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Whole-Genome Effects of Ethyl Methanesulfonate-Induced Mutation on Nine Quantitative Traits in Outbred *Drosophila melanogaster*

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ABSTRACT

We induced mutations in *Drosophila melanogaster* males by treating them with 21.2 mm ethyl methanesulfonate (EMS). Nine quantitative traits (developmental time, viability, fecundity, longevity, metabolic rate, motility, body weight, and abdominal and sternopleural bristle numbers) were measured in outbred heterozygous F_3 (viability) or F_2 (all other traits) offspring from the treated males. The mean values of the first four traits, which are all directly related to the life history, were substantially affected by EMS mutagenesis: the developmental time increased while viability, fecundity, and longevity declined. In contrast, the mean values of the other five traits were not significantly affected. Rates of recessive X-linked lethals and of recessive mutations at several loci affecting eye color imply that our EMS treatment was equivalent to ~100 generations of spontaneous mutation. If so, our data imply that one generation of spontaneous mutation increases the developmental time by 0.09% at 20° and by 0.04% at 25°, and reduces viability under harsh conditions, fecundity, and longevity by 1.35, 0.21, and 0.08%, respectively. Comparison of flies with none, one, and two grandfathers (or greatgrandfathers, in the case of viability) treated with EMS did not reveal any significant epistasis among the induced mutations.

KNOWLEDGE of the genomic parameters of spontaneous mutation is essential for resolving several key issues in evolutionary genetics, such as the evolution of sex, recombination, mate choice, and breeding systems, the maintenance of intrapopulation genetic variability, and the long-term viability of small populations (see Barton and Charlesworth 1998; Kondrashov 1998 for reviews). Unfortunately, measuring spontaneous mutation is difficult because of low per nucleotide and per locus mutation rates. Thus, estimates of the genomic deleterious mutation rate U and other key parameters remain controversial (for reviews, see Drake *et al.* 1998; Kondrashov 1998; Keightley and Eyre-Walker 1999; Lynch *et al.* 1999).

In particular, high values of U (≈1) inferred from mutation-accumulation experiments performed over 25 years ago on *Drosophila melanogaster* (Mukai 1964; Mukai *et al.* 1972) have recently been questioned. Estimating U in such experiments involves maintaining a set of isolated strains or a panmictic population under relaxed selection for many generations and measuring the rates of the decline of the mean and of the increase of the variance in one or more fitness-related traits. Several authors (Keightley 1996; García-Dorado 1997; Fry *et al.* 1999; García-Dorado *et al.* 1999; Keightley and Eyre-Walker 1999) claimed that the relative rate of decline of the mean viability reported in the early articles, 1–2% per generation, was overestimated, leading to overestimation of U. A number of new experiments (Keightley and Ohnishi 1998; Fry *et al.* 1999; García-Dorado *et al.* 1999) produced much lower rates of decline of fitness-related traits. In contrast, the results of Shabalina *et al.* (1997) are in excellent agreement with those of Mukai (1964) and Mukai *et al.* (1972).

Measuring parameters of high-rate artificial mutagenesis is much simpler. Knowledge of these parameters can shed light on spontaneous mutation in at least two ways. First, we can measure the impacts of artificial mutagenesis on different traits and predict the ratios of the impacts of spontaneous mutation on the same traits, assuming that these ratios under artificial and spontaneous mutagenesis are similar. Second, if we know the number of generations of spontaneous mutation to which a mutagenic treatment is equivalent, artificial mutagenesis can be used to estimate even the absolute values of parameters of spontaneous mutation.

Artificial mutagenesis is used widely to produce mutant alleles. In contrast, its impact on quantitative traits has been addressed only in a few articles (Mukai 1970; Mitchell 1977; Mitchell and Simmons 1977; Ohnishi 1977a,b,c; Simmons *et al.* 1978; Wijsman 1984; Koivisto and Portin 1987). The analysis of Keightley and Ohnishi (1998), who measured the impacts of a single

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treatment with EMS on nine quantitative traits in *D. melanogaster*, is so far the only application of artificial mutagenesis for estimating the parameters of spontaneous mutation.

In this article, we report the impacts of EMS-induced mutations on the following nine quantitative traits: developmental time, viability, fecundity, longevity, metabolic rate, motility, body weight, and abdominal and sternopleural bristle numbers. In contrast to Keightley and Ohnishi (1998), we used outbred flies and studied the impacts of heterozygous mutations in the whole genome.

MATERIALS AND METHODS

Stem population: In October 1998, we started a panmictic laboratory population of *D. melanogaster* from 50 mated females sampled from a wild population in Ithaca, NY. This population was kept under 16° for three generations before the start of the experiment.

Cultural condition: Unless otherwise specified, flies were grown under a 12/12 light cycle, at 25° and 75% humidity. We used 95 × 25-mm vials with 10 ml of medium containing 10 g agar, 80 g brewer's yeast, 80 g glucose, and 8 ml propionic acid per liter of water, seeded with few grains of live yeast. CO_2 anesthesia was used when handling the flies.

EMS mutagenesis: In January 1999, ∼500 males were sampled from the stem population. Males were kept without food and water for 16 hr and then placed for 20 hr in vials with filter paper soaked with EMS solution (21.2 mm) in 2% sucrose (Ashburner 1989). Six hours after this treatment, these males were "premated" with virgin females, with whom they spent 24 hr, to induce sperm turnover and increase the yield of mutations (Ashburner 1989). These females were discarded, and the males were mated again, to produce offspring for further analysis.

Detecting mutations at five eye-color loci: A total of 200 EMS-treated males were mated with 200 females homozygous for the following five recessive alleles that affect eye color: pr^1, cn^1, bw^1, st^1, and kar^1. We set up 100 vials, each containing 2 males and 2 females. Once ∼100 eggs were laid in a vial, the parents were discarded. Thus, because the carrying capacity of a vial is ∼300 flies, competition among the offspring was weak. The offspring in each vial were counted daily and screened for eye color mutants.

Production of flies for assaying quantitative traits: A total of 200 EMS-treated males were mated individually and randomly with wild-type virgin females from the stem population (T matings). Simultaneously, 200 untreated males and 200 virgin females from the stem population were also mated individually and randomly (U matings). Parents of T and U matings were removed after 3 days and 3–4 offspring of each sex were collected separately from each sibship. The offspring from different T and U matings were mated individually and randomly to make all four possible types of crosses: T × T, T × U, U × T, and U × U (the first symbol corresponds to the origin of a mother, and the second symbol corresponds to the origin of a father). Also, in some cases we made the crosses between sibs produced in the same U mating ($U \times U_{Inbr}$). Parents were kept together for 3 days, after which they were transferred to fresh vials and kept there for 1 day to lay eggs. The offspring from these crosses (F-flies) were used for all fitness assays, except the developmental time. Thus, they were handled with care, using very light CO_2 anesthesia.

Developmental time: The parents used to make the four types of crosses (see above) were then merged into sets of 10 females and 10 males per vial, and each group was allowed to lay eggs for 2 hr. These eggs were then put under either 25° or 20° and permanent light. For each vial, we counted the emerging flies every hour.

TABLE 1

Numbers of recessive X-linked lethal mutations in F-females

Cross type	*N*	No. of lethal	Proportion of lethal
T × T	193	36	0.187
T × U	144	25	0.174
U × T	116	10	0.086
U × U	153	8	0.052

Viability: We followed the procedures of Shabalina *et al.* (1997) with some modifications. Two- to 3-day-old virgin F-flies from the four crosses were mated with males from the stem population and kept at low density for 3 days. The reference line used for competition with all the experimental larvae was marked with the homozygous bw^1 allele, outbred, and was genetically similar to our stem population. Females from the reference line were of the same age and were treated in the same way but were mated with the reference line males. Previously mated 5- to 6-day-old F-females and the reference line females were placed together, without males, for 2 days. Each vial contained either 4 or 8 females of each kind. At noon of day 3, females from each vial were transferred, without anesthesia, into narrow vials (95 × 20 mm) containing poor food (20 g brewer's yeast, 30 g glucose, 10 g agar and 0.2% propionic acid per liter of water), and allowed to lay eggs for 24 ± 0.1 hr. On day 4 at noon, females from each small vial were again transferred to a big vial (95 × 28 mm) containing the standard good food for 4 ± 0.1 hr to lay eggs before being discarded. When the offspring of these females started to eclose, numbers of wild-type and brown-eyed flies were daily counted and removed from each vial for 3 days.

Fecundity: Virgin F-flies from each of the four crosses were kept under low density (∼25 flies per vial) for 3 days. After this, each female was mated individually with two males from the stem population and kept with them for 3 days. Then, these families were transferred, in the course of 40 min and without anesthesia, into fresh vials and females laid eggs for 24 ± 0.1 hr before being removed. Male and female offspring of each female were counted.

Lethal mutations on X chromosome: The data on fecundity were also used to detect lethal mutations. We concluded that a sibship was produced by a mother heterozygous for an X-linked recessive lethal if the number of male offspring was so low that the hypothesis that the sex ratio is 1:1 can be rejected with 95% confidence.

Longevity: Virgin F-flies were collected and kept separately under low density and optimal conditions for 3–4 days. After this, they were transferred to Plexiglas boxes. Each 150 mm × 150 mm × 150 mm box contained 300 flies of the same sex. Flies were kept at 25° and 70% humidity. The food was provided in a small petri dish at the bottom of the box and changed daily. Dead flies were removed and recorded daily.

Metabolic rates: The 5- to 6-day-old F-flies were anesthetized lightly using N_2, sexed, and placed in vials. We then sealed each vial with a rubber stopper. Flies quickly recovered and were able to fly within 5 min of being anesthetized. The vials were flushed for 15 sec at a flow of 90 ml/min with CO_2-free water-saturated (100% relative humidity) room air. The flies were left sealed in the chambers for 1 hr at 24.5°. A 1.1-ml (standard temperature and pressure) gas sample was then

TABLE 2

Means and standard errors of developmental time (in hours) of F-flies

Cross type	Female at 20°	Male at 20°	Female at 25°	Male at 25°
T × T	364.58 ± 1.05 (130)[a]	375.53 ± 0.82 (133)	225.37 ± 0.51 (250) [0.032][b]	232.97 ± 0.61 (172) [0.076]
T × U	354.08 ± 0.62 (236)	365.11 ± 0.75 (192)	224.74 ± 0.37 (266) [0.019]	233.79 ± 0.50 (219) [0.091]
U × T	358.24 ± 0.85 (100)	367.67 ± 0.96 (104)	224.92 ± 0.54 (157) [0.006]	232.69 ± 0.55 (159) [0.013]
U × U	347.06 ± 0.65 (117)	358.86 ± 0.85 (109)	221.78 ± 0.31 (162) [0]	228.47 ± 0.48 (157) [0.006]

[a] Numbers in parentheses are the sample sizes.
[b] Numbers in brackets are the numbers of uneclosed flies (only flies developed at 25° were counted).

removed from the vial with a syringe and injected into a Sable System TR-2 carbon dioxide gas respirometry system. The vial was then reflushed with CO_2-free air and a second sample was taken 1 hr later. The amount of CO_2 produced by each fly was calculated using DATACAN software. The average of these two measurements was used.

Motility: F-males were kept under low density and optimal conditions for 3 days, after which their motility was assayed by escape response as described in Shabalina *et al.* (1997). In short, the escape response was measured as follows. A set of 10 males was placed in a small compartment at the top of the experimental tube (500 mm × 40 mm × 40 mm), and 30 sec later the tube was turned upside down, and the sliding wall separating the compartment from the rest of the tube was removed. The time for each male to climb 100 mm was recorded.

Body weight: The flies used in the metabolic rate study were later weighed to the nearest microgram on a microbalance.

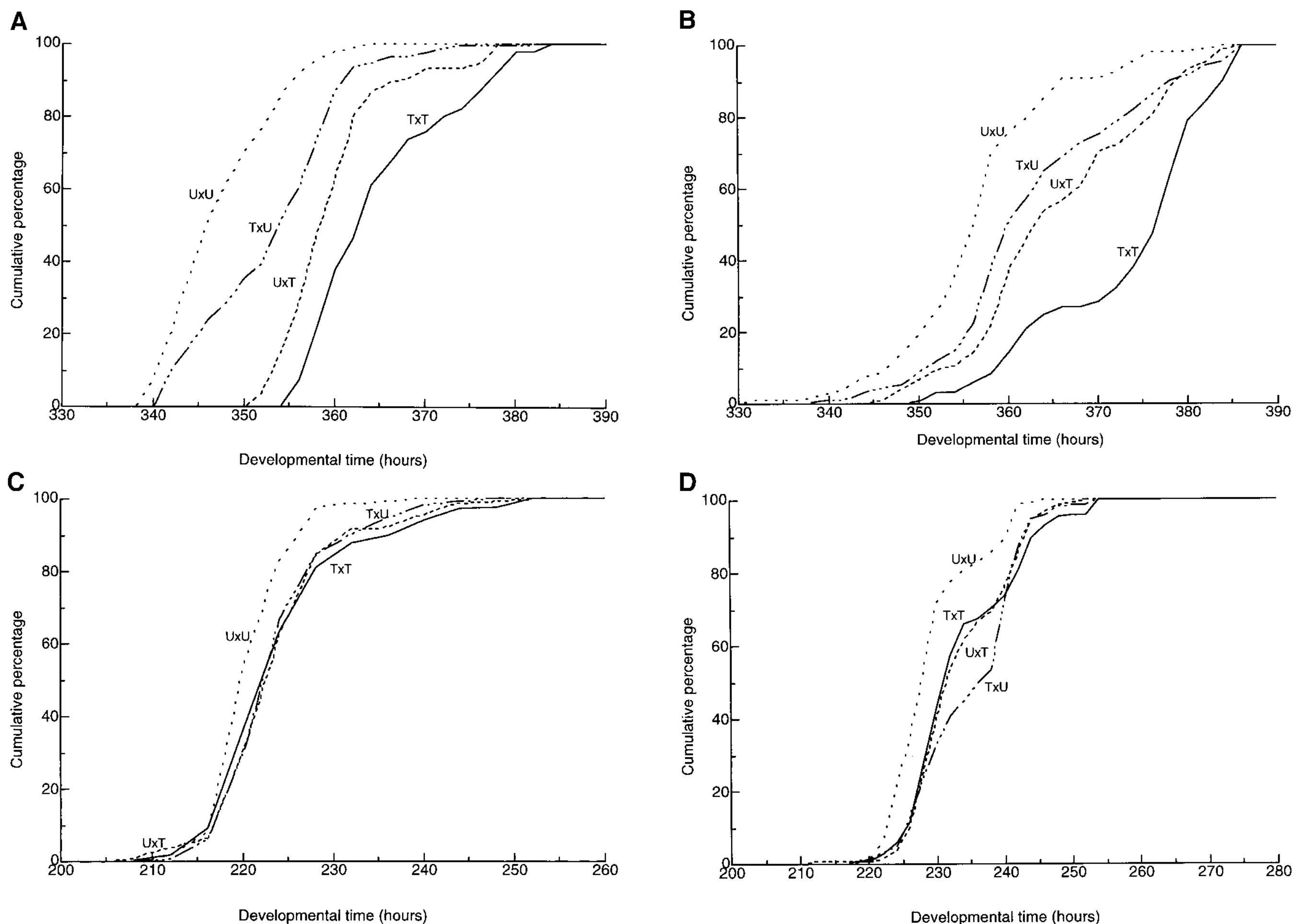

Figure 1.—Cumulative frequency distribution of developmental time of F-flies at two temperatures. (A) Females developed at 20°; (B) males developed at 20°; (C) females developed at 25°; and (D) males developed at 25°.

TABLE 3

Means and standard errors of viability of offspring of F-flies

Cross type	*N*	Poor food/ high density	Poor food/ low density	Good food/ high density	Good food/ low density
T × T	38	4.71 ± 0.85	2.22 ± 0.19	1.85 ± 0.18	1.55 ± 0.13
T × U	39	4.95 ± 0.62	2.38 ± 0.21	1.77 ± 0.14	1.79 ± 0.21
U × T	32	5.24 ± 1.03	3.13 ± 0.35	2.02 ± 0.19	2.00 ± 0.34
U × U	37	6.46 ± 0.86	2.91 ± 0.18	2.36 ± 0.22	1.42 ± 0.11

Bristle numbers: Numbers of sternopleural (both sides) and abdominal (the fifth segment) bristles of females that were used for fecundity assays were recorded.

Statistical analysis: Distributions of all the traits did not deviate significantly from normality. The only exception was viability, in which case log-transformation was used. The impact of EMS mutagenesis on the mean of each trait was recorded as a regression coefficient of the trait values on the fraction of EMS-treated genome. The confidence limits of these impacts were determined using the *t*-test. Possible epistasis among mutations could have been detected by significant deviations from linearity in the dependency of the mean value of a trait on the fraction of the genome treated with EMS. The impact of EMS mutagenesis on the variance of a trait shown as the differences in variances of the trait distributions in F-flies obtained in U × U and T × T crosses was compared. The software package JMP was used for most of the analysis.

RESULTS

EMS-induced mutations at the five eye-color loci: In 10,881 offspring from the EMS-treated males and females carrying the five recessive alleles affecting eye color, we observed 11 whole-bodied *bw* mutants, 30 *bw* mosaics, 2 whole-bodied *kar* mutants, 4 *kar* mosaics, 3 whole-bodied *pr* mutants, and 8 whole-bodied *cn* and *st*

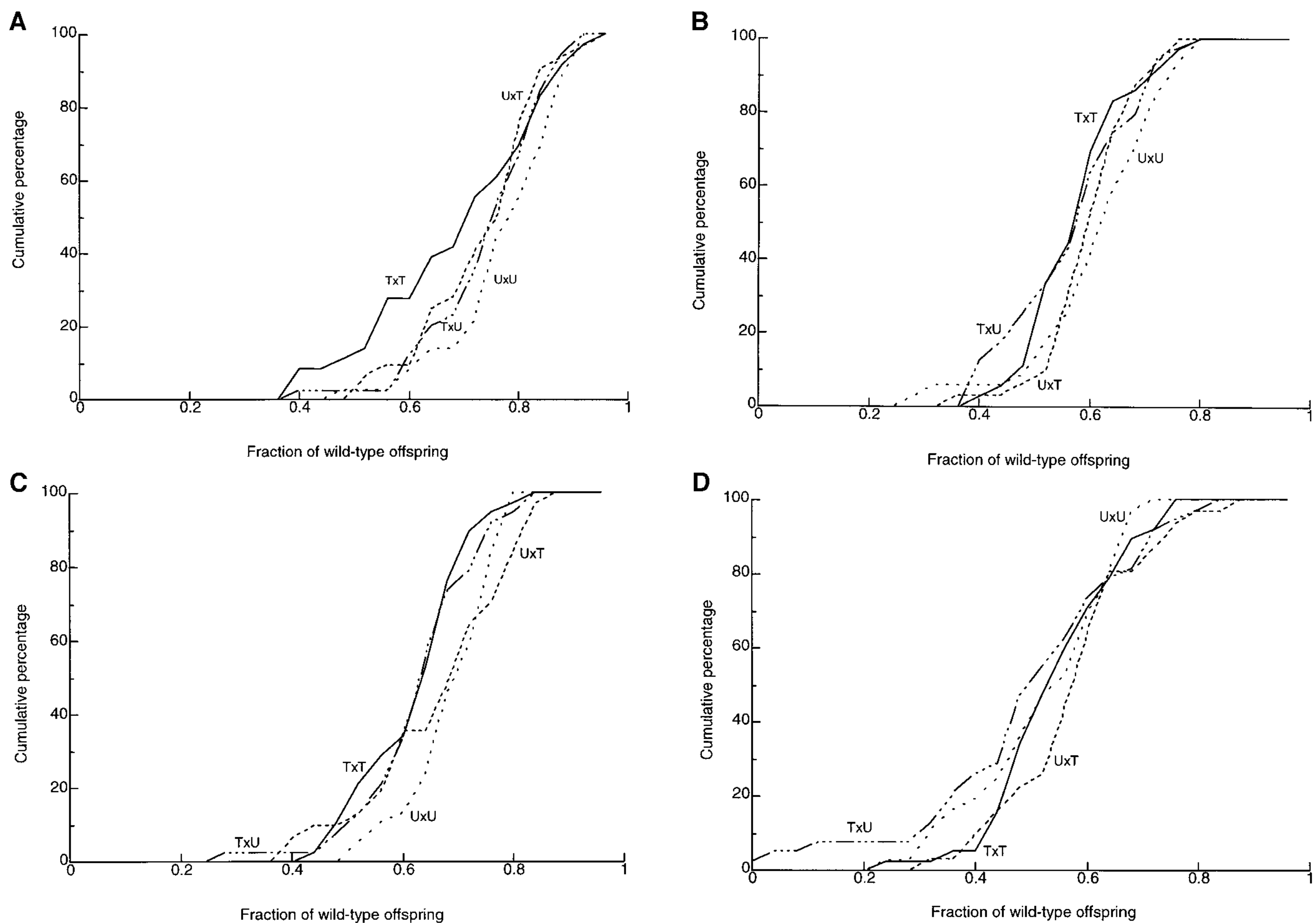

FIGURE 2.—Cumulative frequency distribution of viability of offspring of F-flies under four kinds of conditions. (A) High density and poor food; (B) high density and good food; (C) low density and poor food; and (D) low density and good food.

TABLE 4

Means and standard errors of fecundity of female F-flies

Cross type	*N*	No. of female offspring	No. of male offspring
T × T	193	42.38 ± 0.98	39.03 ± 1.15
T × U	144	47.47 ± 0.88	42.88 ± 1.06
U × T	116	47.52 ± 1.08	46.85 ± 1.15
U × U	153	46.14 ± 1.01	45.25 ± 0.99

mutants. No genetic analysis of the mutants was performed, so that we could not discriminate phenotypically identical *cn* and *st* mutants. Because *pr*, *cn*, and *st* encode soluble enzymes, only *bw* and *kar* mosaic could possibly have been detected. Almost all the mosaics had ~50% of their eye surface mutant and ~50% wild type, which is to be expected since mosaicism after EMS treatment occurs as the result of mismatched nucleotide pairs in the sperm of treated males. No clusters of mutants were found, presumably because EMS affected the terminal stages of spermatogenesis (Ashburner 1989).

Thus, counting a mosaic as one-half of a mutant, the average per locus mutation rate in the offspring from EMS-treated males was 7.5×10^{-4}. Measuring spontaneous mutation rates at the same loci in the flies extracted from the same wild population, we screened ~1,000,000 offspring and observed 28 mutants in total, leading to the estimate of ~6.0×10^{-6} (with 95% confidence interval of 2×10^{-6} to ~15×10^{-6}) per locus per generation (Yang *et al.* 2000), in good agreement with other estimates (Schalet 1960; Woodruff *et al.* 1983). This implies that our EMS treatment was equivalent to ~100 generations of spontaneous mutation.

EMS-induced X-linked recessive lethals: The frequencies of X-linked recessive lethals after the EMS treatment are presented in Table 1. Extra lethals appeared only in females whose maternal grandfathers were treated since they originated from T × T and T × U crosses. In these females, 50% of their X-linked genes were EMS-treated. Thus, the rate of EMS-induced X-linked recessive lethals L can be estimated as two times the excess of recessive lethals in females from T × T and T × U crosses, $L = \{(0.187 + 0.174)/2 - (0.086 + 0.052)/2\}*2 = 0.224$. The spontaneous rate of X-linked recessive lethals is ~0.002 (Crow and Simmons 1983). Thus, our EMS treatment is equivalent to ~100 generations of spontaneous mutation.

TABLE 5

Means and standard errors of longevity (in days) of F-flies

Cross type	Male longevity	Female longevity
T × T	66.70 ± 0.56 (578)	61.08 ± 0.39 (596)
T × U	68.43 ± 0.50 (579)	63.05 ± 0.45 (593)
U × T	71.13 ± 0.42 (574)	58.33 ± 0.63 (290)
U × U	71.79 ± 0.39 (577)	63.06 ± 0.43 (605)

Numbers in parentheses are the sample sizes.

Developmental time: Data on developmental time under 20° and 25° are shown in Table 2 and in Figure 1. Among those individuals that failed to hatch under 25°, 4% were very close to completing their development (wings were clearly visible within their pupae), and 77% were males.

Viability: Data on competitive viability during the larval stage, measured as the ratio of the numbers of pupated offspring of F-flies (wild type) to that of the reference line (*bw*), are shown in Table 3 and in Figure 2.

Fecundity: Data on fecundity, measured as numbers of offspring that reached adulthood, are shown in Table 4. We call this trait fecundity since mortality of offspring was not high. The high fecundity of the females obtained in U × U crosses reflects the high genetic quality of our flies and good experimental conditions (Shabalina *et al.* 1997). In ~10% of sibships produced by females originated in T × T and T × U crosses, the number of male offspring was only ~50% of the number of female offspring, presumably due to the presence of X-linked recessive lethals (see above). Thus, we analyzed our data in two different ways: characterizing the fecundity of a female either by the total number of her offspring or only by the number of her female offspring.

Longevity: Data on longevity of F-flies are shown in Table 5.

Metabolic rate: Data on metabolic rates of F-flies, measured as the CO_2 output rate in both males and females, are shown in Table 6.

Motility: Data on motility of F-flies, measured as the escape response of male F-flies, are shown in Table 6.

Body weight: Data on body weight of F-flies are shown in Table 6.

Bristle number: Data on bristle numbers on the left and the right sternopleural plates and on the fifth abdominal segment are shown in Table 7.

Summary of the data: Table 8 summarizes the data on the mutational impacts on the means and variances of the nine quantitative traits. The estimate of percentage of change of the mean is calculated by standardizing the difference of trait means between U × U and T × T with the mean of U × U and dividing it by number of generations equivalent to spontaneous mutation (assuming that our treatment with EMS was equivalent to 100 generations of spontaneous mutation). *P* values for testing percentage of change of mean as different from zero are also shown. As far as the variance is concerned, we present the increments of mutational coefficient of variation (CV%; Houle 1992) due to the EMS treatment, computed as $100\%(V_{TXT} - V_{UXU})^{-1/2}/M$, where V_{TXT} and V_{UXU} are the trait variances in F-flies from T × T and U × U crosses, and M is the trait mean.

TABLE 6

Means and standard errors of metabolic rates, motility, and body weight of F-flies

Cross type	Metabolic rates (μl/hr)		Motility (sec)	Body weight (g)	
	Male	Female		Male	Female
T × T	66.70 ± 0.56 (578)	61.08 ± 0.39 (596)	5.363 ± 0.366 (11)	0.741 ± 0.021 (47)	1.465 ± 0.026 (46)
T × U	68.43 ± 0.50 (579)	63.05 ± 0.45 (593)	5.104 ± 0.222 (10)	0.777 ± 0.015 (43)	1.452 ± 0.037 (42)
U × U	71.13 ± 0.42 (574)	58.33 ± 0.63 (290)	5.602 ± 0.467 (10)	0.780 ± 0.015 (46)	1.395 ± 0.022 (43)
$U \times U_{\mathrm{Inbr}}$	71.79 ± 0.39 (577)	63.06 ± 0.43 (605)	5.593 ± 0.411 (10)	0.776 ± 0.022 (40)	1.388 ± 0.022 (42)

Numbers in parentheses are the sample sizes.

DISCUSSION

The experimental design: Natural populations are outbred, and autosomal deleterious mutations are selected against mostly in heterozygotes (Crow and Simmons 1983). The rationale for the design of our experiment, as well as for the middle class neighborhood design used in the mutation-accumulation experiment of Shabalina *et al.* (1997), was to imitate nature to obtain realistic estimates of the mutational impact on the mean values of quantitative traits. Thus, we used outbred flies whose ancestors were extracted from nature only a few generations ago, and introduced EMS-treated genomes only in the heterozygous state. Also, we tried to assay one of our traits, viability, under a variety of conditions, including the most harsh and competitive conditions feasible. Such conditions should increase the observed mutational impact (Kondrashov and Houle 1994) since many mutations that are deleterious in nature are effectively neutral (cryptic; Davies *et al.* 1999) under benign laboratory conditions.

In contrast, the study of the impact of EMS mutagenesis by Keightley and Ohnishi (1998), as well as the mutation-accumulation experiments of Mukai (1964; Mukai *et al.* 1972), used sets of strains of homogeneous genetic background, kept each strain inbreeding for many generations, and compared flies heterozygous for *Cy* balancer chromosomes and those homozygous for EMS-treated chromosomes, so that the impacts of homozygous deleterious mutations only on the second chromosome were studied. We believe that our approach is superior as far as measuring the mutational impacts on the mean values of quantitative traits is concerned. However, the strain-based design may be superior for measuring the mutational impact on the variance, which can lead to meaningful estimates of the genomic deleterious mutation rate U, as long as all mutations that are deleterious in nature affect the studied traits under experimental conditions (which is usually not the case; Davies *et al.* 1999). We did not attempt to estimate U and concentrated instead on measuring the mutational impacts on the means, since even their values remain controversial (Fry *et al.* 1999; Garcia-Dorado *et al.* 1999; Keightley and Eyre-Walker 1999; Lynch *et al.* 1999).

Applying EMS mutagenesis to studying spontaneous mutation: Two types of evidence, from the rate of X-linked recessive lethals and from the rates of mutation at five eye-color loci, suggest that our treatment with EMS induced as many loss-of-function mutations as ~100 generations of spontaneous mutation (Table 1). Keightley and Ohnishi (1998) reached a similar conclusion (84 generations) from the data on the rate of recessive lethals in chromosome 2, although they used a substantially lower concentration of EMS. Because the efficiency of EMS mutagenesis strongly depends on seemingly minor details of treatment (Ohnishi 1977a), differences between the rates of induced mutation per millimole of EMS obtained in different experiments are not surprising. The diploid genomic rate of decline of viability per millimole of EMS inferred from our data, ~5% (21.2 mm EMS was used and viability of flies that had one-quarter of their diploid genomes treated since they were sired by T × T fathers was reduced by 27% relative to U × U control; Table 1), was higher than

TABLE 7

Means and standard errors of sternopleural and abdominal bristle numbers of female F-flies

Cross type	N	Sternopleural (left)	Sternopleural (right)	Abdominal
T × T	106	9.57 ± 1.20	9.67 ± 1.08	20.33 ± 3.32
T × U	105	9.48 ± 1.34	9.58 ± 1.06	20.81 ± 3.05
U × T	81	9.51 ± 1.13	9.59 ± 1.16	21.00 ± 2.58
U × U	76	9.39 ± 1.01	9.33 ± 1.05	20.88 ± 2.37

TABLE 8

Summary of impacts of EMS mutagenesis on the trait means and variance

Traits		% changes per generation	*P* value	CV% per generation
Developmental time	20°, male	0.087 ± 0.007	<0.0001	0.15 ± 0.14
	25°, male	0.041 ± 0.007	<0.0001	0.34 ± 0.13
	20°, female	0.097 ± 0.001	<0.0001	0.34 ± 0.15
	25°, female	0.033 ± 0.006	<0.0001	0.44 ± 0.12
Viability	High density/poor food	−1.29 ± 0.49	0.010	13.38 ± 13.30
	High density/good food	−1.43 ± 0.75	0.061	−5.80 ± 5.11
	Low density/poor food	−0.90 ± 0.52	0.007	2.74 ± 3.92
	Low density/good food	−1.36 ± 2.23	0.543	5.59 ± 5.18
Fecundity	No. of female offspring	−0.21 ± 0.07	0.0015	1.62 ± 1.31
	Total no. of offspring	−0.18 ± 0.06	0.0032	2.38 ± 1.26
Longevity	Male	−0.101 ± 0.002	0.0000	1.45 ± 0.49
	Female	−0.067 ± 0.003	<0.0001	0.41 ± 0.58
Motility	Male	−0.110 ± 0.200	0.550	−0.34 ± 0.40
Metabolic rate	Male	0.067 ± 0.188	0.756	0.41 ± 1.70
	Female	0.316 ± 0.092	0.075	0.00 ± 0.58
Body weight	Male	−0.18 ± 0.16	0.371	1.50 ± 0.99
	Female	−0.31 ± 0.078	0.058	1.20 ± 0.74
Bristles	Sternopleural (L)	0.039 ± 0.041	0.351	0.99 ± 0.91
	Sternopleural (R)	0.076 ± 0.038	0.046	0.34 ± 0.63
	Abdominal	−0.065 ± 0.046	0.158	1.57 ± 0.74

~1% reported in Simmons *et al.* (1978; 5mm EMS reduced viability of flies heterozygous for a treated second chromosome by ~1%) but lower than 14% implied by the data of Keightley and Ohnishi (1998, Table 1) under the assumption that induced mutations have intermediate dominance.

In contrast, data on variance in the number of abdominal bristles imply that EMS treatment by Keightley and Ohnishi (1998) is equivalent to >300 generations of spontaneous mutation. However, since the increase in the variance of the number of bristles under spontaneous mutation may be retarded by stabilizing selection (Nuzhdin *et al.* 1995), and since the variance in a quantitative trait is more difficult to measure than the frequency of lethals or of visible phenotypes, this figure is less reliable and is probably overestimated.

In fact, even ~100 generations of spontaneous mutation may be an overestimation of the efficiency of EMS treatment in both our experiment and that of Keightley and Ohnishi (1998), as far as its impact on quantitative traits is considered. Indeed, Mukai (1970) and Ohnishi (1977b) have shown that the ratio of lethal to minor mutations induced by EMS is approximately two times higher than among spontaneous mutations. Certainly, the molecular natures of EMS-induced and spontaneous mutations are not the same. EMS mostly causes transitions (Pastink *et al.* 1991) and large aberrations (Ashburner 1989). The molecular nature of spontaneous mutation in Drosophila is poorly known, although it is already clear that deletions and insertions play a substantial role (Nitasaka *et al.* 1995; ten Have *et al.* 1995; Petrov and Hartl 1998; Yang *et al.* 2001).

The impact of EMS mutagenesis on quantitative traits: EMS-induced mutation has different impacts on different quantitative traits. The mean values of the four life-history-related traits—developmental time, viability, fecundity, and longevity (Table 8), were affected substantially. By far the strongest impact was on viability, the only trait assayed under harsh, competitive conditions. If our treatment with EMS was equivalent to 100 generations of spontaneous mutation, a generation of spontaneous mutation reduces competitive viability by 1.35% [the average decline of viability under high density/poor food (1.29%) and high density/good food (1.43%), Table 8].

In contrast, the impacts of mutation on the means of the traits that are not directly related to life history, *i.e.*, body weight, motility, metabolic rate, and the numbers of abdominal and sternopleural bristles, are much smaller and mostly statistically insignificant. Keightley and Ohnishi (1998) observed the same pattern, although even these smaller impacts were significant in their data. This pattern is to be expected if life history-related traits are under directional selection, while the other traits are under stabilizing selection. With relaxation of selection, mutations can move the mean of traits under directional selection toward the opposite direction of selection, while for traits under stabilizing selection, change of trait means may not be significant, but variance of the traits should increase by mutations. Mutation increased the development time and decreased viability, fecundity, and longevity, which is consistent with this explanation. Also, the mutational targets for life-history-related traits are usually larger than for

other traits (Houle 1992; Houle *et al.* 1996). No detectable impact of EMS mutagenesis on the means of motility and metabolic rate might be partially explained by the difficulty of measuring these traits.

Although studying the variance was not our main goal, we did detect a general tendency of EMS mutagenesis to increase the variance. For developmental time, competitive viability (only under high density and poor food condition), fecundity, male longevity, body weight, and number of abdominal bristles, these increases are significant.

In agreement with Kondrashov and Houle (1994), and Shabalina *et al.* (1997), we have found that the mutational impact on viability under harsh conditions (high density of larvae and poor quality of food) is an order of magnitude higher than that on viability under benign conditions and on the three other life history-related traits measured under benign conditions. Perhaps this is because many mutations remain cryptic under benign conditions (Davies *et al.* 1999). The difference between the mutational impact on viability observed in our study under harsh conditions and that reported by Keightley and Ohnishi (1998; 1.35% *vs.* 0.21% per generation of spontaneous mutation) may be due to the more benign conditions used by Keightley and Ohnishi. A smaller impact on longevity observed in our experiment [0.08% *vs.* 0.51%, where 0.08% is the average decline of longevity in males (0.10%) and females (0.067%), Table 8] might be due to substantial recessivity of new deleterious alleles that reduce longevity since Keightley and Ohnishi (1998) observed the impacts of homozygous mutations while we observed the impacts of heterozygous mutations.

Implications for the parameters of spontaneous mutation: Different authors reported different values of the impacts of spontaneous mutation on the mean values of life history-related traits in *D. melanogaster*. Since chromosome 2 constitutes 40% of the *D. melanogaster* genome, the data of Mukai (1964) and Mukai *et al.* (1972) imply that viability declines by ~2% per generation, assuming that deleterious mutations in their experiments were partially recessive. Shabalina *et al.* (1997) reported a close figure for viability under harsh competitive conditions. In contrast, Keightley and Ohnishi (1998), Fry *et al.* (1999), and García-Dorado *et al.* (1999) reported significantly lower figures, well below 1%, for the decline of viability.

Obviously, our results on viability under harsh conditions are much closer to the higher figures. In fact, if the comparison of recessive lethal rates overestimates the impact of EMS mutagenesis relative to spontaneous mutation by a factor of ~2 (Mukai 1970; Ohnishi 1977b), our data imply that viability declines by ~2% per generation and are fully consistent with the results of Mukai (1964), Mukai *et al.* (1972), and Shabalina *et al.* (1997). This is important, because the design of our experiment is free from several features that might lead to overestimation of the decline of the mean value of a fitness-related trait. First, the tricky issue of control flies, which must remain invariant in the course of many generations of accumulation of mutations, was irrelevant, since the whole experiment took just three generations and untreated flies are a perfect control. Second, we did not use any ambiguous phenotypes (it was suspected that misidentifying *Cy* could have been a problem in Mukai's studies; Keightley and Eyre-Walker 1999). Finally, our results cannot be due to destruction of linkage disequilibria (Keightley *et al.* 1998), since the number of generations was very small, and the control and the experimental flies had the same pedigrees. Total decline of fitness is certainly substantially higher (Mitchell 1977; Mitchell and Simmons 1977; Simmons *et al.* 1978) but is much more difficult to measure precisely.

Thus, our data support the view that rapid decline of viability reported in the early experiments was caused by high rate of spontaneous mutation ($U \approx 1$) in *D. melanogaster*, so that selection against deleterious mutations is a crucial evolutionary factor (Barton and Charlesworth 1998; Kondrashov 1998). Still, the issue is not settled yet, in particular, because estimating U requires difficult measurements of the increase in the variance of fitness-related traits due to new mutations, as well as the parameters of the distribution of the effects of individual mutations. EMS mutagenesis may be a useful tool for such research.

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