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Mutation accumulation and the effect of *copia* insertions in *Drosophila melanogaster*

DAVID HOULE[1]* AND SERGEY V. NUZHDIN[2]
[1] *Department of Biological Science, Florida State University, Tallahassee, FL 32306-1100, USA*
[2] *Section of Evolution and Ecology, University of California, Davis, Davis, CA 95616, USA*

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Summary

Repeated efforts to estimate the genomic deleterious mutation rate per generation (U) in *Drosophila melanogaster* have yielded inconsistent estimates ranging from 0·01 to nearly 1. We carried out a mutation-accumulation experiment with a cryopreserved control population in hopes of resolving some of the uncertainties raised by these estimates. Mutation accumulation (MA) was carried out by brother–sister mating of 150 sublines derived from two inbred lines. Fitness was measured under conditions chosen to mimic the ancestral laboratory environment of these genotypes. We monitored the insertions of a transposable element, *copia*, that proved to accumulate at the unusually high rate of 0·24 per genome per generation in one of our MA lines. Mutational variance in fitness increased at a rate consistent with previous studies, yielding a mutational coefficient of variation greater than 3%. The performance of the cryopreserved control relative to the MA lines was inconsistent, so estimates of mutation rate by the Bateman–Mukai method are suspect. Taken at face value, these data suggest a modest decline in fitness of about 0·3% per generation. The element number of *copia* was a significant predictor of fitness within generations; on average, insertions caused a 0·76% loss in fitness, although the confidence limits on this estimate are wide.

1. Introduction

Selection created by deleterious mutations might help to explain a very wide range of evolutionary phenomena including the maintenance of genetic variation, sex and recombination, mate choice, species ranges, extinction of small populations, and inbreeding depression (for a recent review, see Lynch *et al.*, 1999). The general importance of deleterious mutations depends on two things: the genomic rate of deleterious mutation (U) and the distribution of their selection coefficients. For example, deleterious mutations can cause extinctions of small populations if $U>0{\cdot}1$ and the average selection coefficient (s) is less than the reciprocal of the population size (Lynch *et al.*, 1995; Schultz & Lynch, 1997); drive substantial 'good-genes' sexual selection if $U>0{\cdot}2$ (Houle & Kondrashov, 2002); and account for the maintenance of sexual reproduction if U is on the order of 1 or more (Kondrashov, 1988).

* Corresponding author. Tel: +1 850 645 0388. Fax: +1 850 644 9829. e-mail: dhoule@bio.fsu.edu

The importance of mutational parameters has led to many attempts to estimate them. Unfortunately, estimates in multicellular organisms still range over an order of magnitude for species in which estimates are available (Drake *et al.*, 1998; García-Dorado *et al.*, 1999; Keightley & Eyre-Walker, 1999; Lynch *et al.*, 1999). Recent efforts have multiplied uncertainty rather than converged on general answers. Paradoxically, these uncertainties are greatest for *Drosophila melanogaster*, the species for which the most estimates are available.

In the traditional mutation-accumulation (MA) approach, natural selection is partially suspended on replicates of an initially homozygous genotype. A variant of this approach uses a heterozygous initial population (Shabalina *et al.*, 1997). The numbers and effects of the mutations can then be estimated from the fitnesses of the replicates by means of a model of the mutational effects. For example, the Bateman–Mukai model (Bateman, 1959; Mukai *et al.*, 1972) assumes that only deleterious mutations occur and uses the change in mean relative to the change in genetic

variance to infer limits on U and s. Other models also assume that the distribution of mutant effects is partially known (Keightley, 1994, 1996; Keightley & Bataillon, 2000). The minimum-distance approach (García-Dorado, 1997) uses only the distribution of mutant-line means to infer U and s. The MA approach has been used most frequently in *D. melanogaster* but has also been applied in bacteria, yeast, *Arabidopsis*, *Daphnia* and *Caenorhabditis*.

A weakness of this approach is the difficulty of maintaining a control for MA in species with no resting stage to the life cycle, such as *D. melanogaster*. Consistent with these difficulties, estimated rates of decline in fitness or fitness components are variable, ranging from several percent per generation (Shabalina *et al*., 1997) to 0·1% per generation (Chavarrías *et al*., 2001). Several authors have proposed that biases of various sorts have led to an overestimate of the actual rate of decline in *D. melanogaster* (Keightley, 1996; García-Dorado, 1997; Fry *et al*., 1999) and consequently to an overestimate of U, although some of these claims have been contradicted (Fry, 2001). Analyses that use data showing rapid declines in the mean lead to estimates of U on the order of 0·5 per genome per generation, whereas those that do not generally produce estimates on the order of 0·02 per genome per generation.

Other available methods of estimating mutation parameters also have their difficulties. Estimates of U based on differences in the rate of base-pair substitutions between species (Kondrashov & Crow, 1993) tend to underestimate U because of variation in the neutral rate of substitution. This approach can also not detect insertion–deletion variation, which must be addressed by other means (Keightley & Eyre-Walker, 2000). Direct sequencing gives estimates of mutation rate an order of magnitude higher than do interspecific comparisons, suggesting that the bias is large (Howell *et al*., 1996; Parsons *et al*., 1997; Denver *et al*., 2000). However, it is not clear that direct sequencing is necessarily more accurate, because the impact of sequencing errors has yet to be adequately addressed. The Morton–Charlesworth method and its extensions (Charlesworth *et al*., 1990; Deng & Lynch, 1996; Deng, 1998) assumes that the genetic variation in a population is maintained by mutation–selection balance. There is considerable reason to doubt that this assumption is correct for outbred populations (Drake *et al*., 1998).

With these difficulties as background, we investigated two variants of the MA method for estimating U in *D. melanogaster*. We accumulated mutations by brother–sister matings of flies of two inbred lines, similar to the approach taken by Chavarrías *et al*. (2001). First, we used a cryopreserved sample (Houle *et al*., 1997) of the original population as a control, which was revived before each fitness assay. Second, we used *de novo* insertions of the transposable element (TE) *copia* to detect directly a substantial proportion of the spontaneous deleterious mutations occurring in one of our lines.

2. Materials and methods

The base population for this experiment, IV*e*, was descended from the Ives population (IV+) (Charlesworth & Charlesworth, 1985). IV+ has been maintained in ten half-pint bottles since 1975 and was rendered homosequential in 1978. In 1992, a spontaneous *ebony* mutation (Lindsley & Zimm, 1992) was found at appreciable frequency in one replicate of IV+, and homozygotes for this recessive allele were backcrossed to IV+ flies in four rounds to form a homozygous *ebony* population on an outbred IV+ background (Houle *et al*., 1997). The IV+ population has a cytotype of R and weak P (Houle *et al*., 1994*b*). The Ives populations and the experimental flies were all maintained on a medium consisting of agar, killed brewer's yeast, sucrose and corn flour, with propionic acid as a preservative. Live yeast was not used except in rare cases to maximize fecundity of poorly performing isolates. Antibiotics or Nipagin were added to the medium as necessary to control bacterial or fungal contamination. Experiments and maintenance were carried out at 25 °C, on a 20 : 4 light : dark cycle.

To establish the base genotypes for these experiments, we derived 50 inbred lines from IV*e* by brother–sister mating. Lines with poor fitness were culled progressively, and the final sample of about 15 inbred lines was subjected to the fitness assay used below after 35 generations of inbreeding. Insertion sites of the TEs *copia*, *roo* and *297* (Lindsley & Zimm, 1992) were checked in the two inbred lines with the highest fitness, IV*e*-33 and IV*e*-39, after 40 generations of full-sib inbreeding. The *297* TE was fixed in all replicates, and *roo* segregated in some, whereas *copia* showed a striking difference (discussed in detail below). This pattern was most consistent with a small amount of ongoing transposition within essentially completely inbred lines.

From each of IV*e*-33 and IV*e*-39, 75 MA sublines were established. These sublines were independently maintained by full–sib mating of virgin flies. We minimized selection within vials by allowing single pairs to lay eggs for a short time and choosing offspring to initiate the next generation at random with respect to development time.

Because pairs of flies frequently fail to produce offspring, five vials of each subline were set up. Two vials (A and B) were set up with single pairs of flies and two (C and D) with sets of four female and four male parents. Every other generation, four pairs from vials C and D were transferred to a fifth vial (E), placed at 17 °C, and used to replace sublines that

failed in the next generation. Flies for establishment of the next generation were all taken from only one of these vials, which were used in the order A, B, C, D and, finally, E if necessary. A vial that did not produce the necessary 12 flies was considered to have failed. Over the first 90 generations of accumulation, single-pair IV*e*-33 vials failed at the rate of 14·1% per generation, whereas those of IV*e*-39 failed 28·5% of the time. Overall, IV*e*-33 vials were founded from one of the four-pair vials (C, D and E) 6·4% of the time; the rate for IV*e*-39 was 13·74%.

Sublines were occasionally lost when no pairs of flies were produced in any of the vials set up. Only one subline was lost in the first 21 generations of the experiment, but the rate of loss was relatively high from generations 22 through 65, when a total of 53 sublines were lost. The rate of loss was higher in IV*e*-39 (36 sublines lost as opposed to 17 from line 33). From generation 65 to generation 90, only eight sublines were lost. Logistic regression of failure rate on generation showed no evidence for an increase in failure rate of either vials or sublines over time, consistent with the results of Chavarrías *et al*. (2001).

Examination of *copia* positions (as described below) suggested that one subline of line 39 had become contaminated before generation 22 and that two became contaminated before generation 47. These sublines were discarded.

(i) *Cryopreserved controls*

Shortly before the establishment of the MA lines, samples of each line were cryopreserved as embryos (Houle *et al*., 1997). Each line therefore had to be expanded to approximately 10 000 flies to provide many eggs at the proper age. A few generations before each of the first three fitness assays described here, a sample of cryopreserved flies was revived and used to found two or three control lines. These were rapidly expanded to large size for the assays. Survival rate of recovered embryos was less than 5% at all time intervals. No line-33 embryos could be recovered after generation 18. At generation 47, all remaining line-39 embryos were needed to provide a sufficient sample for use, so no control was available for later generations of the experiment.

(ii) *Fitness assays*

The fitness assay was chosen to mimic the normal culture of the IV*e* base populations. These were maintained in bottles at 25 °C, and all surviving offspring were transferred to new bottles after 14 days. We assayed fitness by placing the offspring of mated *ebony* experimental females in competition with those of wild-type IV+ flies. Three generations before fitness assays, each control and MA line was split into two replicates. Simultaneously, wild-type flies from an IV+ population were also removed from their culture bottles and reared in parallel with the experimental flies. All flies were reared for one generation in vials, from four male and four female parents in each vial. Their offspring were then placed in groups of 12 males and 12 females in bottles. The identities of IV*e* genotypes were coded so that lab workers did not know the identities of the flies they were working with. The next generation's offspring (the parents of the experimental flies) were held for 2 days in groups of about 200 flies for mating. The males were then removed and 25 IV+ and 25 IV*e* (MA or control) flies placed in each experimental bottle. The parents were discarded after 3 days, and the emerging offspring counted daily until day 14 after the establishment of the bottles. Generation-18 assays of line 33 were carried out in two blocks 1 month apart. Generation-47 assays of line 39 were carried out in blocks 2 weeks apart.

The proportion of *ebony* offspring eclosing is our measure of fitness. It captures variation in female fecundity, including variation owing to the ability to store sperm; the viability of the offspring under competitive conditions; and the probability of eclosion within the 14-day time limit enforced in the ancestral environment.

Mutational variance at each generation was estimated with the VARCOMP procedure in SAS (SAS Institute, 1990), using restricted maximum likelihood.

The rates of the decline in mean (ΔM) and increase in among-line variance (ΔV) for fitness were calculated by weighted least-squares regression. We first standardized the data to place these estimates on a convenient scale. For ΔM the data were standardized to a generation-0 intercept of 1. The inverses of the standard deviations of the differences between control and MA means were used as weights. To estimate mutational variance, we calculated the subline variance components from data standardized to a generation mean of 1. Regression of these estimates on generation, with the total degrees of freedom of each experiment as the weight, then yielded estimates of the rate of increase in among-line variance, ΔV. On this scale, the mutational coefficient of variation, CV_M (Houle *et al*., 1996), is the square root of ΔV. Multiplication by 100 transforms CV_M into a percentage.

(iii) *Determination of* copia *copy number by* in situ *hybridization*

The *copia* insertion sites were located by *in situ* hybridization of the plasmid cDM5002 containing a full-length *copia* transposable element (Finnegan *et al*., 1978) to polytene salivary-gland chromosomes of third-instar larvae (Shrimpton *et al*., 1986). Probes were labelled with biotinylated dATP (bio-7-dATP,

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Table 1. *Means, standard deviations (SD) and sample sizes for female fitness in line 39 at each generation. The lower part of the table gives the linear regression estimates for trait values on generation*

	Control		Mutation accumulation		
Generation	Bottles	$W \pm$SD	Sublines	Bottles	$W \pm$SD
13	8	0·398 ± 0·053	30	58	0·345 ± 0·082
22	36	0·539 ± 0·138	39	73	0·449 ± 0·149
47-1	36	0·323 ± 0·094	30	98	0·270 ± 0·107
47-2	42	0·233 ± 0·060	32	121	0·225 ± 0·078
73	0	–	25	88	0·227 ± 0·112
Regression on generation					
Intercept		0·672 ± 0·134*			0·431 ± 0·014**
Slope		−0·0083 ± 0·0034*			−0·0033 ± 0·0003**

* $P < 0{\cdot}05$.
** $P < 0{\cdot}001$.

Table 2. *Means, standard deviations, and sample sizes for female fitness from each experiment with line 33 flies*

	Control		Mutation accumulation		
Generation	Bottles	$W \pm$SD	Sublines	Bottles	$W \pm$SD
13	16	0·441 ± 0·075	30	59	0·426 ± 0·055
18-1	20	0·236 ± 0·034	39	73	0·267 ± 0·077
18-2	57	0·230 ± 0·082	39	130	0·260 ± 0·093
Regression on generation					
Intercept		0·986 ± 0·069			0·850 ± 0·041*
Slope		−0·0419 ± 0·0040**			−0·0327 ± 0·0024**

* $P < 0{\cdot}01$.
** $P < 0{\cdot}001$.

BRL) by nick translation. Hybridization was detected with the Vectastain ABC kit (Vector Labs) and visualized with diaminobenzidine. The element locations were determined at the level of cytological bands on the standard Bridge's map.

(iv) *Measurements of transposition rate*

Transpositions were detected as described by Pasyukova & Nuzhdin (1993) and Nuzhdin *et al.* (1996). Briefly, one male of the tested line was crossed with the Oregon R females (fixed for a set of known *copia* sites), and *copia* insertion sites in multiple progeny larvae were scored by *in situ* hybridization. Each of the F_1 progeny inherits one chromosome set from the maternal Oregon R and the other chromosome set from the tested male parent. The positions of *copia* in the father can thus be reconstructed from the segregation of sites in the F_1 *copia* transpositions, and excisions in the germ lines of tested males are detected by the appearance of non-parental *copia* insertion sites or the loss of parental insertion sites in the progeny larvae. The transposition rate in a given male is calculated as (number of transpositions) ÷ (number of gametes analysed × *copia* copy number).

3. Results

(i) *Female fitness*

Mean fitnesses for line 39 are shown in Table 1, along with the sample sizes for controls and MA lines. Mean fitness declined significantly over the course of the experiment in both the control and MA populations, as demonstrated by the significant regression slope with generation in each case (Table 1). The means for line 33 are shown in Table 2. Mean fitness declined significantly between generations 13 and 18. Unexpectedly, the absolute fitness of the control populations declined more rapidly than those of both MA lines.

If natural selection were responsible for some of the losses of sublines after generation 21, we would expect fitness estimates to predict the probability of loss in the subsequent generations of MA. Logistic regression of subline survival on fitness at a previous assay time was not significant for either line over any time period (analyses not shown).

Relative fitnesses of the MA sublines and the cryopreserved control are shown in Fig. 1. For line 39, when each generation was analysed separately, a one-sided test for a decline yielded P values of 0·072, 0·001 and 0·061 for generations 13, 22 and 47, respectively.

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Table 3. *Estimates of the rate of change in fitness and the variance in fitness ± standard error* (*SE*). P *values for the slopes for one-sided tests that* ΔM *is negative or that* ΔV *is positive*

Parameter	Line	Standardization	Intercept	Slope
ΔM	39	Control	Constrained to 1	−0·00341 ± 0·00145**
	33	Control	Constrained to 1	0·00105 ± 0·00475
	Both	Control	Constrained to 1	−0·00285 ± 0·00145*
	39	Control	0·841 ± 0·059	0·00092 ± 0·00181
	Both	Control	0·965 ± 0·082	−0·00173 ± 0·00310
ΔV	39	MA mean	Constrained to 0	0·00116 ± 0·00013‡
	39	MA mean	−0·0103 ± 0·0175	0·00136 ± 0·00037**
	33	MA mean	Constrained to 0	0·00099 ± 0·000372
	Both	MA mean	Constrained to 0	0·00115 ± 0·00011‡‡
	Both	MA mean	−0·0076 ± 0·0084	0·00132 ± 0·00021‡

* $P<0·10$.
** $P<0·05$.
‡ $P<0·01$.
‡‡ $P<0·001$.

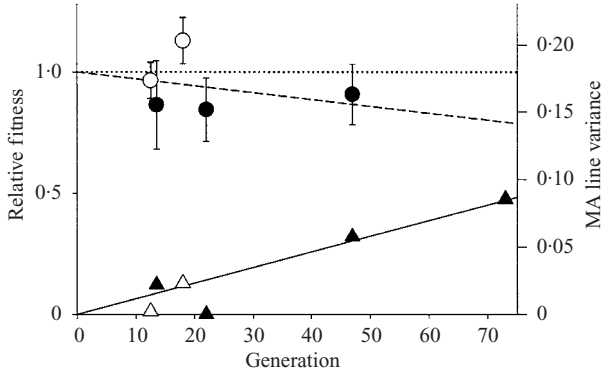

Fig. 1. Mean fitness relative to control means at each assay (circles) and subline variance in fitness (triangles). Line 33, open symbols; line 39, closed symbols. Confidence limits on the mean fitnesses are for the difference between controls and mutation-accumulation (MA) lines, based on Student's *t* test. The dashed line gives the regression of mean fitness on generation in both lines combined with the intercept constrained to 1. The solid line gives the regression of among-subline variance on generation in both lines with the intercept constrained to 0. The dotted line shows mean fitness = 1.

For line 33, the corresponding *P* values for one-sided tests at generations 13 and 18 were 0·26 and 0·99. Evidence therefore supports a decline in fitness relative to the control in line 39 but not line 33.

(ii) ΔM

The rate of decline in the mean fitness, ΔM, was estimated in two ways (Table 3). When the control and MA flies were assumed to have the same fitness at generation 0, ΔM for line 39 was 0·341%, which was significant by a one-sided test at $P=0·0498$. Line 33 showed a non-significant increase in mean with time. Analysis of the combined data revealed a decline of 0·285%, which was almost significantly different from 0 ($P=0·053$). For both lines, the evidence in favour of a decline relative to the control means was ambiguous, because the estimated decline at the final generation was less than that at the earlier times. When the intercepts for the regressions were allowed to vary, the intercepts were not different from 1, and the slopes were not significantly different from 0 (Table 3). This result suggests that our estimates of ΔM are suspect. For this reason, we do not present Bateman–Mukai estimates of *U*.

(iii) ΔV

The among-subline mutational variance estimates are shown as triangles in Fig. 1. In line 39, significant added variance among lines was evident at generations 13, 47 and 73 (*P* values 0·023, 0·89, <0·0001 and 0·006 for generations 13, 22, 47 and 73, respectively). In line 33, added subline variance was not significant at generation 13 ($P=0·24$) but was at generation 18 ($P=0·011$).

In contrast to estimates of ΔM, estimates of ΔV were consistent regardless of whether the intercept was constrained or which data were used. All estimates were within 10% of 0·0013. CV_M in these lines were between 3·1% and 3·6%, toward the high end of the range of values observed for fitness correlates (Houle *et al*., 1996), as expected given that our fitness measure covered a large proportion of the life cycle (Houle, 1998).

(iv) *Transpositions of* copia *in line 39*

When the lines were initially checked for homozygosity by means of TE insertion sites, the two differed markedly in *copia*, which segregated in most derivatives of IV*e*-39 but not in IV*e*-33 (data not shown). We verified that transposition of *copia* in IV*e*-39 was the cause of this difference by directly observing

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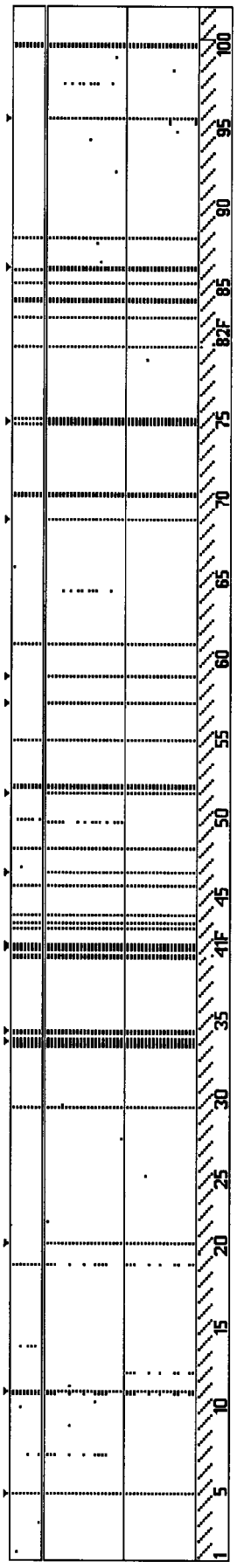

transpositions in the offspring of two males of inbred line IV*e*-39, which was segregating for *copia* but not for *roo*, crossed with Oregon R females. Cytological analysis revealed 11 *copia* transpositions in 18 progeny larvae of the first and five transpositions in 19 progeny of the second male (Fig. 2, boxes 2, 3).

Consequently, we monitored the positions of *copia* elements in IV*e*-39 during the process of MA. At the beginning of the MA experiment, *copia* positions were scored in eight full-sib brothers and sisters of the IV*e*-39 founders. Most *copia* positions were detected in each larva. Most of these sites are likely to be fixed, but segregation would be difficult to detect because the hybridization signal is dominant. Eight segregating *copia* insertion sites were detected (Fig. 2, box 1).

Keightley *et al*. (1993) have argued that residual selection can substantially bias the rate of MA. To compare the rates of *copia* transposition and accumulation, we measured the transposition rate directly after ten generations of MA in ten replicates of IV*e*-39 (Table 4). In 179 progeny, 138 *copia* transpositions were found, for a transposition rate of 0·88 per gamete. Because *copia* does not transpose in females (Pasyukova & Nuzhdin, 1992), the rate averaged over sexes was 0·44 per gamete per generation (standard error 0·09). No significant correlation was found between *copia* transposition rate and copy number. This result is expected given the small variation in copy number among these closely related lines, although such a correlation has been observed among lines differing substantially in copy number (Nuzhdin *et al*., 1996; Pasyukova *et al*., 1998).

After 22 generations of MA, two or three females of each of 67 replicate MA sublines were crossed with Oregon R males, and one offspring per female was scored for *copia* position (Fig. 3). Similarly, after 47 generations, 35 replicate MA sublines were analysed, with two or three progeny per female and one or two females per replicate (data not shown). A summary of copy number over time is shown in Fig. 4. The *copia* copy number increased by 0·24 (standard error 0·05) per genome per generation ($t=4{\cdot}7$, $P<0{\cdot}0001$). Although the rate of accumulation (0·24 per generation) is lower than the rate of transposition (0·44 per generation), the difference is not statistically significant. Confidence limits are consistent with anything from complete suspension of selection on TE

Fig. 2. Positions of *copia* along cytological map at the beginning of the experiment. Each row of dots represents the positions in a single individual. The top section of the figure shows *copia* positions in eight siblings of the founding parents of IV*e*-39. The next two sections show positions in heterozygous progeny of an individual IV*e*-39 male and Oregon R females. Sites detected in one or two progeny originate from transpositions in the male germ cells. Triangles depict sites fixed in Oregon R stock.

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Table 4. *The rate of* copia *transposition in line IV*e-*39*

Subline	Copy number	Transpositions*		Transposition rate‡ ($\times 10^{-2}$)
		X chromosome	Autosomes	
39-2	35·7	2 (7)	29 (18)	5·85
39-6	34·2	3 (9)	3 (18)	1·54
39-7	39·3	8 (11)	15 (19)	3·86
39-8	35·0	1 (4)	9 (17)	2·47
39-9	34·6	2 (14)	19 (22)	2·91
39-10	35·3	1 (10)	11 (16)	2·23
39-11	34·9	3 (8)	22 (20)	4·23
39-13	42·5	1 (10)	2 (18)	0·28
39-14	33·8	1 (10)	3 (15)	0·78
39-16	34·8	0 (3)	3 (16)	0·30

* Transpositions detected (chromosomes examined).
‡ The rate was calculated for X chromosomes and autosomes separately and then averaged.

insertions to the loss of almost half the new insertions to residual selection. We also tested whether the loss of line 39 sublines was caused by *copia* accumulation, by comparing the numbers of *copia* elements in lines that survived with those in lines lost between generations 22 and 47 and between generations 47 and 73. In neither period was there significant evidence for such an effect (data not shown).

The rate of *copia* excision (owing to either precise or imprecise deletions) is interesting because of its rarity (Biemont *et al*., 1987; Pasyukova & Nuzhdin, 1993; Nuzhdin & Mackay, 1994). We detected the first *copia* excision from site 30A in generation 22 (no hybridization in six larvae of one MA replicate). This site was occupied in all founder flies (Fig. 2) and in all other replicates at generation 10. The presence of the *e* visible marker and of *copia* in all other expected positions in this replicate argues against contamination (data not shown). Other cases of apparent excisions [e.g. Fig. 3, box 22 (34F) and box 30 (75B and 75D)] were seen in the later generations of the experiment. Most of them are disappearances of single *copia* positions in individual lines; these are likely to be excisions. Some of the sites that appeared to be fixed at the beginning of the experiment were not observed in a substantial number of sublines in later generations (e.g. Figs 2, 3, 85D and 88C). This result could be explained either by heterozygosity in the base population or by unusually high rates of excision.

(v) *Fitness decline because of* copia *accumulation*

To analyse the effects of *copia* insertions on fitness within each assay, we standardized fitnesses to a mean of 1 at each assay time. These standardized data are shown in Fig. 5 and the regression results in Table 5. At generation 22, the regression of fitness on *copia* copy number was significantly negative by a one-tailed test, whereas the regression was very close to significance at generation 47. Combined analysis yielded a significantly negative slope of $-0{\cdot}76 \pm 0{\cdot}32\,\%$.

A quadratic term was added to the regression models as a check for evidence of epistasis. The quadratic term was significantly negative by two-tailed test at generation 47 (linear $0{\cdot}0181 \pm 0{\cdot}093$, $P = 0{\cdot}06$; quadratic $-0{\cdot}00021 \pm 0{\cdot}00009$, $P = 0{\cdot}04$) but almost significantly positive in generation 22 (linear $-0{\cdot}0605 \pm 0{\cdot}031$, $P = 0{\cdot}06$; quadratic $0{\cdot}00064 \pm 0{\cdot}00037$, $P = 0{\cdot}09$). The quadratic term was not significant in the combined generation-22 and -47 analysis (linear $-0{\cdot}0022 \pm 0{\cdot}0155$, $P = 0{\cdot}89$; quadratic $-0{\cdot}00005 \pm 0{\cdot}00017$, $P = 0{\cdot}78$). On balance, the evidence for epistasis is weak.

The total number of excisions detected in a line was positively related to fitness in generations 22 and 47. Combined analysis showed that this effect was just significant by a one-tailed test when it was the only effect in the model (slope $5{\cdot}83 \pm 3{\cdot}49\,\%$, $P = 0{\cdot}05$) but not when the number of sites was also included in the model (sites $-0{\cdot}66 \pm 0{\cdot}33\,\%$, $P = 0{\cdot}024$; excisions $4{\cdot}03 \pm 3{\cdot}52\,\%$, $P = 0{\cdot}128$).

From the above analysis of insertions, *copia* is expected to have accounted for 0·24 (inserts per generation) $\times$ 0·76 % (drop in fitness per insert) = 0·18 % decline in fitness each generation. This estimate is comparable to that of the overall decline in fitness, 0·34 %, in line 39 with the control fitness as a standard and that of 0·73 % with the raw fitness data (Table 3).

4. Discussion

We have studied the properties of deleterious mutations using two rather different approaches. We estimated the rate of increase in genetic variance, ΔV,

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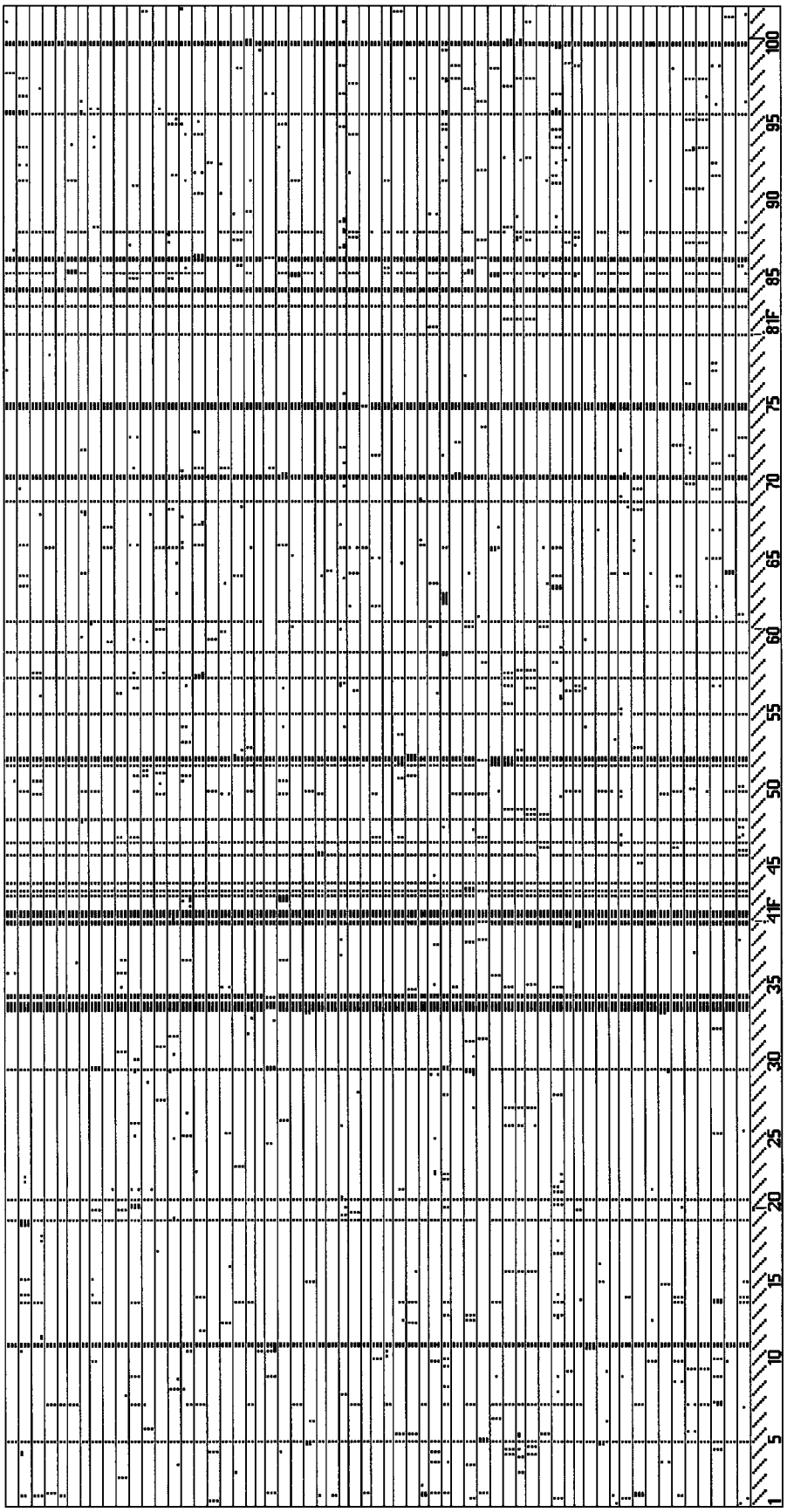

Fig. 3. Positions of *copia* in IV*e*-39 at generation 22. Each division of the figure corresponds to a different subline. Two larvae were scored per subline.

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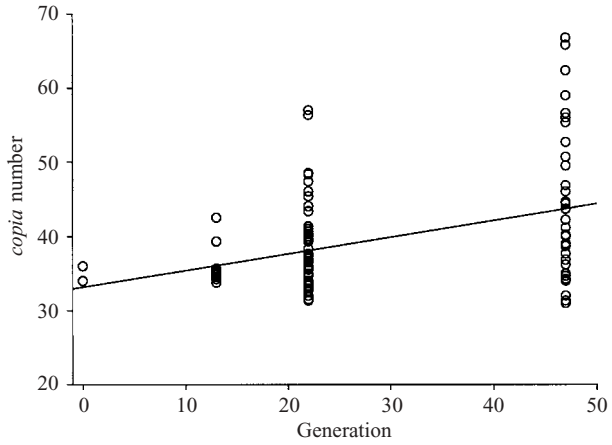

Fig. 4. Copy number of *copia* in line IV*e*-39 as a function of generations of mutation accumulation.

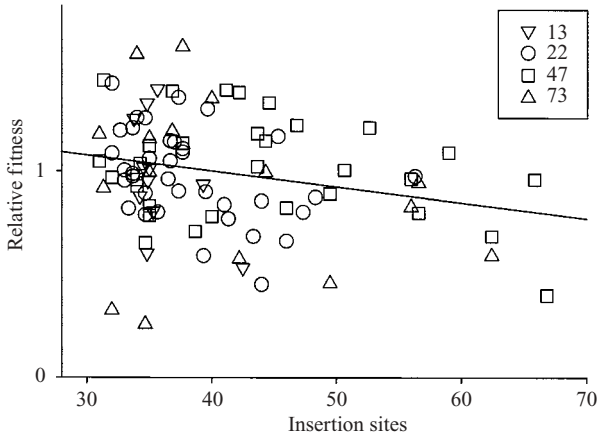

Fig. 5. Relationship between fitness estimates in generations 13, 22, 47 and 73 (standardized to a within-generation mean of 1) and the number of insertion sites detected. The regression line is for data from generations 22 and 47 only.

in female fitness and the rate of decline in mean fitness, ΔM, in hopes of making traditional Bateman–Mukai estimates of the deleterious mutation rate, U. Our experiment differed from previous ones in its use of a cryopreserved control. We also tracked the insertions of a transposable element, *copia*, which proved to transpose at an unusually high rate in one of our MA lines. Element number was a significant predictor of fitness within generations, allowing us to estimate the impact of these known mutations on fitness.

(i) *Cryopreserved control*

Much of the uncertainty over mutational parameters in *D. melanogaster* reflects uncertainty over the rate of decline in mean fitness under MA. Some experimental results suggest that the rate of decline is of the order of 1% or more per generation (Mukai, 1964; Mukai *et al*., 1972; Shabalina *et al*., 1997; Fry, 2001), whereas, in another experiment, the rate of decline is much lower (Fernández & López-Fanjul, 1996; Chavarrías *et al*., 2001; Ávila & García-Dorado, 2002). In addition, several authors have raised the possibility that the experiments finding higher rates of decline are flawed in allowing adaptation (Keightley, 1996; García-Dorado, 1997) or contamination (Houle *et al*., 1994*a*) of the control population or that misscoring of flies inflated fitness estimates (Fry *et al*., 1999).

We used a cryopreserved control in a standard MA experiment in hopes that it might help to resolve these uncertainties. In the event, our estimates of the decline in mean fitness proved to be variable and not always consistent with the expectation that deleterious mutations were accumulating. However, we obtained very consistent estimates of the increase in among-line variance, which sampling theory suggests should be the more difficult parameter to estimate.

This uncertainty about the decline in the mean is probably the result of our fitness assay, which compounded the effects of female fecundity and offspring viability. In theory, this is an advantage over assays that only assess offspring viability, but the control, standard competitor and MA genotypes are necessarily treated differently until two generations before the assays. This interval might have been insufficient for the elimination of cross-generation environmental effects.

Some unknown environmental or genetic effect of cryopreservation or the revival process could also be a source of such variation. The low success we had in reviving cryopreserved embryos certainly implies that the process could have been stressful to the survivors. The fitness of populations founded by the cryopreserved offspring did improve subsequent to the assays reported here (data not shown), but this result could also be due to adaptation as a result of advantageous mutations.

Several alternative explanations for the inconsistent performance of the controls seem to be less likely. Mutation during cryopreservation is unlikely to have been important. We estimated that the lethal mutation rate during the cryopreserved generation was equal to that in a normal generation (with an upper 95% limit of an increase by a factor of two; Houle *et al*., 1997), making mutation an unlikely cause of fitness decline. Viability selection during cryopreservation should not affect fitness because our controls were inbred lines that would have very little genetic variation. Contamination would tend to increase fitness and not cause it to be low and variable. On balance, insufficient standardization of rearing conditions in the generations leading up to these fitness assays seems to be the most likely explanation for the variability in relative fitness of the control lines.

It is unclear whether our difficulties have general implications for the use of cryopreservation. All of our

Table 5. *Statistics from regression of standardized fitness on copy number*

Data set	*N*	Slope (SE)	*P**	Intercept (SE)
Generation 22	36	−0·0159 (0·0062)	0·008	1·590 (0·241)
Generation 47	31	−0·0069 (0·0040)	0·055	1·311 (0·189)
Generations 22, 47	67	−0·0076 (0·0032)	0·010	1·304 (0·133)

SE, standard error.
* Probabilities are one-tailed.

samples were stored in a single Dewar flask, which might have suffered from an event such as depletion of liquid nitrogen at some point during storage. In previous work, rates of success in reviving embryos of a particular test genotype were as high as 45% (Steponkus & Caldwell, 1993). Our results (here and Houle *et al*., 1997) are the only attempts we know of to revive embryos from cryopreservation after more than a few days. Additional attempts to use the method are needed before it is discarded.

(ii) *Use of TE insertions to estimate mutational effects*

The high rate of transposition of *copia* in one of our lines provided us with a convenient opportunity to assess the effects of transposon insertions on fitness. The *copia* copy number increased at the rate of about 0·24 insertions per genome per generation, which could be anywhere from one-quarter of the normal rate to about the normal transposition rate of all transposons combined (Nuzhdin & Mackay, 1994, 1995; Maside *et al*., 2000). The relationship between fitness and copy number within generations allowed us to estimate that the average *copia* insertion fixed in this experiment caused a loss of fitness of 0·76 ± 0·32%. It should be borne in mind that insertions with lethal or near-lethal effects do not persist to be measured.

This estimate is in general agreement with the estimates of the hemizygous effects of *P*-element insertions by Eanes *et al*. (1988), who regressed fitness on the number of elements and estimated the mean homozygous effect as 1·4%, not significantly different from our estimates. By contrast, Mackay and coworkers (Mackay *et al*., 1992; Lyman *et al*., 1996) estimated the mean viability effect of a *P*-element insertion to be as high as 13%. There are several possible explanations for this discrepancy. Eanes *et al*. only estimated the effect of insertions on the X chromosome, whereas Mackay *et al*. examined the effects of insertions on each major chromosome. The estimated effects of X-chromosome insertions in the Mackay *et al*. study are much closer to those of Eanes *et al*. than were autosomal effects. Second, Eanes *et al*. mobilized endogenous *P* elements, whereas Mackay *et al*. used a *P*-element construct four times larger than a natural full-length *P*-element that might also have differed in the pattern of insertion. Finally, Mackay *et al*. might have overestimated effects, because aborted transpositions were undetectable (C. Langley, pers. commun.). Eanes *et al*.'s (1988) estimates are less subject to this bias because, in their design, effects of most undetected mutations entered the error variance.

(iii) *TEs in mutation-accumulation experiments*

If our estimates of the effects of *copia* insertions and change in mean fitness are taken at face value, as much as half of the fitness decline observed in our line IV*e*-39 could be due to *copia* insertions, even though base-pair mutations, deletions and other kinds of insertions were all presumably occurring as well. The large impact of *copia* is unusual because of the elevated transposition rate in the line studied, but elevated *copia* activity has been observed before. In one study of *D. melanogaster*, 60% of 327 transpositions detected in 17 TE families involved *copia* (Nuzhdin & Mackay, 1995). In another line, *copia* accounted for ten out of 27 transpositions detected (Maside *et al*., 2000). The ease with which genotypes of *D. melanogaster* with unusually high rates of transposition have been found suggests that MA experiments vary in the relative importance of insertions.

Changes in transposition or excision rates may also occur during a MA experiment. For example, Mukai (1969) estimated that the rate of fitness decline accelerated in one experiment, as would be expected if the accumulation of retrotransposons is a primary source of the decline (Nuzhdin *et al*., 1997). By contrast, divergence of sublines slowed during two other experiments (Ohnishi, 1977; Mackay *et al*., 1995), a result that could in principle be explained by a decrease in transposition or an increase in excision rate over time. We observed reversions of some previously fixed insertions in our experiment that might have caused partial rescue of fitness. Excisions of *copia* have been seen in one other experiment (Biemont *et al*., 1987). Excisions of other retrotransposons also seem to be rare (Belyaeva *et al*., 1982; Kim & Belyaeva, 1991; Nuzhdin & Mackay, 1994; Maside *et al*., 2000). No

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convincing mechanism for retrotransposon excision has been offered, so we can offer no concrete scenario for a change in excision rate.

Even though the rate of insertion observed in one of our lines was unusually high, several kinds of evidence suggest that the normal impact of transpositions on the mutation rate in *D. melanogaster* is substantial. There are close to 50 other TE families in *Drosophila* (Charlesworth *et al*., 1992*a*, *b*). About half of the major morphological mutations in flies are caused by TE insertions (Finnegan *et al*., 1978). The mutation rate toward loss-of-function (null) alleles of several proteins is approximately ten times higher than that toward active alleles with changed electrophoretic mobility (Mukai & Cockerham, 1977; Voelker *et al*., 1980; Harada *et al*., 1993), which is to be expected if insertions are a more common source of mutations than nucleotide changes. Although the observed rate of transposition is unusual, our estimates of the average effect of an insertion might be widely applicable.

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