Age-Specific Properties of Spontaneous Mutations Affecting Mortality in *Drosophila melanogaster*

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Manuscript received June 9, 1997
Accepted for publication September 24, 1997

ABSTRACT

An analysis of the effects of spontaneous mutations affecting age-specific mortality was conducted using 29 lines of *Drosophila melanogaster* that had accumulated spontaneous mutations for 19 generations. Divergence among the lines was used to estimate the mutational variance for weekly mortality rates and the covariance between weekly mortality rates at different ages. Significant mutational variance was observed in both males and females early in life (up to ~30 days of age). Mutational variance was not significantly different from zero for mortality rates at older ages. Mutational correlations between ages separated by 1 or 2 wk were generally positive, but they declined monotonically with increasing separation such that mutational effects on early-age mortality were uncorrelated with effects at later ages. Analyses of individual lines revealed several instances of mutation-induced changes in mortality over a limited range of ages. Significant age-specific effects of mutations were identified in early and middle ages, but surprisingly, mortality rates at older ages were essentially unaffected by the accumulation procedure. Our results provide strong evidence for the existence of a class of polygenic mutations that affect mortality rates on an age-specific basis. The patterns of mutational effects measured here relate directly to recently published estimates of standing genetic variance for mortality in Drosophila, and they support mutation accumulation as a viable mechanism for the evolution of senescence.

As the source of all genetic variance, mutation provides the basis for both variation and response to selection. Knowledge about the properties of spontaneous mutation is crucial to understanding the maintenance of genetic variance within populations and the genetic divergence between them (Simmons and Crow 1977; Clark and Hulleberg 1995; Houle *et al.* 1996; Keightley 1996). The question of whether mutation is sufficient to maintain the levels of genetic variance observed for many quantitative characters in natural populations is the subject of much debate (Lande 1976; Turelli 1984; Barton 1990; Kondrashov and Turelli 1992; Mackay *et al.* 1994; Curtsinger and Ming 1997). Of primary importance in resolving this debate is information about the genetic properties of new mutations, including the numbers of mutable loci affecting the trait, mutation rates at these loci, and homozygous effects of mutations on the trait and fitness (Barton and Turelli 1989; Fry *et al.* 1995). Furthermore, the mechanisms behind the maintenance of variation are important in determining the relationship between mutation and evolutionary change. In considering the evolution of multiple characters, the pleiotropic effects of mutations interact with natural selection to determine the short-term selection response and the long-term equilibrium values of each character (Clark 1987).

The number of empirical studies investigating the properties of spontaneous mutations is growing (Mackay *et al.* 1992b; Santiago *et al.* 1992; Lopez and Lopez-Fanjul 1993a,b; Mackay *et al.* 1994; Mackay and Fry 1996). Much of this work is concerned with estimating mutational effects on morphological characters, but some important experiments have examined effects on life history traits (Mukai 1964; Mukai *et al.* 1972; Lynch 1985; Houle *et al.* 1994). For these experiments, the character of ultimate interest is lifetime fitness, measured as fitness components: viability, lifetime fecundity, and/or mean life span. Age-specific effects of new mutations have generally been ignored, however, in spite of the fact that theories about the evolution of life history traits are often based on assumptions about age-dependent patterns of survival and reproductive output (Charlesworth 1990; Stearns 1992).

Genetic models that combine age-structure and natural selection to predict age-related changes in life history characters are highly dependent on assumptions about age-specific mutational effects (Charlesworth 1990; Partridge and Barton 1993). Currently, there are two areas where this dependence is most evident: (1) predictions about levels of standing genetic vari-

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ance for mortality rates, and (2) hypotheses about the evolution of senescence and patterns of mortality at very old ages.

Genetic variance for mortality and reproduction has been shown to vary as a function of age in Drosophila (Engstrom *et al.* 1989; Hughes and Charlesworth 1994; Promislow *et al.* 1996; Tatar *et al.* 1996; Charlesworth and Hughes 1996). Hughes and Charlesworth (1994) and Promislow *et al.* (1996) estimated age-specific components of genetic variance for mortality within laboratory populations of Drosophila assumed to be near mutation-selection balance. Although both studies found a considerable increase in additive genetic variance with age at early ages, Promislow *et al.* (1996) documented a decline in the level of additive variance late in life, while Hughes and Charlesworth (1994) observed a monotonic increase in genetic variance with age. The discrepancy is likely to result from the differences in sample sizes and in the statistical techniques used to analyze the data (F. Shaw, unpublished data). However, standard genetic models do not predict an initial increase and then decrease of genetic variance in mortality with advancing age (Charlesworth 1990; Hughes and Charlesworth 1994; Promislow *et al.* 1996), although it is likely that spontaneous mutations with pleiotropic effects on mortality rates at different ages could produce such patterns.

Age-specific patterns of mutational effects also have a profound effect on predictions about the evolutionary dynamics of mortality. Although it is clear that senescence, defined as an increase in age-specific mortality rates with age, can be explained as a consequence of the age-related decline in the intensity of natural selection, it cannot evolve without the age specificity of genetic effects (Charlesworth 1994). Selection experiments designed to modify survival and fecundity at one age and identify genetically correlated changes in another are difficult to interpret, and they have produced conflicting results (Rose 1984; Partridge and Fowler 1992). The essential question remains: To what extent do spontaneous mutations with effects on mortality at one particular age influence mortality at another?

Recent life history investigations using large sample sizes find that mortality rates in very old individuals level off and may even decline (Carey *et al.* 1992; Curtsinger *et al.* 1992; Fukui *et al.* 1993). This phenomenon is not an artifact of the decline in density with age (Curtsinger 1995; Khazaeli *et al.* 1995). In fact, mortality deceleration has been shown to have a genetic basis (Promislow *et al.* 1996), but it is unknown whether the deceleration results from genetic constraints on mortality rates late in life or from the inability of mutation to produce further increases without insuring the death of the organism. Information concerning the properties of mutations that affect mortality at these late ages is vital to the development of detailed genetic models of senescence.

Here, we present results from a large demographic study—with observations of 109,860 *Drosophila melanogaster*—designed to estimate the age-specific properties of spontaneous mutations on mortality rates. Data have been obtained from 29 inbred lines derived from one isogenic population that accumulated spontaneous mutations for 19 generations, and from three control lines representing the base population before mutation accumulation. We present estimates of mutational variances and covariances for mortality rates at various ages and investigate the age-specific effects of spontaneous mutations that produced large changes in mortality. The large number of animals permits accurate estimates of mortality rates and genetic variance components throughout life, even after a large proportion of the flies have died (Promislow *et al.* 1996).

MATERIALS AND METHODS

Stocks: Mutation accumulation lines were provided by David Houle, who maintained 100 independent lines of *D. melanogaster* derived from a single isogenic stock. The isogenic population was derived from a laboratory stock, the Ives population, which was started with 400 isofemale lines in 1975 (Charlesworth and Charlesworth 1985) and has been maintained in half-pint bottles on a 14-day generation schedule. At the beginning of Houle's experiment, the isogenic stock had been maintained by full-sib mating for >40 generations. Examination of transposable element insertion sites showed no detectable heterozygosity, confirming the completely inbred status of the population (D. Houle and S. Nuzhdin, personal communication). The isogenic line and all mutation accumulation lines carry the *ebony* (Lindsley and Zimm 1992) mutation to guard against contamination from exogenous flies.

The inbred isogenic stock was used as a base population for founding 100 independent lines. Each generation, three vials per line were set up: two with a single male and female in each, and a third with four flies of each sex. Progeny from the first of the single-pair vials were used to found the next generation whenever possible. If the first vial did not contain enough flies, progeny from the second and then third vial were used. The overall failure rate for A vials (the target vial for collection) was 8.1% (91/1125 subline generations); 1.5% (17/1125) of the time, this required going to the C or four-pair vial because the second single-pair vial (the B vial) also failed. Logistic regression of the failure rates on generation, with B vials scored as 1, C as 2, and A vials as 0, showed a significant positive effect of generation on failure rate (analysis in Proc Logistic of SAS; $\chi^2 = 17.66$, 1 d.f., $P < 0.0001$). This may reflect mutation accumulation in the sublines but possibly environmental factors. There was no significant overall effect of subline on failure rate (analysis in Proc Catmod in SAS; $P > 0.9$). Such failures allow natural selection to influence the allele frequencies of nonneutral mutations, leading to biased estimates of mutational properties. The few times this occurred and the apparently random distribution among accumulation lines suggest this effect is small. No lines were lost during the 19 generations of accumulation. To control for further mutation during experiments and to control for effects of common environment, at generation 19 of mutation accumulation, samples of flies from each line were used to generate two replicates per line. These replicates were maintained separately and with large population sizes.

Control populations were constructed through the use of cryopreservation. Concurrent to the initiation of the 100 mutation accumulation lines, a large number of embryos were sampled from the base population and cryopreserved at Cornell University (see Houle *et al.* 1997). A sample of embryos (100) was withdrawn at generation 19 of the accumulation, and two males and three females were successfully revived. A single female was used to found each of three replicate control populations. One male was mated with two of the females, and the second was mated to the third female.

To insure that cross contamination did not occur between the mutation accumulation lines or between the mutation accumulation lines and the controls, analyses of transposable element positions were carried out on the control populations as well as 20 of the accumulation lines—10 with the highest fitness and 10 with the lowest (D. Houle, personal communication). Two lines were identified as potentially contaminated and are not included in the analysis.

The base population and all mutation accumulation lines bear the R and weak P cytotype (Houle *et al.* 1994), suggesting that a high activity of transposable elements is suppressed. Although active transposable elements can generate significant levels of genetic variation by insertional mutation (Engels 1989; Mackay *et al.* 1992a), observed rates of mutation are expected to be similar to that in natural populations which are also usually of the P cytotype (Mackay *et al.* 1992a; Clark and Hulleberg, 1995).

Culture conditions in Minnesota: At generation 19 of mutation accumulation, a sample of each control population and of 41 randomly chosen accumulation lines (each consisting of two replicates) was sent from the Houle lab at the University of Toronto to the University of Minnesota. Of the 41 accumulation lines, a random sample of 31 lines was chosen for mortality analysis. Flies from each control population and from each of the two replicates of each accumulation line were transferred into half-pint milk bottles containing standard agar-yeast-molasses-cornmeal medium. Bottles were kept for three generations in a constant temperature (24°) and constant light incubator at ~68% relative humidity. During this time, each experimental and control population was expanded into six half-pint milk bottles to generate sufficient numbers of flies for the mortality measurements.

Mortality measurement: For mortality measurements, flies were kept in 3.8-liter plastic "population cages" designed specifically for estimating mortality rates (Fukui and Kirscher 1993; Promislow *et al.* 1996). The cages are formed from clear plastic jugs with a screened window in the side to allow for air exchange and a small, covered opening to allow for instrument access. The opening of the jug is covered with a removable fine mesh screen. Fly medium is placed in the lid of the jug and covered with a single layer of absorbent gauze (USP type III 28/24; Professional Medical Products, Greenwoods, SC). The jug is then inverted and placed on the lid and securely fastened using two rubber bands. The medium is accessible to the flies for feeding and egg laying through the screen and gauze. Each day the food was removed; the exterior of the screen was cleaned; and dead flies were removed from each cage, sexed, and recorded. A new food lid was provided for each cage every other day. Each cage in the experiment was randomly assigned to one of four groups, and each day, cages from one of the groups were cleaned, and their screens were changed.

For each of the two replicates for the 31 mutation accumulation lines, ~1600 flies emerging within a 30-hr period were collected without anesthesia. Flies were weighed to estimate the ~800 individuals that were placed into each of two cages. Nearly equal numbers of males and females were placed in each cage although there was a slight but consistent female bias. For each of the three control populations, six cages were established. In total, 142 cages—averaging 775 flies—and 109,860 flies were involved in the experiment. The large numbers of genetically identical flies in each cage allows accurate measures of mortality at each age and makes possible the estimation of mortality rates late in life after a large proportion of the flies have died. With reference to a more standard genetic analysis, each cage is treated as a single observation with an associated mortality rate at each age (Promislow *et al.* 1996). As a result, our estimates for environmental variance are an underestimate of the true environmental variation among individuals (Promislow *et al.* 1996).

Mortality estimation: Each day, all cages were examined for dead flies, which were removed, sexed, and recorded until the last death occurred. Summing over the duration of the experiment provides the number of individuals in the initial cohort, N_0, as well as the number alive at the start of each day, N_x. The probability of surviving from age x to age $x + 1$ given the individual is alive at the start of age x is $\hat{P}_x = N_{x+1}/N_x$. The age-specific rate of mortality is estimated as:

$$\hat{\mu}_x = -\ln(\hat{P}_x) \tag{1}$$

(Lee 1992). Note that $\hat{\mu}_x$ expresses the instantaneous rate of mortality or "hazard." It is not a probability measure; therefore, it is not bounded above (Pletcher 1997).

Age-specific variance components analysis: All age-specific variance component analyses were carried out on the natural logarithm of mortality. The transformation has two important effects: (1) within an age class, log mortality rates are normally distributed, and (2) the logarithmic transformation normalizes the variance within age classes (Promislow *et al.* 1996). A Shapiro-Wilks test was used to insure that log mortality rates were distributed normally (Lindgren 1993). To reduce the random daily variation in mortality rates and to reduce the number of ages we analyze, thereby lessening the multiple testing problem (see below), mortality rates were calculated on a weekly basis and were log transformed for analysis. Sexes were analyzed separately. For weeks in which there were zero deaths in a cage (because all individuals in the cage had died) $\mu_x = 0$ and $\ln(\mu_x)$ is undefined. For these occasions, we considered the observation a missing value.

Relevant components of (co)variance were estimated using maximum likelihood procedures. For each week, the data were analyzed using a modified version of the module *nf3.p* of *QUERCUS*, a software package produced by R. and F. Shaw (Shaw 1987) that provides maximum likelihood estimates of the variance components. Maximum likelihood methods are preferred over the standard analysis of variance techniques because the estimates produced by maximum likelihood have known asymptotic distributions, even for unbalanced data (Searle *et al.* 1992), and therefore provide hypothesis tests for the variance components. In particular, we chose the restricted maximum likelihood (REML) option in *nf3.p* because of its unbiased nature in the balanced case (Searle *et al.* 1992). Our model for observation in each age period was a simple nested design:

$$y_{ijk} = \mu + \alpha_i + \beta_{j(i)} + \varepsilon_{k(ij)}, \tag{2}$$

where μ is the mean mortality rate, α_i ($i = 1, 2, \ldots, 29$) is the line random effect, $\beta_{j(i)}$ ($j = 1, 2$) is the random effect for the two genetic replicates nested within each line, and $\varepsilon_{k(ij)}$ ($k = 1, 2$) is the residual error corresponding to the mortality rates in the *ijk*th cage.

The design allows the total variation in mortality rates at any given age to be partitioned into three sources of variation. Parallel analyses were carried out for components of covariation. Thus, the phenotypic variation in log mortality at age x,

$V_{p,(x)}$, is the sum of variation between lines, $V_{l,(x)}$, between replicates within a line, $V_{r,(x)}$, and between cages within a replicate, $V_{e,(x)}$. The same representation stands for covariance components between ages x and y by replacing $V_{i,(x)}$ with $Cov_{i,(x,y)}$. Identical procedures were used to estimate the covariance in mortality rates between males and females of the same age.

Likelihood ratio tests were used to test hypotheses concerning between-line variances and covariances. To test the hypotheses $V_{l,(x)} = 0$, we calculated log likelihoods for Equation 2 with the between-line component of variance either free or constrained to zero. Twice the difference in the log likelihoods is asymptotically distributed as χ^2 with 1 d.f. when parameters are tested one at a time. To test the hypothesis $V_{l,(x)} = V_{l,(y)}$ $(x \neq y)$, we used the module *pcrf1.p* in *QUERCUS*. This module was designed to compare variance components from two separate, genetically independent populations. In the present case, mortality rates at each age are clearly not independent, so significance levels from this analysis should be viewed with caution (Promislow *et al.* 1996). In cases where the algorithm failed to converge when the parameter of interest was constrained to zero, we used the asymptotic standard error of the estimated value, derived from the Fisher information matrix, to test whether the parameter was significantly different from zero (Searle *et al.* 1992).

Estimates of mutational variance were obtained from the between-line components of variance, assuming the mutations were neutral, additive, and of small effect. In this case, for each age x,

$$V_{l,(x)} = 2\sigma^2_{m,(x)}\{t - 2N_e[1 - \exp(-t/2N_e)]\}, \quad (3)$$

where $\sigma^2_{m,(x)}$ is the mutational variance at age x, t is the number of generations of divergence, and N_e is the effective population size in the accumulation lines through the experiment (Lynch and Hill 1986; Lynch 1994). In our case, $N_e = 2$, and Equation 3 is well approximated by:

$$V_{l,(x)} \sim 2t\sigma^2_{m,(x)} \quad (4)$$

(Lynch 1994). Mutational covariances can be obtained from the above formulae by substituting $Cov_{m,(x,y)}$ for $\sigma^2_{m,(x)}$ and $Cov_{l,(x,y)}$ for $V_{l,(x)}$. Mutational correlations between mortality at ages x and y ($r_{m,(x,y)}$) are calculated as $Cov_{m,(x,y)} / (\sigma_{m,(x)}\sigma_{m,(y)})$.

Variance components for mean longevity: To allow a comparison of results between the data presented here and other mutation accumulation experiments, variance components were estimated for average longevity. Relevant variance components were estimated using the *varcomp* procedure in S-Plus (MathSoft, Cambridge, MA). The data consist of the age at death for 95,721 individual flies (control lines are not included in this analysis). In this case, there is an additional level of variance representing variation in age at death among flies within the same cage. Mutational variance is again calculated using Equation 4. The distribution of these data is very nearly normal, and because of the large sample size, hypothesis tests concerning the values of the estimates are based on the asymptotic standard errors provided by the inverse of the information matrix (Searle *et al.* 1992).

Variance components analysis for parameters of mortality models: A number of mathematical formulae have been proposed for describing the relationship between age and mortality rates (Gompertz 1825; Gavrilov and Gavrilova 1991; Fukui *et al.* 1993). These mortality models often contain a small number of parameters, and as a result, they can be used to identify systematic differences in mortality rates among cohorts. Two specific models are common in the literature and will be examined. The first is the Gompertz equation, which assumes that mortality rates increase exponentially with age:

$$\mu_x = \alpha e^{\beta x} \quad (5)$$

where α describes mortality rate at birth, and β is the rate of exponential increase in mortality with age. Recent experimental work involving large cohorts has documented a significant deceleration of mortality rates in advanced ages (Carey *et al.* 1992; Curtsinger *et al.* 1992; Fukui *et al.* 1993). In such cases, the Gompertz equation is a poor description of mortality dynamics. To correct for departures from Gompertz, the logistic frailty model was also examined:

$$\mu_x = \frac{\alpha e^{\beta x}}{1 + s\frac{\alpha}{\beta}(e^{\beta x} - 1)} \quad (6)$$

(Vaupel 1990). Early in life (x near zero), mortality increases exponentially at a rate determined by α and β. The parameter s determines the extent to which mortality rates decelerate late in life. Higher values of s indicate greater decleration. Note that when $s = 0$, Equation 6 reduces to Equation 5.

Genetic and phenotypic components of variance for the parameters of these two models were estimated using maximum likelihood methods (S. D. Pletcher, unpublished results). For each population cage, the parameters of the appropriate mortality model were estimated by maximizing the likelihood over individual deaths. Because likelihood estimates are asymptotically normally distributed, components of variance for each parameter were estimated using *QUERCUS*, and hypothesis testing was carried out in the same manner as described for measures of age-specific mortality. Estimates of mutational variance for the parameters were calculated using Equation 4.

Estimation of age-specific mutational effects: The effects of spontaneous mutations on age-specific mortality were examined by comparing mortality rates in 3-day intervals between the control lines and each mutation accumulation line. Mortality rates for each line were determined by calculating the arithmetic average, on a log scale, of the mortality rates in each replicate population cage (four cages for each accumulation line and 16 cages for the control lines). There are two complications in testing differences in mortality rates at any particular age. First, because each individual contributes to observed levels of mortality throughout its lifetime, mortality rates at different ages are not independent. Therefore, treating each 3-day interval as a separate character is not justified. Second, within any age interval, there are 29 comparisons (one for each accumulation line) to the control, and with a type I error rate of 0.05, we would expect one line to exhibit "significantly" different mortality by chance alone.

To alleviate these problems, a bootstrapping procedure (Efron and Tibshirani 1993) was used to produce simultaneous 99.5% confidence intervals on 3-day mortality rates for all lines in the analysis. This was done in the following manner: For each accumulation line, one "bootstrapped sample" was generated by sampling with replacement four population cages from the four cages in the original data. The mortality rates within each cage were then calculated by sampling with replacement, from the data of that cage, a number of ages at death equal to the number in the original cohort. The mean mortality rate (on a log scale) for each interval was then calculated using the four "resampled" cages. This procedure was repeated 50,000 times, and the appropriate quantiles were examined to determine the confidence intervals. The data were not resampled at the level of the replicate. Because there are only two replicates at this level, resampling can only reduce the variance. An identical procedure was used for the control lines, with the exception that there were 16 cages per line.

Mutation accumulation lines with confidence intervals that did not overlap those of the control in at least two age in-

tervals were considered as having mutations affecting mortality. Although it controls for the dependence in mortality rates between ages and for the large number of comparisons at each age, the nonparametric determination of the confidence intervals makes this procedure quite conservative in detecting mortality differences.

To identify small mutational effects on mortality throughout life, log mortality rates, in 3-day intervals, were calculated for each of the four cages within each mutation accumulation line and for the 16 control line cages. Log mortality was then regressed on age using time as a nonlinear covariate. Thus, for each mutation accumulation line and for each sex, we used the following model:

$$y_{ij} = \mu + s(t) + \beta_i + \varepsilon_{j(i)}, \quad (7)$$

where μ is the average mortality rate at age zero, β_i ($i = 1, 2$) is the treatment-fixed effect (MA or control), $\varepsilon_{j(i)}$ ($j = 1, \ldots, 4$) is the residual error corresponding to the 3-day mortality rates in the ijth cage, and $s(t)$ is the nonlinear covariate estimated using a lowess smooth (Hastie and Tibshirani 1990; Cook and Weisberg 1994). A significant β term provides evidence for the existence of mutations that increase/decrease mortality rates throughout life.

RESULTS

Divergence of mortality rates: Daily estimates of age-specific mortality rates, averaged over replicate cages, are presented for the 29 mutation accumulation lines and three control lines in Figure 1. Although mutational effects in individual lines are difficult to discern, some trends are clear. For both males and females, there is greater variation in mortality rates early in life than at older ages. In addition, female mortality curves in the accumulation lines are nearly all above the control lines early in life, suggesting that, on average, spontaneous mutations tend to increase mortality. This is not so in males because there are approximately the same number of accumulation lines with higher mortality as there are with lower mortality. Lastly, mortality rates for all lines in the experiment show a marked deceleration late in life, as evidenced by the departure from linearity in the mortality curves. Interestingly, the point of deceleration is nearly coincident with the reduction in variation; mortality curves converge at older ages.

During the experiment, two of the 142 population cages exhibited high (>20 times the average) mortality beginning at eclosion. In large experiments like this, we often see a small number of anomalous cages. This is likely to be caused by local problems with the cage environment during set up. Such cages are treated as outliers and removed from the analysis. Also, on day 3 of the experiment, a technical problem with the fly medium caused abnormally high male mortality in 17 of the 142 cages (12%). High mortality was random with respect to genotype and position in the incubator, and cages experiencing this mortality increase did not show any lasting effects. Female mortality was unaffected.

Age-specific variance components: The REML procedures require that the data be normally distributed, and after log transformation, weekly mortality rates are normally distributed ($P > 0.10$, Shapiro-Wilks test). In one case (females week 6) $P = 0.01$, but this is not significant after correcting for multiple hypothesis tests. The log transformation forced us to consider cages with zero deaths during any period as undefined, and omitting zero mortality cages early in life can introduce a significant bias in the estimation of genetic variance for mortality (Promislow *et al.* 1997). The large sample size, together with the calculation of mortality rates in weekly intervals, insured that non-zero mortality rates were observed in all cages early in life (through week 5), with the exception of one cage for males in week 2.

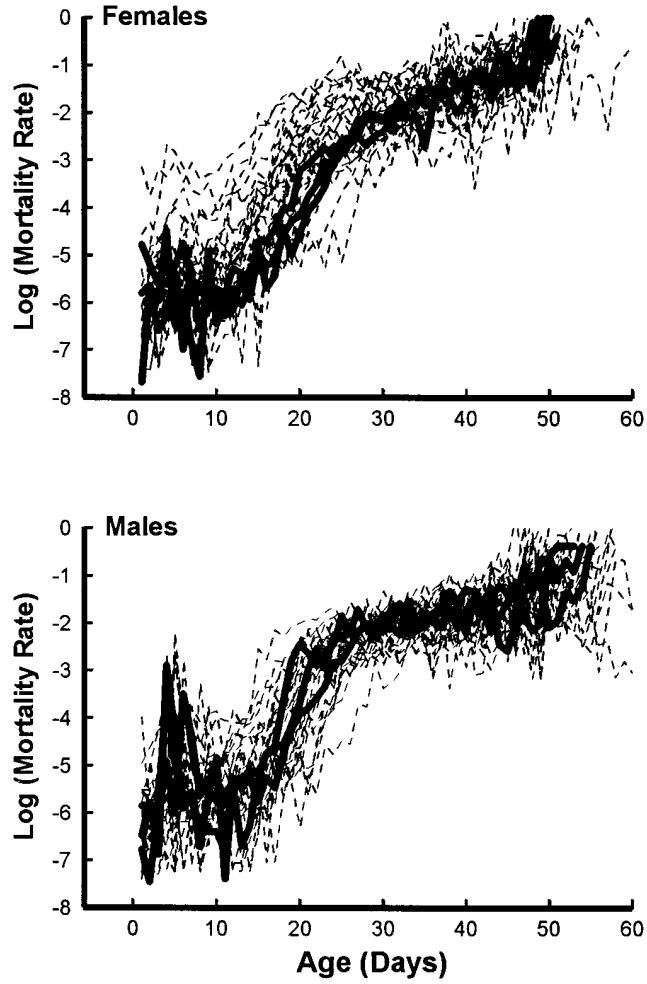

Figure 1.—Mortality rate curves, plotted on a log scale, for 29 lines allowed to accumulate spontaneous mutations for 19 generations (dashed lines) and three control lines genetically representative of the population before accumulation (solid lines). Mortality rates were averaged over four cages for each accumulation line and over six cages for each control line. Each accumulation (control) line curve is based on average of 1676 (2526) females or 1403 (2187) males. Notice the decline in variation among the accumulation lines and the general convergence of the mortality curves late in life.

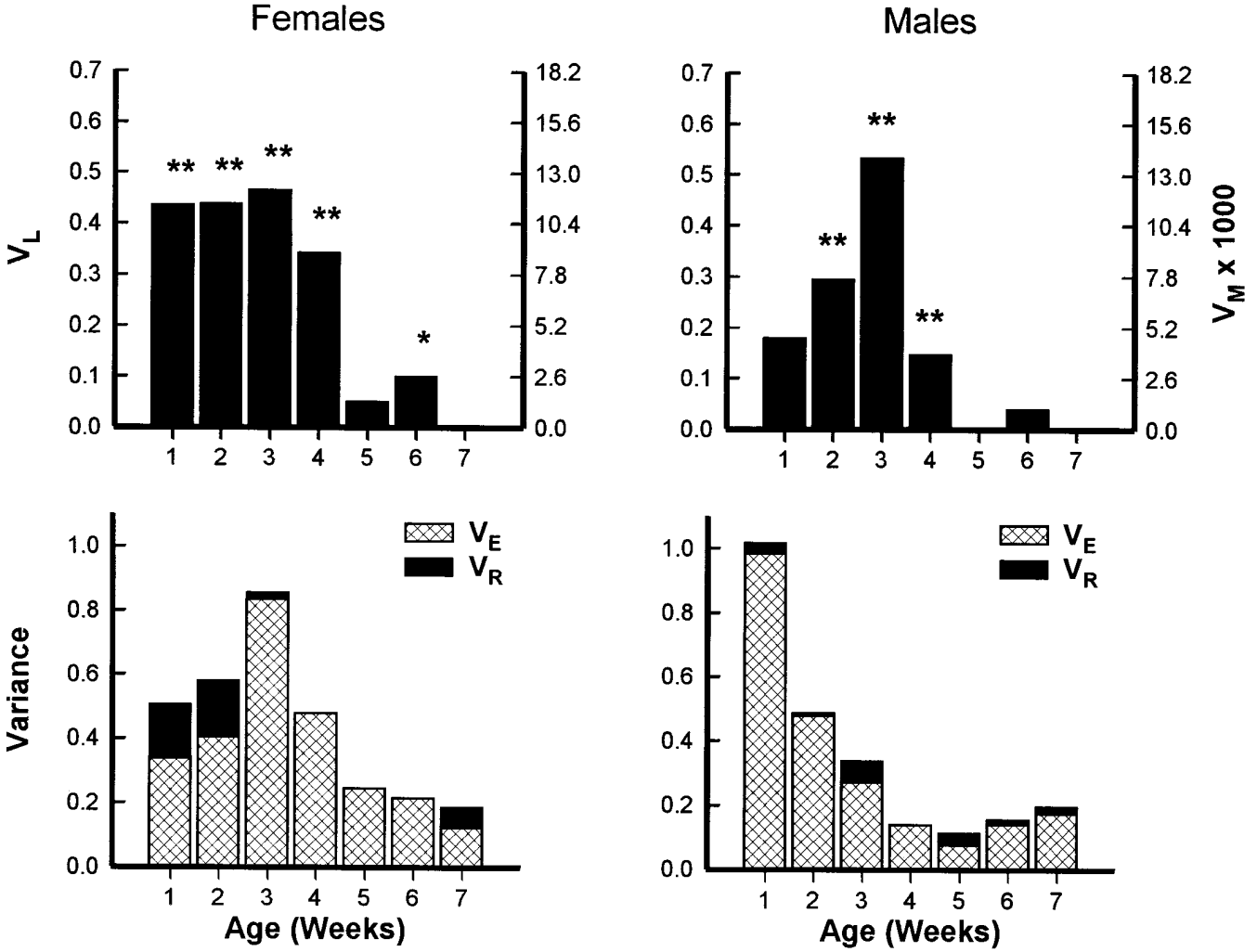

Figure 2.—Age-specific components of variance in weekly log mortality rates for males and females. Restricted maximum likelihood was used to estimate variance components. Genetic variation in log mortality rates resulting from the accumulation of spontaneous mutations is presented in the upper panels. The left *y* axis depicts the actual between-line variance, while the right *y* axis is scaled to show the level of mutational variance (see text). Lower panels show variance resulting from genetic replicates nested within mutation accumulation lines (V_r) and environmental variance (V_e). $^{**}P < 0.007$, $^{*}P < 0.05$. After correcting for multiple comparisons using the Bonferroni criterion, ages marked with double asterisks remain significant.

The pattern of age-specific genetic variance in log mortality rates created by 19 generations of mutation accumulation is consistent with the pattern seen in Figure 1. Females show significant genetic variance in mortality over the first four weeks, after which there is a sudden decline in variance (Figure 2). Males show significant genetic variance in mortality from week 2 through week 4, after which genetic variance is not significantly different from zero (Figure 2). These ages remain significant after a Bonferroni correction for multiple tests ($P < 0.007$). Although we were unable to detect significant genetic variance for males in week 1, this is most likely caused by the large amount of random mortality that occurred on day 3 of the experiment.

We used the *pcrf1.p* module of *QUERCUS* to compare variance in log mortality rates between two separate ages. All comparisons between genetic variance in mortality rates early in life (<5 wk) and late in life were corrected for 15 multiple comparisons using the Bonferroni criterion. For an $\alpha = 0.05$, this requires a $P < 0.003$ to indicate significance. In females, the genetic variance for log mortality is significantly higher in the first 4 wk of life than it is in weeks 5 or 7. The pattern is less clear for comparisons involving week 6 mortality. The algorithm did not converge for comparisons between week 1 with week 6 and week 2 with week 6, and comparisons between week 3 and week 4 with week 6 were not significant after correction for multiple comparisons ($P = 0.02$ and $P = 0.05$, respectively). In males, genetic variance in mortality rates during weeks 2 and 3 is significantly greater than it is for weeks 5–7 ($P < 0.003$), and variance in week 3 is significantly greater than variance in week 4. There is some evi-

dence for greater genetic variance in week 4 mortality over weeks 6 or 7, but these comparisons are not significant after correction for multiple comparisons ($P = 0.04$ and $P = 0.009$, respectively). There is weak evidence for an initial increase in genetic variance from week 1 to week 3 ($P = 0.09$).

Variance component analyses were also carried out on weekly control line mortality. Total phenotypic variation in mortality rates was partitioned into variance between the three control lines and error variance. In both males and females, there were no instances of significant between line variance in weekly mortality rates ($P > 0.20$; data not presented). In most cases, this estimate was essentially zero, *i.e.*, $<10^{-8}$. Error variance was qualitatively very similar to that estimated in the analysis of the mutation accumulation lines (data not presented). This provides evidence that the cryopreservation process did not induce random mutations that affect mortality rates, but it does not rule out the unlikely possibility that freezing results in very specific and consistent changes in the genome (also see Houle *et al.* 1997).

Age specificity of mutational effects: The genetic correlation in weekly mortality rates generated by 19 generations of mutation accumulation is presented in Table 1. One striking feature of the correlation structure is the preponderance of positive correlations. For both males and females, mortality rates are highly correlated between ages separated by 1 or 2 wk. Although in both sexes there is strong evidence for a number of correlations being greater than zero, none remained significant after a Bonferroni correction for 21 multiple comparisons (see Table 1). Point estimates of the genetic correlation decline essentially monotonically, and they are not significantly different from zero ($\alpha = 0.05$) between ages separated by ≥4 wk. Furthermore, maximum likelihood methods designed to test for a decline in genetic correlation over time in an age-dependent character provide strong evidence in favor of a significant decline in both sexes (Pletcher and Geyer, manuscript in review). Although in females there is a small number of negative genetic correlations involving mortality in weeks 6 and 7, these are not significantly different from zero ($P > 0.10$). Assuming a normal distribution of random error, we expect half of the estimates not different from zero to give negative point estimates by chance. This expectation is borne out because five of nine estimates are negative in sign. The genetic variance from mutation in male mortality during week 5 was estimated to be zero; therefore, we are unable to estimate correlations involving this trait.

The genetic correlation in log mortality rates between males and females of the same age were large and positive for early ages (Table 2). Correcting for multiple tests, significant positive correlations were detected in weeks 3 and 4 ($P \leq 0.01$). Correlations in week 1 and week 2 were marginally significant ($P < 0.10$). Male mutational variance was estimated to be zero in week 5, precluding the calculation of between sex genetic correlations involving this trait.

For each sex, each of the 29 mutation accumulation lines was examined for significant differences in mortality rates (3-day intervals) from the control lines. In females, 12 lines were identified as exhibiting significantly different mortality over at least two age intervals. Another two lines showed significantly different mortality in one age class. For males, six lines were identified with significantly different mortality in at least two age

TABLE 1

Mutational correlations between age-specific mortality rates

	Week					
Week	2	3	4	5	6	7
1	**0.97**	**0.68**	*0.46*	0.30	0.10	0.05
2		**0.83**	**0.66**	**0.78**	0.30	—
3			**0.96**	**0.93**	−0.32	−0.82
4				**0.95**	−0.07	−0.76
5					*0.19*	—
6						−0.66
1	*0.68*	**0.86**	*0.56*	—	0.36	0.99
2		**0.77**	**0.69**	—	0.04	0.32
3			**0.92**	—	0.09	0.39
4				—	*0.55*	—
5					—	—
6						—

Entries are genetic correlations generated by 19 generations of mutation accumulation. Hypothesis tests were conducted to determine if each entry is significantly different from zero. Significance was determined using log-likelihood ratio tests of unconstrained covariance relative to covariance constrained to zero (see text). Bold values indicate $P < 0.01$, and italics indicate $0.01 < P < 0.10$. A dash indicates either lack of additive variance for one age (in the case of week 5 males) or failure of the likelihood algorithm to converge.

TABLE 2

Mutational correlations across sexes for age-specific mortality

Week	Mutational correlation	*P*
1	0.89	0.080
2	0.95	0.030
3	0.86	0.004**
4	0.60	0.010**
5	—	—
6	0.53	0.11
7	NC	—

Significance values were determined by likelihood ratio test. ** indicate significance after correction for multiple comparisons; a dash indicates lack of additive genetic variance. NC, likelihood algorithm failed to converge.

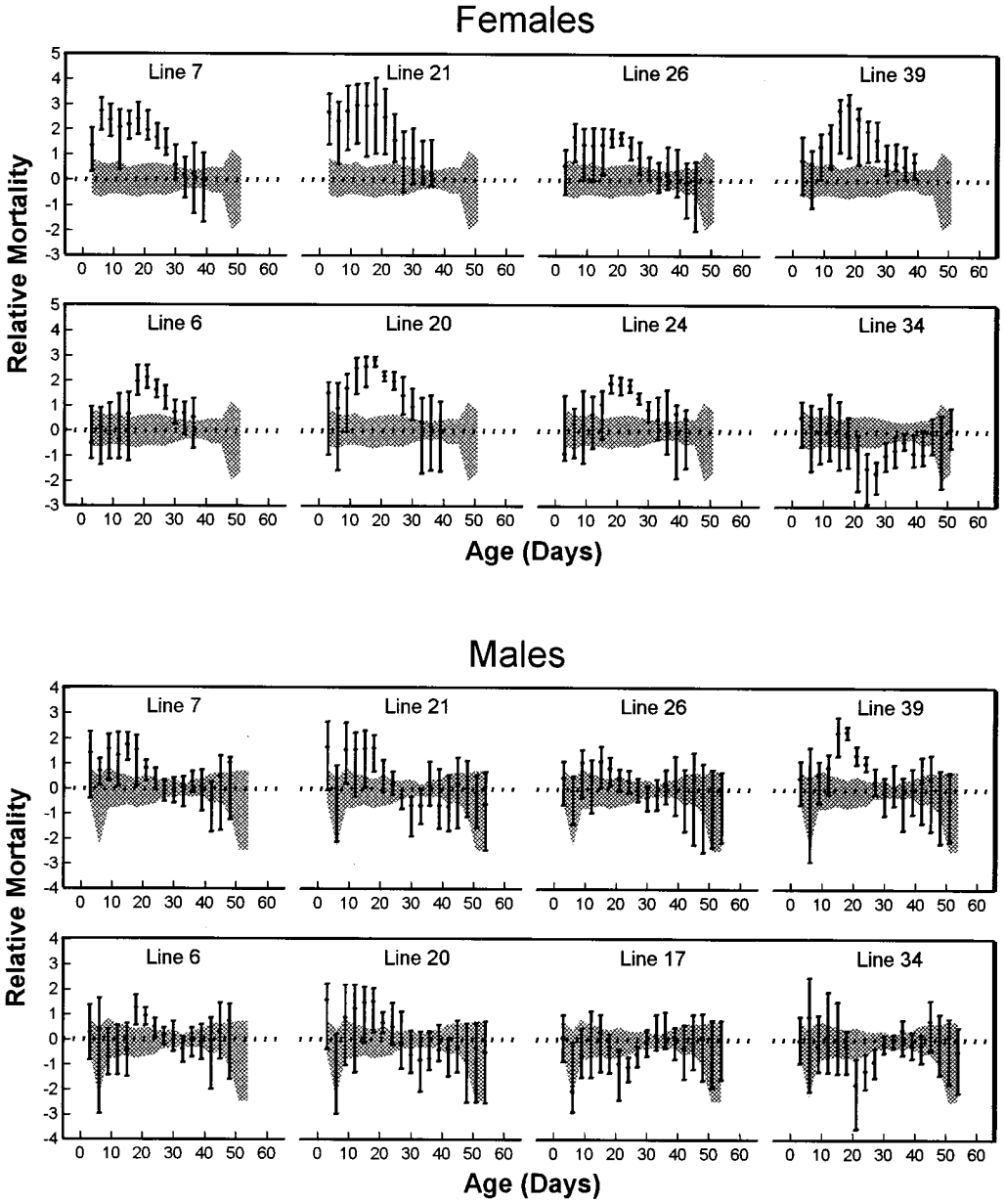

Figure 3.—Mutation accumulation lines showing age-specific effects of spontaneous mutations on mortality. Mortality rates (and 99.5% confidence intervals) for each of the accumulation lines are calculated from deaths occurring in 3-day intervals, and they are plotted as the difference from control line mortality at each age. The shaded region represents the 99.5% confidence region for control line mortality. Confidence intervals were generated from a bootstrap procedure and were based on 50,000 bootstrapped samples. Ages in which confidence intervals are not overlapping are considered to represent ages in which control line mortality is significantly different than accumulation line mortality.

classes, and one additional line was found with different mortality at a single age. Six of these seven lines were also observed to have significant age-specific effects in females. A sample of mortality estimates relative to the control lines for females from eight lines and males from eight lines is presented in Figure 3. Most of the mutations affect mortality rates early in life, and a few show significant effects on middle ages. No lines were identified as showing mortality effects of mutation late in life, *i.e.*, >37 days.

Evidence for mutations that have effects on mortality throughout life were identified in both sexes (Table 3). Although there were no lines that remained significant after a strict Bonferroni correction for the 29 hypothesis tests in each sex, we would expect only 1.45 lines to show significance at the $P = 0.05$ level by chance alone. In females, eight accumulation lines showed mortality rates consistently higher than controls, and one showed lower mortality throughout life (at the $P = 0.05$ level). Of these eight lines, five were also identified as having significant effects at specific ages. The evidence is less convincing in males: Two lines were considered to have higher mortality than the controls throughout life, while one line showed a consistent decrease in mortality. The two lines with higher mortality also exhibited significant effects of mutation in at least two age intervals (Table 3).

Mean mortality curves for the 29 mutation accumulation lines and the three control lines are presented in Figure 4. The prevalence of mutations increasing early-age mortality in females is reflected in the significantly greater average mortality for the accumulation lines.

TABLE 3

Mutation accumulation lines with significant changes in mortality throughout life

Sex	Number of lines	Higher mortality	Lower mortality
Males	3 (2)	2	1
Females	9 (4)	8	1

Entries are the number of mutation accumulation lines (out of a possible 29) that show significantly different mortality rates throughout life for each sex and whether the difference was toward increased or decreased mortality. Significance ($P \leq 0.05$) was determined by least-squares regression of log mortality rate on age with time as a nonlinear covariate (see text). Numbers in parentheses indicate the number of lines that also show significant differences at specific ages (see Figure 3).

The mutational bias toward effects that increase mortality is not seen at older ages. In males, the nearly identical average mortality rates in the accumulation and control lines is evidence for a lack of mutational bias toward mutations that increase mortality at any age.

Mortality models: We find significant mutational variance for the baseline mortality parameter and the rate of increase parameter for the Gompertz model (Equation 5) for females only (Table 4). Males show no significant effects of mutations on these general mortality patterns. Although the Gompertz model is commonly used to analyze mortality data, it is clear that our data do not follow the log-linear Gompertz trajectory (Figure 1). The logistic frailty model (Equation 6), which predicts a deceleration of age-specific mortality late in life, produced a better fit (likelihood ratio test, $P < 0.01$) for males in 99/116 cages (85%) and for females in 92/116 cages (79%). Under this model, females have significant mutational variance in baseline mortality as well as in the rate of mortality increase with age (Table 4). There is marginal evidence for genetic variance in the s parameter of the logistic model ($P = 0.08$), suggesting that mutations are affecting the rate of deceleration of mortality at older ages. Males show no significant effects of mutations on any parameters of the logistic model.

Mean longevity: The amount of genetic variance for longevity created each generation by mutation is 0.30 for males and 0.46 for females (Table 5). The ratio V_m/V_e for female mean longevity is twice that of males (7.3×10^{-3} and 3.5×10^{-3}, respectively), but both are of the order of magnitude commonly reported for quantitative traits (Lynch 1988; Houle *et al.* 1994). Houle *et al.* (1994) report estimates of V_m/V_e of 0.8×10^{-3} and 1.2×10^{-3} for male and female longevity, respectively. The mutational coefficients of variation (Houle 1992) for female and male longevity are 2.0 and 2.6, respectively. This is higher than the mutational coefficients of variation for average longevity (1.14 for females and 1.80 for males) reported by Houle *et al.* (1994). There is a significant reduction in longevity for females as a result of mutation accumulation ($P < 0.01$), but not for males. Because the data for average longevity are measurements from individuals (as opposed to age-specific mortality; see materials and methods), the estimates of environmental variance are accurate representations of the random variation in longevity.

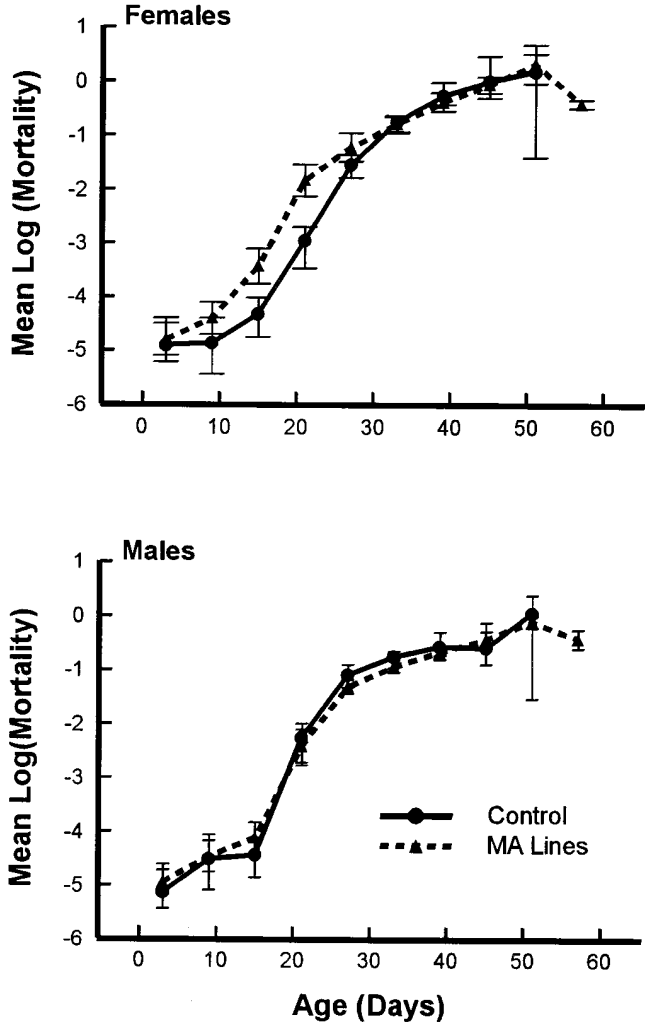

Figure 4.—Overall mean mortality curves and 95% confidence intervals for mutation accumulation lines and control lines for both sexes. Means were calculated by averaging mortality rates (3-day intervals) at each level of the design. Confidence intervals were calculated as twice the standard deviation of the mean mortality rates for each line at each age ($n = 3$ for control lines, $n = 29$ for mutation accumulation lines). Mutations in female mortality rates show a significant bias toward increased mortality early in life but not at older ages. Mutations in male mortality show no bias throughout life.

DISCUSSION

Significant genetic variance for weekly mortality rates caused by recent spontaneous mutations was ob-

TABLE 4

Mutational variance for parameters of mortality models

			Males		Females	
Model	Parameter		Mean	V_m	Mean	V_m
Gompertz	Baseline	(α)	0.0052	4×10^{-7}	0.0043	2.1×10^{-4}***
	Rate	(β)	0.11	1.0×10^{-4}	0.14	3.0×10^{-4}***
Logistic	Baseline	(α)	0.0025	$<10^{-5}$	0.0019	1.4×10^{-5}***
	Rate	(β)	0.22	2.0×10^{-4}	0.24	5.5×10^{-3}***
	Deceleration	(s)	0.97	0.082	0.67	0.065*

Entries are means and mutational variances estimated by using maximum likelihood procedures for both the Gompertz and logistic mortality models. ***$P < 0.001$; *$P < 0.10$

served in both males and females early in life (up to approximately day 30) but not at older ages. Mutational correlations are highly positive between mortality rates separated by a week or two, and they decline monotonically as the ages in question become further separated in time. Correlations are not significantly different from zero between ages separated by ≥4 wk. An examination of individual mutation accumulation lines provides strong evidence for a class of mutations with age-specific effects on mortality. Surprisingly, late-life mortality was essentially unaffected by the mutation accumulation procedure.

Age-specific properties of mutations

Our results provide strong evidence for the existence of a class of polygenic mutations that differ with respect to their effects on mortality at various ages. The consistent decline in genetic correlation between increasingly separated ages suggests that mutations with temporary effects are not uncommon and may even form the dominant component of the spectrum of mutations that affect mortality. Mutations affecting mortality throughout life were also identified, but their contribution to measured levels of mutational variance was small. All mutation accumulation lines exhibiting significant effects of mutation showed changes in mortality over a range of adjacent ages. We did not find evidence for the occurrence of "antagonistic" mutations with beneficial effects on mortality early in life and detrimental effects in later ages (or vice versa).

The pattern of age-specific mutational variance suggests the following: (1) loci that affect mortality at early ages are more likely to experience mutations, (2) a larger proportion of the genome influences mortality at earlier ages, and/or (3) the average effects of spontaneous mutations are larger in loci that influence mortality at early ages. It is interesting to note that our estimates of V_m/V_e for mean longevity are the same order of magnitude as those previously reported for longevity (Houle *et al.* 1994) and those reported for a number of other quantitative traits (Houle *et al.* 1996). This is despite the fact that V_m/V_e for early age mortality (weeks 1–4 average 0.026 and 0.038 for females and males, respectively) is quite large in comparison with other life history characters (Houle *et al.* 1996). Unfortunately, a direct comparison of mutational variance in mortality with variance in other characters is problematical be-

TABLE 5

Estimates of the mean, the mutational variance, and the mutational variance scaled to environmental variance for longevity

Sex	Control mean	Mutation accumulation mean	$V_m \times 1000$	V_e	$(V_m/V_e) \times 1000$
Male	26.8 (0.47)	27.2 (0.06)	301.7 (92.7)	85.5 (0.58)	3.5
Female	29.0 (0.31)	25.7 (0.83)	455.7 (148.5)	62.7 (0.39)	7.3

Means are estimated by averaging at each level of the design. Standard errors of the mean are presented in parentheses and are calculated from the variance of the mean longevities for each line ($n = 3$ for control, $n = 29$ for mutation accumulation). Variance components were estimated using REML, and the corresponding standard deviations of the estimates are presented in parentheses. Standard deviations were calculated from the asymptotic distributions of the estimates.

cause estimates of environmental variance for mortality rates are underestimates of the variation among individuals (preventing the usual scaling for comparison; V_m/V_e), and mean mortality rates on a log scale are negative (precluding the calculation of coefficients of variation).

Many of the lines identified as showing age-specific effects of mutations have large effects in both sexes (Figure 3). This observation is consistent with the high positive mutational correlations between sexes (Table 2). In all cases, mutation-induced increases (decreases) in female mortality was mirrored by increases (decreases) in male mortality (Figure 3). The age range of effects seen in each line is roughly equivalent in both males and females, although the magnitude of the mortality changes generated by mutation is larger in females than males. This result is consistent with the larger estimates of mutational variance seen in females (Figure 2) and with previous reports of sex-specific genetic variance for mortality rates in laboratory populations of Drosophila (Promislow *et al.* 1996).

There is no evidence for antagonistically pleiotropic effects of mutations across sexes for mortality rates at the same age. At any particular age, there were no accumulation lines with a significant increase in mortality in one sex and decrease in the other, and all genetic correlations across sexes were positive (Table 2). It is possible that, for example, high male mortality early in life might generate relatively high female mortality at older ages because of the deleterious effects of reproductive activity on females (Chapman *et al.* 1995). Such age-specific pleiotropic effects are difficult to detect given the extremely large number of comparisons involved in the analysis, *e.g.*, the genetic correlation of each male age with each female age. We did not detect significant ($P < 0.05$) mutational correlations across sexes for weeks 1 and 2 with weeks 5 and 6 (data not presented). A more detailed analysis of the effects of mutations across sexes will be presented elsewhere.

The deceleration and convergence of mortality rates at old ages in the mutation accumulation and the control lines is probably not caused by a decline in density as the flies age. It has been argued (Graves and Mueller 1993) that if mortality is in part brought about by interactions between individuals, then this source of mortality will wane with time, and mortality rates would decelerate late in life. Several experiments rule out density as the causal factor in mortality deceleration (Khazaeli *et al.* 1995, 1996) Specifically, Khazaeli *et al.* (1996) studied mortality rates at constant adult densities by replacing dead flies with young, marked mutants. Late-life mortality deceleration was observed equally in both supplemented and nonsupplemented populations.

Mutations affecting genes with age-dependent patterns of expression provide a possible mechanism for generating the range of mutational effects in our data. Using enhancer trap-marked genes that express β-galactosidase when transcriptionally active, Helfand *et al.* (1995) were able to identify a number of genes with varying temporal patterns of expression in adult Drosophila. A few of the marked genes were expressed constitutively, but several were active only during early ages (Helfand *et al.* 1995; Rogina and Helfand 1995). Others were expressed from approximately age 10–35 days (Helfand *et al.* 1995; Rogina and Helfand 1995, 1996). It seems plausible that mutations in genes with temporally localized expression might produce age-specific changes in mortality. In addition, at least 86% of the genes examined were expressed at eclosion, and of these genes, 31% showed a steady decline in expression throughout life (Helfand *et al.* 1995). If expression in a large number of genes is much lower at older ages under "normal" circumstances, we might expect spontaneous mutations that disrupt transcription to have relatively little effect late in life.

The mutation accumulation lines used in this experiment were initiated from the Ives stock (Charlesworth and Charlesworth 1985), a stock maintained according to a standard 2-wk population cycle for ~400 generations. In such a culture regime, mutations whose effects are limited to ages greater than ~8 days after eclosion will experience no selection (D. Houle and L. Rowe, unpublished data). It is therefore possible that the lack of genetic variance for mortality rates at older ages is a result of an already high late-age mutational load generated by the 2-wk cycle. However, the force of selection at later ages in nature may not be very different from that in the Ives transfer schedule. A daily adult mortality rate of only 10% yields an average lifespan of ~8 days. We are not aware of any estimates of adult mortality rates in wild *D. melanogaster*, but it seems likely that many populations would experience mortality rates this high or higher.

In addition to mutational effects on mortality *per se*, we can envision four other factors that have the potential to influence our results. First, there may be a reproduction effect. Because we measured mortality on flies kept in mixed-sex cages, we cannot distinguish between mutations that affect mortality directly and those that influence mortality through costs of reproduction (Promislow *et al.* 1996). Mutations affecting reproductive activity may account for the greater genetic variance in females for the first 2 wk after eclosion and the lack of variance late in life when nearly all lines have ceased reproducing. Measurements of female egg production do not support this hypothesis for the following two reasons: (1) mutational variance for egg production was significant for the first 3 days after eclosion but not for days 4–15 (S. D. Pletcher, unpublished data), and (2) evidence from previous studies (A. Khazaeli, personal communication) and observations during this experiment (S. D. Pletcher, personal observation) indicate that most lines ceased egg produc-

tion well before the observed decline in variance. If reproductive costs are both immediate and delayed, however, mutations affecting reproduction may influence mortality for some time after reproduction has ceased.

Second, cryopreservation may have introduced unmeasured genetic changes. Our estimates of mutational effects rely on the cryopreserved control lines to accurately represent the genetic aspects of age-specific mortality in the base population before mutation accumulation was started. At the present time, there is no detailed information on the genetic consequences of freezing Drosophila embryos, but the lack of a significant "between-line" variance in control mortality is evidence against cryopreservation, causing random genetic changes (see also Houle *et al.* 1997).

Third, nongenetic and developmentally acquired variation may have age-specific effects. Environmentally induced variation in overall physiological quality can lead to age-specific changes in observed variance components and to departures from log-linear (Gompertz) mortality dynamics (Vaupel and Yashin, 1985). Currently, there are no methods for quantifying the level of heterogeneity for mortality within cages, but empirical (A. Khazaeli, unpublished data) and simulation results (S. D. Pletcher and J. W. Curtsinger, unpublished data) suggest that the amount of induced variation required to generate both the abrupt decline in genetic variation and the corresponding deceleration of mortality is much greater than normally observed within or between distinct genotypes. Additional theoretical and experimental work to assess the significance of this effect is in progress.

Fourth, the reduced sample size at older ages may result in a loss of statistical power to detect significant effects of mutations. Although deaths in each cohort (population cage) result in a progressive reduction in sample size, the power to detect age-specific genetic variation in mortality rates depends on—in addition to the number of flies alive at a particular age—the observed mortality rate at each age and the number of mortality observations at each age along with their genetic relationships. To investigate the effect of declining sample size and increasing mortality rate on our ability to detect significant genetic variance, we conducted a large number of computer simulations in which genetic variance was held constant for all ages and our ability to detect it was evaluated (details of the simulation algorithm are given in the appendix). Three different combinations of between-line (genetic), between-replicate, and error variance were used in the simulations. A high-variance combination used values similar to those measured at early ages ($V_l = 0.4$, $V_r = 0.2$, and $V_e = 0.3$), a low-variance set of parameters was similar to values observed at late ages ($V_l = 0.2$, $V_r = 0.1$, and $V_e = 0.2$), and the third set consisted of a combination of the two ($V_l = 0.2$, $V_r = 0.2$, and $V_e = 0.3$). Simulated data sets were generated using the observed average female mortality rate at each age and the average number of females alive at each age (see appendix). For each age and combination of variance parameters, 500 data sets were generated. Variance component estimation was then carried out on each set using the *varcomp* procedure in S-Plus (MathSoft). Asymptotic standard errors on the estimates allowed us to determine the fraction of simulated data sets that would have resulted in the detection of significant genetic variance. In addition, the average of the 500 estimates was obtained to determine if we would expect an age-related bias in the observed levels of genetic variance.

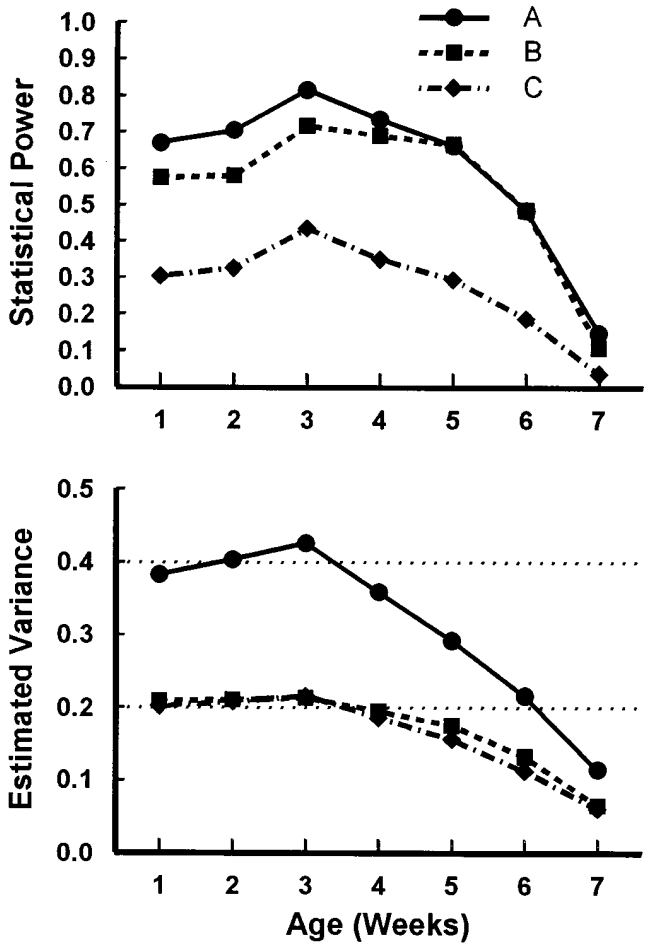

Figure 5.—Estimates of statistical power for detecting significant ($P < 0.05$) genetic variance (top) and average estimated genetic variance (bottom) for age-specific mortality rates. Estimates are based on the results of computer simulation (see appendix). Each point is based on the analysis of 500 replicate simulations. Data were simulated using three distinct parameter sets of between line (genetic), between replicate, and error variance. Values for the three sets are as follows: (A) $V_l = 0.4$, $V_r = 0.2$, $V_e = 0.3$; (B) $V_l = 0.2$, $V_r = 0.1$, $V_e = 0.2$; and (C) $V_l = 0.2$, $V_r = 0.2$, $V_e = 0.3$. For all three levels of variation, statistical power remains relatively constant until week 7. Error bars on the average estimated genetic variance are smaller than the symbols and are not displayed.

For all three combinations of variance parameters, the power to detect genetic variance in log mortality is greatest at intermediate ages, and it is essentially equivalent among ages 1 and 2 wk and age 6 wk (Figure 5). This is because the average mortality rate increases as the cohort ages, and as a result, smaller numbers of flies are required to obtain accurate estimates of mortality. Such is the case until week 7, when the average number of flies in each cage becomes too small (<20), and there are few cages with intermediate levels of mortality (many cages become extinct and must be treated as missing data). Genetic variance in log mortality tends to be underestimated at older ages (Figure 5). As a proportion of the actual parameter value, the average estimated genetic variance underestimates the actual variance by 24, 10, and 12% in the high, low, and combination simulations, respectively. A major source of bias in these cases is the generation of large mortality rates late in life (after week 4) causing many cages to become extinct; this is reflected in the larger underestimates in the high-variance simulations. In our actual data, there were no extinctions of cages before week 6, suggesting that this source of bias is minimal. In summary, the power to detect any fixed level of genetic variance does not decline dramatically in our experiment until week 7, and the age-related bias in the estimates of genetic variance, though considerable, are not sufficient to generate the magnitude of age-specific changes seen in our data. Therefore, it is not likely that the observed decline in genetic variance after week 4 is an artifact of declining sample size in each cohort.

Mortality models: Mathematical models are useful for summarizing general differences in age-specific mortality between experimental cohorts. For example, under the Gompertz model, mortality variation can be divided into differences in baseline mortality and differences in the rate of aging. The logistic frailty model adds a third term describing the degree of mortality deceleration, and, therefore, differences in this aspect of age-specific mortality can be examined as well. The large mutational variance in mortality for female mortality rates early in life (Figure 2) likely accounts for the significant genetic variation detected in the baseline mortality parameter of the Gompertz and logistic models (Table 4). Since mortality rates converge at older ages, this variation would be translated into variation in the rate parameter of these models. Furthermore, despite strong evidence for age-specific effects of mutations in males (Figure 2), we fail to detect significant mutational variance for patterns of mortality under either of the models (Table 4). It is not clear how transient, age-specific changes in mortality rates would affect the parameters of these models. It is likely that age-specific "bumps" in the mortality curves would result in somewhat unstable estimates of model parameters. New mathematical mortality models that describe how mortality rates should change with age and that incorporate the possibility of age-specific changes in mortality are needed to further investigate different classes of mutational effects.

Mutation and life histories: The primary motivation behind this study was to determine how spontaneous mutations affect mortality. Is there a class of mutations that affect mortality in only a subset of ages? What is the pleiotropic structure of mutations in regard to mortality rates at various ages? If mutations do have age-specific properties, are mutations with effects at one age as likely to occur as mutations with effects at another? These questions relate directly to current thought about the evolution of life history characters in general and the evolution of senescence in particular. Investigations into the genetics of age-specific characters are revealing inconsistencies between theoretical predictions and experimental data (Promislow *et al.* 1996; Tatar *et al.* 1996). When this happens, it is often the assumptions of the theoretical models that come under scrutiny. In many cases, evolutionary models of life history characters are quite specific about how spontaneous mutations are assumed to affect age-specific mortality, and such assumptions can be critical to predictions about equilibrium levels of genetic variance and about the evolution of senescence.

Relationship between mutation and levels of standing genetic variance: The high mutational variance early in life and low mutational variance at older ages provide insight into the factors that influence estimates of variance in equilibrium populations. Observed levels of genetic variance and covariance in outbred laboratory populations of Drosophila result from an interaction between selection and mutation (Clark 1987). In age-structured populations, the intensity of natural selection declines with advancing age (Medawar 1952; Hamilton 1966), and assuming that mutations with age-specific effects on mortality occur at a constant rate independent of the age of effect, we would expect standing genetic variance to be greatest at the oldest ages (Charlesworth 1990). Promislow *et al.* (1996) argue that observed genetic variance for mortality increases at early ages but declines later in life. Our results are consistent with this observation, suggesting that levels of genetic variance early in life are kept low by natural selection, but the absence of significant effects of mutation at older ages is responsible for the decline of variance late in life.

Additional information concerning the nature of standing levels of genetic variance for mortality can be obtained by examining a response to selection. A number of laboratories have selected for increased longevity in Drosophila and have observed a significant response within 15–20 generations (Luckinbill *et al.* 1984; Rose 1984; Partridge and Fowler 1992; Engstrom *et al.* 1992; Zwaan *et al.* 1995). Our analysis of age-specific mortality in the lines selected by Luckinbill reveals that the increased longevity has resulted from a nearly

proportional decrease in mortality throughout life (Curtsinger *et al.* 1995; Pletcher 1997). Interestingly, both selected and control lines exhibit a deceleration in mortality rates at older ages, and although this leveling-off occurs later in the selected lines, the absolute level of mortality at this point is unchanged (Curtsinger *et al.* 1995). Given our findings concerning the age-specific effects of mutations, this observation is consistent with the idea (Tatar *et al.* 1996) that the selection response in these experiments is largely caused by the removal of deleterious alleles whose effects are expressed after 1 wk posteclosion rather than by the change of allele frequencies at loci with antagonistic effects on mortality at different ages.

The evolution of senescence: Currently, there are two predominant evolutionary models of senescence—mutation accumulation (MA) and antagonistic pleiotropy (AP) (Partridge and Barton 1993; Charlesworth 1994; Curtsinger *et al.* 1995). Antagonistic pleiotropy postulates the existence of genetic constraints such that an increase in early life fitness components results in a reduction of fitness late in life (Williams 1957). As a result of the early fitness benefits, the optimal genotype will evolve to exhibit senescence (Partridge and Barton 1993). Mutation accumulation assumes that, because the intensity of natural selection declines with advancing age, mutations with specific effects late in life will reach a higher frequency in mutation-selection balance the later the age at which they act (Partridge and Barton 1993; Charlesworth and Hughes 1996).

The MA hypothesis requires that there be a substantial input into a population of deleterious mutations that affect mortality and that there is a class of such mutations whose effects are restricted to a narrow range of ages (Partridge and Barton 1993; Charlesworth 1994). Research into the effects of mutation on quantitative traits has shown that there is a substantial input of deleterious mutations into populations (Simmons and Crow 1977; Kondrashov 1988; Mackay *et al.* 1992b; Houle *et al.* 1994; Mackay *et al.* 1994). The relatively high estimates of mutational variance for early life mortality reported here provides direct support for this assumption. Our observations of spontaneous mutations with age-specific effects on mortality and their relative frequencies among those measured provide the first evidence in support of the second assumption of this hypothesis. Specifically, mutational changes in mortality early in life are uncorrelated with changes at older ages. In contrast, Houle *et al.* (1994) found a moderately high, positive mutational correlation ($Cov_m = 0.6$) between early and late fecundity in Drosophila, and they argued the apparent lack of age specificity was evidence against the MA hypothesis.

The AP theory predicts the existence of mutations with opposite effects on fitness at two separate ages. Our failure to observe mutations of this kind cannot be used to reject this hypothesis for two reasons. First, because mutations that increase early fitness at the expense of late can be extremely rare and still contribute to senescence (Partridge and Barton 1993), it is possible we simply failed to sample enough mutations. Second, we only measured variation in mortality, and it is conceivable that some mutations that affect mortality have opposite, pleiotropic effects on other components of fitness. While no evidence for or against AP arises from this study, however, there are many reasons to be skeptical of its general validity (Curtsinger *et al.* 1994, 1995).

Results from this study and others examining the genetics of age-specific life-history characters (Promislow *et al.* 1996; Tatar *et al.* 1996) suggest that, under their present formulations, neither MA nor AP is sufficient to explain observed age-dependent patterns of mortality. Assuming reasonable effects of new mutations, neither model is consistent with mortality rates leveling off at a relatively low value (usually $\sim$0.2) late in life (Pletcher and Curtsinger 1997, manuscript in review). Furthermore, the lack of significant mutational effects on mortality rates at older ages, coupled with the absence of standing genetic variance at these ages in equilibrium populations, present a serious challenge to the classical interpretation of these models (Promislow *et al.* 1996; Curtsinger *et al.* 1995). Models of senescence that make clear the factors influencing mortality rates throughout life might provide a clue as to why mortality rates late in life are not affected by mutation. Death is the failure of a complicated system, and the genetic effects of mutation must be interpreted within the context of this system.

Future prospects in the evolutionary study of aging: Ideally, we are interested in estimating the rates of mutation at loci with age-specific effects and the age-specific changes in mortality resulting from mutation at a single locus. Traditional inferences of minimum mutation rate and maximum gene effect using models of equal gene effect (Mukai 1964) are not useful for age-specific data. The inability to observe the number of mutations that occur in each accumulation line leaves open the possibility that mortality changes at one age are generated by the combined contributions (possibility antagonistic) of mutations whose major effect is focused at a different age. Experiments inducing single mutational events are important for investigating the age dependence of the average mutation. Such information is vital to the development of a statistical model capable of estimating mutational properties based on data from all ages.

Despite statistical difficulties, recent work investigating the genetic properties of age-specific characters has produced a number of exciting and unexpected results (Hughes and Charlesworth 1994; Hughes 1995; Promislow *et al.* 1996; Tatar *et al.* 1996; Charlesworth and Hughes 1996). Many of these discoveries

are not consistent with the predictions of either of the evolutionary models of senescence in their present from. We echo Promislow *et al.* (1996) in calling for the development of more realistic biological models of senescence. These models might incorporate the average effect of mutations across ages observed here and the relatively weak influence of mutation on mortality at older ages. When combined with the age-dependent nature of the intensity of natural selection, they might predict an initial increase and then decrease in age-specific genetic variance, as well as a leveling-off of the probability of death late in life at a value significantly below unity.

We thank A. Khazaeli, D. Promislow, R. Shaw, M. Simmons, M. Tatar, and two anonymous reviewers for comments on the manuscript. Thanks especially to K. Broz, C. Gendron, G. Kantor, and K. Kelly for their technical assistance and endless patience in counting dead flies. This work was supported by National Institutes of Health grants AG-0871 and AG-11722 to J.C.

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Communicating editor: A. G. Clark

APPENDIX

This appendix describes the procedure used to calculate the statistical power to detect levels of age-specific genetic variance in mortality rates. The general approach was based on a suggestion provided by D. Promislow.

Each simulated data set was created according to a three-step procedure. First, log mortality rates were generated for each of the 116 cages (29 distinct mutation accumulation lines each with two genetic replicates and each genetic replicate represented by two population cages; see materials and methods) according to specific levels of between-line (genetic), between-replicate, and error variance. Second, the probability of death for each individual in each population cage was calculated from the simulated mortality rates. Random numbers were drawn for each individual to determine if the individual died in the age interval in question, and the resulting "observed" mortality rates were calculated. Third, variance component analysis was carried out on the "observed" mortality rates to estimate the level of between-line variance and to determine if between-line variance was significant ($P < 0.05$).

Log mortality rates were simulated using the covariance matrix for all cages in the experiment. Because each of the 29 mutation accumulation lines was composed of two observations from each of two genetic replicates, the phenotypic covariance matrix of log mortality rates at any specific age can be written as follows:

$$\mathbf{P} = [\mathbf{I_{29}} \otimes \mathbf{J_4}]\sigma_l^2 + [\mathbf{I_{58}} \otimes \mathbf{J_2}]\sigma_r^2 + \mathbf{I_{116}}\sigma_e^2, \qquad \text{(A1)}$$

where $\mathbf{I_p}$ is a $p \times p$ identity matrix, $\mathbf{J_q}$ is a $q \times q$ matrix of ones, $\otimes$ represents the Kronecker product, σ_l^2 reflects the genetic variance, σ_r^2 is the between-replicate variance, and σ_e^2 is the random environmental variance (see materials and methods). The covariance matrix, **P**, is then decomposed, using the Cholesky factorization, into the lower triangular matrix **L** such that $\mathbf{P} = \mathbf{LL^T}$, where T denotes matrix transposition. A sequence, **x**, of 116 independent, standard normal deviates is then generated, and the vector **y** is calculated according to $\mathbf{y} = \mathbf{Lx}$. The vector **y** now has covariance matrix $Cov[\mathbf{y}] = \mathbf{L}Cov[\mathbf{x}]\mathbf{L^T} = \mathbf{LI_{116}L^T} = \mathbf{P}$. For each age, the mean female mortality rate was added to each component of **y** to obtain the proper simulated mortality rates for that particular age.

Values used to simulate data were obtained from the actual female data set. Three distinct levels of variance were used. The first, $V_l = 0.4$, $V_r = 0.2$, and $V_e = 0.3$, was based on the levels of variance estimated for mortality rates early in life. The second, $V_l = 0.2$, $V_r = 0.1$, and $V_e = 0.2$, represented variance estimated from older ages (> week 5), and the third, $V_l = 0.2$, $V_r = 0.2$, and $V_e = 0.3$, was a combination of the two. Mean log mortality rates used for generating mortality values at each age were −3.65, −3.27, −1.55, −0.371, 0.188, 0.385, and 0.673 for weeks 1–7, respectively.

Once log mortality rates were obtained for each cage (i) and for each age (x), the probability of death for individuals in that cage during that age interval, q_x^i, was calculated according to the following equation:

$$q_x^i = 1 - \exp[-e^{y_i}] \quad \text{(A2)}$$

Then, for each individual alive at the start of age x, a uniform random number was drawn. If the number was less than q_x^i, the individual was considered to have died during the age interval; otherwise, it survived. Thus, for each cage, we were able to calculate the "observed" mortality rate according to Equation 1. These values were then log transformed for the variance component analysis. The number of individuals assumed to be alive at the start of each age was calculated from the actual data as the average number of females alive in each cage at the start of each week. These values are 419, 402, 380, 285, 154, 60, and 16 for weeks 1–7, respectively.

Variance components were then estimated for each data set using the *varcomp* procedure in S-Plus (MathSoft). This procedure also provides the asymptotic variance of the estimates. To determine statistical power, each data set was considered to show significant genetic variation if the point estimate of between-line variance was larger than twice the standard deviation of the estimate (indicating that it was significantly >0 at the 0.05 level). For each age and combination of variance parameters, the fraction of data sets out of 500 that indicated significance was calculated and is reported as statistical power in Figure 5. In addition, for each set of 500 simulations, the mean estimate of between-line variance was calculated to investigate the potential for age-related bias in parameter estimation (Figure 5).