On the Rate and Linearity of Viability Declines in Drosophila Mutation-Accumulation Experiments: Genomic Mutation Rates and Synergistic Epistasis Revisited

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Manuscript received July 9, 2003
Accepted for publication November 11, 2003

ABSTRACT

High rates of deleterious mutations could severely reduce the fitness of populations, even endangering their persistence; these effects would be mitigated if mutations synergize each others' effects. An experiment by Mukai in the 1960s gave evidence that in *Drosophila melanogaster*, viability-depressing mutations occur at the surprisingly high rate of around one per zygote and that the mutations interact synergistically. A later experiment by Ohnishi seemed to support the high mutation rate, but gave no evidence for synergistic epistasis. Both of these studies, however, were flawed by the lack of suitable controls for assessing viability declines of the mutation-accumulation (MA) lines. By comparing homozygous viability of the MA lines to simultaneously estimated heterozygous viability and using estimates of the dominance of mutations in the experiments, I estimate the viability declines relative to an appropriate control. This approach yields two unexpected conclusions. First, in Ohnishi's experiment as well as in Mukai's, MA lines showed faster-than-linear declines in viability, indicative of synergistic epistasis. Second, while Mukai's estimate of the genomic mutation rate is supported, that from Ohnishi's experiment is an order of magnitude lower. The different results of the experiments most likely resulted from differences in the starting genotypes; even within Mukai's experiment, a subset of MA lines, which I argue probably resulted from a contamination event, showed much slower viability declines than did the majority of lines. Because different genotypes may show very different mutational behavior, only studies using many founding genotypes can determine the average rate and distribution of effects of mutations relevant to natural populations.

THE genomic rate of deleterious mutations and the way in which the mutations interact have important evolutionary consequences. Mutation rates on the order of one or more per zygote per generation could cause the extinction of small populations (Gabriel and Bürger 1994; Lande 1995; Lynch *et al.* 1995) and could cause severe reductions in fitness (genetic loads) even in large populations (Crow and Simmons 1983). Synergistic epistasis among deleterious mutations increases the efficacy of selection against them and therefore reduces the mutational genetic load (Crow 1970). High deleterious mutation rates with synergistic epistasis also create conditions favorable for the evolution of sexual reproduction (Kondrashov 1988; Charlesworth 1990).

An early experiment on Drosophila by Mukai and co-workers (Mukai 1964, 1969; Mukai and Yamazaki 1968) seemed to give evidence that the genomic rate of deleterious mutations, U, is indeed high and that mutations synergize each others' fitness effects. Mukai propagated a set of mutation-accumulation (MA) lines in which the second chromosome was protected from recombination and most selection. The viability decline over the first 25 generations, together with the increase in among-line variance, gave rise to an estimate of the rate of viability-reducing mutations of nearly one per zygote per generation (Mukai 1964). In later generations, the viability decline accelerated, suggesting that mutations occurring in already mutation-laden backgrounds had greater effects than those that occurred initially (Mukai and Yamazaki 1968; Mukai 1969). Subsequent experiments by Mukai *et al.* (1972) and Ohnishi (1977a) appeared to support Mukai's mutation rate estimate, although Ohnishi's was a bit lower. These later experiments were of shorter duration (40 generations) and therefore had less power to detect an acceleration of the rate of decline; nonetheless, the viability decline in Ohnishi's experiment appeared to decelerate, inconsistent with synergistic epistasis (see also García-Dorado and Caballero 2000; Fry 2001).

In the last several years, however, Mukai and Ohnishi's conclusions have been called into question (Keightley 1996; García-Dorado 1997; García-Dorado and Caballero 2002). The central problem is that none of the three experiments had a satisfactory control for the viability declines observed in the MA lines. Thus it is possible that some or all of the declines had nonmutational causes, leading to an overestimate of the genomic mutation rate. Possible nonmutational causes of viability

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declines include subtle changes of rearing conditions, evolution of the balancer chromosome (Keightley 1996), or scoring errors (Fry *et al.* 1999). Furthermore, a nonmutational decline occurring late in an MA experiment would give the false impression of synergistic epistasis, while one occurring early would have the reverse effect. Skepticism of Mukai's original conclusions has been bolstered in recent years by MA experiments on Drosophila giving lower estimates of *U* (García-Dorado *et al.* 1998; Fry *et al.* 1999; Chavarrías *et al.* 2001; Fry 2001) and studies of diverse species giving little evidence for synergistic epistasis (de Visser *et al.* 1997; Elena and Lenski 1997; Elena 1999; Peters and Keightley 2000; but see Whitlock and Bourguet 2000).

It is tempting simply to dismiss Mukai and Ohnishi's experiments as flawed. Mutation-accumulation experiments, however, are difficult to perform, and the magnitude of *U* and the prevalence of synergistic epistasis are still unresolved (Lynch *et al.* 1999). Therefore any method for estimating viability declines relative to a suitable control from Mukai and Ohnishi's data sets would be valuable.

Here, I point out that such a method exists. Both Mukai and Yamazaki (1968) and Ohnishi (1977b) simultaneously estimated viability of not only homozygous MA lines, but heterozygotes created by crossing different MA lines. If mutations are recessive or partly so, then the viability of homozygotes relative to that of heterozygotes should decline as the lines accumulate mutations. The absolute rate of viability decline of homozygotes relative to the progenitor chromosome can be estimated by using the difference between heterozygous and homozygous viability and an estimate of the average dominance (ratio of heterozygous to homozygous effects) of mutations. Such estimates are reported by Mukai and Yamazaki (1968) and can be calculated from data in Ohnishi's (1974) thesis (García-Dorado and Caballero 2000). Even without dominance estimates, the data on homozygous and heterozygous viabilities allow one to examine the viability declines for linearity, assuming only that the average degree of dominance remained constant throughout the experiment.

Applying these methods yields two unexpected conclusions. First, Ohnishi's (1977b) experiments with both spontaneous and ethyl methanesulfonate (EMS)-induced mutations show accelerating viability declines, consistent with synergistic epistasis. The acceleration of the viability declines may have been obscured by a nonmutational viability decline occurring early in the experiments (*cf.* García-Dorado and Caballero 2000). Second, genomic deleterious mutation rates estimated from the two studies differ strongly. While the results support Mukai's (1964) original, high estimate of *U*, the estimate from Ohnishi's study is an order of magnitude lower. I argue that the different *U* estimates most likely reflect true differences in mutational activity between the strains studied; indeed, as García-Dorado and Caballero (2002) have recently pointed out, mutation rates appear to even have been heterogeneous among the lines in Mukai's experiment.

MATERIALS AND METHODS

Overview of MA experiments: In each of the experiments of Mukai (1964; Mukai and Yamazaki 1968) and Ohnishi (1974; 1977a,b), mutations were accumulated on second chromosomes descended from a single wild-type progenitor chromosome (+). Each experiment was initiated by crossing a single *Pm*/+ male to females from a *Cy/Pm* balanced lethal stock; here *Pm* is the dominant marker *Plum*, and *Cy* denotes a balancer (a chromosome with multiple inversions to suppress recombination) bearing the dominant marker *Curly*. *Pm*/+ progeny were then crossed individually to *Cy/Pm* stock females to create multiple MA lines, each of which was propagated by repeating the cross with a single *Pm*/+ male each generation. Deleterious mutations are expected to accumulate on the + chromosomes in such lines due to their very small effective population size and the lack of recombination in males. In addition to the spontaneous mutation treatment, Ohnishi created two treatments in which males were fed 0.1 and 0.5 mM EMS each generation.

At intervals, homozygous viability was estimated by intercrossing $Cy/+_i$ males and females, where $+_i$ denotes a chromosome from the *i*th MA line. Both sets of authors used the percentage of wild-type flies emerging from the crosses as their measure of viability; if *Cy*/+ and +/+ genotypes have equal viability, this is expected to be 33.3% (the *Cy*/*Cy* combination is lethal). The authors also conducted two types of crosses to estimate heterozygous viability, "coupling" and "repulsion." For our purposes, the repulsion crosses are more useful; in these, $Cy/+_i$ females were crossed to $Cy/+_j$ males, where $+_i$ and $+_j$ come from different MA lines. Averaged over the entire set of crosses, the same set of *Cy* competitor genotypes is produced by these crosses as in the homozygous crosses. Moreover, in both types of crosses, each line was used once as a female parent and once as a male parent, so maternal and paternal effects contribute equally to the cross types. In the coupling crosses, $Cy/+_i$ males were crossed to females of a standard stock. Unlike the repulsion crosses, the coupling crosses are not equivalent to the homozygous crosses in terms of competitor genotype or maternal and paternal effects; therefore the coupling viabilities are disregarded here, except where noted below. Details specific to each experiment are considered below.

Examining viability declines for linearity: The percentage of wild-type flies tends to underestimate true viability differences (Latter and Sved 1994). To determine whether viabilities declined linearly over time, it is preferable to use relative viability, defined as RV = 2(no. of wild-type flies)/(no. of *Cy* flies). If the genotypes have equal viability and the number of *Cy* flies is not small, RV has an expected value of one. The mean percentages of wild-type flies reported by the authors were therefore converted to relative viabilities. The ratio of mean relative viability of homozygotes to that of repulsion heterozygotes estimates the corresponding ratio of mean absolute viabilities (*i.e.*, the viabilities of the *Curly* genotypes cancel). Under the assumptions that genes act multiplicatively (*i.e.*, there is no epistasis on a logarithmic scale), the log of this ratio should decline linearly over time.

Estimating genomic mutation rates: A lower bound for the diploid genomic mutation rate is given by the formula of Bateman (1959) and Mukai (1964):

$$U_{BM} = \frac{5(\Delta M)^2}{\Delta V} \leq U. \quad (1)$$

Here, ΔM is the per-generation rate of viability decline, and ΔV is the rate of increase of among-line variance; the 5 scales the estimate to the entire genome. Variation in mutational effects causes U_{BM} to systematically underestimate U; the two are equivalent only when all mutations have equal effects (Mukai *et al.* 1972). Similarly, an upper-bound estimate of S, the average mutational effect, can be obtained as

$$S_{BM} = \frac{\Delta V}{\Delta M} \geq S. \quad (2)$$

Improved estimates of U_{BM} and S_{BM} can be obtained by replacing Mukai and Ohnishi's ΔM estimates with estimates calculated from heterozygous and homozygous viabilities. Because ΔV estimates are available only on the authors' original scale, this scale is retained. Letting $P_h(t)$ represent the mean percentage of wild-type flies at generation t in the homozygous crosses, we have

$$P_h(t) = P(0) - t\sum_{loci} u_i s_i = P(0) - t\Delta M. \quad (3)$$

Here, u_i and s_i are the mutation rates and effects, respectively, at the ith locus. Similarly, letting $P_r(t)$ be the percentage of wild-type flies from the repulsion crosses, we have

$$P_r(t) = P(0) - t\sum_{loci} 2u_i h_i s_i = P(0) - 2th_s\Delta M, \quad (4)$$

where h_i is the dominance coefficient at the ith locus, and $h_s = \Sigma u_i h_i s_i / \Sigma u_i s_i$, the average dominance coefficient weighted by s. Subtracting (3) from (4) and rearranging gives

$$(1 - 2h_s)\Delta M = \frac{P_r(t) - P_h(t)}{t}. \quad (5)$$

Thus ΔM can be estimated if an estimate of h_s is available.

Mukai and Yamazaki (1968) and Ohnishi (1977b) both reported estimates of h_s, but these are based on the authors' ΔM estimates; therefore they cannot be used without circularity. Fortunately, estimates of a related quantity, not dependent on the authors' ΔM estimates, are available for both experiments. This is h_s^2, the average dominance weighted by s^2 rather than by s; it can be estimated by dividing the covariance between repulsion heterozygote viability and midparent (homozygous) viability by the among-line variance for homozygous viability (Mukai *et al.* 1972). If h and s are negatively correlated, as is sometimes believed to be the case, h_s^2 would underestimate h_s. As the following example shows, the difference between the two quantities is likely to be slight. Suppose that there is an extreme negative correlation between h and s, such that h declines linearly from 0.5 to 0 as s, expressed as a proportion of original viability, increases from 0 to 0.5. (Values of $s > 0.5$ do not need to be considered, because the data sets to be analyzed include only "quasi-normal" lines, those with viability at least half of the starting viability.) If s is assumed to have a triangular distribution that falls off from 0 to 0.5, then its probability density function is given by $4 - 8s$. Using $h = 0.5 - s$ and integrating gives $h_s = E(hs)/E(s) = 1/4$, and $h_s^2 = E(hs^2)/E(s^2) = 1/5$. Thus with a more realistic, less extreme negative relationship between h and s, the difference between h_s^2 and h_s is likely to be trivial. In any case, any bias caused by using h_s^2 in place of h_s would be in the same direction as the inequalities already present in (1) and (2).

Mukai and Yamazaki's data set: Mukai and Yamazaki (1968) measured viability of homozygotes and repulsion heterozygotes at generations 32 and 52. They report means and among-line variance estimates for all quasi-normal lines (their Tables 1 and 2) and for "group 2" lines only (their Table 4).

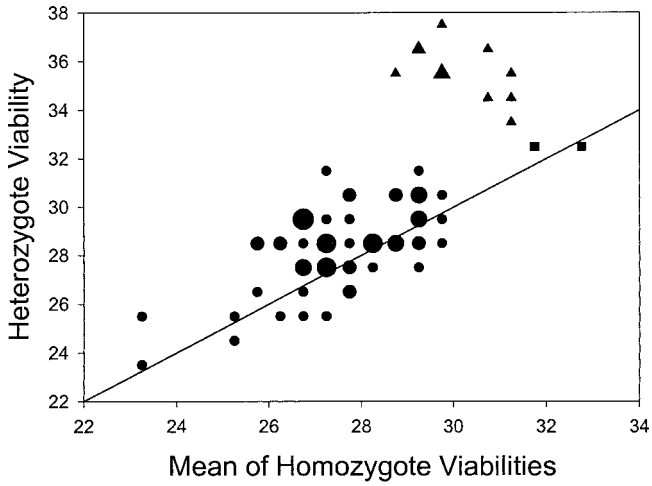

Figure 1.—Relationship between viability of repulsion heterozygotes and the mean viability of the parental homozygotes in the MA experiment of Mukai and Yamazaki (generation 32). Symbol sizes are proportional to the number of observations (one to five) with the given combination of values. The circles represent crosses between group 2 lines. The authors state that the cluster of 11 points (14 crosses) in the top right were crosses involving at least one group 1 line. Because crosses were between lines with sequential numbers, and only two pairs of group 1 lines had sequential numbers (91 and 92; 15 and 16), two of these crosses must have been between group 1 lines and the remaining 12 between a group 1 and group 2 line. Because group 1 lines had higher homozygous viability than group 2 lines, one can infer that the crosses between group 1 lines are the two rightmost points (squares), while the rest (triangles) were crosses between a group 1 and group 2 line. The line represents equal viability of heterozygotes and homozygotes. This was redrawn from Figure 6 in Mukai and Yamazaki (1968).

The authors made the distinction between the group 2 ($N = 72$) and group 1 ($N = 8$) lines after noticing puzzling differences between the two groups. First, while viability of the group 2 lines declined steadily throughout the experiment, that of the group 1 lines remained essentially constant (Mukai and Yamazaki 1968; see also García-Dorado and Caballero 2002). Second, in repulsion crosses, there was a positive correlation between homozygous and heterozygous viability when only crosses involving group 2 lines were considered, but a negative correlation when crosses involving group 1 lines were considered (Figure 1). In the discussion, I argue that the best explanation for these results is that the group 1 lines derived from a contamination event that occurred early in the experiment. For this reason, I restrict my analysis to the group 2 lines. The authors reported estimates of h_s^2 for these lines at generations 32 and 52 in their Table 4. The same table presents among-line variances in homozygous viability, which were divided by generation number to give ΔV estimates.

Ohnishi's data set: Ohnishi (1977b) presents viability means of repulsion heterozygotes and the parental homozygotes at generations 10, 20, 30, and 40. In addition, he presents means at generations 3, 7, 15, and 25 for the 0.1-mm EMS treatment and at generations 3, 7, and 13 for the 0.5-mm treatment. To test for linearity of the viability declines, the log of the ratio of homozygous RV to heterozygous RV was regressed against generation number for each treatment, forcing the regression through the origin (before any mutations had time to occur, heterozygotes and homozygotes would

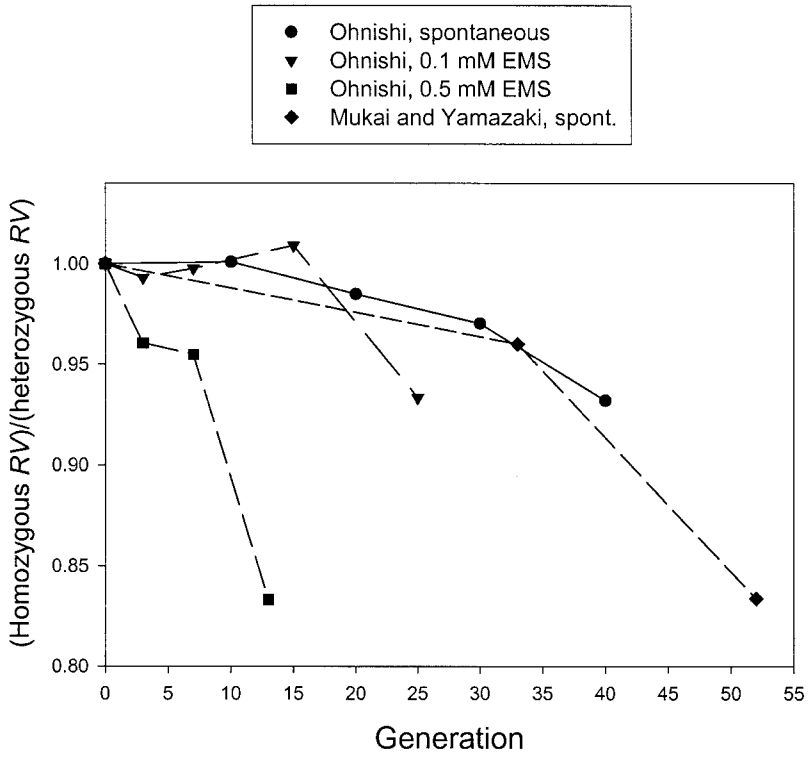

FIGURE 2.—Ratios of relative viability of homozygous MA lines to that of repulsion heterozygotes in the experiments of MUKAI and YAMAZAKI (1968) and OHNISHI (1977b). Data from Ohnishi are for nonlethal lines (quasi-normal + deleterious).

have been identical). The improvement in fit from adding a quadratic term was investigated. This was done for both quasi-normal lines and the broader category of nonlethal lines.

Ohnishi did not present estimates of h_s^2, but GARCÍA-DORADO and CABALLERO (2000) noted that it is possible to estimate h_s^2 for quasi-normal lines from data in his thesis (OHNISHI 1974). Ohnishi reported correlations between heterozygous and homozygous viabilities, r_{xy}; from these, García-Dorado and Caballero estimated the covariance between heterozygous viabilities as $r_{xy}s_x s_y$, where s_x and s_y are the standard deviations of homozygous and heterozygous viability means, respectively. They estimated s_x and s_y from mean squares reported by OHNISHI (1974), but noted that these mean squares were for a broader set of lines than those used to calculate the correlations. However, OHNISHI (1974) reported s_x and s_y for the relevant set of repulsion heterozygotes and their parental homozygotes in his Table 14; for coupling heterozygotes, the same quantities can be easily calculated from data in his Table 13. I used these s_x and s_y values in place of the ones used by GARCÍA-DORADO and CABALLERO (2000) to calculate revised h_s^2 estimates, otherwise following their procedure. Among-line variances in homozygous viability were taken from OHNISHI (1977a).

RESULTS

Linearity of viability declines: In MUKAI and YAMAZAKI's (1968) experiment, the decline in homozygous RV/heterozygous RV between generations 32 and 52 is much greater than what occurred up until generation 32 (Figure 2). One caveat is that while the authors excluded four lines with <20% wild type (RV < 0.5) at generation (G)32, no such criteria were applied at G52, when many lines had <20% wild type. This would enhance the apparent acceleration of the viability decline. Including the four low-viability lines probably would not have had a great effect on the G32 means, however, because they comprised only 5% of the total.

In all three of OHNISHI's (1977b) experiments, there was also a tendency for viability declines to accelerate (Figure 2). For the spontaneous mutation treatment, adding a quadratic term significantly improves the fit of the regression of log(homozygous RV/heterozygous RV) against time (Table 1). For the two EMS treatments, adding a quadratic term does not significantly improve the fit, but in all cases a model with a quadratic term alone gives a higher R^2 than a model with a linear term alone (Table 1). Due to the small number of points, all of the tests have low power.

Genomic mutation rate estimates: MUKAI and YAMAZAKI (1968) present three estimates of h_s^2, 0.361 at G32 and 0.410 and 0.465 at G52. Combining these with the heterozygous and homozygous viability means reported by the authors gives ΔM estimates of 0.09 and 0.47, respectively (Table 2; the units are the percentage of wild-type flies). The corresponding U_{BM} estimates are 0.37 and 2.1 (Table 2), roughly in keeping with MUKAI's (1964) original estimate of 0.71 from the same experiment.

It is possible to calculate four estimates of h_s^2 for repulsion heterozygotes from data in OHNISHI's (1974) thesis, one each from G10, -20, -30, and -40 (see MATERIALS

TABLE 1

Tests for linearity of viability declines in Ohnishi's experiment

	Simple linear regression		Adding quadratic term		Quadratic term only	
	Probability	R^2	Probability	Multiple R^2	Probability	R^2
Nonlethal lines						
Spontaneous	0.009	0.88	0.025	0.99	0.0009	0.98
0.1 mM EMS	0.063	0.60	0.16	0.88	0.046	0.78
0.5 mM EMS	0.015	0.94	0.43	0.98	0.013	0.97
Quasi-normal lines						
Spontaneous	0.016	0.83	0.053	0.98	0.004	0.95
0.1 mM EMS	0.20	0.24	0.45	0.47	0.29	0.35
0.5 mM EMS	0.067	0.75	0.78	0.78	0.12	0.78

Log(homozygous relative viability/heterozygous relative viability) was regressed against generation number. In simple linear regressions, all estimated slopes were negative. Quadratic terms were likewise always negative (downward curvature).

and methods). These are 0.16, 0.22, 0.20, and 0.03, respectively. Estimates can also be calculated from the coupling crosses; these are −0.02, 0.21, 0.14, and 0.08. All differ only slightly from the estimates calculated by García-Dorado and Caballero (2000). Because the coupling and repulsion estimates are similar, their means are used in Table 2. The resulting estimates of ΔM and U_{BM} are 0–0.027 and 0–0.061, respectively (Table 2). The U_{BM} estimates are considerably lower than Ohnishi's (1977a) estimate of 0.29.

Estimates of U_{BM} and S_{BM} for both studies are shown for a range of dominance estimates in Figures 3 and 4. The difference in U_{BM} estimates between studies stems almost entirely from the different dominance estimates (Figure 3). The estimates become quite sensitive to slight differences in h_s as it approaches 0.5; for this reason, the estimate from G52 of Mukai and Yamazaki's experiment (Table 2) should not be taken too seriously. Nonetheless, if one ignores the nonindependence of different estimates from the same lines, the dominance estimates in Table 2 are significantly different between studies ($t = 4.3$, d.f. = 4, $P = 0.013$). The same is true for the U_{BM} estimates (after log transformation, ignoring the G10 estimate: $t = 4.1$, d.f. = 3, $P = 0.027$). This gives evidence that the different results from the two studies cannot be explained by sampling error alone.

DISCUSSION

A new method to estimate the rate of mutational decline in viability in two Drosophila mutation-accumulation experiments yields two surprising conclusions. First, although homozygous viability means in Ohnishi's (1977a) MA experiments showed decelerating declines over time, the ratio of homozygous to heterozygous viabilities indicates that the declines accelerated (Figure 2), consistent with synergistic epistasis. Second, applying the method to Mukai and Yamazaki's (1968) and Ohnishi's (1974) data gives dramatically different estimates of the minimum rate of spontaneous deleterious mutations per generation. Mukai's (1964) original estimate of nearly one mutation per zygote is supported, while that from Ohnishi's experiment is an order of magnitude lower, consistent with some recent estimates (Fry *et al.* 1999; Chavarrías *et al.* 2001; Fry 2001). These results are discussed in turn.

TABLE 2

Estimates of mutational parameters for quasi-normal lines

Study	Generation	h_s^2	ΔM	ΔV	U_{BM}	S_{BM}
Mukai and Yamazaki (1968)	32	0.361	0.092	0.115	0.370	1.25
	52	0.437	0.470	0.526	2.10	1.12
Ohnishi (1974, 1977a,b)	10	0.071	0[a]	0.070	0	—
	20	0.216	0.015	0.080	0.014	5.32
	30	0.171	0.016	0.069	0.018	4.42
	40	0.054	0.027	0.060	0.061	2.22

ΔM, ΔV, and S_{BM} are given on the percentage of wild-type scale.
[a] Heterozygous viability was slightly lower than homozygous viability at this generation.

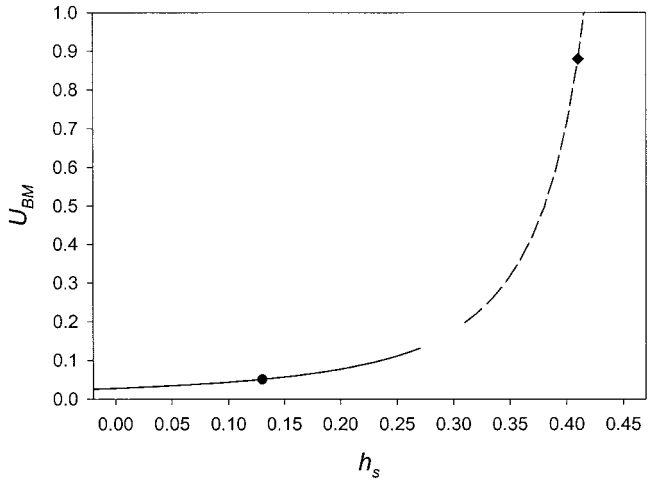

FIGURE 3.—The Bateman-Mukai estimator of mutation rate, U_{BM}, as a function of the average dominance of mutations, h_s, for generation 32 of MUKAI and YAMAZAKI's (1968) experiment (dashed line) and for generations 0–40 in OHNISHI's (1974, 1977a,b) experiment (solid line). OHNISHI's (1977a) regression estimate of ΔV was used, and the per-generation increment of the difference between heterozygous and homozygous viabilities was estimated by regressing the differences against generation number, forcing the regression through the origin. Solid symbols correspond to mean dominance estimates from the two studies.

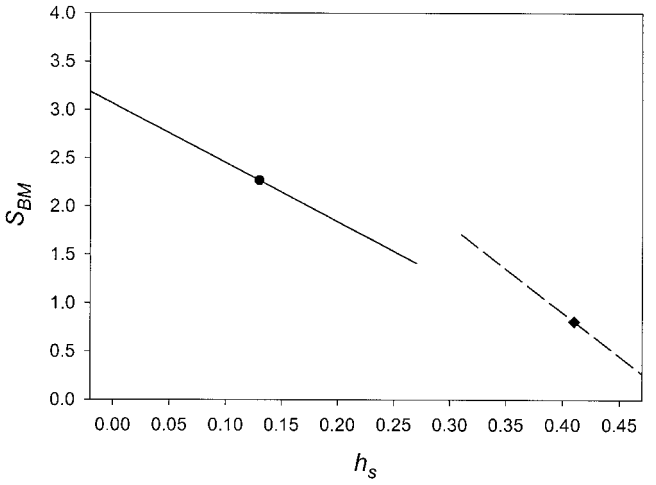

FIGURE 4.—The Bateman-Mukai estimator of the average effect of mutations, S_{BM}, as a function of the average dominance of mutations, h_s, in the experiments of Mukai and Yamazaki (dashed line) and Ohnishi (solid line). See Figure 3 legend for details.

Synergistic epistasis: An acceleration of fitness decline was observed in all four independent MA experiments considered, including the two EMS treatments of Ohnishi. Because the number of time points sampled was small, statistical tests for nonlinearity are either not possible or of low power. Nonetheless, the nonlinearity in Ohnishi's spontaneous MA experiment was significant, and the occurrence of the same pattern in the other three experiments gives evidence that the pattern is real. Unfortunately, data from later experiments in which mutations were accumulated on Drosophila second chromosomes do not give information on the linearity of the declines, either because no appropriate control was available (MUKAI *et al.* 1972) or because viability assays were performed only once (FRY *et al.* 1999; FRY and HEINSOHN 2002).

The simplest explanation for the accelerating declines is that mutations had greater effects when they occurred in backgrounds already containing multiple mutations than when they occurred in relatively mutation-free backgrounds. An alternative explanation is that mutation rates increased over time. As suggested by NUZHDIN *et al.* (1997), increasing mutation rates in MA experiments could result from transposable element (TE) activity, because the rate of new insertions of a particular TE family is likely to be positively related to copy number. The increasing mutation rate hypothesis leads to the prediction that lethal mutation rates should have increased during the experiments. This was clearly not the case in Ohnishi's experiments (OHNISHI 1977a, Figure 7). Unfortunately, MUKAI and YAMAZAKI (1968) do not present data on lethal mutation rates after generation 32, although lethal rates appeared to have been roughly constant up until that time (MUKAI 1964; MUKAI and YAMAZAKI 1968).

One argument against invoking synergistic epistasis to explain the nonlinear viability decline in Ohnishi's spontaneous MA experiment is that the U_{BM} estimate for this experiment, 0.011 mutations per haploid second chromosome per generation, implies that the average number of mutations per line at G40 was considerably less than one. U_{BM} is well known to underestimate U if mutational effects vary, however (MUKAI *et al.* 1972). In addition, as discussed below, synergistic epistasis itself causes U_{BM} to underestimate U.

Although WHITLOCK and BOURGUET (2000) found evidence for synergistic epistasis among visible mutations in Drosophila, several recent studies of other organisms have given little evidence for the phenomenon. In an approach comparable to Ohnishi's EMS treatments, PETERS and KEIGHTLEY (2000) subjected sets of *Caenorhabditis elegans* to zero, one, or two rounds of EMS mutagenesis. For four of five traits examined, mutations did not have a significantly greater effect when occurring in an already mutagenized background, but there was a trend toward synergistic epistasis for most traits. Three studies (DE VISSER *et al.* 1997; ELENA and LENSKI 1997; ELENA 1999) tested for interactions among a set of known mutations in microbes and found no evidence that synergistic epistasis is more common than diminishing-returns epistasis. It is possible that the prevalence of synergistic epistasis increases with organismal complexity; indeed, the high U in mammals suggested

by molecular data (Eyre-Walker and Keightley 1999; Keightley and Eyre-Walker 2000; Nachman and Crowell 2000) would pose a severe challenge to the persistence of populations in the absence of synergistic epistasis. More studies on the prevalence of synergistic epistasis in higher eukaryotes are needed.

Mutation rate estimates: The estimated mutation rate from Ohnishi's experiment reported here is severalfold lower than Ohnishi's (1977a) own estimate. This difference stems from different estimates of ΔM. García-Dorado and Caballero (2000) have suggested that a nonmutational viability decline occurred early in Ohnishi's experiment, causing him to overestimate ΔM. The analysis reported here lends support to that conclusion; while the raw viability means in Ohnishi's experiment showed an initial rapid decline followed by a much slower decline, no such pattern is observed when comparing heterozygous and homozygous viabilities. The nonmutational viability decline invalidates Ohnishi's (1977b) estimates of average dominance of spontaneous and EMS-induced mutations (*cf.* García-Dorado and Caballero 2000; Fry and Nuzhdin 2003; Peters *et al.* 2003), as well as his mutation rate estimates.

One caveat concerning the mutational parameter estimates reported here is that the Bateman-Mukai method, like other available estimation methods, assumes additive interactions among loci. The evidence for synergistic epistasis therefore potentially complicates interpretation of the estimates. In the appendix, I show that in an equal-effects model, synergistic epistasis causes the Bateman-Mukai method to underestimate the number of mutations per line. The degree of underestimation depends on both the number of mutations per line and the strength of the epistasis. This provides another reason, in addition to the likely presence of variation in mutational effects, to regard the U_{BM} estimates reported here as underestimates of the true mutation rates.

The difference in mutation rate estimates between the studies could be explained by differences in methodology or by real differences in mutation rates between the strains used. The former explanation seems unlikely. Both studies used the same method for accumulating mutations, and although Mukai and Yamazaki (1968) used a higher density than Ohnishi (1977a,b) used for the viability assays, Fry and Heinsohn (2002) found that lowering the assay density increased rather than decreased U_{BM} estimates, primarily by decreasing the mutational variance.

In contrast, the difference between the group 1 and group 2 lines in Mukai and Yamazaki's (1968) study (see materials and methods) suggests that different chromosomes can show large differences in rates of mutational viability decline even within the same experiment. In the next section, I take up the issue of the origin of the two groups of lines. I argue that the group 1 lines probably resulted from a contamination event early in the experiment, with the contaminating chromosome having a lower mutation rate than the original chromosome.

TABLE 3

Mean homozygous viabilities of group 1 and group 2 lines in Mukai's MA experiment

	Mean % wild type (no. of lines)	
Generation	Group 1	Group 2
10	34.06 (5)	<31.47 (90)
15	31.72 (5)	<27.68 (89)
20	32.25 (5)	<30.85 (81)
25	32.91 (6)	<28.00 (76)
32	32.57 (8)	27.54 (72)
52	32.35 (8)	19.97 (67)

The group 1 lines were numbers 15, 16, 37, 44, 58, 72, 91, and 92 (Mukai and Yamazaki 1968). The G32 and G52 values come from Mukai and Yamazaki (1968). For G10–G25, viabilities of a subset of group 1 lines (the "order method" controls) and the mean of all quasi-normal lines are given by Mukai (1964). For example, for G10, the viabilities of lines 15, 37, 72, 91, and 92 are given; these average 34.06. The mean of all 98 quasi-normal lines was 31.60. The mean of the 93 lines not including the 5 above was therefore 31.47; these included three group 1 lines, so that the mean of the group 2 lines alone would have been slightly lower.

Overdominant mutations or contamination? Mukai and co-workers obtained a puzzling array of results that seemed to indicate that new mutations were overdominant. In the coupling crosses, in which all lines were crossed to a single high-viability line (no. 92), there was a negative correlation between heterozygous viability and parental homozygous viability, as if mutations that decreased homozygous viability increased heterozygous viability (Mukai *et al.* 1964; Mukai and Yamazaki 1968). In the repulsion crosses, as shown in Figure 1, there was a positive correlation between heterozygous and homozygous (midparent) viability for much of the range of midparent viability, but a negative correlation at the highest values (Mukai and Yamazaki 1968). The authors termed the set of lines showing the positive correlation the group 2 lines; these lines were assumed (apparently accurately) to carry new deleterious mutations. In contrast, the set of lines showing a negative correlation included eight lines that they termed group 1 lines; these lines retained high viability throughout the experiment, as shown in Table 3, and were therefore assumed to carry few or no mutations. The group 1 lines included line 92, the common parent of the coupling crosses. Mukai and Yamazaki's (1968) interpretation of the results in Figure 1 (as well as similar results at generation 52) was that overdominance of new mutations was exhibited only when the mutations were present on one homolog; they called this a "coupling-repulsion" effect. This would explain why strong heterosis was observed in crosses between group 1 and group 2

lines (Figure 1, triangles), but not in crosses within each group (Figure 1, circles and squares).

The coupling-repulsion hypothesis is biologically implausible, and Ohnishi's (1977b) crosses gave no evidence for either overdominance of mutations or a coupling-repulsion effect. A much simpler hypothesis, parenthetically suggested recently by García-Dorado and Caballero (2002), is that the group 1 lines resulted from a contamination event occurring early in the experiment. For example, in the founding generation, some of the balancer stock females may have been fertilized by a male from a stock that had a second chromosome unrelated to the founding chromosome of the group 2 lines. The contaminating second chromosome apparently had a lower mutation rate than the original chromosome and may have had higher homozygous viability initially.

This hypothesis can easily explain most of the puzzling results that Mukai and Yamazaki (1968) obtained. A large number of studies have shown that heterozygotes for unrelated, nonlethal-bearing second chromosomes have much higher viability, ~27% on average, than the parental homozygotes (summarized in Charlesworth and Charlesworth 1987). In the coupling crosses, two types of genotypes would have been produced, I/II heterozygotes and I/I homozygotes. The latter were derived from lines with higher homozygous viability, but the former were heterozygotes for unrelated chromosomes and hence had higher viability. In the repulsion crosses, three types of genotypes would have been produced, I/I, I/II, and II/II. The viability relationships expected under the contamination hypothesis are I/II > I/I > II/II, as observed (Figure 1). The contamination hypothesis also explains the much higher genetic variance among repulsion heterozygotes than among coupling heterozygotes (Mukai and Yamazaki 1968): the former contained relatively low-viability II/II genotypes, while the latter lacked this group.

The heterosis in crosses between group 1 and group 2 lines can be estimated from both the coupling and repulsion crosses and is remarkably close to the expected 27% for crosses between unrelated chromosomes. Considering the 12 repulsion crosses inferred to be between group 1 and group 2 lines (Figure 1), heterozygous and homozygous means estimated from Figure 1 are 35.6 and 30.1%, respectively. On the relative viability scale, these are 1.106 and 0.861, respectively, for a 28% increase of heterozygous over homozygous viability. Although Mukai and co-workers do not give the results of the coupling crosses broken down by which group the parental lines belonged to, Table 3 in Mukai *et al.* (1964) gives heterozygous and homozygous means from G32 for five sets of 16 lines each, ranked by homozygous viability. It can be safely assumed that all the group 1 lines were in the highest-ranking set. Line 92 had homozygous relative viability of 0.945 (32.08% wild type; Mukai *et al.* 1964), and mean homozygous and heterozygous (when crossed to line 92) relative viabilities of the lowest four sets were 0.745 and 1.067, respectively, for a 26% increase of heterozygous over midparent viability. In contrast, crosses among the group 2 lines at G32 showed only 4% heterosis (Figure 2), and the two crosses between group 1 lines showed no evidence for heterosis (Figure 1).

In seeming support of Mukai and Yamazaki's (1968) overdominance hypothesis, however, there is evidence for negative correlations between heterozygous and homozygous viabilities when only crosses generating I/II heterozygotes are considered. The correlation between heterozygous and homozygous viability among the 12 putative crosses between group 1 and group 2 lines in Figure 1 is -0.56 ($P = 0.06$). In addition, the four low-ranking sets of lines from the coupling crosses at G32 (Mukai *et al.* 1964) showed a negative correlation between mean heterozygous and homozygous viabilities ($r = -0.98$, $P = 0.02$). The former result, if real, could be explained by one or two group 1 lines having been misclassified as group 2 lines; these would have relatively high viability for group 2 lines, and so would be among the rightmost triangular points in Figure 1, but would not give rise to as much heterosis as the other lines. One or two incorrectly classified lines would not have a major effect on the mutation rate estimates presented above. The latter result is probably a coincidence. The range of heterozygous viabilities was small (RV = 1.06–1.08), and no similar correlations were observed when the same sets of lines were crossed to two additional unrelated chromosomes (Mukai *et al.* 1965) or in crosses to line 92 at G60 (Mukai and Yamazaki 1968, Figure 5).

The contamination hypothesis has important implications for interpretation of the above mutation rate estimate from the group 2 lines. If the hypothesis is correct, two founding chromosomes, when crossed to the same balancer stock, showed very different rates of mutational decline of viability. Therefore the high mutation rate estimated for the group 2 lines is apparently not a general property of *Drosophila melanogaster* second chromosomes, even under the conditions of Mukai's experiment. The analysis of Ohnishi's data presented above and the results of two recent MA experiments (Fry *et al.* 1999; Fry 2001; Fry and Heinsohn 2002) add support to this conclusion.

The different behavior of the two groups raises the question of what sort of mutations were responsible for the rapid viability decline of the group 2 lines. That these lines may have had unusually high TE activity is supported by two indirect pieces of evidence. First, Fry and Nuzhdin (2003) present evidence that TE insertions have greater average dominance in their viability effects than do base substitutions; the relatively high dominance of mutations in the group 2 lines is therefore consistent with high TE activity. Second, in two MA experiments where different progenitor second chro-

mosomes were crossed to the same balancer stock, we found the retrotransposable element *copia* to be active in one set of lines (Fry and Nuzhdin 2003) but not in the other (the "Experiment 2" lines of Fry and Heinsohn 2002; J. Fry and S. Nuzhdin, unpublished data). The two sets of lines did not differ in rates of viability decline, but the results illustrate that differences in TE activity can be controlled by the second chromosome in experiments using Mukai's design.

Conclusion: Both Ohnishi's MA lines and the group 1 lines of Mukai and Yamazaki appear to have experienced considerably lower rates of deleterious mutations than Mukai and Yamazaki's group 2 lines. The different mutation rates are most plausibly explained by differences in the founding chromosomes themselves. Taken together, the published MA experiments in Drosophila for which reasonably credible estimates of ΔM can be obtained have been based on at most eight founding genotypes. These are the progenitors of Mukai and Yamazaki's group 2 lines and Ohnishi's lines, the two founder chromosomes used by Fry (2001) and Fry and Heinsohn (2002), the founding inbred line used by López-Fanjul and co-workers (Fernández and López-Fanjul 1996; García-Dorado *et al.* 1998; Chavarrías *et al.* 2001), and the three founder chromosomes used by Mukai *et al.* (1972), which arguably should not be included on the list (García-Dorado and Caballero 2002). If there is large variation among genotypes in deleterious mutation rates, as seems to be the case, then the number of genotypes sampled is too small to make generalizations about mutational parameters applicable to natural Drosophila populations. Only future experiments that use many founding genotypes can determine whether the average rate of deleterious mutations in Drosophila populations is closer to the surprisingly high estimate of Mukai (1964; $U \approx 1$), the surprisingly low estimate of García-Dorado *et al.* (1998; $U \approx 0.01$), or an intermediate value ($U \approx 0.1$; Fry 2001; Fry and Heinsohn 2002).

This work was supported by National Science Foundation grant DEB-0108730.

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Communicating editor: D. Begun

APPENDIX: U_{BM} UNDERESTIMATES U WHEN SYNERGISTIC EPISTASIS IS PRESENT

I assume a set of MA lines with a Poisson distribution of mutations per line, with mean n. The fitness of a line with X mutations, relative to the nonmutant ancestor, is $W(X) = -aX - raX^2$, where a and r are constants. We can assume without loss of generality that $a = 1$, simply by choosing the appropriate scale. The average fitness of the lines then becomes

$$E(W) = \sum_{x=0}^{\infty} \frac{(-x - rx^2)e^{-x}n^x}{x!} = -n(nr + r + 1). \quad \text{(A1)}$$

The average squared fitness is

$$E(W^2) = \sum_{x=0}^{\infty} \frac{(-x - rx^2)^2 e^{-x}n^x}{x!} = n[n^3r^2 + (1 + r)^2 + 2n^2r(1 + 3r) + n(1 + 6r + 7r^2)]. \quad \text{(A2)}$$

The variance among lines in fitness is

$$\mathrm{VAR}(W) = E(W^2) - [E(W)]^2 = n[1 + (2 + 4n)r + (1 + 6n + 4n^2)r^2]. \quad \text{(A3)}$$

The Bateman-Mukai estimator of the number of mutations per line, N_{BM}, is simply the square of the mean divided by the variance, which works out to $n \times \beta(n, r)$, where

$$\beta(n, r) = \frac{1 + (2 + 2n)r + (1 + 2n + n^2)r^2}{1 + (2 + 4n)r + (1 + 6n + 4n^2)r^2}. \quad \text{(A4)}$$

Note that as long as $r > 0$ (*i.e.*, synergistic epistasis is present), $\beta(n, r) < 1$. Thus N_{BM} will underestimate the average number of mutations per line. For example, with $n = 10$ and $r = 0.01$ (relatively mild epistasis), $\beta(n, r) = 0.84$. With $r = 0$, $\beta(n, r) = 1$, as expected. As $r \to \infty$, so that fitness depends only on the square of the number of mutations, (A4) becomes

$$\beta(n, \infty) = \frac{1 + 2n + n^2}{1 + 6n + 4n^2}. \quad \text{(A5)}$$

For large n, this approaches $1/4$, the maximum degree of underestimation that can occur in this model.

The above calculations were performed with the aid of Mathematica software (Wolfram 1996).