the value of $\hat{V}_m/\hat{V}_e$ for genes influencing viability (MUKAI 1964). AQUADRO *et al.* (1990) repeated the analysis of 47 lines that had accumulated mutations for 300 generations and found that the lines had already accumulated as much variation in activity as is found within either major electromorph in natural populations. The variation was found to be unassociated with transposable element variation in the neighborhood of the *Adh* structural gene. TACHIDA *et al.* (1989) found significant variation in Amylase activity among Mukai's mutation-accumulation lines, but their analysis of restriction site variation around *Amy* revealed a clear case of double recombination with the balancer chromosome. When the recombinant line was removed from the analysis, no significant increase in variance was detected.

The theoretical work on pleiotropy has raised the challenge to quantify experimentally the magnitude of pleiotropic effects of new mutations. Pleiotropic effects of mutations have been acknowledged in reports of mutation-accumulation experiments (MACKAY *et al.* 1992; HOULE *et al.* 1994), and there is need for parameterization of models that incorporate pleiotropy in predicting equilibrium variances and covariances. In addition, by examining simple metabolic traits such as enzyme activities, it is hoped that the molecular basis for pleiotropic effects will be experimentally accessible. The motivating questions behind this study include the following: at what rate do mutations influencing enzyme activity inflate the variance among lines, do the among-line variances and covariances increase linearly over time (as predicted by the infinitesimal model), how does the distribution of mutational effects compare with the normal distribution and what is the correlation structure of novel mutations?

MATERIALS AND METHODS

Drosophila stocks: The basic design of this experiment, which was done in collaboration with Drs. BRIAN CHARLESWORTH, DEBRA HOFFMASTER and DAVID HOULE, was to allow mutations to accumulate in a series of 200 lines of *D. melanogaster* that were all derived from a single original line. Following the design of MUKAI (1964), mutations accumulated on the second chromosome by being maintained in a heterozygous state over the *SM1* marked balancer chromosome. Every 11 generations, test crosses were done to produce progeny that were homozygous for the wild second chromosomes. CHARLESWORTH's laboratory quantified several components of age-specific fitness in these lines, including total fitness (HOULE *et al.* 1992, 1994).

The initial line of wild flies came from the Ives population, which had been started with 400 iso-female lines in 1975 and had been maintained in 10 half-pint bottles with mixing and transferring every 14 days. As described in detail by HOULE *et al.* (1994), 50 second chromosomes were extracted from the Ives population using a balancer stock that was *SM1, Cy/ bw^D; spa^{pol}/ spa^{pol}*. *SM1* is a multiply inverted balancer second chromosome marked by the dominant *Curly* (*Cy*) wing mutation. bw^D has a dominant eye color phenotype, and spa^{pol} is a fourth chromosome eye mutation used to identify stock contamination. The balancer stock had been intercrossed to the Ives population so as to have a common background of chromosome *I* and *III* variation. The extracted lines were tested for viability and reproductive fitness, and line 55, one of the best performers, was chosen for the remainder of the experiment.

An important aspect of the *SM1/ bw^D; spa^{pol}/ spa^{pol}* and the

Ives-derived lines is that both bear the R and weak P cytotypes (HOULE *et al.* 1994), so high rates of transposition associated with hybrid dysgenesis is avoided (ENGELS 1986). However, if they do move, these transposable elements can generate significant levels of quantitative genetic variance by transpositional mutation (MACKAY 1984, 1985), and rates of transposition in such lines have been estimated at $\sim 3 \times 10^{-3}$ per generation (EGGLESTON *et al.* 1988). Whereas this rate is perhaps three orders of magnitude greater than the spontaneous per-locus mutation rate, the rate of mutation of polygenes affecting viability is yet another two orders of magnitude higher (MUKAI 1964; MUKAI *et al.* 1972). It will not be possible to determine the relative rates of mutation induced by *P*-element transposition and other spontaneous mutations, but the rates of transposition should in fact be similar to that in natural populations of *D. melanogaster*, which are generally of the P cytotype.

After the $+/+$; spa^{pol}/spa^{pol} founder line was chosen, a single male was crossed to three virgin females of $SM1/bw^{D}$; spa^{pol}/spa^{pol} to generate 200 lines that were all initially $+/SM1$; spa^{pol}/spa^{pol}. These 200 lines were maintained by repeated backcrossing of single males of $+/SM1$; spa^{pol}/spa^{pol} to virgin females that were $SM1/bw^{D}$; spa^{pol}/spa^{pol}. Any mutations that occur on the + chromosome, are transmitted to the next generation and, with the exception of strong dominant effects, selection is virtually eliminated. Single males were used to avoid the possibility of selection acting through differential mating success. By crossing to virgin females from a common balancer stock, the design assures that the genetic background of the 200 lines remains uniformly variable on chromosomes *I* and *III*. Recall that the balancer stock is segregating in chromosomes *I* and *III* for variation in common with the Ives population. The repeated backcrossing to this balancer stock, which was maintained at a large population size, should retain the variation in the genetic background. If the balancer stock loses variation by genetic drift, then that loss will be propagated to all the mutation-accumulation lines as well, and not be manifested as among-line divergence. Mutations can accumulate on the *SM1* balancer chromosome of the balancer line, but natural selection can act to eliminate some of these mutations. Effects of mutations on the balancer also will be common to all lines, and hence will not inflate the variance among lines. Lines that acquired lethal mutations were eliminated from the experiment. A control population initiated with the single founder line was maintained in parallel with the mutation-accumulation lines, but at one point during the experiment, the control population was passed through a bottleneck (HOULE *et al.* 1994) during which time mutations could have become fixed. In this study, sufficient numbers of the initial line were tested that the analysis could be done on the basis of divergence among lines without reference to the potentially corrupted control line.

Preparation of homogenates: At generations 0, 11, 22, 33, and 44, a sample of mutation-accumulation lines was sent from the CHARLESWORTH lab at the University of Chicago to Penn State. In our laboratory, three or four newly emerged pairs of flies were transferred to fresh medium and reared through one complete generation. Flies from each line were reared in at least three vials on each of 2 days. Upon emergence, two replicate groups of five males were collected from each vial and transferred to fresh, yeasted medium for 6 days of aging. Homogenates of 6-day-old males were prepared as described in CLARK and KEITH (1989). Each sample was weighed to the nearest 1 μg and placed in a coded microcentrifuge tube. The entire process of homogenizing, centrifugation and pipetting was performed in a 4° room. Each homogenate was distributed into the same position in 96-well microtiter plates (15 plates). Homogenates were stored in microtiter plates at −70° until the day of assay. The number of lines tested at each generation was: generation 0 (1 line), generation 11 (30 lines), generation 22 (77 lines), generation 33 (41 lines) and generation 44 (52 lines). The basic sampling design was (5 generations) × (N lines) × (2 days) × (3–5 vials) × (2 replicates), but the sampling was not perfectly balanced.

Assays of enzyme activities: For each sample of flies, live weight and 15 biochemical quantities were measured. A microtiter plate reader was used for the enzyme kinetic assays, and details of the procedures appeared in CLARK and KEITH (1989) and CLARK and WANG (1994). Each microtiter plate had homogenate samples as well as standards and controls. The 15 identical plates were used to assay triacylglycerol (TRI), glycogen (GLY), total protein (PRO) and the activities of fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PD), α-glycerophosphate dehydrogenase (GPDH), glycogen phosphorylase (GP), glycogen synthase (GS), hexokinase (HEX), malic enzyme, (ME), 6-phosphogluconate dehydrogenase (6PGD), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), trehalase (TRE) and alcohol dehydrogenase (ADH). Mutations accumulated on the second chromosome. Traits whose structural gene map to the second chromosome were ADH, HEX-C, PGI and TRE, whereas G6PD, 6PGD and HEX-A are on the *X*, and GPDH, ME and PGM are on the third chromosome (the remaining enzyme loci are unmapped). The units of TRI and GLY are micrograms per fly, and the units of the enzyme activities are nanomolar NADP (or NAD) reduced per fly per minute. The microtiter plate reader (VMAX, Molecular Devices Inc.) was programmed to record 6–12 optical densities of each well at prescribed time intervals depending on the assay. Each such run was recorded in a separate data file. Altogether the project involved 319,680 optical density readings from 555 microtiter plates (37 sets of 15 plates).

Univariate statistical analysis: For each generation, the data were organized into the linear model as follows: Day and Line main effects, Day crossed with Line and Vial nested within Line and Day. The first null hypothesis of genetic interest is the equivalence of the line means for each character at each generation. This was tested by nested analysis of variance (ANOVA) of each of the 16 characters and by nested analysis of covariance (ANCOVA), using live weight and total protein as covariates. The two linear models used were

$$Y_{ijkl} = \mu + D_i + L_j + (D*L)_{ij} + V_{ijk} + \epsilon_{ijkl}$$

and

$$Y_{ijkl} = \mu + D_i + L_j + (D*L)_{ij} + V_{ijk} + \beta_w(w_{ijkl} - \bar{w}) + \beta_p(p_{ijkl} - \bar{p}) + \epsilon_{ijkl}$$

where μ is the grand mean, D_i is the effect of the ith day, L_j is the effect of the jth line, $(DL)_{ij}$ is the day by line interaction, V_{ijk} is the effect of the kth vial nested in the jth line and ith day, and ϵ_{ijkl} is the residual error. The covariates in the ANOVA were weight (w_{ijkl}) and protein content (p_{ijkl}), whose means were $\bar{w}$ and $\bar{p}$, and whose regression coefficients were β_w and β_p, respectively. The first model makes no attempt to adjust for the effects of different weights or protein contents and allows for the possibility that changes in size during the course of the mutation-accumulation might be caused by newly acquired mutations. The second model is an analysis of covariance that removes the effects that variation in weight

and protein content among lines may have on the other variables. In applying the latter model, we are assuming implicitly that variation in weight and protein is the cause of variation in enzyme activities. Mutations that affect weight will directly affect other characters. It is also possible that mutations whose direct effect is on lipid storage also may affect weight, so removing the covariance with weight from the lipid effect may not be appropriate. Both models were used because these linear models cannot reveal which character is causal.

To test whether mutational effects have a Gaussian distribution, the normality of line means was tested with the Shapiro-Wilk statistic (the UNIVARIATE procedure of SAS), and normal probability plots gave a visual verification of these tests. The linear models were fitted with the routine GLM in the statistical package SAS (SAS Institute, 1990). Hypothesis testing of this model (which has crossed and nested terms) required calculation of adjusted denominator mean squares (GROSSMAN and GALL 1968). In addition to testing whether lines were homogeneous, the nested ANOVA indicated other levels in the experiment at which significant heterogeneity was introduced. The procedure VARCOMP in the SAS package was used to partition the total variance in each character into components of Line, Day, Vial and error. The error term is actually the variance between samples taken from the same vial. Further details concerning the application of linear models appears in RESULTS.

Estimation of mutational variance: From the distributions of line means at generations 0, 11, 22, 33, 44 the among-line component of variance was estimated for all 16 characters. Linear regression then was applied to determine the per-generation rate of increase of variance among lines. The within-line component of variance provided a estimates of environmental variance. Environmental variance was not expected to increase over generations, and regression was used to test this hypothesis. Estimates of V_m/V_e then were made by dividing the rate of increase of among-line variance (V_m) by pooled estimates of environmental variance.

To determine standard errors of $\hat{V}_m/\hat{V}_e$ and to conduct hypothesis tests using the estimates, a bootstrapping procedure was devised. Bootstrap samples (1000) of the data were drawn with replacement in such a way that the same number of lines, days and vials were drawn, retaining the imbalance of the complete data (as in SPITZE *et al.* 1991; HOULE *et al.* 1994). $\hat{V}_m/\hat{V}_e$ estimates were calculated as described above, and tail probabilities and standard errors were extracted from these bootstrap distributions.

Mutational covariance: As mutations accumulate, pairs of traits may exhibit covariance among line means due to pleiotropic effects of mutations. Just as variance was partitioned into among-line and within-line components, covariance was partitioned into an among-line component, which is a mutational covariance, and a within-line component, which is environmental. As mutations accumulate, the among-line covariance, like the variance, should increase linearly. This was tested by regression (of among-line covariance on generation) and the slope provided an estimate of the per-generation rate of increase of covariance (or mutational covariance, Cov_m). Environmental covariance is again not expected in increase with time, and regression was used to test this hypothesis. If environmental covariance is homogeneous across generations, then a pooled estimate (Cov_e) can be made, as can an estimate of mutational covariance scaled by the environmental covariance (Cov_m/Cov_e). From the estimates of mutational covariance and variance for each trait, mutational correlation was estimated. Mutational correlation was estimated, and regression was used to test whether the mutational correlation increased over generations. It is expected that the among-line variances and covariances should accumulate at the same rate as the lines diverge, so correlations should be stable. If tests indicate that correlations are stable, then it is appropriate to make a single estimate, pooling over generations.

The same bootstrapping procedure as described above was used to obtain confidence intervals on each element in the correlation matrix. From each bootstrap sample, mutational correlation was calculated as the among-line covariance divided by the square root of the product of among-line variances. Bootstrap distributions gave tail probabilities for the one-tailed test of correlations being different from 0. To determine experiment-wide estimates of significance, the sequential Bonferroni method was applied (RICE 1989). Caveats to this approach are explored in RESULTS.

RESULTS

Univariate analysis: Inspection of the descriptive statistics for the characters at generations 0, 11, 22, 33, and 44 revealed some clear trends (Table 1). Mean activities and storage quantities appeared to increase in some characters and decrease or remain unchanged in others, but the variances exhibit consistent increases. The distributions of activities at each generation show a clear spreading in variance as time proceeded. The consistency of the increase in variance among lines was assessed graphically in Figure 1. The center of the figure represents the among-line variance at the beginning of the experiment, and, because all lines derived from a single line, the among-line variance at generation 0 is set to 0. The figures are scaled such that the circle represents the among-line variance observed at generation 44. If all traits accumulate variance linearly in time (the expectation under the infinitesimal model), the figures would show circles of increasing size. In general, however, the figures show a monotonic increase in variance, although there appears to be some departure from linearity. Linearity is more easily assessed in Figure 2, and is tested statistically by regression below.

Variance was partitioned into components with the SAS procedure VARCOMP using the two linear models detailed above. The component of among-line variance is reported as a percentage of the nonerror variance at each generation for each model in Table 2. Error variances were stable over the course of generations, but varied across traits, so reporting among-line variance as a proportion of nonerror variance allows easier comparison across traits. The among-line variance exhibits a clear increasing trend for most traits, consistent with the idea that accumulated mutations make the line means diverge.

ANOVA and ANCOVA were performed on the generation 44 data to assess the significance of the accumulated among-line variance (Table 3). There was a significant Vial effect in every test, consistent with previous observations; this demonstrated that small variation in

TABLE 1

Characters after 0, 11, 22, 33 and 44 generations of mutation accumulation

Generation	L	N	A	WT	PRO	TRI	GLY	FAS	G6PD	GP	GPDH	GS	HEX	ME	PGD	PGI	PGM	TRE	ADH
0	1	80	40.0	0.721	43.700	56.936	13.195	4.070	3.580	2.028	56.758	2.951	3.203	7.251	1.710	11.070	60.160	17.449	—
				± 0.037	± 3.898	± 5.266	± 1.959	± 1.320	± 0.412	± 0.644	± 5.584	± 0.322	± 0.622	± 1.335	± 0.297	± 1.859	± 9.72	± 0.911	
11	30	293	4.9	0.733	43.854	55.788	13.020	4.084	3.501	2.094	55.526	2.914	3.267	7.256	1.694	11.078	59.012	17.838	—
				± 0.042	± 3.985	± 5.816	± 2.056	± 1.325	± 0.456	± 0.673	± 6.482	± 0.346	± 0.649	± 1.470	± 0.301	± 1.881	± 10.41	± 0.963	
22	77	710	4.6	0.743	43.935	54.751	12.898	4.094	3.404	2.153	54.486	2.890	3.307	7.262	1.673	11.100	57.121	18.260	—
				± 0.046	± 4.086	± 6.361	± 2.153	± 1.328	± 0.502	± 0.692	± 7.893	± 0.381	± 0.665	± 1.664	± 0.305	± 1.882	± 10.85	± 1.028	
33	41	410	5.0	0.758	44.262	53.969	13.019	4.105	3.283	2.119	52.696	2.841	3.372	7.059	1.643	11.014	56.567	18.946	—
				± 0.050	± 4.340	± 6.866	± 2.254	± 1.314	± 0.548	± 0.682	± 8.817	± 0.398	± 0.731	± 1.675	± 0.310	± 1.926	± 10.97	± 1.064	
44	52	927	8.9	0.777	43.933	52.790	12.455	4.107	3.183	2.256	53.029	2.853	3.444	6.872	1.613	11.106	56.122	19.230	5.795
				± 0.046	± 3.093	± 7.293	± 2.310	± 1.400	± 0.589	± 0.793	± 9.006	± 0.411	± 0.708	± 1.664	± 0.310	± 1.989	± 12.02	± 1.101	± 1.068

Values are means ± SD. L, no. of lines assayed; N, total no. of samples examined; A, average no. of samples per line. ADH was not scored in generations before 44. The units are milligrams for weight, micrograms/fly for protein and triacylglycerol and nanomoles NADP/fly per minute (or nmoles NAD/fly/min) for the enzyme activities. PGI activities were multiplied by 0.1, and trehalase activities were multiplied by 0.001 throughout.

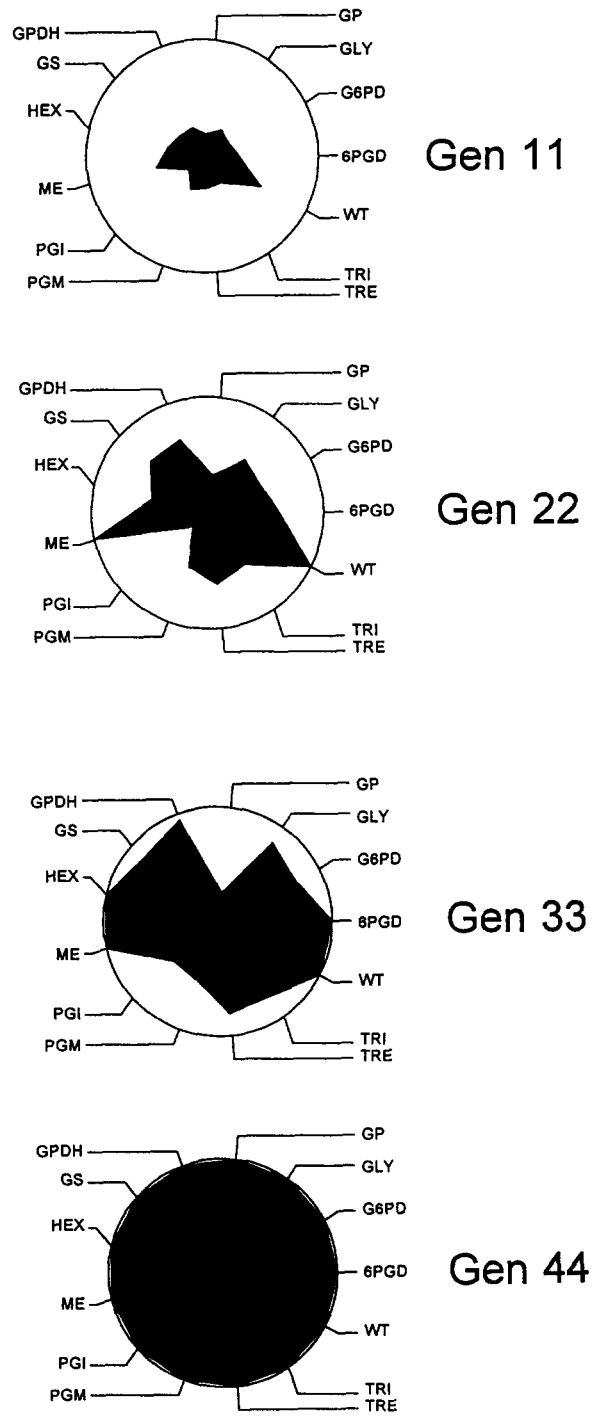


FIGURE 1.—The solid area in each star chart represents the magnitude of the among-line variance for each character. A radius is drawn for each trait with a length corresponding to the fraction of the generation 44 variance observed at the generation corresponding to each figure. The radii are scaled such that the center of each plot represents generation 0 (0 among-line variance), and the circle represents the among-line variance at generation 44.

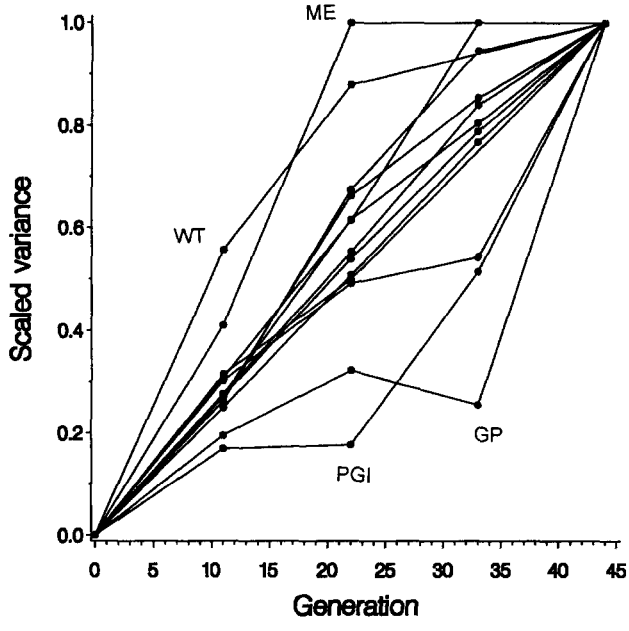


FIGURE 2.—Total variance in each trait plotted against generation of mutation accumulation. Variances are taken from Table 1 and are scaled to the generation 44 value.

rearing conditions influence these quantitative traits. Many of the traits also showed a significant Day effect, which may include both the effects of rearing and day-to-day variation in homogenate preparation and kinetic assays. Significant Day and Vial effects were included in the denominator mean squares in testing significance of Line and Day × Line interactions. All of the traits except GP, GPDH, PGD and PGI showed highly significant differences among lines at generation 44. Despite the observation that Weights were significantly heterogeneous at generation 44, the ANCOVA results were very similar to the ANOVA results. One exception was ME, which was found to exhibit significant heterogeneity in the raw data, but not in the weight-adjusted data (Table 3).

Normal probability plots provide a quick check for departure from normality of the deviations among line means (Figure 3). Some models of evolutionary quantitative genetics assume that mutational effects are normally distributed, and their cumulative effects also are expected to be normally distributed. There were clear outlying lines in the traits HEX, PGI and ADH. The high activity outlier in HEX and ADH was the same mutation-accumulation line, suggesting a major pleiotropic effect. The Shapiro-Wilk statistic indicated a departure from normality in the characters PRO, GS, HEX, PGI, PGM and ADH (details not shown). When the outliers were removed, HEX, PGI and ADH fit the normal distribution adequately.

Estimation of mutational variance: The variance among lines is expected to increase in a linear fashion if the effects of new mutations are additive across loci. Regression coefficients describing the rate of increase of among-line variance per generation were used as estimates of mutational variance. These estimates are $\hat{V}_m$ per second chromosome, which is 40% of the genome, so the estimate of per-genome $\hat{V}_m$ is 2.5 times this. An important parameter for determining properties of mutation-selection balance of quantitative traits is V_m/V_e. This ratio was estimated by dividing the esti-

TABLE 2

Among-line variance components

	Unadjusted				Weight adjusted			
Gen	11	22	33	44	11	22	33	44
WT	0.0	0.096 ± 0.032	0.128 ± 0.049	0.164 ± 0.042	—	—	—	—
PRO	0.0	0.086 ± 0.031	0.0	0.075 ± 0.026	—	—	—	—
TRI	0.0	0.027 ± 0.022	0.058 ± 0.035	0.053 ± 0.021	0.056 ± 0.042	0.312 ± 0.063	0.303 ± 0.084	0.365 ± 0.082
GLY	0.0	0.048 ± 0.025	0.029 ± 0.029	0.070 ± 0.024	0.0	0.293 ± 0.061	0.150 ± 0.053	0.292 ± 0.068
FAS	0.0	0.0	0.0	0.007 ± 0.012	0.187 ± 0.073	0.317 ± 0.064	0.429 ± 0.109	0.784 ± 0.168
G6PD	0.084 ± 0.049	0.085 ± 0.031	0.062 ± 0.036	0.130 ± 0.036	0.324 ± 0.105	0.312 ± 0.064	0.414 ± 0.106	0.407 ± 0.089
GP	0.0	0.0	0.0	0.006 ± 0.013	0.022 ± 0.034	0.256 ± 0.055	0.098 ± 0.043	0.204 ± 0.051
GPDH	0.0	0.0	0.0	0.015 ± 0.014	0.260 ± 0.089	0.120 ± 0.035	0.271 ± 0.077	0.332 ± 0.074
GS	0.0	0.062 ± 0.028	0.0	0.249 ± 0.059	0.071 ± 0.045	0.394 ± 0.076	0.082 ± 0.039	0.696 ± 0.142
HEX	0.074 ± 0.047	0.0	0.002 ± 0.024	0.079 ± 0.027	0.306 ± 0.101	0.166 ± 0.042	0.101 ± 0.043	0.394 ± 0.088
ME	0.0	0.103 ± 0.033	0.0	0.054 ± 0.021	0.103 ± 0.053	0.347 ± 0.068	0.204 ± 0.064	0.253 ± 0.059
PGD	0.0	0.0	0.011 ± 0.026	0.028 ± 0.017	0.288 ± 0.095	0.208 ± 0.049	0.215 ± 0.066	0.257 ± 0.061
PGI	0.058 ± 0.044	0.046 ± 0.028	0.0	0.0	0.291 ± 0.098	0.258 ± 0.062	0.266 ± 0.076	0.402 ± 0.094
PGM	0.036 ± 0.036	0.0	0.0	0.100 ± 0.033	0.158 ± 0.065	0.119 ± 0.035	0.204 ± 0.064	0.388 ± 0.088
TRE	0.015 ± 0.033	0.036 ± 0.025	0.098 ± 0.043	0.212 ± 0.052	0.542 ± 0.156	0.648 ± 0.114	0.264 ± 0.076	0.582 ± 0.122
ADH	—	—	—	0.360 ± 0.097	—	—	—	0.787 ± 0.181

Figures are the restricted maximum likelihood estimates of the percentage ± SE of the nonerror variance attributed to LINE as determined by the SAS procedure VARCOMP.

TABLE 3

Analysis of variance and analysis of covariance of all traits after 44 generations of mutation accumulation

Trait	Day	Line	Day * Line	Vial	Error
		Unadjusted Mean Squares			
WT	0.04*	0.004‡	0.006‡	0.003‡	0.001
PRO	94.59‡	18.28‡	9.92	11.34‡	10.42
TRI	387.71†	112.94‡	74.08	79.38‡	32.75
GLY	0.63	12.43‡	10.79‡	8.49†	2.06
FAS	4.78†	2.48‡	1.66	2.19‡	1.74
G6PD	14.44‡	1.23‡	0.75‡	0.45‡	0.13
GP	59.81‡	0.46	0.44	1.19‡	0.11
GPDH	4154.29‡	124.28	46.83	137.49‡	33.23
GS	4.02	0.92‡	0.18	0.19‡	0.07
HEX	2.69	1.30‡	0.95‡	0.84‡	0.17
ME	214.52‡	5.61‡	4.48‡	4.09‡	1.01
PGD	3.75‡	0.09	0.18‡	0.12‡	0.05
PGI	21.72‡	2.58	3.25	7.76‡	1.39
PGM	23148.03‡	607.50‡	150.55	187.48‡	42.41
TRE	0.79	5.08‡	1.51	1.74‡	0.39
ADH	14.23‡	5.56‡	1.29‡	1.18‡	0.46
		Weight-adjusted Mean Squares (ANCOVA)			
TRI	802.20‡	88.12‡	62.25	66.44‡	30.93
GLY	10.55	9.34‡	7.82‡	6.49‡	1.73
FAS	4.66	2.47‡	1.66	2.19‡	1.75
G6PD	8.82‡	0.90‡	0.61‡	0.38‡	0.12
GP	43.06‡	0.40	0.42	1.10‡	0.10
GPDH	2566.94‡	128.24	43.29	132.87‡	30.92
GS	2.39‡	0.81‡	0.13	0.17‡	0.07
HEX	0.18	1.14‡	0.59	0.72‡	0.14
ME	130.45‡	3.98	3.59‡	3.59‡	0.88
PGD	1.98‡	0.09	0.15‡	0.11‡	0.05
PGI	6.38	2.34	2.45	7.65‡	1.32
PGM	18518.40‡	568.88‡	109.89	179.12‡	40.39
TRE	1.01	3.60‡	0.91	1.41‡	0.32
ADH	6.57†	5.23‡	1.10‡	1.05‡	0.45

WT was a significant covariate for all traits except FAS. PRO was a significant covariate in all but FAS, GS, and ADH. Degrees of freedom for all traits were Day (1), Line (51), Day*Line (37), Vial (346) and Error (791). * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

mate of $\hat{V}_m$ by an estimate of within-line variance ($\hat{V}_e$) pooled across generations. This calculation was appropriate because $\hat{V}_e$ was found to be homogeneous across generations. As described in MATERIALS AND METHODS, a resampling approach was used to obtain standard errors of estimates of $\hat{V}_m/\hat{V}_e$. Table 4 presents the results of these estimation procedures and illustrates the magnitude of deviation in $\hat{V}_m$ among traits. The bootstrap confidence intervals suggest significant increases in among-line variance, consistent with the ANOVA and ANCOVA of Table 3.

Accumulation of genetic covariance: Consistent with the idea that many of the mutations had pleiotropic effects, by generation 44 significant covariance had accumulated for many of the pairs of traits. By plotting the line means for pairs of characters for the initial founder line and for the generation 44 data, the pattern of dispersion and covariance can be seen (Figure 4). Although among-line covariance is expected to increase over time, within-line error variance is not expected to increase. Figure 5 reports the pattern of significant deviations from 0 for six different statistics whose bootstrap distributions were scored. First is the mutational covariance, Cov_m, calculated as slope of among-line covariance *vs.* generation. Many estimates of Cov_m deviated significantly from 0, with a tendency toward positive covariance, but many significantly negative pairs also were seen. The regression coefficient of within-line covariance (second panel) generally did not deviate from 0. The pooled within-line covariance, calculated by pooling across generations, showed many significant departures from 0, and it is striking to note that every

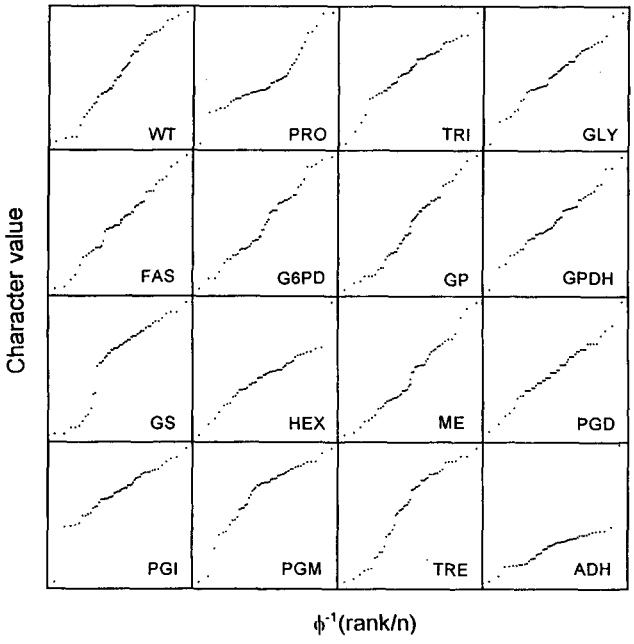


FIGURE 3.—Normal probability plots of all 16 characters at generation 44 of mutation accumulation. Each point represents a line mean. The *X*-axis is the inverse cumulative distribution function for the rank and the *Y* axis is the sorted rank.

TABLE 4

Estimates of mutational variance ($\hat{V}_m$) and the mutation variance scaled to environmental variance ($\hat{V}_m/\hat{V}_e$)

Trait	$\hat{V}_m \times 1000$	$\hat{V}_e$	$\hat{V}_m/\hat{V}_e \times 1000$
WT	0.0	0.0014 ± 0.0001	9.4 ± 1.3
PRO	49.9 ± 23.4	13.107 ± 2.343	3.8 ± 1.6
TRI	241.0 ± 40.9	32.875 ± 1.418	7.3 ± 1.2
GLY	24.5 ± 3.4	3.543 ± 0.132	6.9 ± 0.9
FAS	7.2 ± 2.1	1.613 ± 0.116	4.5 ± 1.4
G6PD	1.5 ± 0.2	0.196 ± 0.008	7.6 ± 01.3
GP	1.6 ± 0.4	0.410 ± 0.027	3.9 ± 1.1
GPDH	293.3 ± 54.0	47.077 ± 2.346	6.3 ± 1.3
GS	1.2 ± 0.2	0.119 ± 0.006	9.7 ± 01.4
HEX	1.6 ± 0.5	0.384 ± 0.025	4.1 ± 1.3
ME	6.5 ± 1.8	1.734 ± 0.079	3.7 ± 1.1
6PGD	0.2 ± 0.1	0.078 ± 0.004	2.6 ± 1.2
PGI	1.3 ± 2.3	3.013 ± 0.106	0.4 ± 0.8
PGM	564.9 ± 96.8	90.367 ± 3.690	6.3 ± 1.1
TRE	3.0 ± 5.0	1.323 ± 0.230	2.8 ± 4.0
ADH	12.1 ± 1.3	0.429 ± 0.066	28.9 ± 5.6

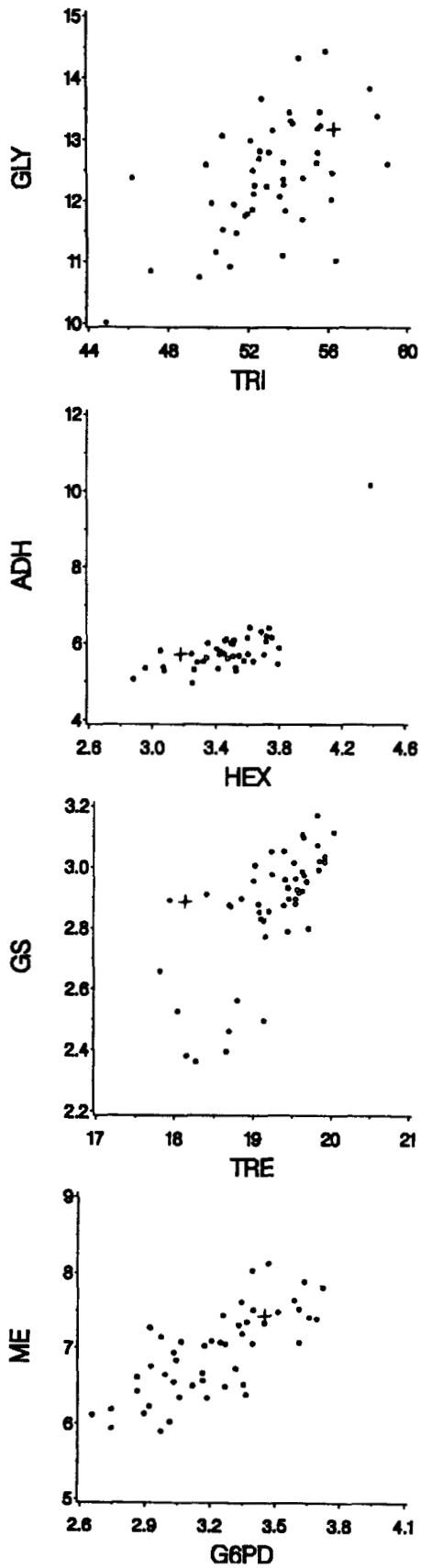
$\hat{V}_m$, slope of among-line variance *vs.* generation and represents the inflation of variance per generation among lines; $\hat{V}_e$, within-line environmental variance averaged across generations; $\hat{V}_m/\hat{V}_e$, ratio of $\hat{V}_m$ to $\hat{V}_e$. Values are mean ± SD across the 1000 bootstrap samples.

significant environmental covariance is positive. The bottom left panel of Figure 5 is $\text{Cov}_m/\text{Cov}_e$, the scaled mutational covariance. Next, the mutational correlation, r_m exhibited a striking lack of change across generations. The last panel in Figure 5 shows that many pairs of traits exhibited significant mutational correlation.

DISCUSSION

Crude enzyme activities are influenced by variation in any of a number of steps in gene expression, including transcription, transcript processing, message stability, translation, posttranslational processing and the catalytic activity of the final enzyme. This observation makes it more plausible that the polygenic mutation rate of enzyme activities can be as high as the data indicate. Although most mutations in a structural gene reduce the efficacy of the enzyme product, mutations that affect enzyme activity may do so by influencing gene expression at any of several levels. These effects can occur on positively or negatively acting regulatory pathways, and there is no way *a priori* to know which is more likely to suffer mutations. The data show significant increases in among-line variances with very little change in the mean activities, suggesting a nearly equal chance for positive and negative regulatory changes. The characters in this study differed widely in the rate at which mutational variance accumulated, as quantified by estimates of V_m/V_e. The magnitude of variation in mutational parameters suggests that the genes encoding the enzymes differ dramatically in the structure of their regulatory variation. The rapid accumulation of covariance among characters further supports the idea that most of the mutations have pleiotropic effects on many of the characters. The distributions of mutational effects in this study were acceptably fitted by a normal distribution in 10 of the 16 characters. Three of the exceptions were leptokurtic, and the remaining exceptions had significant outliers (elimination of outliers resulted in acceptable fits to normal distributions). Inference of the distribution of effects per mutation requires fitting a model, and KEIGHTLEY (1994) provided the machinery to obtain parameter estimates by maximum likelihood. He found that the distribution of mutational effects for viability was highly leptokurtic, but estimates of mutation rates were strongly confounded with other parameters in the model, such as those specifying the distribution of mutational effects.

The heritability of these characters varies from 0.14 to 0.57 (CLARK 1990), so the range of V_m/V_e is expected to reflect the range of V_m/V_g across characters. Wide variation in estimates of V_m/V_g suggests that the force of apparent selection differs among characters, because the intensity of apparent selection is approximately equal to the ratio of the variance introduced by mutation each generation to the equilibrium genetic variance (KONDRASHOV and TURELLI 1992). It should be



noted, however, that this claim is true for only some models of pleiotropy.

The pleiotropic effects of mutations can be inferred directly from the accumulation of covariance among characters in a mutation-accumulation experiment. In the absence of selection and assuming a population sufficiently large that effects of genetic drift can be ignored, the equilibrium covariance should be proportional to that seen among new mutations. Many investigators have sought to infer pleiotropic or epistatic interactions among traits from the patterns of genetic covariance, but this is not as simple as it might seem (HOULE 1991). For example, two traits may be determined by two interacting pleiotropic loci but have 0 genetic covariance at equilibrium (GIMELFARB 1986). Where the population falls in the parameter space of mutation rates and mutational effects also can be important. With pleiotropic mutations and stabilizing or directional selection, the "house-of-cards" and continuum-of-alleles models produce very different patterns of equilibrium genetic covariance (SLATKIN and FRANK 1990).

Several studies have quantified variation and covariation in enzyme activities in experiments designed to estimate parameters of quantitative genetics. LAURIE-AHLBERG *et al.* (1980, 1982, 1985) examined sets of second and third chromosome replacement lines of *D. melanogaster* and found that most of the enzymes showed significant variation in activity. Most of the variation was found to occur at sites distinct from the structural locus. A number of significant genetic correlations were detected (WILTON *et al.* 1982), and it was noted that enzymes sharing glucose-6-phosphate as a substrate were positively intercorrelated. In the pentose phosphate shunt, the positive correlation between G6PD and 6PGD activities is one of the most consistently observed (COCHRANE *et al.* 1983; MIYASHITA and LAURIE-AHLBERG 1984; CLARK and KEITH 1988). The observation of correlation between G6PD and 6PGD activity, when the mutational correlation did not differ from 0, suggests that natural selection shaped the bivariate distribution of these two activities. Studies of extant variation provide an excellent backdrop against which to compare the distribution of mutational effects.

If the mutational covariance matrix and the genetic covariance matrix as estimated from an equilibrium population differ from one another by a degree greater than expected under a model of neutral genetic drift, we might infer that the departure is caused by natural selection. This motivates a formal statistical comparison

FIGURE 4.—Scattergrams of line means at generation 44 of mutation accumulation. Means of each independent mutation-accumulation line are shown (•) as is the mean of the initial founder line (+).

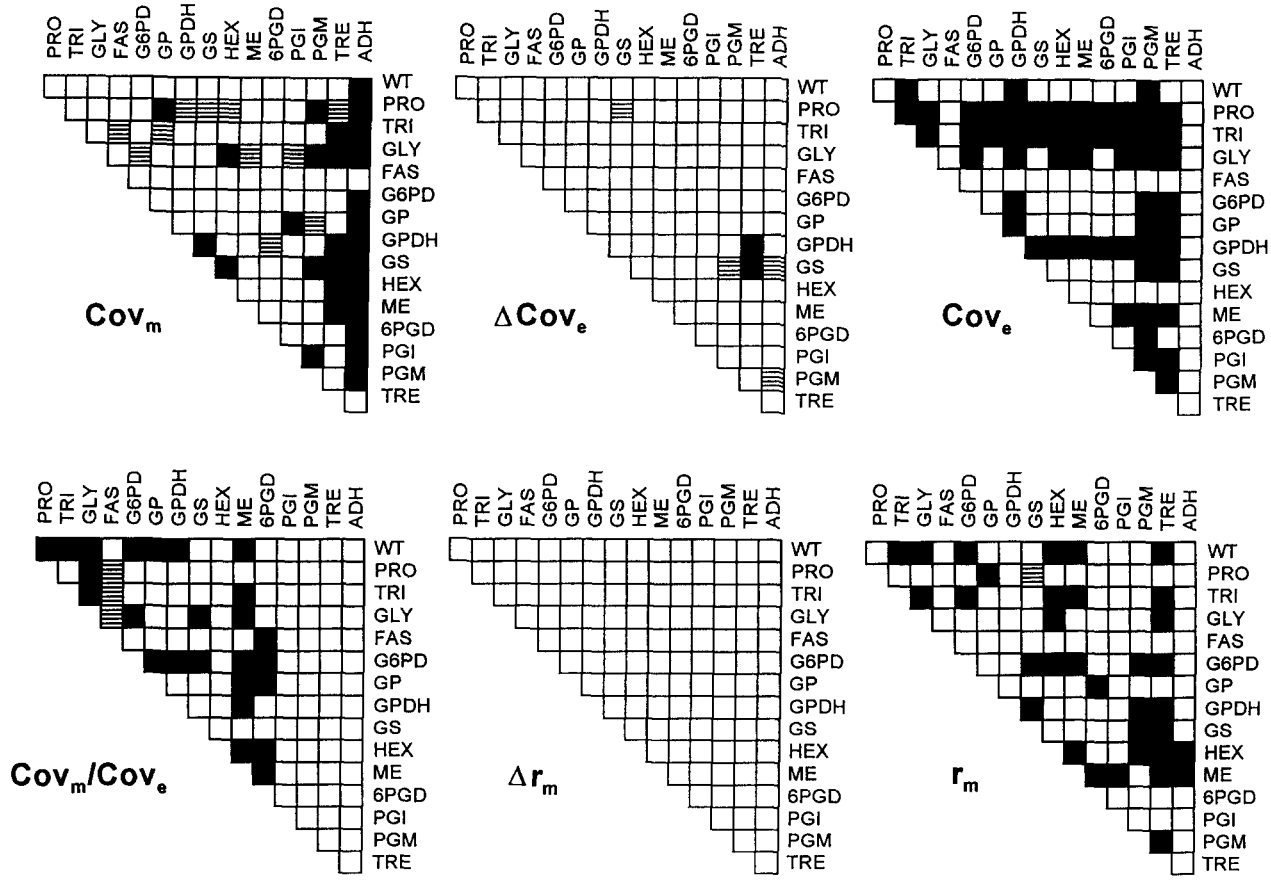


FIGURE 5.—Estimates of several quantities related to the genetic covariance of newly arisen mutations. Cov_m is the mutational covariance, estimated as the slope of the covariance over generations. ΔCov_e is the rate of change of environmental covariance, estimated as the slope of the within-line covariance regressed on generation. Cov_e is the estimate of environmental covariance pooled over generations. Cov_m/Cov_e is the analog to V_m/V_m, and is the mutational covariance scaled to the environmental covariance. Δr_m is the rate of change of mutational correlation (expected to be stable over generations) and r_m is the genetic correlation of new mutations pooled over generations. For all plots, positive correlations (■) and negative correlations (▤) are shown with $P < 0.05$ in a sequential Bonferroni test of experiment-wide significance.

of covariance matrices. One caveat in comparing mutational and equilibrium covariance matrices is that genetic covariance is a property of the genetic variation sampled from a particular population.

In previous reports, we have tried to eliminate the effects of weight and protein content as covariates that were not themselves of intrinsic interest (CLARK and KEITH 1988, 1989; CLARK 1989, 1990). Novel mutations may alter the normal allometric relations between body size and enzyme activities, so in this report analyses were done with and without correcting for size. The mutation-accumulation lines showed significant deviation in weight and total protein by generation 44, and several of the traits exhibited significant correlations with these two traits. In tests of variation among lines in single characters, adjusting for effects of size changed the qualitative conclusion in only the case of HEX, which was significantly heterogeneous in raw scores but not in weight-adjusted scores. When weight and total protein were treated as covariates, the partial correlations among traits were only slightly decreased from the unpartialed correlations. Allometric slopes with weight are less than one for all characters, and the slopes differ significantly among traits (CLARK and WANG 1994). Furthermore, the allometry between lines is different from that within lines. In the ANCOVA, weight explains more of the variance than does protein, suggesting that the lines differ more in water content than in protein content. Finally, if one considers that the mutation-accumulation lines are suffering mutations that decrease fitness, it is clear that the fecundities will vary from line to line (HOULE *et al.* 1994). Our only control for density effects was to rear flies in vials with the same number of adults, so it could well be that the significant weight effects we saw were merely a consequence of more fecund lines producing more

crowded cultures, and hence smaller adults. Additional hypotheses relating the characters measured in this study to components of fitness will be tested when our analyses are merged with those of HOULE *et al.* (1994).

The observation of frequent, pleiotropic mutations has interesting consequences for metabolic characters. Simple models for the evolution of enzyme activity based on principles of metabolic control theory suggest that, in situations like bacterial chemostats where metabolic flux might be maximized by selection, enzyme activities evolve to the point where further changes in activity have negligible change in flux (HARTL *et al.* 1985; DEAN *et al.* 1988). The relationship between enzyme activity and flux has been demonstrated in higher organisms as well. A particularly clear case is that of G6PD activity and pentose phosphate shunt flux in Drosophila (LABATE and EANES 1992). It has been argued that stabilizing selection also may be important to metabolic characters, and whether flux or metabolite concentration is selected, the equilibrium distribution of allelic effects is expected to be skewed (CLARK 1991). Such skewness has important consequences to the equilibrium genetic variance maintained in mutation-selection balance (BARTON and TURELLI 1987).

Just as the relationship between activity and metabolic flux can provide an explanation for dominance (KACSER and BURNS 1981), quantitative genetic models of metabolic control can provide a means of generating stabilizing selection on enzyme activity when the target of selection is a property of the pathway (BEAUMONT 1988). The same mechanism that results in dominance of a single metabolic trait also is expected to produce consistent degrees of dominance for all pleiotropically related metabolic traits (KEIGHTLEY and KACSER 1987). An empirical test of this prediction requires an analysis of the dominance of the accumulated mutations. These experiments are underway. Although metabolism seems to involve a highly interactive system of characters, the equilibrium epistatic variance is expected to be very small (KEIGHTLEY 1989). Metabolic control theory provides a simple model for mapping many discrete characters (enzyme activities) into a global property (such as flux), which may be associated with fitness (CLARK 1991). Two mutations are most likely to be synergistic in their effects on fitness if they both influence the same enzyme (SZATHMARY 1993), providing a case in which pleiotropy influences the likelihood for epistasis. A likely cause for pleiotropy in metabolic characters is variation in transcription factors common to more than one gene. In the case of two classic examples of enzyme activity variation, variation in transcription was shown to be the cause among different lines bearing Adh^S alleles of *D. melanogaster* (THOMPSON *et al.* 1991), and in *Ldh-B* expression in different populations of *Fundulus heteroclitus* (CRAWFORD and POWERS 1992).

A population in mutation-drift balance would be expected to exhibit a pattern of correlations that reflects the mutational correlations. A very small population, undergoing pronounced random drift, or a population subjected to natural selection may have an equilibrium correlation pattern that differs from the mutational correlation pattern. Previous studies revealed that G6PD and 6PGD exhibit a strong positive phenotypic and genetic correlation (WILTON *et al.* 1982), yet we find no evidence for such correlated effects among new mutations. There was also a strong pattern of positive correlation among enzymes that share G6P as a substrate (PGI, PGM, HEX and G6PD), although this pattern is much weaker among new mutations. These observation motivate a formal statistical test for the differences between estimates from an equilibrium population and the mutational patterns seen here.

The importance of pleiotropy in determining the properties of a mutation-selection balance depend on the covariance of mutational effects on each trait with fitness (KEIGHTLEY and HILL 1988, 1992). Although there is an extensive literature reporting analyses of associations between enzyme activities and fitness-related phenotypes (recently reviewed by WATT 1994), none of these studies report measures of fitness effects of newly arisen mutations. Comparison of these results from an extant, equilibrium population with those of newly arisen mutations, including *P*-element generated mutations (CLARK *et al.* 1995), may provide important insights into the past operation of natural selection.

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