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Fitness decline in spontaneous mutation accumulation lines of *Caenorhabditis elegans* with varying effective population sizes

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The rate and fitness effects of new mutations have been investigated by mutation accumulation (MA) experiments in which organisms are maintained at a constant minimal population size to facilitate the accumulation of mutations with minimal efficacy of selection. We evolved 35 MA lines of *Caenorhabditis elegans* in parallel for 409 generations at three population sizes ($N = 1$, 10, and 100), representing the first spontaneous long-term MA experiment at varying population sizes with corresponding differences in the efficacy of selection. Productivity and survivorship in the $N = 1$ lines declined by 44% and 12%, respectively. The average effects of deleterious mutations in $N = 1$ lines are estimated to be 16.4% for productivity and 11.8% for survivorship. Larger populations ($N = 10$ and 100) did not suffer a significant decline in fitness traits despite a lengthy and sustained regime of consecutive bottlenecks exceeding 400 generations. Together, these results suggest that fitness decline in very small populations is dominated by mutations with large deleterious effects. It is possible that the MA lines at larger population sizes contain a load of cryptic deleterious mutations of small to moderate effects that would be revealed in more challenging environments.

KEY WORDS: **Deleterious mutation, epistasis, fitness, genome-wide mutation rate, productivity, survivorship to adulthood.**

Mutation induces genetic variation, which in turn, fuels evolutionary change. Although mutationally induced variation is a prerequisite for evolution, the majority of spontaneous mutations have detrimental effects on organismal fitness (Lynch et al. 1999). The rate and fitness effects of new mutations are central to an understanding of a multitude of evolutionary phenomena, including the maintenance of genetic variation (Lynch et al. 1999), the contribution to quantitative trait variation (Caballero and Keightley 1994), the evolution of sex, mating systems, and recombination (Pamilo et al. 1987; Kondrashov 1988), inbreeding depression (Charlesworth and Charlesworth 1987), the evolution of senescence (Hamilton 1966), the persistence of gene duplicates (Force et al. 1999), and the evolution of ploidy level (Kondrashov and Crow 1991), among others. Recently, there has been a renewed interest in the consequences of spontaneous mutations for long-term human health (Crow 1997; Eyre-Walker et al. 2006; Lynch 2010) with similarly somber implications for the maintenance of threatened plant and animal populations at small sizes (Lynch and Gabriel 1990; Lande 1994).

Mutation accumulation (MA henceforth) studies, although labor- and time-intensive, have served as an exemplar means to elucidate the basic properties and spectrum of both spontaneous and induced mutations (Halligan and Keightley 2009, and references therein). In MA experiments, multiple replicate lines derived from an inbred ancestral stock population are allowed to evolve independently under conditions of extreme bottlenecking each generation, which severely diminishes the efficacy of natural selection and promotes evolutionary divergence due to the accumulation of deleterious mutations by random genetic drift. The vast majority of MA studies have maintained the focal

EVOLUTION
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organism at a constant and minimal effective population size (N_e henceforth) (Halligan and Keightley 2009). The loss or fixation of mutations and their consequences for population fitness depend upon the selection coefficients (s) associated with individual mutations and the N_e. The fates of mutations with selection coefficients much less than the reciprocal of the N_e [$|s| \ll 1/2N_e$ for diploids] are primarily influenced by genetic drift. Conversely, the dynamics of mutations with $|s| \gg 1/2N_e$ are largely determined by natural selection. Deleterious mutations with very large effects are unlikely to pose a long-term threat to population fitness as they are rapidly eradicated and unlikely to reach fixation; those with very small effects would be effectively neutral. Although the effective strength of selection is dependent on the N_e, the prevailing opinion is that the most detrimental class of mutations influencing long-term population fitness comprise slightly deleterious or nearly neutral mutations (Lande 1994). Such mutations would be eradicated via purifying selection at high N_e, but can behave in an "effectively neutral" fashion and reach fixation by genetic drift at low N_e (Lynch and Gabriel 1990; Lande 1994).

The parallel creation of experimental evolution lines at varying N_e would provide significant advances over past studies in this classical field of enquiry. First, the varying N_e treatment offers a powerful framework to assess the interaction between mutation and natural selection. Second, phenotypic measurements of fitness-related traits can elucidate the trajectory of fitness decline due to spontaneous MA as a function of N_e. Only two studies thus far have used this approach of MA experiments with varying N_e. However, both studies opted to accelerate the rate of mutations by either gene-knockout or chemical mutagenesis. Estes et al. (2004) used a mutant strain of *Caenorhabditis elegans* possessing a complete knockout of the mismatch–repair gene *msh-2* in two different sets of experiments. Another study mutagenized ϕX174 phage populations maintained at $N = 3$, 10, 30, and 100 through 90 transfers (Silander et al. 2007). Although both experiments have provided important insights into the distribution of fitness effects (DFEs) of mutations, there is evidence that the DFE occurring under these nonspontaneous, elevated regimes of mutagenesis is quite different from that under spontaneously incurred mutations (Keightley and Ohnishi 1998; Wloch et al. 2001). As such, conclusions about the rate, spectrum, and fitness effects of such artificially induced mutations may not accurately represent the characteristics of spontaneous mutations in nature.

To address the limitations of past MA studies and gain fundamental insights into the molecular and fitness consequences of spontaneous mutation, we evolved 35 long-term spontaneous MA lines of the nematode *C. elegans* in parallel at varying population sizes ($N = 1$, 10, 100 individuals). These lines represent the lengthiest spontaneous MA experiment to date for any species at differing population sizes spanning ~410 consecutive MA generations. Furthermore, our experimental lines represent a wider range of population size treatments ($N = 1$, 10, and 100) compared to the *msh-2* knockout experiment in *C. elegans* with $N = 1$, 5, and 25 (Estes et al. 2004). Phenotypic assays for two fitness-related traits on all extant MA lines were conducted under standard, benign laboratory conditions at four time intervals. Our goal is to provide the first and most comprehensive cross-generation analysis of fitness decline in populations of varying N_e under a regime of spontaneous MA and elucidate how smaller populations are compromised by MA via drift, with important implications for conservation biology and captive breeding programs.

Methods

STUDY SYSTEM

Caenorhabditis elegans is a self-fertilizing soil nematode where the majority of individuals are hermaphrodites. The species has a short generation time of 3.5 days from egg to egg-laying adult at 20°C in the laboratory. *Caenorhabditis elegans* is particularly amenable to MA studies as selfing rapidly drives populations to homozygosity (Vassilieva et al. 2000; Baer et al. 2005; Katju et al. 2008). Another immense advantage of this system is the ability of nematode cultures to survive long-term cryogenic storage at −86°C, enabling direct comparisons between experimentally evolved lines and ancestral genotypes (Lewis and Fleming 1995).

SPONTANEOUS MA EXPERIMENT IN *C. ELEGANS* AT VARYING N_e

We initiated an independent, long-term spontaneous MA experiment in *C. elegans* comprising 35 *C. elegans* populations maintained in parallel at varying population size treatments of $N =$ 1, 10, and 100 hermaphrodites per generation. The experiment was conducted over a time span of four and a half years and initiated with a single wild-type Bristol (*N2*) hermaphrodite originally isolated as a virgin L4 larva (Fig. 1) with termination at 409 MA generations. The F1 hermaphrodite descendants of this single worm were further inbred by self-fertilization before establishing 35 MA lines and cryogenically preserving thousands of excess animals at −86°C for use as future ancestral controls. Twenty of these 35 lines were established with a single worm and propagated at $N = 1$ per generation. Ten lines were initiated with 10 randomly chosen L4 hermaphrodite larvae and subsequently bottlenecked each generation at $N = 10$. Five lines were initiated and subsequently maintained each generation with 100 randomly chosen L4 hermaphrodite larvae ($N = 100$). A new generation was established every four days. The worms were cultured using standard techniques with maintenance at 20°C on Nematode Growth Medium (NGM) agar in (1) 60 × 15 mm Petri dishes seeded with 250 μL suspension of *Escherichia coli* strain OP50 in YT media ($N = 1$ and $N = 10$ lines) or (2)

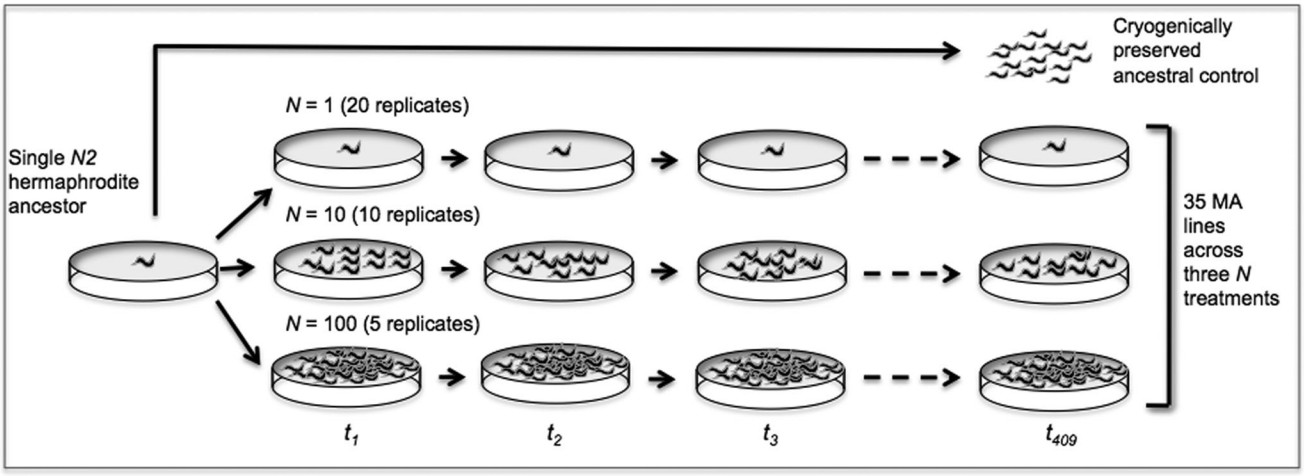

Figure 1. Schematic of *C. elegans* spontaneous MA experiment with three treatments of varying population size (*N*). All 35 lines are descended from a single *N2* hermaphrodite whose additional descendants were expanded for two generations and frozen as ancestral, pre-MA controls. The maintenance of lines at varying *N* enables manipulation of the strength of selection. After *t* generations, the lines are expected to have independently accumulated mutations, leading to a mean decline in fitness relative to the ancestral control and increased among-line variance.

90 × 15 mm Petri dishes seeded with 750 μL suspension of *E. coli* strain OP50 in YT media ($N = 100$ lines). Stocks of the MA lines were cryogenically preserved at −86°C at approximately every 50 additional MA generations. The experiment was terminated following 409 MA generations because the $N = 1$ lines displayed a highly significant fitness decline. Three lines were already extinct due to the accumulation of a significant mutation load and five additional lines were on the verge of extinction (displaying great difficulty in generation to generation propagation).

THEORETICAL UNDERPINNINGS

The fitness effect of a mutation can range continuously, from lethal to deleterious to neutral to beneficial. Loss or fixation of mutations and their consequences for population fitness depend upon the selection coefficients (s) associated with individual mutations and the effective population size, N_e. For sexually reproducing diploids, the dynamics of mutations with $|s| << 1/2N_e$ are dictated entirely by random genetic drift (Eyre-Walker and Keightley 2007). Conversely, the dynamics of mutations with $|s| >> 1/2N_e$ are governed by natural selection. Moreover, given the predominantly self-fertilization mode of reproduction in hermaphroditic species such as *C. elegans*, complete selfing may additionally result in further reduction of N_e relative to the census population size (N or N_C) (Crow and Kimura 1970; Pollak 1987; Charlesworth 2009). Hence from the perspective of population-genetic theory while taking selfing into account, the genetic effective population sizes of our three treatments ($N = 1$, 10, and 100 individuals) correspond to $N_e = 1$, 5, and 50 individuals (Pollak 1987). For lines bottlenecked each generation at $N_e = 1$, 5, and 50 individuals, mutations with selection coefficients less than approximately 0.5, 0.1, and 0.01 (50, 10, and 1%) are expected to contribute to mutational degradation, respectively, given that they will accumulate at the neutral rate although they may not necessarily be neutral with respect to absolute fitness (Fig. 2). Therefore, small populations subjected to attenuated selection and an increased magnitude of genetic drift can potentially accumulate mutations with extremely large effects in addition to ones with moderate to very slight effects (Eyre-Walker and Keightley 2007).

BENIGN FITNESS ASSAYS OF MA LINES

The fitness assay procedures largely follow previous protocols for *C. elegans* MA lines (Vassilieva et al. 2000) with some modifications. All extant experimental MA lines were assayed under standard *benign* laboratory conditions at 20°C in parallel with ancestral controls for two life-history traits, namely (1) *survivorship to* adulthood (survivorship henceforth), and (2) *productivity* following 100, 172, 300, and 409 MA generations. Frozen stocks of the MA lines and the ancestral control line were thawed and one and 20 individuals, respectively, were selected and expanded into five replicates to establish within-line replication. The replicates were then maintained by transferring single L4 (fourth larval stage) hermaphrodite individuals for two additional generations. A single third-generation descendant from each thawed replicate was employed in the actual assay to ensure a clean estimate of genetic divergence without contributions of maternal and grandmaternal environmental effects to the among-line component of variance (Lynch 1985). This protocol yielded 275 lines across four treatments ($N = 1$, $N = 10$, $N = 100$, and ancestral control).

The entire assay was conducted at 20°C, an optimal temperature for *C. elegans* growth. For each line, 10 L1 (first larval stage)

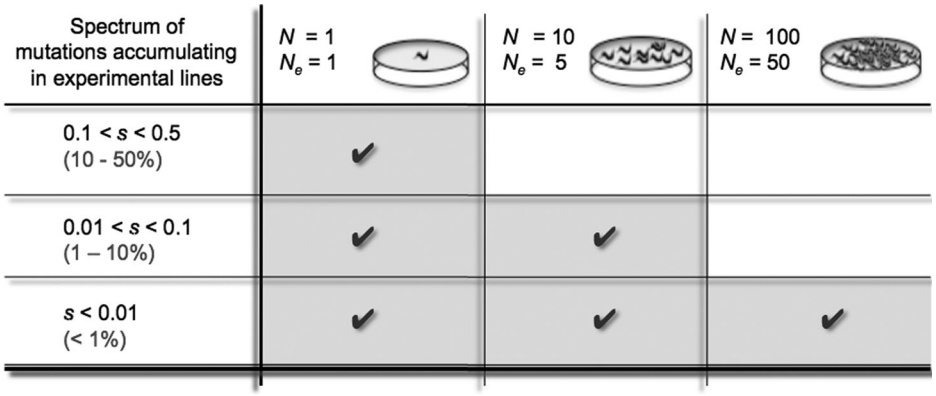

Figure 2. **The maintenance of experimental lines at varying N_e permits the manipulation of the strength of selection across different treatments. This enables the subdivision of the spectrum of mutational effects into a wide range of successively narrower classes across the three N_e treatments. Lines maintained at $N_e = 1$ are expected to accumulate mutations with $s < 0.5$ (very large effect to neutral). $N_e = 5$ lines experience minimal levels of selection and are predicted to accumulate mutations with $s < 0.1$ (moderately large effect to neutral). $N_e = 50$ lines are subjected to the greatest intensity of selection and are predicted to accumulate mutations with $s < 0.01$ (slight effect to neutral).**

progeny, where possible, were randomly selected upon hatching and isolated on a separate Petri dish with an *E. coli* OP50 lawn for measuring survivorship. Thirty-six hours after isolation, the number of individuals surviving past the L4 larval stage to reach adulthood was quantified. Survivorship values can range from 0 to 1, and are calculated by dividing the number of adult worms by the number of L1 larvae originally sequestered. This survivorship assay procedure is different from earlier *C. elegans* MA studies wherein survivorship and productivity were measured on the same worm and survivorship was scored as 1 if the worm produced a minimum of one progeny, otherwise as zero (Vassilieva et al. 2000). Measurements on a pool of 10 worms that were full siblings of the single worm entering the productivity assay enabled us to gain a more robust range of values for estimating survivorship of the line.

A single hermaphrodite, a full sibling of the 10 individuals assayed for survivorship, was randomly selected as an L4 larva and transferred to a new Petri dish with an *E. coli* OP50 lawn for measuring productivity. Every 24 h $\pm$ 30 min thereafter, each alive individual was transferred to a fresh Petri dish. In the first assay at MA generation 100, we conducted daily transfers until the worm was dead, sometimes extending to 14 consecutive days. Data analysis of this assay revealed that progeny production is negligible following eight days of transfer (2–5% of lines may produce at the most one to three additional progeny). Subsequently, we conducted the productivity assay by transferring live worms onto new plates for eight days after reaching the L4 (last) larval stage. Transfers were terminated if the worm was found dead prior to the completion of these eight days. Following each daily transfer of the assayed individuals, plates with eggs were placed at 20°C for an additional 24-h period to enable hatching. The plates were then stored at 4°C to kill the larvae and enable progeny counts. Progeny counts were conducted by staining the agar pad and *E. coli* lawn with a 0.075% water dilution of toluidine blue, which rendered the dead worms transparent and visible on the contrasting purple background for the ~2–5 min period required for counting. Productivity was calculated as the total number of progeny produced; nonreproductive individuals were scored as having zero productivity. Preceding *C. elegans* MA experiments only conducted the productivity assay for four days, based on the argument that 90% of total progeny production was completed within this time period (Vassilieva et al. 2000). This procedure was found to be error prone as we had instances where individuals frequently produced 25–80 progeny on days 5–6 accounting for 10–32% of total progeny production. In one instance, a worm produced 95 of a total 361 progeny on days 5–6, accounting for 26.3% of its total productivity. In another instance, 134 of a total 160 progeny (~84%) were produced on days 5–8. In our experience, the termination of the productivity assay after day 4 in preceding studies both underestimates and leads to erroneous estimates of total productivity because delayed reproduction is not uncommon in lines that have accumulated a significant mutational load. Given the extended time span of our productivity assays, our productivity counts are higher than some previous studies.

STATISTICAL ANALYSES OF MEANS AND VARIANCES OF LIFE-HISTORY TRAITS

A two-level nested Model I ANOVA with unequal sample sizes (Sokal and Rohlf 1995) was employed to partition the total phenotypic variance into among- and within-line components. The highest level of classification tested for a treatment effect (four treatments: (1) MA at $N = 100$, (2) MA at $N = 10$, (3) MA at $N = 1$, and (4) the ancestral control). The next level of hierarchy tested for a subgroups variance component

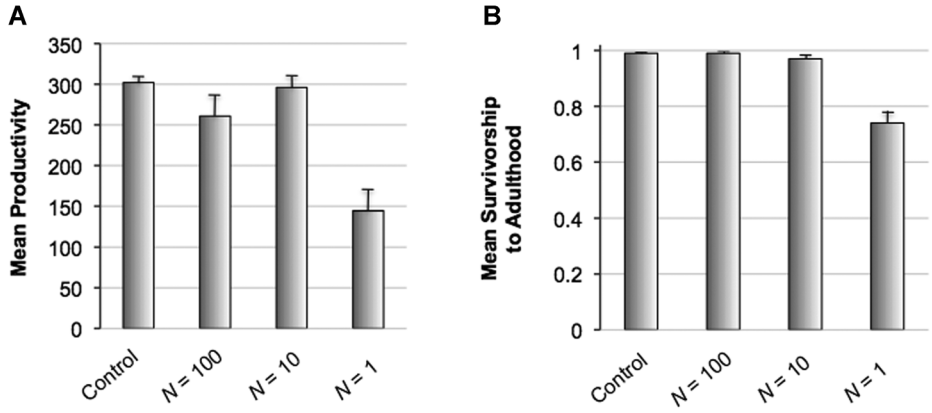

Figure 3. **Trait means for varying population-size treatments following 409 consecutive generations of mutation accumulation. Phenotypic assays were conducted for two fitness-related traits: (A) productivity and (B) survivorship to adulthood. Each bar represents the mean phenotype for a specified population-size treatment and ancestral control listed on the *x*-axis. Errors bars represent one standard error.**

(differences among independent lines within a treatment). The last hierarchical level estimated the intraline variance. A Satterthwaite approximation was used to account for unequal sample sizes (Sokal and Rohlf 1995). The GT2 method was used to conduct multiple pairwise mean comparisons based on unequal sample sizes at a 5% experiment-wise error rate (Hochberg 1974).

MAXIMUM-LIKELIHOOD ESTIMATION OF MUTATIONAL PARAMETERS

The program MLGENOMEU (Keightley 1994; Keightley and Ohnishi 1998) was used to estimate the genome-wide deleterious mutation rate (U) and parameters of the distribution of effects of mutations in the $N = 1$ populations relative to the control. At the termination of the experiment at 409 MA generations, the extant 17 $N = 1$ lines had been subjected to an average of 375 MA generations, given frequent failed transfers due to a presumed increase in mutation load and associated fitness decline. We assumed that mutation effects are gamma distributed with shape parameter β and mean $E(a)$. Profile likelihoods were fitted for a series of fixed values of β, while maximizing log-likelihood with respect to all of the other parameters fitted in the model. Data were also analyzed for the limiting case of equal mutational effects ($\beta \to \infty$). For both productivity and survivorship to adulthood, controls were assumed to be mutation-free and analyzed along with the MA line data. In the case of productivity, replicate measures for each MA line were analyzed, and separate means and error variances were estimated for the controls and MA lines. For survivorship, the individual measures depart substantially from normality, so the means of the replicates for each pseudocontrol line and MA line were analyzed. In this case, equal means and error variances in controls and MA lines needed to be assumed.

Results

SIGNIFICANT DECLINE IN FITNESS OF $N = 1$ LINES FOLLOWING 409 GENERATIONS OF SPONTANEOUS MA

The productivity and survivorship of all extant experimental lines across three population size treatments following 409 spontaneous MA generations was compared with that of 20 lines of the cryopreserved ancestral control. Three $N = 1$ MA lines had gone extinct by the time we commenced with this final fitness assay (line 1H at MA generation 293; 1S at MA generation 328; 1T at MA generation 309) and were excluded from the dataset. The final phenotypic fitness assay comprised measurements on five replicates each, where possible, of 20 control lines (C1–20), five $N = 100$ lines (100A-E), 10 $N = 10$ lines (10A-J), and 17 $N = 1$ lines (1A-G, 1I-R).

The mean productivity of $N = 100$, 10, and 1 and the ancestral control lines were 261, 296, 172, and 302 offspring, respectively (Fig. 3A). $N = 1$ lines showed a 44% decline in productivity relative to the control following 409 spontaneous MA generations. The mean survivorship of $N = 100$, 10, and 1 and ancestral control lines were 99, 97, 87, and 99%, respectively (Fig. 3B). $N = 1$ lines showed a 12% decline in survivorship relative to the ancestral control.

Following 409 MA generations, ANOVA analyses found a significant variance component for both productivity ($F_S{}' = 13.62$; $P < 10^{-5}$) and survivorship ($F_S{}' = 5.16$; $P = 0.0035$) among the four treatments (Table 1). In addition, there was a significant among-line divergence component within treatments for productivity ($F_S = 7.73$; $P = 0$) and survivorship ($F_S = 7.53$; $P = 0$), which is a hallmark of the stochasticity inherent in the MA process (Table 1).

Table 1. Two-level nested Model I ANOVA for productivity and survivorship to adulthood of ancestral control and MA lines of *C. elegans* following 409 successive generations of bottlenecking at $N = 1$, 10, and 100 hermaphrodites.

Source of variation	df	SS	MS	F_S	F_S'
			Productivity		
Among groups	3	885,283.9	295,094.6	13.64	13.62***
Among lines	48	1,038,113.7	21,627.4	7.73***	
Within lines (error)	207	579,431.4	2799.2		
Total	258				
			Survivorship to adulthood		
Among groups	3	0.7	0.226	5.17	5.16**
Among lines	48	2.1	0.044	7.53***	
Within lines (error)	207	1.2	0.006		
Total	258				

*Significance level of 0.05.
**Significance level of 0.01.
***Significance level of 0.001.

Comparisons among pairs of means based on unequal sample sizes using the Tukey–Kramer HSD method found no significant difference in the mean productivity or survivorship of MA lines maintained at $N = 10$ and 100 individuals relative to each other as well as to the ancestral control. In contrast, the mean productivity and survivorship of $N = 1$ was significantly lower than that of the other three treatments ($N = 100$, $N = 10$, and control) (Tables 2 and 3).

INCREASED AMONG-LINE VARIANCE IN THE $N = 1$ LINES UNDER MA

A significant divergence among lines within a treatment is in itself diagnostic of MA. After 409 MA generations, the among-line variance in mean productivity is significantly higher than that of the ancestral control for all population size treatments ($F_{N\,=\,1,\mathrm{Control}} = 13.20$, $P = 4.5 \times 10^{-7}$; $F_{N\,=\,10,\mathrm{Control}} = 2.79$, $P = 0.029$; $F_{N\,=\,100,\mathrm{Control}} = 4.27$, $P = 0.012$; Fig. 4A). For mean survivorship to adulthood, the among-line variance was significantly higher for $N = 1$ and $N = 10$ ($F_{N\,=\,1,\mathrm{Control}} = 199.51$, $P = 0$; $F_{N\,=\,10,\mathrm{Control}} = 6.95$, $P = 2.01 \times 10^{-4}$; $F_{N\,=\,100,\mathrm{Control}} = 2.26$, $P = 0.104$; Fig. 4B).

DELAY IN THE ONSET OF REPRODUCTION OF $N = 1$ LINES

Apart from the overall decline in productivity, $N = 1$ MA lines additionally exhibited a slight but significant delay in reproduction. For both controls and $N = 1$ MA lines, the majority (~70%) of progeny production occurs on days 2–3, with peak production on day 3 (41%). However, $N = 1$ lines produce a smaller proportion of progeny on day 1 but greater proportions on days 4–8

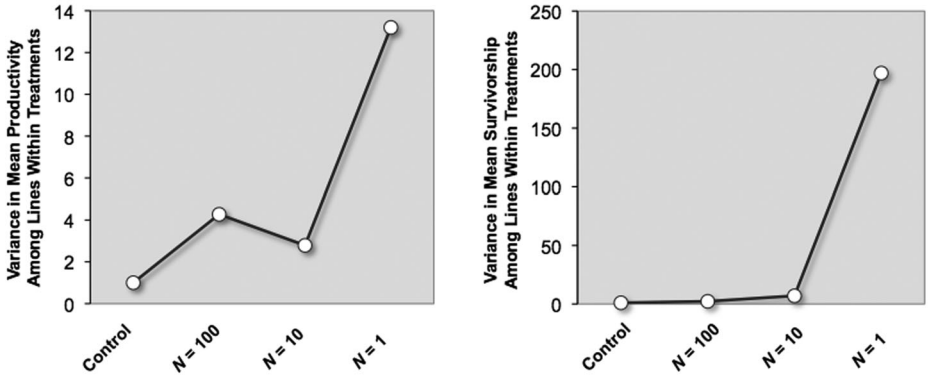

Figure 4. Among-line variance in (A) mean productivity and (B) mean survivorship to adulthood for the ancestral control and three population size treatments ($N = 1$, 10, and 100 individuals) following 409 consecutive generations of mutation accumulation. Lines within the $N = 1$ treatment exhibit the greatest among-line variance, implicating significant divergence amongst the lines with respect to both fitness traits due to the stochastic occurrence of mutations and high intensity of genetic drift operating under extreme bottlenecking conditions.

Table 2. Tukey–Kramer HSD method of multiple comparisons among pairs of means in a productivity assay of pre-MA controls and three sets of *C. elegans* experimental lines ($N = 100$, 10, and 1) following 409 successive generations of MA.

	Treatment 1 (Control)	Treatment 2 ($N = 100$)	Treatment 3 ($N = 10$)	Treatment 4 ($N = 1$)
Treatment 1 (Control)	—	47.32	62.58	73.56
Treatment 2 ($N = 100$)	41.33 *ns*	—	61.87	0.52
Treatment 3 ($N = 10$)	6.09 *ns*	35.24 *ns*	—	55.30
Treatment 4 ($N = 1$)	132.05*	90.72*	125.96*	—

Absolute differences among all pairs of productivity means *i* and *j* are listed below the diagonal and their critical MSD_{ij} values above the diagonal. A pair of means is declared significantly different at $\alpha = 0.05$ if their absolute difference equals or exceeds their MSD values (indicated by an asterisk).
ns indicates no significant difference between two means.
*A pair of means that are significantly different at an experiment-wise error rate of $\alpha = 0.05$.

Table 3. Tukey–Kramer HSD method of multiple comparisons among pairs of means in a survivorship to adulthood assay of pre-MA controls and three sets of *C. elegans* experimental lines ($N = 100$, 10, and 1) following 409 successive generations of MA.

	Treatment 1 (Control)	Treatment 2 ($N = 100$)	Treatment 3 ($N = 10$)	Treatment 4 ($N = 1$)
Treatment 1 (Control)	—	0.128	0.082	0.036
Treatment 2 ($N = 100$)	0.004 *ns*	—	0.128	0.015
Treatment 3 ($N = 10$)	0.020 *ns*	0.016 *ns*	—	0.002
Treatment 4 ($N = 1$)	0.123*	0.119*	0.103*	—

Absolute differences among all pairs of survivorship means *i* and *j* are listed below the diagonal and their critical MSD_{ij} values above the diagonal. A pair of means is declared significantly different at $\alpha = 0.05$ if their absolute difference equals or exceeds their MSD values (indicated by an asterisk).
ns indicates no significant difference between two means.
*A pair of means that are significantly different at an experiment-wise error rate of $\alpha = 0.05$.

compared to the ancestral controls. A total of 2.6% of the total progeny of $N = 1$ MA lines were produced on day 1 compared to 4.7% in the controls. In contrast, 28.5% of the MA progeny production occurred on days 4–8, compared to 22.9% in the controls. The ratio of relative proportions of progeny produced by the MA and control lines during the productivity assay following 409 MA generations is represented in Figure 5. The difference in the proportion of progeny per day between the $N = 1$ MA lines and the controls was highly significant (G-test, $G = 223.1$, $P < 0.001$).

ESTIMATES OF MUTATIONAL PARAMETERS

$N = 1$ lines allowed us to estimate the mutational parameters for both productivity and survivorship to adulthood under a regime of spontaneous MA largely uninfluenced by natural selection. A maximum-likelihood (ML) approach implemented in the program MLGENOMEU (Keightley 1994, 1998; Keightley and Ohnishi 1998) was used to estimate mutational parameters from phenotypic data generated from the last fitness assay following 409 MA generations (Table 4). Like other MA studies, we assumed mutational effects to be exclusively negative with respect to fitness. A multigenerational ML approach has been developed to estimate mutational parameters from MA experiments in which phenotypic assays are conducted at regular, multiple time intervals as was done in this study (Keightley and Bataillon 2000). However, this method assumes equal effects, which precludes the testing of different distributions of effects. Moreover, most of the relevant information to infer the DFEs is expected to occur in the later generations of the MA experiment. Indeed, it has been shown that there was little benefit for inferring the mean effect of a mutation by adding more generations in terms of reducing the variance of the estimate (Keightley and Bataillon 2000).

For productivity, the haploid genomic mutation rate per gamete per generation, U, was estimated to be 0.007. The likelihood of a model with a free β value was not significantly better than a model assuming equal effects ($\beta \to \infty$), similar to a previous study (Keightley and Caballero 1997). The upwardly biased average mutational effect, $E(a)$, for productivity was -49.55 progeny (or -16.4% of the control mean) and is within the range of values generated by preceding MA experiments for this species (Keightley and Caballero 1997; Vassilieva et al. 2000; Baer et al. 2005). Our estimate of $E(a)$ for productivity implies the accumulation of mutations with strong negative selection coefficients contributing to decline of this fitness trait under extreme bottlenecking conditions.

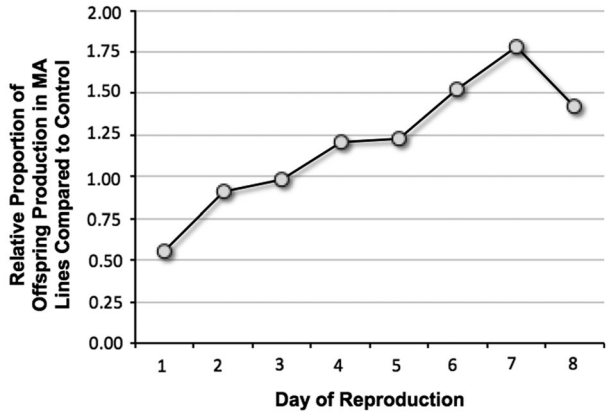

Figure 5. The proportion of offspring produced per day in $N = 1$ mutation accumulation lines following 409 MA generations relative to the ancestral control. Each value in the graph is the ratio of the proportion of offspring produced any given day in the mutation accumulation lines compared to the proportion produced on the same day in the ancestral control lines.

Table 4. Maximum-likelihood (ML) estimates for spontaneous mutation parameters affecting two life-history traits, productivity and survivorship to adulthood, in *Caenorhabditis elegans*.

	Fitness-related trait	
	Productivity	Survivorship to adulthood
$\bar{z}_{control}$	302.16 (6.25)	0.992 (0.0027)
$\bar{z}_{MA}$	172.00 (24.47)	0.870 (0.04)
U	0.007 (0.0019, ∞)	0.0028 (0.0014, ∞)
$E(a)$	−0.164 (−0, −0.51)	−0.118 (−0, −0.21)

All estimates are for the $N = 1$ population-size treatment within a 409 generation spontaneous MA experiment comprising three population size treatments ($N = 1$, 10, and 100 individuals). Three lethal-bearing lines of the original 20 MA lines within the $N = 1$ treatment were excluded from the analyses. Estimates of the initial mean phenotype ($\bar{z}_{control}$), the mean phenotype of the $N = 1$ lines subjected to the specified number of mutation accumulation generations ($\bar{z}_{MA}$), per generation genomic mutation rate (U_{min}), and the average mutational effect $E(a)$ are calculated from phenotypic data generated in the final fitness assay following 409 consecutive spontaneous MA generations. One standard error or 95% confidence intervals are shown in parentheses following each estimate.

With respect to survivorship, ML analysis excluded a model assuming mutations of equal effect. A gamma distribution assuming a β value of 1.8 gave the best fit to the data, providing evidence that mutations of variable effects contributed to a decline in survivorship. For survivorship, U was estimated to be 0.0028, which is similar to Vassilieva et al.'s rate of 0.003 for the *C. elegans* N2 strain (Vassilieva et al. 2000). However, $E(a)$ for survivorship was −0.118 (or −11.8%), which is threefold lower than a previous estimate of −0.39 (Vassilieva et al. 2000).

These estimates of U and the parameters of DFE of mutations for productivity and survivorship are consistent with the small changes in the means of these traits observed under MA at the two higher population sizes. By transition matrix iteration, we calculated the reduction in fitness explained by segregating and fixed deleterious mutations of effect s occurring at rate U per haploid genome in each of $t = 409$ generations in a Wright–Fisher population of size N, integrating over the estimated DFE for survivorship, or assuming a fixed selection coefficient for productivity. Predicted reductions for $N = 10$ are 5% and 2% for productivity and survivorship, respectively, and are close to 0% for both traits for $N = 100$.

TIME-SERIES ANALYSES OF FITNESS DECAY AS A FUNCTION OF POPULATION SIZE

Fitness of all the experimental lines relative to the ancestral controls was also assayed at three additional earlier time intervals, at MA generations 100, 172, and 300. ANOVA analyses found a significant variance component for both fitness traits among the four treatments as well as a significant among-line divergence component during each of these assays (data not shown). In addition, comparisons among pairs of means using the Tukey–Kramer HSD method found a significantly lower mean productivity and survivorship of $N = 1$ lines relative to the other three treatments ($N = 100$, $N = 10$, and control) following MA generations 100, 172, and 300 but no significant difference in mean fitness of MA lines maintained at $N = 10$ and 100 relative to each other as well as to the ancestral control (data not shown). In summary, all four fitness assays through the course of the MA experiment detected a significant decline in the fitness of the $N = 1$ lines relative to the ancestral control and two larger population size treatments ($N = 10$ and 100). The trajectories of decline in productivity and survivorship are displayed in Figures 6 and 7, respectively. The average decline per generation in productivity and survivorship in $N = 1$ lines was 0.12% and 0.03%, respectively (44% and 12% decline in productivity and survivorship, respectively, across an average 375 MA generations for $N = 1$ lines).

We additionally conducted ANCOVA to test the null hypothesis that all four treatments (ancestral control, $N = 100$, $N = 10$, $N = 1$) have similar slopes for their (1) productivity and (2) survivorship response with respect to the number of MA generations. The regression lines of the four treatments did not differ significantly in their intercepts with respect to the two fitness traits. We found a significant interaction between population size and the number of MA generations. In concordance with the ANOVA results, only the $N = 1$ experimental lines exhibited significantly different regression slopes from the ancestral controls and other two MA treatments at larger population sizes (productivity of $N = 1$ lines vs. controls, $P = 0.003$; survivorship of $N = 1$ lines vs. controls, $P = 0.037$). Therefore, the benign fitness assays

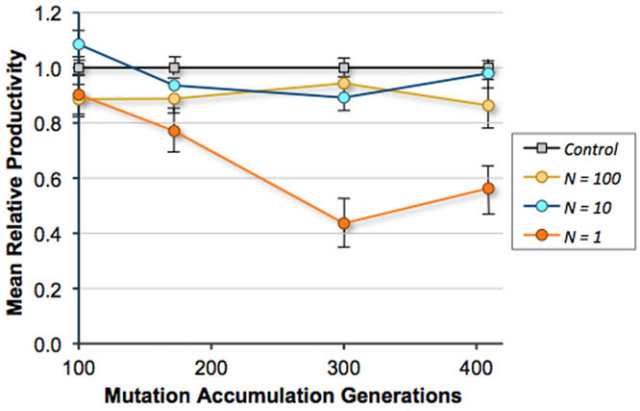

Figure 6. Time series of mean relative productivity across 409 spontaneous mutation accumulation generations at varying population size treatments ($N = 1$, 10, and 100 individuals). The mean relative productivity of the ancestral control has been scaled to a value of 1. The $N = 1$ lines showed a significant decline in mean productivity relative to the control at each time interval.

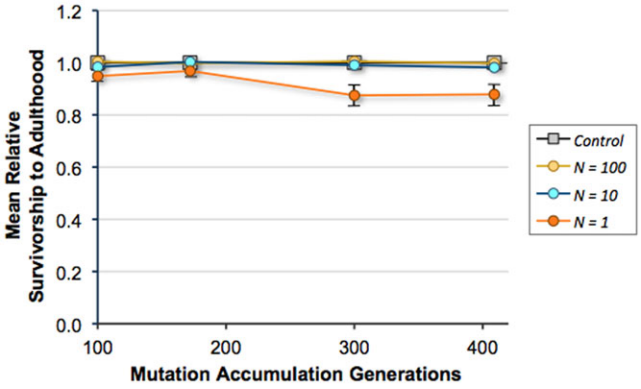

Figure 7. Time series of mean relative survivorship to adulthood across 409 spontaneous mutation accumulation generations at varying population size treatments ($N = 1$, 10, and 100 individuals). The mean relative survivorship of the ancestral control has been scaled to a value of 1. The $N = 1$ lines showed a significant decline in mean survivorship to adulthood relative to the ancestral control at each time interval.

could not distinguish the fitness of $N = 10$ and $N = 100$ lines from the ancestral control. That is, larger population size treatments exhibited fitness levels comparable to ancestral controls, thereby displaying no discernible impact of MA.

EPISTASIS BETWEEN NEW MUTATIONS CONTRIBUTING TO FITNESS DECLINE?

The rate and trajectory of fitness decay in bottlenecked populations can provide some insight into the genetic architecture of new spontaneous mutations, specifically the nature of interactions between them under the assumption that the mutation rate is constant. The plot of log mean productivity for $N = 1$ lines as a function of number of MA generations appears to exhibit a concave upward curvature, which would be suggestive of diminishing returns epistasis (Fig. 8A) for this fitness-related trait under the assumption that the mutation rate remained constant through the experiment. A simple linear regression model is highly significant (P-value $= 2.45 \times 10^{-7}$). However, a quadratic model does not significantly improve the fit to the data (increase in R^2 from a linear to a quadratic model, $P = 0.32$). A similar plot for survivorship to adulthood exhibits a nearly linear decline in the trait, implicating first-order, multiplicative action in the decline of the associated phenotype (Fig. 8B). As in the case of productivity, a simple linear regression model is highly significant ($P = 0.00056$) but a quadratic model does not significantly improve the fit to the data ($P = 0.785$). Hence, we conclude that decline in both fitness-related traits is best explained by multiplicative action between accumulated mutations.

Discussion

MA studies have served as an exemplary means to investigate the rate, molecular properties, and phenotypic consequences of both spontaneous and induced mutations (Halligan and Keightley 2009, and references therein) and served to highlight the genetic and phenotypic consequences of maintaining populations at small sizes. In addition to deterministic threats, small populations are affected by various stochastic factors pertaining to their demographics and environment. Yet another one of these threats is genetic stochasticity. It is readily apparent that small populations are subject to inbreeding depression and loss of genetic variation if maintained under prolonged bottlenecks. Moreover, the fixation of new, mildly deleterious mutations may further increase the risk of extinction for small, threatened populations (Lynch and Gabriel 1990; Lande 1994). This is because the loss or fixation of mutation and their consequences for population fitness are dictated not only by the selection coefficients (s) of individual mutations but also the effective population size, N_e.

The main goal of this study was to provide the first comprehensive time-series analysis of fitness decline in eukaryotic populations of varying N_e under a regime of spontaneous MA exceeding 400 generations. We aimed to determine (1) how MA directly impacts and compromises differently sized populations due to varying intensities of natural selection and genetic drift, and (2) to gain insight into the DFEs. $N_e = 1$ lines exhibited significantly higher among-line variance and a significant decline in both mean productivity and survivorship to adulthood relative to ancestral controls. Average productivity and survivorship in the $N_e = 1$ lines declined at a rate of 0.12% and 0.03% per generation, respectively, with productivity declining at a fourfold higher rate than survivorship, demonstrating the erosion of fitness in populations subjected to the vagaries of genetic drift and largely devoid

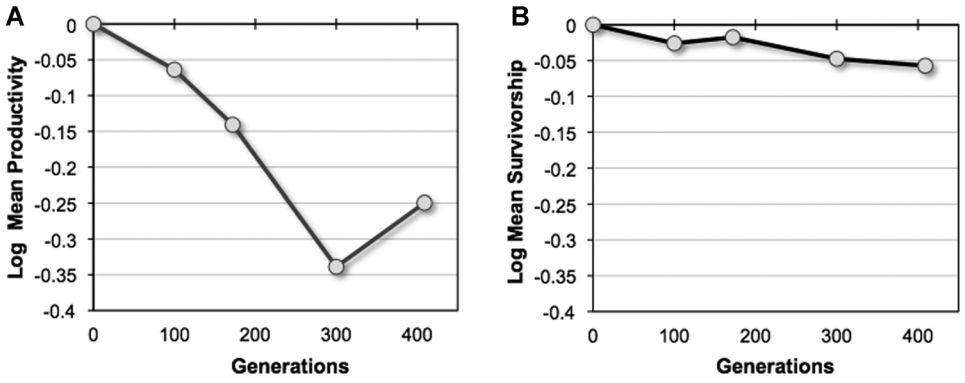

Figure 8. **Changes in log mean fitness of 17 $N = 1$ lines over 409 generations of spontaneous mutation accumulation for two fitness-related traits, (A) productivity and (B) survivorship to adulthood. Three experimental lines that went extinct during the course of the experiment were excluded. Productivity decline exhibits a concave upward curvature suggestive of diminishing returns epistasis. The decline in survivorship to adulthood is manifested as a nearly linear function.**

of natural selection. Our estimated rates of decline in the means of two fitness-related traits in the $N_e = 1$ lines are concordant with those of other spontaneous MA experiments in *C. elegans* (Keightley and Caballero 1997; Vassilieva et al. 2000; Baer et al. 2005) and fall within Vassilieva et al.'s computed range of 0.04–0.21% per generation for this species (Vassilieva et al. 2000). $N_e = 1$ lines also exhibited a delayed shift in the temporal spectrum of their reproductive output relative to the ancestral control, which was also observed in a preceding spontaneous MA study in *C. elegans* (Keightley and Caballero 1997). A delay in reproduction may be inconsequential for fitness under benign laboratory conditions with unlimited food supply but may incur strongly detrimental fitness costs in the wild for a species whose ecology is likely dominated by patchy resource environments.

Our benign fitness assays could not distinguish between the mean fitness of $N = 10$ and $N = 100$ treatment lines (corresponding to $N_e = 5$ and 50 individuals, respectively) relative to the ancestral controls. Likewise, mutator lines of *C. elegans* subjected to MA only found fitness decline under benign condition in populations subjected to extreme bottlenecks of $N = 1$ and 2 (Estes et al. 2004). In our experiment, the fact that only populations maintained at extreme bottlenecks of $N_e = 1$ individuals showed a significant decline would suggest that a few mutations of very large effects are primarily responsible (Keightley and Caballero 1997; Halligan et al. 2003; Estes et al. 2004; Halligan and Keightley 2009). This inference is further supported by our estimates of the average mutational effects *E(a)*, which exceed 10% for both assayed traits.

The upwardly biased average mutational effect, *E(a)*, for productivity was −0.164 and falls within the range of −0.09 to −0.46 values reported from preceding spontaneous MA experiments in *C. elegans* and related nematodes (Keightley and Caballero 1997; Vassilieva et al. 2000; Baer et al. 2005). The ML analysis could not reject an equal-effects model as providing the best fit to the data for productivity, as observed previously in *C. elegans* (Keightley and Caballero 1997) and in the ciliate *Tetrahymena thermophila* (Long et al. 2013). In mismatch repair knockout mutant lines of *C. elegans*, the average effects of mutations affecting productivity was estimated to be −0.106 (Estes et al. 2004), which falls within the range generated by spontaneous MA lines and is most similar to our estimate. Clearly, diverse assay procedures of investigators contribute to these differences in *E(a)* for productivity. We measured productivity over eight days given our results that mutationally degraded MA lines exhibited a lag in reproduction, whereas preceding MA studies measured productivity over the first four days following onset of reproductive maturity. It is possible that total productivity measured by preceding studies is significantly lower than our study's (no data for productivity for days 5–8), which would lead to their higher estimates for the average effect of mutations influencing productivity.

Our estimate of *E(a)* for survivorship to adulthood was −0.118, which is ∼two- to threefold lower than that of two studies that measured survivorship in *C. elegans*. Vassilieva et al. estimated a >threefold higher average mutational effect of −0.39 for survivorship to adulthood (Vassilieva et al. 2000). This discrepancy is in part due to Vassilieva et al. generating a Bateman–Mukai (BM) estimate of *E(a)* while assuming unidirectional mutations with equal effects. If mutational effects vary, the BM method will underestimate *U* but overestimate the average mutational effect, *E(a)*. Indeed, our ML analysis excluded an equal-effects model, finding contrary evidence that mutations of variable effects best explain the observed decline in survivorship. Estes et al.'s MA experiment with mutator *C. elegans* lines estimated *E(a)* for survival to be −0.232 (Estes et al. 2004), which is still twofold higher than our estimate. This may reflect an actual difference in the mutational properties of the two strains due to

different genetic background or may be due to different procedures used by us to measure survivorship. Both of these preceding studies measured both survivorship and productivity on a single hermaphrodite worm, by assigning a survivorship value of 1 or 0 if the worm did or did not produce offspring, respectively. We isolated 10 additional worms as L1 larvae and determined how many developed into reproductively mature adults, thereby enabling a finer scale of measurement for survivorship. Regardless of the differences in the *E(a)* for survivorship among these three studies, the general emerging conclusion is that the decline in survivorship is predominantly due to mutations of large effect (>10%).

This class of mutations with high negative selection coefficients would be detected and eradicated by natural selection even in small to moderately sized populations; therefore, they would not comprise a substantial threat to population fitness in the long term except under extreme and prolonged bottlenecking conditions. Alternatively, the larger sized populations in our experiment may have accumulated a cryptic load of genetic mutations with moderate to minor selection coefficients whose fitness effects may be obscured under benign laboratory conditions but may manifest and erode fitness in harsher, more competitive natural conditions (Davies et al. 1999). It is also possible that the $N = 100$ ($N_e = 50$) populations have accumulated a cryptic mutation load due to recessive deleterious alleles that could threaten populations under future inbreeding conditions. From a biological conservation standpoint, it would therefore be premature to conclude that even moderate population sizes of tens to hundreds of individuals are sufficient to curb or impede an erosion of genetic integrity due to the accumulation of a mutational load. This may be especially germane when captive populations are reintroduced into the wild. *Competitive* or *stress* fitness assays under harsher environmental conditions in the laboratory will be required to reveal the cumulative effects of slightly deleterious mutations, if any, which would be otherwise obscured in *benign* assays but may be expressed as a fitness decline at moderate population sizes.

Our estimate for the haploid genomic mutation rate per gamete per generation, U, for productivity in *C. elegans* was 0.0064, which is ~twofold higher than Keightley and Caballero's rate of 0.0026 (Keightley and Caballero 1997) and Baer et al.'s average estimate of 0.00375 for two independent *C. elegans* lines (Baer et al. 2005), but eightfold lower than Vassilieva et al.'s rate of 0.0489 (Vassilieva et al. 2000). Point estimates for U for productivity in two strains of the congeneric species, *C. briggsae*, are >10-fold higher with an average of 0.0495 (Baer et al. 2005). Hence, estimates of U for productivity across nematode species *C. elegans*, *C. briggsae*, and *Oeschius myriophila* range from 0.0026 to 0.0495, representing a 19-fold difference. Our point estimate of U for survivorship to maturity in *C. elegans* was 0.0026, which is very similar to the preceding estimate of 0.003 (Vassilieva et al. 2000). In general, estimates of U in *C. elegans* and the ciliate *T. thermophila* (Long et al. 2013) tend to be an order of magnitude lower than those in plants (Shaw et al. 2000; Schoen 2005). A direct comparison with *Drosophila* MA studies would suggest *Drosophila* point estimates of U exceeding those of *C. elegans* by as much as two orders of magnitude (Mukai 1964; Fry et al. 1999; Gong et al. 2005, among others but see Garcia-Dorado 1997). However, it has been pointed out that the majority of *Drosophila* MA experiments used balancer chromosomes and competitive fitness assays, which likely serve to reveal more deleterious mutations (Halligan and Keightley 2009). Furthermore, the number of germ cell divisions per generation is higher in *Drosophila* relative to *C. elegans* (Drost and Lee 1995; Drake et al. 1998) and is another likely factor contributing to higher deleterious mutation rates in *D. melanogaster*.

What role might second-order interactions between mutations play in phenotypic change pertaining to fitness? We could not reject a simple linear regression model as providing the best fit to the trajectories of decline for productivity and survivorship to adulthood. We therefore conclude no evidence for strong epistasis (synergistic or diminishing returns) for both fitness-related traits in *C. elegans*, in concordance with two preceding studies (Vassilieva et al. 2000; Baer et al. 2005).

The spontaneous nucleotide base substitution rate in the *N2* strain of *C. elegans*, averaged across seven independent MA lines, was 1.3×10^{-9} mutations per nucleotide site per generation (Denver et al. 2012). Using this nucleotide substitution rate and our estimate of the deleterious mutation rate for productivity to estimate the genomic mutation rate, ~5% of all novel mutations contribute to a large reduction in productivity. However, most spontaneous mutations would result in a slight or no reduction of fitness-related traits. Hence, the DFEs of new mutations might be multimodal, or at least bimodal, with some new mutations having large effects ($s > 0.1$), most mutations having very small effects, and an insignificant proportion of mutations with moderate effects. For example, just such a multimodal distribution provided the best fit to SNP data for 11,000 human genes (Boyko et al. 2008).

In conclusion, MA lines maintained under extreme bottlenecking conditions of $N_e = 1$ individuals exhibited a significant decline in fitness due to erosion of genetic integrity via accumulation of deleterious mutations. $N = 10$ (corresponding to $N_e = 5$) lines are theoretically expected to accumulate mutations of moderate and small effects ($s < 0.1$) at the neutral rate. The *benign fitness* assays could not distinguish the fitness of $N = 10$ or $N = 100$ (corresponding to $N_e = 5$ and 50, respectively) from the ancestral controls. One explanation for these observations is that mutations of very strong deleterious effects contribute to fitness decline; as such, the maintenance of natural populations at moderate sizes may suffice to stave off extinction due to the accumulation of a deleterious mutation load. However,

it is possible that even moderately sized populations bear the potential to accumulate a cryptic load of genetic mutations of smaller fitness effects that cumulatively erode fitness but can only be revealed under harsher environmental conditions. Under this latter hypothesis, the observed drastic decline in fitness of the $N_e = 1$ lines under *benign* conditions may be an underestimate. From the perspective of biological conservation, our study reinforces the need for *competitive* or *stress* fitness assays to provide an accurate representation of the trajectory of fitness decline, if any, due to the accumulation of slightly deleterious mutations at moderate population sizes.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.v5012.

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