BRIEF COMMUNICATIONS

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VARIATION IN PLEIOTROPY AND THE MUTATIONAL UNDERPINNINGS OF THE **G**-MATRIX

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Abstract.—The pattern and extent of pleiotropic gene action can contribute substantially to the internal structure and shape of the additive genetic variance-covariance matrix (**G**)—a key determinant of evolutionary trajectories. We use data from our study (Estes et al. 2004) on the univariate effects of mutation in a mismatch-repair-defective strain, *msh-2,* of *Caenorhabditis elegans* to address the impact of increasing levels of selection on the magnitude and pattern of genetic covariance due to new mutations. Mutational covariances between three life-history traits are shown to exhibit a weak pattern of decline with increasing population size (increasing selection), while the orientation of mutational matrices remains reasonably constant. This suggests that mutations with smaller effects on fitness may tend to be slightly more confined in their influence than large-effect mutations (i.e., small-effect mutations reduce the magnitude of covariation between characters), but do not change the direction of this covariation.

Key words.—Mismatch repair, *msh-2*, mutation accumulation, mutational covariance, population size, selection.

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Interest in how character complexes evolve has persisted since the first formal attempts at treating the issue by F. Galton and W. Weldon, which involved measuring correlations between morphological characters within species—correlations that Weldon proposed would ultimately shed light on the evolutionary relationships among higher taxa (Provine 1971). Now, over a century later, the usefulness of **G**, the matrix of genetic variances and covariances that reflects the genetic architecture and evolutionary history of character complexes, for forecasting responses to and inferring historical patterns of selection has emerged as a major problem in evolutionary biology (e.g., Arnold and Phillips 1999; Steppan et al. 2002). The primary obstacle concerns the extent to which patterns of genetic covariance remain constant over evolutionary time and will thus continue to reflect relationships among organisms. Addressing this question will require a vastly improved understanding of the behavior and evolution of the underpinnings of **G** (e.g., Barton and Turelli 1989; McGuigan 2006; Phillips and McGuigan 2006).

Determinants of the off-diagonal components of **G**-matrices reflect the patterns of pleiotropic allelic effects and gametic-phase disequilibrium generated by physical linkage and/or selection. Genetic associations due to linkage disequilibrium are expected to erode over time, leaving pleiotropic mutation as the most enduring source of covariance among phenotypic traits (Lande 1980, 1984). The pattern of pleiotropy will therefore ultimately impact multivariate responses to both genetic drift and selection—either facilitating or precluding evolution depending on details of the adaptive landscape (e.g., Lande 1979; Jones et al. 2003; A. G. Jones, S. J. Arnold, and R. Bürger, unpubl. ms.). In addition, mutational correlations can provide a window into the modular organization of genotypes and phenotypes.

The contributions to **G** by pleiotropic mutation are described by **M**, the matrix of mutational variances and covariances (Lande 1980; Camara et al. 2000; Phillips and McGuigan 2006). **M** can be estimated using classical quantitative genetic approaches involving phenotypic variance partitioning in conjunction with mutation accumulation or selection experiments (e.g., Estes et al. 2005). The limited data available indicate that the distribution of mutational effects is probably L-shaped with the most frequently occurring mutations producing only minor effects on fitness (Estes et al. 2004 and references therein). Although we have some knowledge about the covariance patterns of the most readily detected mutations (those with large effects) (Estes et al. 2005), we need to know whether the pattern of pleiotropy is comparable between mutations of large and small effect. For example, if mutations of large effect also produce more widespread effects (i.e., influence a greater number of characters) than those of smaller effect as might be expected, this will have important implications for the predicted progression of evolutionary walks (e.g., Otto 2004).

Because most mutation accumulation experiments begin at a high fitness state, the majority of new mutations will be deleterious, potentially strongly so. If these mutations have generalized effects on many traits (i.e., the organism is simply ''sick'') then differential accumulation of multiple mutations among lines (particularly mutations of large effect) will tend produce a positive correlation among traits (Fig. 1) (see also the discussion in Estes et al. 2005). We call this ''general pleiotropy'' because, although these are pleiotropic mutations in the strict sense, they do not reflect the direct functional associations that are often implied by pleiotropic gene action. In addition to these widespread effects, there is an alternative pattern of mutational pleiotropy that could occur because of the direct functional linkage generated by new pleiotropic mutations (Fig. 1). We call this ''specific pleiotropy'' because these mutations are more likely to reflect specific associations between traits rather than generalized effects across the whole organism. General pleiotropic mutations of large detrimental effect would be expected to be rapidly eliminated by natural selection, but both types of pleiotropy are still relevant for theories that deal with deleterious mutation per se (e.g., the evolution of sex) and for understanding the evolution of genetic covariance structure,

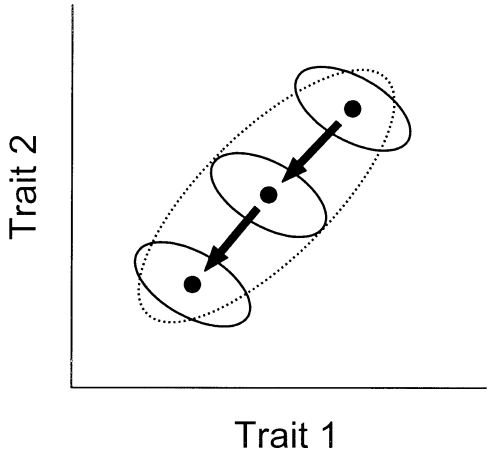

FIG. 1. Distinguishing general versus specific pleiotropy in mutation accumulation experiments. Because mutation accumulation experiments start at a point of relatively high mean fitness (first dot on right), mutations that affect the general state of the organism will drive down the values of all traits (arrows), leaving a generally positive estimate of mutational covariance (dashed oval). However, there may be another, more subtle pattern of mutational covariance generated by mutations with effects that are specific to the suite of traits under study (solid ovals). Allowing selection to eliminate the general-effect mutations potentially allows the specific-effect mutations to be revealed.

as for example reflected in Lande's (1980) use of the mutational covariance parameter (Phillips and McGuigan 2006). If the potentially more subtle effects of specific pleiotropies cannot be well estimated in traditional mutation accumulation experiments as suggested by some (Keightley et al. 2000), then how are we to understand their role in the long-term evolution of **G**?

We endeavor to answer this question using data from a previous experiment (Estes et al. 2004) that uses mutation accumulation in populations of different sizes to variably cull the mutations of large effect that are most likely to generate general pleiotropy, to more fully reveal the entire spectrum of pleiotropic mutational effects. Specifically, we ask whether such selection alters the magnitudes of mutational covariances between trait pairs or the orientation or overall shape of **M**.

MATERIALS AND METHODS

Mutation Accumulation Lines

We use data from our recent study (Estes et al. 2004) in which replicate lines of a mismatch-repair-defective strain of *Caenorhabditis elegans* were maintained in an array of population sizes to assess the influence of selection on newly arising mutations and thereby infer the distribution of their effects on fitness. Briefly, two mutation accumulation (MA) experiments were conducted using the mutator strain, *msh-2*, with a (now) known molecular mutation rate and spectrum of effects. (Denver et al. [2004, 2005] found an increased rate of indels in A:T microsatellites in *msh-2* compared to repair-proficient worms and, for nonmicrosatellite sequences, a higher proportion of base substitutions than indels—opposite the pattern observed in DNA repair-proficient worms. However, a comparable level of transition bias was found to exist for base substitutions in both *msh-2* and repair-proficient strains.) The first experiment was initiated with three sets of 50 lines of *C. elegans* propagated by self-fertilization at population sizes of 1, 5, and 25 individuals for 10 generations. In the second experiment, lines were maintained in the same manner for 20 generations in population sizes of either 1, 2, 3, or 10 individuals. At the end of the divergence periods lines were assayed for four fitness-related characters: progeny production both early and late in the life cycle, *r*, and survival to maturity. ''Early productivity'' is the number of offspring produced on the first two days of reproduction combined, whereas ''late productivity'' is the number of offspring produced on the third and fourth days of reproduction. See Estes et al. (2004) for further details.

TABLE 1. Means and average within-line variances for each character measured for control populations in the 10- and 20-generation fitness assays.

Trait	Early	Late	*r*	Survival
	Assay 1 (10 generations)			
Mean	95.02	40.99	0.869	0.930
Variance	3559	839.5	0.087	0.055
	Assay 2 (20 generations)			
Mean	77.41	43.05	0.792	0.850
Variance	3740	1106	0.155	0.133

Analyses

Mutational (co)variance estimation.—Mutational covariances and their standard errors for each pair of traits were obtained by least-squares estimation of covariances among recombinant inbred lines with a delete-one-family jackknife procedure. The mutational (co)variances reported are thus the averages of the jackknifed estimates for the dataset. Prior to obtaining mutational covariance estimates, data from the experimental lines were normalized to the environmental (average within-control line) variation present for each trait, so that the covariances would be comparable to one another. Specifically, trait values were divided by the ancestral control standard deviation for that trait (Table 1). All covariance component estimations were carried out using the software package H2jack (Phillips 2002).

Matrix comparisons.—For a more refined comparison of the correlated effects of mutations across population size, we used a method of matrix comparison based on Flury's (1988) model of common principal components that allows us to evaluate at which level in a hierarchy of possible relationships two or matrices can no longer be considered similar (Phillips and Arnold 1999). Comparisons of mutation matrices were conducted using the software package CPCrand (Phillips 1998), which provides significance testing of the Flury hierarchy via a randomization procedure. Briefly, the program determines the eigenstructure of the two or more mutational matrices being compared, then randomizes experimental lines between treatments and asks whether the original matrices are more similar than would be expected by chance. For a complete view of mutational matrix relationships, we evaluated at each level of the hierarchy: (1) matrices for all pop-

ulation-size treatments simultaneously for each assay, and (2) matrices for the $N_e = 1$ treatment compared with that of the other population sizes for both assays. We use the ''jump-up'' approach (Phillips and Arnold 1999) with all hypotheses of matrix relationship tested against a model of unrelated structure.

Finally, to visualize and compare the mutational covariance patterns of different population-size treatments, 95% confidence ellipses of the bivariate variance-covariance matrices were constructed for each pair of traits for all population-size treatments as in Phillips et al. (2001). Principal components of the mutation matrices were calculated using jackknifed mutational (co)variance estimates from transformed data (see above) and used to orient the ellipses on a plane. Distance along each principal axis was calculated as 1.96 times the square root of the eigenvalue associated with that particular axis. This procedure allows us to evaluate two-dimensional projections of the mutational matrices.

Results

Mutational Variance

As previously established (Estes et al. 2004), significant levels of mutational variance had accumulated in lines maintained by single individual bottlenecking after both 10 and 20 generations of divergence (Table 2). Puzzling however, is that estimates of mutational variance for the $N_e = 1$ treatment after 20 generations are consistently lower, though often of the same order, than those for only 10 generations. (This is true for variances calculated from both transformed and raw data.) The reason for this is unknown, but it is consistent with deleterious mutation accumulation in the control population between the 10- and 20-generation experiments. The 20-generation assay was conducted second such that the ancestor of lines in this assay experienced additional single-individual bottlenecks prior to the initiation of the experiment. Both the control mean phenotypes and among-control line variances were lower for this assay than for the 10-generation assay (Table 1; see also fig. 3 in Estes et al. 2004). Experimental lines in the second experiment being initiated from a less fit ancestor could result in the mutated lines being less different from the average line, thus exhibiting reduced accumulated variance. In other words, because mutations tend to decrease these life-history traits, and there is a definite lower bound of no reproduction, moving the mean closer to zero reduces the amount of total variation on an absolute scale. (Note that values for V_m [mutational variance] reported here are scaled by the number of generations of mutation accumulation—10 or 20—and are thus not directly comparable to the among-line variances reported in fig. 3 of Estes et al. [2004] which are unscaled.) Significant mutational variances were also detected for all traits in the $N_e = 2$, 3, and 5 treatments, but only for a few traits in the larger population-size treatments (Table 2).

Mutational Covariance

Significant estimates of mutational covariance were detected for all pairs of traits in the $N_e = 1$ and 2 treatments, but for very few trait pairs in the larger population size lines (Table 2). Like the mutational variances, mutational covariances for the $N_e = 1$ treatment in second assay tend to be slightly lower than those for $N_e = 1$ lines in the 10-generation experiment. The covariances of mutational effects are positive without exception, but exhibit a pattern of modest increase between $N_e = 1$ and 2, and moderate decline in magnitude thereafter with increasing population size in both experiments (Fig. 2). This pattern mirrors that of the mutational variance. This is true for all trait pairs, but the trend is much less pronounced for early-late productivity—our only character combination certain to be uninfluenced by overlapping measurement. (Since r incorporates progeny production and survival, positive covariance between these two pairs of traits will be inflated to some degree.) Mutational covariance between early and late productivity begins at a relatively low value in the $N_e = 1$ lines and remains low in the larger population-size treatments, although there is considerable noise associated with this pattern. Note, however, that mutational covariances are generally not significantly different than zero in the $N_e = 3$ to 25 range (Table 2).

Matrix Comparisons

We find general support for shared structure among mutation matrices estimated for different population-size treatments (Table 3). For the second experiment, the hypothesis of shared principal components (full CPC) cannot be ruled out ($P > 0.057$ for all trait pairs), whereas proportionality is rejected, although sometimes only marginally ($P < 0.035$ for all character combinations). However, a scenario of matrix equality is strongly rejected. We thus find the most support for a scenario in which mutation matrices share principal components, but have different eigenvalues. These findings hold true whether all matrices are compared simultaneously, or when each population-size treatment is compared to the smallest ($N_e = 1$).

The first experiment is less informative because it spanned half as many generations as the second. In addition, we were forced to omit the $N_e = 25$ treatment from the analysis due to singularities in the matrix. This almost certainly results from insufficient time having elapsed for significant levels of mutational variance to accumulate. The Flury method is incapable of dealing with singular matrices or those with zero or negative eigenvalues. Rather than employ matrix bending (Phillips and Arnold 1999), we omitted this matrix from the analyses. Hence, we compare only the $N_e = 1$ treatment to $N_e = 5$ and are unable to rule out that these matrices share two principal components, but reject the hypothesis of full CPC ($P = 0.021$).

As illustrated in Figure 3, all two-character mutation matrices generally maintain the same orientation as that of the $N_e = 1$ lines. In addition, matrices appear to largely retain their shapes while simply shrinking in at least one dimension with increasing population size. An exception seems to be the $N_e = 3$ treatment, particularly with respect to the early-late productivity and late productivity-r comparisons, which neither appear to correspond with respect to orientation nor to shrink compared to the $N_e = 1$ treatment.

Discussion

We examined the influence of selection on mutational covariances and mutational matrices using data from a previous

TABLE 2. Average mutational matrices for each population-size treatment for both the 10- and 20-generation (gen.) assays. The per-generation increase in mutational covariance for each pair of traits for control variance transformed (raw) data appear above (below) the diagonal; mutational variances calculated using transformed (top) and raw (bottom) data are shown on the diagonal. Standard errors are shown in parentheses. One, two, and three asterisks denote significant differences from zero at the 0.05, 0.01, and 0.001 levels, respectively.

		Trait mean	Early	Late	*r*	Survival
N_e = 1 (10 gens.)	early	58.31	0.028 (0.006)***	0.014 (0.005)**	0.036 (0.007)***	0.037 (0.008)***
			97.42 (21.28)***			
	late	30.35	24.30 (8.864)**	0.020 (0.006)***	0.022 (0.009)*	0.028 (0.013)*
				16.60 (4.983)***		
	r	0.587	0.607 (0.128)***	0.202 (0.057)***	0.055 (0.012)***	0.063 (0.015)***
					0.005 (0.001)***	
	survival	0.657	0.485 (0.119)***	0.184 (0.060)**	0.004 (0.001)***	0.076 (0.020)***
						0.004 (0.001)***
N_e = 1 (20 gens.)	early	40.83	0.007 (0.001)***	0.004 (0.001)***	0.009 (0.002)***	0.008 (0.002)***
			24.51 (5.008)***			
	late	17.36	9.555 (2.699)**	0.004 (0.001)***	0.006 (0.002)***	0.006 (0.002)**
				4.179 (1.080)***		
	r	0.523	0.222 (0.043)***	0.080 (0.021)***	0.014 (0.003)***	0.013 (0.003)***
					0.0022 (0.0004)***	
	survival	0.652	0.186 (0.045)***	0.069 (0.022)**	0.0019 (0.0005)***	0.014 (0.004)***
						0.0019 (0.0006)***
N_e = 2 (20 gens.)	early	44.43	0.007 (0.002)***	0.006 (0.002)*	0.009 (0.002)***	0.009 (0.002)***
			27.56 (7.855)***			
	late	24.57	11.59 (4.657)*	0.007 (0.003)*	0.008 (0.003)**	0.007 (0.003)*
				7.285 (3.089)*		
	r	0.546	0.209 (0.055)***	0.096 (0.033)**	0.013 (0.003)***	0.014 (0.003)***
					0.0020 (0.0005)***	
	survival	0.630	0.190 (0.052)***	0.090 (0.031)**	0.0021 (0.0005)***	0.017 (0.004)***
						0.0023 (0.0006)***
N_e = 3 (20 gens.)	early	58.39	0.003 (0.001)*	0.003 (0.002)	0.004 (0.002)	0.004 (0.002)
			11.93 (5.598)*			
	late	43.19	5.780 (4.948)	0.016 (0.005)**	0.007 (0.003)	0.008 (0.004)*
				17.54 (5.497)**		
	r	0.647	0.095 (0.049)	0.086 (0.043)*	0.006 (0.003)*	0.007 (0.003)
					0.0010 (0.0005)*	
	survival	0.700	0.094 (0.049)	0.092 (0.043)*	0.0010 (0.0005)	0.007 (0.004)*
						0.0010 (0.0005)*
N_e = 5 (10 gens.)	early	80.61	0.021 (0.005)***	0.009 (0.006)	0.021 (0.006)***	0.018 (0.006)**
			73.42 (17.29)***			
	late	50.55	13.63 (10.84)	0.024 (0.012)*	0.006 (0.007)	0.001 (0.008)
				20.26 (10.47)*		
	r	0.772	0.365 (0.101)***	0.105 (0.047)*	0.025 (0.008)**	0.025 (0.009)**
					0.0022 (0.0006)**	
	survival	0.824	0.249 (0.088)**	0.052 (0.039)	0.0017 (0.0006)**	0.026 (0.010)**
						0.0014 (0.0005)**
N_e = 10 (20 gens.)	early	75.20	0.003 (0.002)	0.001 (0.001)	0.002 (0.002)	0.002 (0.002)
			10.38 (6.737)			
	late	33.09	3.373 (2.639)	0.001 (0.001)	0.001 (0.002)	0.000 (0.002)
				1.445 (1.560)		
	r	0.738	0.048 (0.036)	0.005 (0.022)	0.003 (0.003)	0.003 (0.002)
					0.000 (0.000)	
	survival	0.785	0.042 (0.034)	0.002 (0.024)	0.000 (0.000)	0.004 (0.002)
						0.0010 (0.0003)
N_e = 25 (10 gens.)	early	89.06	0.010 (0.004)**	0.010 (0.004)*	0.005 (0.003)	0.003 (0.004)
			34.91 (12.53)**			
	late	44.02	18.14 (7.472)*	0.012 (0.006)*	0.010 (0.004)**	0.011 (0.005)*
				10.47 (5.123)*		
	r	0.851	0.091 (0.054)	0.071 (0.029)*	0.002 (0.002)	0.003 (0.004)
					0.000 (0.000)	
	survival	0.868	0.044 (0.057)	0.052 (0.031)	0.000 (0.000)	0.000 (0.000)
						0.000 (0.000)

study in which replicate lines of a mismatch-repair-deficient strain, *msh-2*, of *C. elegans* were maintained in an array of population sizes and thus increasingly intensive selection. It is probably obvious that the design of this experiment—particularly with regard to the length of the mutation accumulation phase and the choice of only one class of characters—is not optimal to adequately address the distribution of mutational covariance. Nonetheless, some important features of mutational covariance structure are established and groundwork is laid for necessary future work.

First, our results establish that the technique of laboratory mutation accumulation (MA) can provide a reasonable meth-

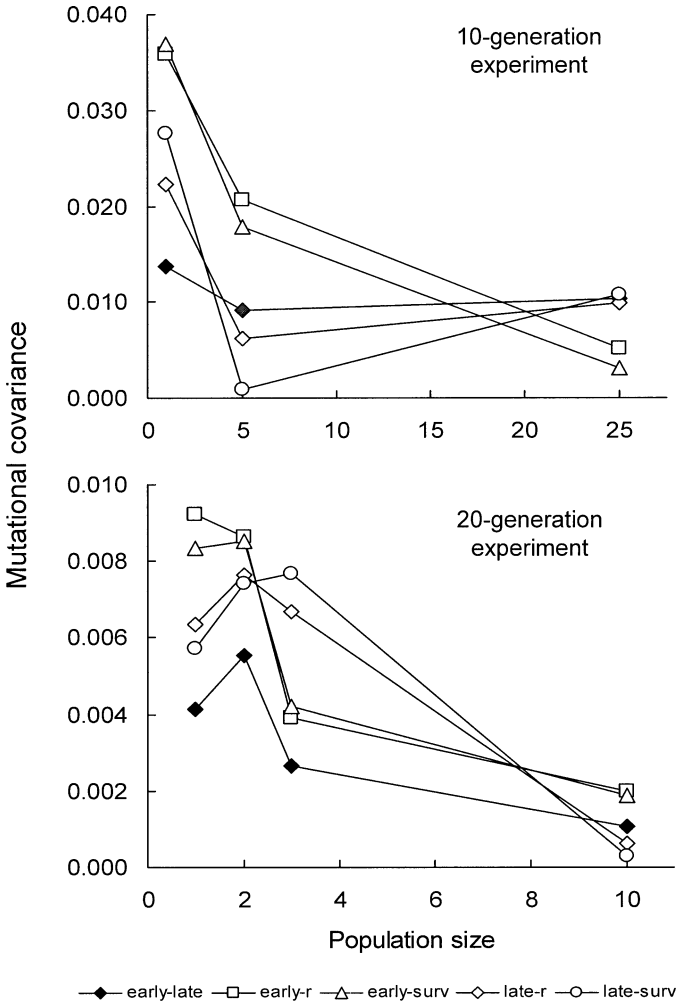

FIG. 2. Pattern of mutational covariance with increasing population size for the 10-generation (above) and 20-generation (below) assays. Note the changes in scale on both axes. The per-generation increase in mutational covariance for each pair of life-history traits was calculated from transformed data (see text). In general, the magnitude of mutational covariance declines with increasing population size.

od of assessing the distribution of covariance effects, just as it does for the univariate distribution of effects. Like the mutational variance (Estes et al. 2004), mutational covariance appears to shrink when selection is allowed; although with our current samples sizes, it is too early to draw any firm conclusion about the exact pattern of this decline. We found mutational covariances to be positive without exception, just as they were with the wild-type (N2) MA lines (Estes et al. 2005; note that the mutational covariance estimates in Estes et al. [2005] are not normalized by the number of generations). This implies that no net negative pleiotropy is inducing trade-offs at this level, neither for the full spectrum of naturally occurring mutations, nor for those generated by mismatch-repair deficiency alone.

As discussed extensively elsewhere (e.g., Phillips and Arnold 1999; Phillips et al. 2001; Steppan et al. 2002), matrices can be related to each other in a variety of ways: they can be identical, proportional, share some or all principal components, or share no principal components and thus have an unrelated structure (see fig. 1 in Steppan et al. 2002). We find general support for the idea that mutations passing through a narrowing selective sieve imposed by increasing population size exhibit reduced magnitudes of covariance, but probably maintain their basic covariance structure. This is supported by visualizing the relationships between matrices (Fig. 3). A caveat to the application of the Flury method employed here is that the significance tests of each level of the hierarchy are not independent; therefore, rejecting a hypothesis does not necessarily mean that the alternative is acceptable (Phillips and Arnold 1999). Additionally, as is true for all statistical tests, we can always fail to reject false hypotheses in the case of insufficient statistical power. However, since we are able to reject full CPC for the $N_e = 1$ and 5 comparison in the 10-generation assay, we should be able to reject the same hypothesis for the other population-size comparisons if the randomized matrices were truly different. These issues are one reason that visualizing matrices, even in simplified form, is useful.

Conclusion

The very high, positive mutational covariances observed in typical MA experiments (e.g., Estes et al. 2005 and references therein) may not provide an entirely accurate reflection of the nature of most mutations (Keightley et al. 2000). However, although estimated mutational covariances do decline somewhat with increasing selection, there seems to be no fundamental difference in the pleiotropic influence of mutations of smaller effect. This indicates that, at least for the fitness traits considered here, typical MA experiments (where

TABLE 3. *P*-values from a Flury hierarchical comparison of mutational matrices for different population-size combinations for the 10- and 20-generation fitness assays. Each hypothesis in the hierarchy is tested against the model of unrelated matrix structure. A significant value (i.e., $P < 0.05$) indicates that the particular hypothesis of matrix similarity is rejected. The hierarchy starts at the bottom (CPC(1)) and moves up from there, stopping when a specific hypothesis of matrix similarity is rejected.

	10-generation assay *P*-values	20-generation assay *P*-values			
Hierarchy	$N_e = 1, 5$	All	$N_e = 1, 2$	$N_e = 1, 3$	$N_e = 1, 10$
Equality	0.01195	0.00049	0.03945	0.00022	0.00745
Proportionality	0.00919	0.00130	0.03417	0.00033	0.02471
Full CPC	0.02114	0.05758	0.46030	0.16479	0.42891
CPC(2)	0.06342	0.16914	0.31709	0.30888	0.31104
CPC(1)	0.47702	0.36682	0.42236	0.16891	0.44028
Unrelated					

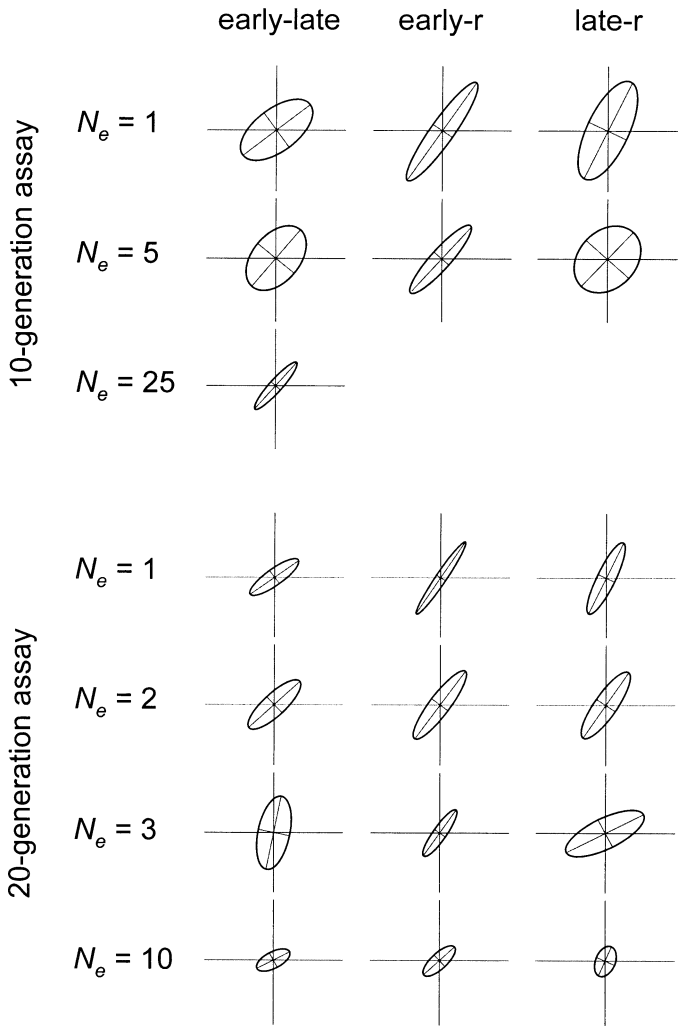

FIG. 3. Mutational covariance structure illustrated as 95% confidence ellipses for each population treatment for both the 10- and 20-generation experiments. No ellipse appears for two character combinations for the $N_e = 25$ group due to singularities in their mutational matrices. Overall, the pattern of mutational covariance is similar despite the increasing efficacy of natural selection with increasing population size.

$N_e = 1$ or 2) do not give too skewed a window into mutational covariance patterns likely to be present in nature. However, elucidating the role of mutation in building phenotypic correlations and their influence for the future evolution of such correlations awaits a bigger experiment that includes a suite of less intrinsically related phenotypic characters.

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