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Fitness decline under osmotic stress in *Caenorhabditis elegans* populations subjected to spontaneous mutation accumulation at varying population sizes

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The consequences of mutations for population fitness depends on their individual selection coefficients and the effective population size. An earlier study of *Caenorhabditis elegans* spontaneous mutation accumulation lines evolved for 409 generations at three population sizes found that $N_e = 1$ populations declined significantly in fitness whereas the fitness of larger populations ($N_e = 5, 50$) was indistinguishable from the ancestral control under benign conditions. To test if larger MA populations harbor a load of cryptic deleterious mutations that are obscured under benign laboratory conditions, we measured fitness under osmotic stress via exposure to hypersaline conditions. The fitness of $N_e = 1$ lines exhibited a further decline under osmotic stress compared to benign conditions. However, the fitness of larger populations remained indistinguishable from that of the ancestral control. The average effects of deleterious mutations in $N_e = 1$ lines were estimated to be 22% for productivity and 14% for survivorship, exceeding values previously detected under benign conditions. Our results suggest that fitness decline is due to large effect mutations that are rapidly removed via selection even in small populations, with implications for conservation practices. Genetic stochasticity may not be as potent and immediate a threat to the persistence of small populations as other demographic and environmental stochastic factors.

KEY WORDS: **Conservation, deleterious mutation, fitness, osmotic stress, productivity, survivorship to adulthood.**

Mutation, as the source of new genetic variants, plays a central role in the evolutionary process. This mutationally induced input of genetic variation initiates intra- and interpopulation divergence in concert with other evolutionary forces such as genetic drift, natural selection, and migration. The loss or fixation of mutations and their consequences for population fitness depend upon both (i) the selection coefficients (s) associated with individual mutations and (ii) the effective population size, N_e. It has been argued that the most detrimental class of mutations influencing long-term population fitness comprise those with small selection coefficients, also referred to as slightly deleterious or nearly neutral mutations (Ohta 1992). 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). This accumulation of a mutation load in small populations is viewed as one of the most consequential genetic threats to the long-term persistence of small, endangered populations and has been formalized as the "mutational meltdown" hypothesis (Lynch and Gabriel 1990; Lynch et al. 1995). An increase in the mutation load is thought to gradually decrease the population size, thereby exacerbating the strength of random genetic drift relative to natural selection and permitting the chance fixation of future deleterious mutations. This synergistic interaction between a gradually increasing mutation load and increased

magnitude of genetic drift at small population size is predicted to have a snowballing effect ultimately manifesting in population extinction.

Several authors have discussed the concept of a critical effective population size, $N_{e,crit}$, wherein genetic drift fixes mutations detrimental to fitness faster than the fixation of beneficial mutations by selection to ameliorate fitness (Franklin 1980; Lande and Barrowclough 1987; Lande 1995; Franklin and Frankham 1998; Lynch and Lande 1998; Whitlock et al. 2003). Accurate estimates of $N_{e,crit}$ are expected to have critically important implications for guiding conservation management practices and policy. A paucity of empirical data on the fitness trajectories of populations maintained at differing N_e has precluded a robust resolution of the debate as to what this elusive $N_{e,crit}$ value may be. If mutations influencing fitness have large deleterious effects on average, they ought to be effectively purged out by selection even at relatively small to moderate population sizes, which in turn would lower the value of $N_{e,crit}$. Conversely, if the vast majority of mutations influencing fitness are nearly neutral with extremely small selection coefficients, the value of $N_{e,crit}$ would have to be scaled up to preclude extinction via erosion of genetic integrity as a consequence of weak efficacy of selection against mutations with small fitness effects.

Mutation accumulation (MA) experiments that comprise one form of experimental evolution studies are conducted under minimal efficacy of natural selection (minimal effective population size N_e) so as to enable the accumulation of the vast majority of spontaneous mutations. Because spontaneous mutation rates are extremely low (Muller 1927), the accumulation of a sufficient number of identifiable mutations requires that experimental lines be consecutively passaged through hundreds of generations under controlled laboratory conditions. In a previously published study assaying the fitness of these experimental lines under *benign* laboratory conditions (Katju et al. 2015), we demonstrated a significant fitness decline in the $N_e = 1$ lines relative to the ancestor but unaltered fitness at larger population sizes ($N_e = 5$ and 50) despite a lengthy regime of consecutive bottlenecks exceeding 400 generations. Our study concluded that the decline in the two fitness traits was predominantly due to mutations of large effect (>10%), a conclusion also arrived at by Estes et al. (2004). If this is indeed the case, this class of mutations with high negative selection coefficients would be detected and eradicated by natural selection even in small to moderately sized populations and a lower $N_{e,crit}$ could suffice to guard against the genetic threat of mutational meltdown from a conservation standpoint. However, *benign* fitness assays may overestimate fitness, thus failing to reveal the more subtle effects of MA and underestimate the U and skew the distribution of fitness effects (DFE) of mutations (Halligan and Keightley 2009). It is possible that the larger sized populations in our experiment had indeed accumulated a cryptic load of genetic mutations with moderate to minor selection coefficients that may manifest and erode fitness in harsher, more competitive natural conditions (Davies et al. 1999). In this study, we developed an *osmotic stress* assay to pose a harsher environmental regime for testing the fitness of our MA lines in an effort to possibly reveal a cryptic load of mutations that may have remained obscured under less challenging *benign* conditions.

Methods

MUTATION ACCUMULATION AT VARYING POPULATION SIZES

We previously conducted a long-term spontaneous MA experiment in *C. elegans* comprising 35 populations maintained in parallel at varying population size treatments of $N = 1$, 10, and 100 hermaphrodites ($N_e = 1$, 5, 50 individuals, respectively) per generation over four and a half years and spanning 409 MA generations (Katju et al. 2015). The 35 experimental lines of the MA experiment were established from the descendants of a single wild-type Bristol (*N2*) hermaphrodite originally isolated as a virgin L4 larva (Fig. S1) with excess animals cryogenically preserved at –86°C as ancestral controls. The MA experiment was conducted under benign laboratory conditions. Additional details on methodology and theoretical underpinnings (Fig. S2) are provided in Katju et al. (2015). Stocks of the MA lines were cryogenically preserved every 50–100 MA generations during the course of the experiment.

OSMOTIC STRESS FITNESS ASSAY

An osmotic stress assay was developed to quantify the extent of fitness decline in *C. elegans* MA lines under a more challenging environmental regime, namely exposure to an increased salt concentration. The canonical benign fitness assay procedure for testing life-history traits in *C. elegans* uses standard NGM plates with a final salt concentration of 50 mM NaCl. Solomon et al. (2004) developed a hyperosmotic assay protocol comprising a 500 mM final NaCl concentration in nematode media to characterize loci involved in the ability of *C. elegans* to detect, adapt and survive in saline environments. Ancestral control and MA lines worms were exposed to six varying salt concentrations representing a tenfold range, namely 50 (standard media), 100, 200, 300, 400, and 500 mM. We selected 200 mM as the final saline concentration for the osmotic stress fitness assay, given it still posed a fitness challenge to the nematodes in terms of reduced productivity and survivorship to adulthood without resulting in complete mortality or a compromised ability to visualize worms.

All extant experimental MA lines were assayed at 20°C in parallel with ancestral controls for two life-history traits, namely (i) survivorship to adulthood (survivorship henceforth), and (ii) productivity following 100, 172, 300, and 409 MA generations.

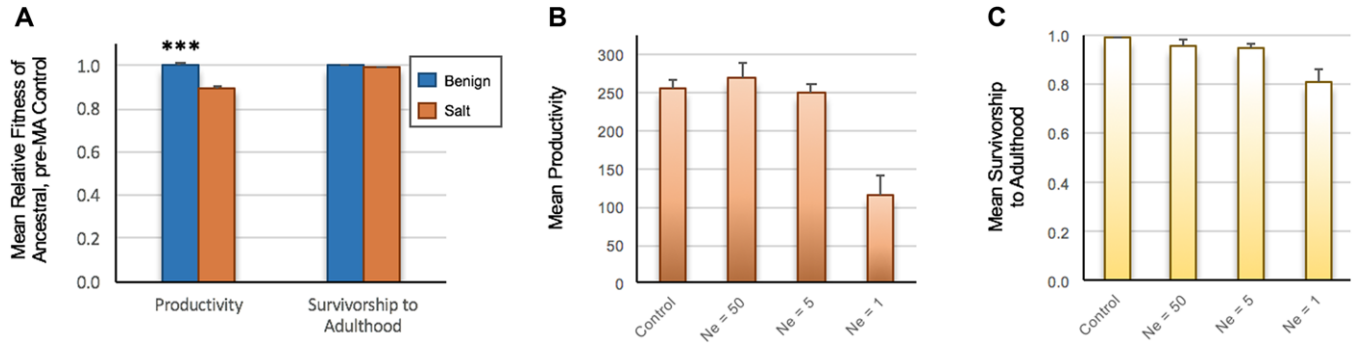

Figure 1. **Trait means of ancestral control and experimental lines under osmotic stress. Error bars represent one standard error. (A) Mean relative fitness of ancestral pre-mutation accumulation control lines under benign laboratory conditions (Katju et al. 2015) versus osmotic stress conditions comprising a fourfold increase in the salt concentration of the growth media. Fitness was quantified via two phenotypic assays for two fitness-related traits, namely (a) productivity and (b) survivorship to adulthood. For simplicity, the mean relative fitness value for each of the two traits in the ancestral control under benign laboratory conditions was scaled to a value of 1. Exposure to osmotic stress decreased the mean relative productivity and survivorship to adulthood of the ancestral control by approximately 10.8 and 0.5%, respectively. (B) Mean productivity for MA lines of varying population size under osmotic stress following 409 consecutive generations of mutation accumulation. (C) Mean survivorship to adulthood for MA lines of varying population size under osmotic stress following 409 consecutive generations of mutation accumulation.**

The fitness assay procedure was identical to the one used in Katju et al. (2015) with one exception, namely the use of NGM plates with a salt concentration fourfold higher (200 mM) than that employed under benign conditions (50 mM). As in our preceding study, all four fitness assays utilized thawed stocks of ancestral controls and experimental lines that had been cryogenically preserved. The statistical analyses of means and variances of life-history traits and maximum likelihood estimation of mutational parameters are detailed in Katju et al. (2015).

Results

Fitness data for two fitness traits (productivity and survivorship) under osmotic stress for control and MA lines at generations 100, 172, 300, and 409 are provided in Data files 1–4.

FITNESS OF ANCESTRAL PRE-MA CONTROL UNDER OSMOTIC STRESS VERSUS BENIGN LABORATORY CONDITIONS

The mean productivity of ancestral control lines under osmotic stress conditions was 272 offspring. Mean productivity of the ancestral control in our preceding study under benign conditions was 304 offspring (Katju et al. 2015). Ancestral control lines exhibited a highly significant 11% decline in productivity under osmotic stress relative to benign growth conditions ($t_s = -4.55$, $df = 115$, $P < 0.0001$) (Fig. 1A). Exposure to osmotic stress resulted in a nonsignificant 0.5% decline ($t_s = -1.25$, $df = 115$, $P = 0.21$) in the survivorship of ancestral control lines, with the mean survivorship to adulthood of benign and osmotic stress conditions being 98.86 (Katju et al. 2015) and 98.37%, respectively (Fig. 1A).

SIGNIFICANT DECLINE IN PRODUCTIVITY OF $N_e = 1$ LINES FOLLOWING 409 GENERATIONS OF SPONTANEOUS MA

Three $N_e = 1$ MA lines had gone extinct by MA generation 409 that was tested in the 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 under osmotic stress comprised measurements on five replicates each, where possible, of 20 control lines (C1–20), five $N_e = 50$ lines (100A–E), ten $N_e = 5$ lines (10A–J), and 17 $N_e = 1$ lines (1A–G, 1I–R). The mean productivity of the ancestral control and $N_e = 50, 5$, and 1 lines following 409 spontaneous MA generations was 256, 271, 250, 116 offspring, respectively (Fig. 1B). $N_e = 1$ lines showed a 55% decline in productivity relative to the ancestral control under osmotic stress, compared to a 44% decline under benign conditions (Katju et al. 2015). We additionally tested the relative mean productivity (ancestral control assigned a value of 1) of each of the 17 $N_e = 1$ lines in benign versus osmotic stress conditions using a matched-pairs comparison approach (Fig. 2A). Mean relative productivity of $N_e = 1$ lines was significantly lower under osmotic stress than in benign conditions (paired t-test, $t = 2.71$, $df = 16$, $P = 0.0155$). This suggests that the osmotic stress conditions were more sensitive than the benign fitness assay in revealing the detrimental fitness effects of spontaneous mutations on productivity. There was no significant difference in the mean relative productivity of $N_e = 5$ lines at benign versus osmotic stress conditions (paired t-test, $t = 0.044$, $df = 9$, $P = 0.966$). Interestingly, $N_e = 50$ lines exhibited significantly higher mean productivity under osmotic stress relative to benign conditions (paired t-test, $t = -3.24$, $df = 4$, $P = 0.032$).

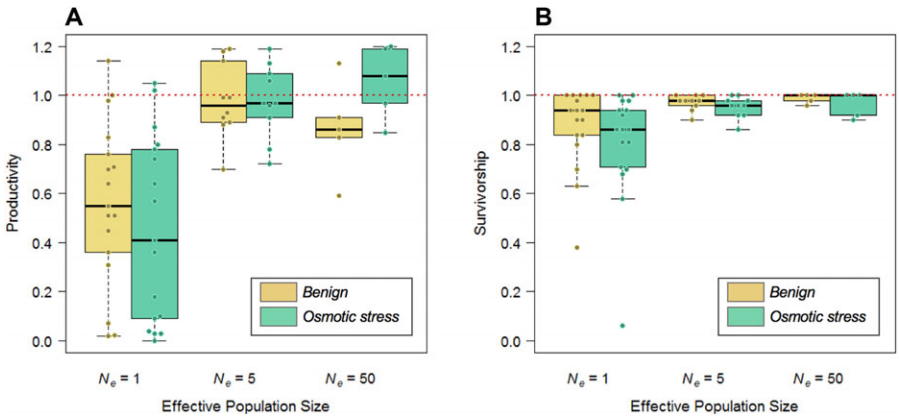

Figure 2. **Fitness measures of mutation accumulation lines in benign (Katju et al. 2015) versus osmotic stress conditions for two traits, (A) productivity, and (B) survivorship to adulthood. Matched pair *t*-tests found a significant decline in both productivity and survivorship of $N_e = 1$ lines under osmotic stress relative to benign conditions. $N_e = 5$ lines exhibited no difference in productivity or survivorship under benign versus osmotic stress conditions. $N_e = 50$ lines exhibited significantly higher productivity under osmotic stress conditions, but no difference in survivorship between the two environmental conditions. For simplicity, the mean relative fitness value for each of the two traits in the ancestral control under benign and osmotic stress conditions was scaled to a value of 1.**

Following 409 MA generations, ANOVA analyses found a significant variance component for productivity under osmotic stress among the four treatments ($F_S' = 15.54$; $P = 3.4 \times 10^{-7}$) and a significant among-line divergence component within treatments ($F_S = 3.58$; $P < 0.0001$) (Table S1, Data file 4). A similar pattern was observed for assays conducted at MA generations 100, 172, and 300 (Data files 1, 2, and 3). Comparisons among pairs of means based on unequal sample sizes using the Tukey–Kramer HSD method found the mean productivity of $N_e = 1$ lines to be significantly lower than that of the other three treatments ($N_e = 50$, $N_e = 5$ and control) but no significant difference in the mean productivity of MA lines maintained at $N_e = 5$, and 50 relative to each other as well as to the ancestral control (Table S2).

An *F*-test for equality of variances was employed to compare the among-line variance in mean productivity across the ancestral control and the three population size treatments at MA generation 409. There was no significant difference in the among-line variance of the larger population size treatments relative to the ancestral control ($F_{Ne\,=\,50,\,\mathrm{Control}} = 0.68$, $P = 0.62$; $F_{Ne\,=\,5,\,\mathrm{Control}} = 0.65$, $P = 0.74$). However, the among-line variance in mean productivity of the $N_e = 1$ lines was significantly higher than that of the ancestral control ($F_{Ne\,=\,1,\,\mathrm{Control}} = 4.17$, $P = 0.0019$). These results support our preceding ANOVA analyses that only discerned a significant decline in productivity of $N_e = 1$ lines relative to the ancestral control.

SIGNIFICANT DECLINE IN SURVIVORSHIP OF $N_e = 1$ LINES FOLLOWING 409 GENERATIONS OF SPONTANEOUS MA

Mean survivorship to adulthood of the ancestral control and $N_e = 50$, 5, and 1 lines following 409 MA generations was 99, 96, 95, and 80%, respectively (Fig. 1C). $N_e = 1$ lines showed a 19% decline in survivorship relative to the ancestral control over the 409 spontaneous MA generations, which comprises an additional 7% greater decline in survivorship under osmotic stress relative to benign conditions (12% decline; Katju et al. 2015), a trend similar to that observed for productivity and demonstrating the greater sensitivity of the osmotic stress assay in revealing the phenotypic decline in survivorship due to deleterious mutation accumulation. Mean relative survivorship of $N_e = 1$ lines was significantly lower under osmotic stress than in benign conditions (paired *t*-test, $t = 2.09$, $df = 16$, $P = 0.026$), suggesting that osmotic stress conditions were more sensitive than the benign fitness assay in revealing the detrimental fitness effects of spontaneous mutations on survivorship (Fig. 2B). The difference in the mean relative survivorship of $N_e = 5$ lines at benign versus osmotic stress conditions was nonsignificant (paired *t*-test, $t = 0.08$, $df = 9$, $P = 0.31$), as was the case for $N_e = 50$ lines (paired *t*-test, $t = 0.86$, $df = 4$, $P = 0.44$).

Following 409 MA generations, ANOVA analyses revealed a significant variance component for survivorship to adulthood among the four treatments ($F_S' = 5.98$; $P = 0.0015$) and a significant among-line divergence component for survivorship within treatments ($F_S = 7.35$; $P = 0$) (Table S1, Data file 4). A similar pattern was observed for assays conducted at MA generations 100, 172, and 300 (Data files 1, 2, and 3). Comparisons among pairs of means based on unequal sample sizes using the Tukey–Kramer HSD method found the mean survivorship of $N_e = 1$ lines to be significantly lower than that of the other three treatments ($N_e = 50$, $N_e = 5$ and control) but no significant difference in the mean survivorship of MA lines maintained at $N_e = 5$, and 50 relative to each other as well as to the ancestral control (Table S3).

Table 1. Maximum-likelihood (ML) estimates for spontaneous mutation parameters affecting two life-history traits, productivity, and survivorship to adulthood, in *Caenorhabditis elegans* under conditions of osmotic stress.

	Generation 100	Generation 172	Generation 300	Generation 409
Productivity				
$\bar{Z}_{control}$	289.64 (5.02)	267.65 (9.43)	274.44 (8.20)	256.02 (10.61)
$\bar{Z}_{MA}$	192.66 (17.12)	200.20 (23.54)	137.96 (19.06)	116.26 (23.50)
U	0.0182 (0.0058,→∞)	0.0042 (0.00075, →∞)	0.0117 (0.0034, →∞)	0.0058 (0.0018, →∞)
$E(a)$	–0.163 (–0.350, →0)	–0.317 (–0.906, →0)	–0.151 (–0.301, →0)	–0.228 (–0.485, →0)
Survivorship to adulthood				
$\bar{Z}_{control}$	0.99 (0.005)	0.98 (0.01)	0.98 (0.004)	0.99 (0.004)
$\bar{Z}_{MA}$	0.90 (0.03)	0.91 (0.04)	0.85 (0.03)	0.80 (0.06)
U	0.0100 (0.0052, →∞)	0.0009 (0.00021, 0.00180)	0.0056 (0.0027, →∞)	0.0044 (0.0018, →∞)
$E(a)$	–0.0015(–0.12, →0)	–0.3790 (–0.540, –0.265)	–0.0930 (–0.173, →0)	–0.0997 (–0.230, →0)

All estimates are for the $N_e = 1$ population-size treatment within a 409-generation spontaneous MA experiment comprising three population size treatments ($N_e = 1$, 5, and 50 individuals). Three lethal-bearing lines of the original 20 MA lines within the $N_e = 1$ treatment were excluded from the analyses. Estimates of the initial mean phenotype ($\bar{Z}_{control}$), the mean phenotype of the $N_e = 1$ 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 osmotic stress fitness assay following 409 consecutive spontaneous MA generations. One standard error or 95% confidence intervals are shown in parentheses following each estimate.

Estimates are provided for four time-intervals, at MA generations 100, 172, 300, and 409.

After 409 MA generations, the among-line variance in mean survivorship was significantly higher in all population size treatments relative to the ancestral control ($F_{Ne\ =50,\ \mathrm{Control}} = 6.64$, $P = 0.0016$; $F_{Ne\ =5,\ \mathrm{Control}} = 5.01$, $P = 0.0015$; $F_{Ne\ =1,\ \mathrm{Control}} = 140.06$, $P < 0.0001$). While the Tukey–Kramer HSD approach for conducting pair-wise mean comparisons failed to detect a significant difference in the survivorship of $N_e = 5$, and 50 lines relative to the control, the observed greater among-line variance in survivorship suggests a discernible impact of accumulated mutations on survivorship even at larger population sizes.

ESTIMATES OF MUTATIONAL PARAMETERS UNDER OSMOTIC STRESS

Estimates of the mutational parameters for both productivity and survivorship to adulthood were generated from the fitness data of $N_e = 1$ lines that were propagated under a regime of spontaneous mutation accumulation 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 in four osmotic stress assays (100, 172, 300, and 409 MA generations (Table 1). Like other MA studies, we assumed mutational effects to be exclusively negative with respect to fitness.

For productivity, estimates of the haploid genomic mutation rate per gamete per generation, U, ranged from 0.0042–0.0182 (average value of 0.01) (Table 1). A gamma distribution assuming approximately equal effects ($\beta \rightarrow \infty$) gave the best fit to the data in each of the four assays. This is in concordance with Keightley and Caballero's (1997) study of *C. elegans* MA lines and our preceding study of this very set of MA lines tested under benign laboratory conditions (Katju et al. 2015). The upwardly biased average mutational effect, $E(a)$, for productivity across the four fitness assays ranged from –41.43 to –84.86 progeny, and corresponding to –15.1 to –31.7% of the control mean (average value of –21.5%) (Table 1). Our estimate of $E(a)$ for productivity using the same set of experimental lines under benign conditions was of a very similar magnitude, namely –16.4% of the control mean. Hence, our investigations into the fitness effects of spontaneous mutations in *C. elegans* under both benign and stressful environmental condition underscore the strong negative selection coefficients associated with spontaneous mutations influencing productivity in this species. The estimates of U and the parameters of DFE of mutations for productivity are consistent with the observed absence of a significant decline in the mean productivity at larger population sizes ($N_e = 5$ and 50). Given an average $E(a)$ of –21.5% for productivity, theory predicts the eradication of these mutations via purifying selection in populations of $N_e \geq 5$.

With respect to survivorship, estimates of the haploid genomic mutation rate per gamete per generation, U, ranged from 0.0009–0.01 (average value of 0.0052) (Table 1). ML analysis excluded a model assuming mutations of equal-effect. A gamma distribution assuming a β value of 0, 30, 1.5, and 0.74 provided the best fit to the data in MA generations 100, 172, 300, and 409, respectively, providing evidence that mutations of variable effects contributed to a decline in survivorship, as had our preceding study of these experimental lines under benign environmental conditions. For survivorship, $E(a)$ estimates ranged from

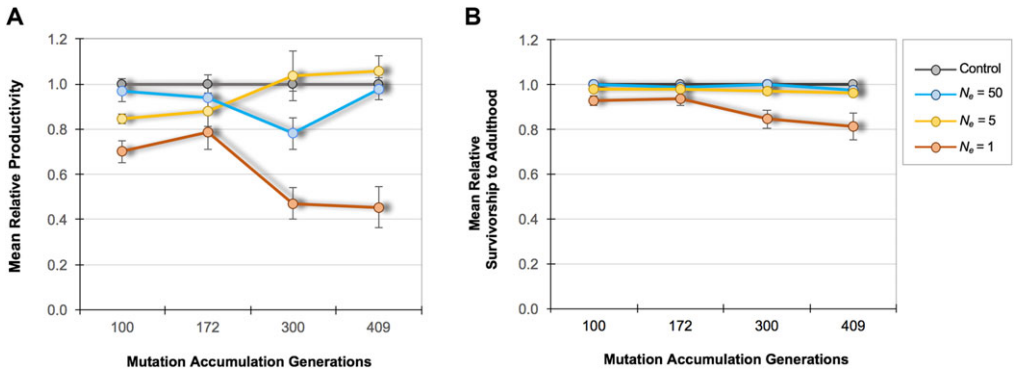

Figure 3. Time-series of mean relative fitness across 409 spontaneous mutation accumulation generations at varying population size treatments (N_e = 1, 5, and 50 individuals) under osmotic stress conditions. Phenotypic assays were conducted at four time-intervals, namely MA generations 100, 172, 300, and 409 for two fitness-related traits: (A) productivity, and (B) survivorship to adulthood. The mean relative fitness trait value of the ancestral control has been scaled to 1. The N_e = 1 treatment showed a significant decline in mean productivity and survivorship to adulthood relative to the ancestral control at each time-interval.

–0.0015 to –0.379, or –0.15–37.9% (average value of –14.33%) (Table 1). Our estimate of $E(a)$ for survivorship to adulthood using the same set of experimental lines under benign conditions was of a very similar magnitude, namely –11.8% of the control mean. As is observed for productivity, spontaneous mutations influencing survivorship in *C. elegans* are associated with strong negative selection coefficients. As was the case for productivity, the estimates of U and the parameters of DFE of mutations for survivorship to adulthood too are consistent with the small changes in the means of these traits observed under mutation accumulation at the two higher population sizes, wherein $N_e = 5$ and $N_e = 50$ experimental lines showed no significant decline in survivorship relative to the ancestral control. Given an average $E(a)$ of –14.3% for survivorship, theory predicts the eradication of these mutations via purifying selection in populations of $N_e \geq 5$.

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 under osmotic stress 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 (Tables S4–S6). The trajectories of decline in productivity and survivorship are displayed in Figs. 3A and B, respectively. The average decline per generation in productivity and survivorship in N_e = 1 lines relative to the ancestral control was approximately 0.15% and 0.05%, respectively (55% and 19% decline in productivity and survivorship, respectively, across an average 375 MA generations for N_e = 1 lines). Under benign assay conditions, the observed average decline per generation in productivity and survivorship in N_e = 1 lines was 0.12% and 0.03%, respectively (Katju et al. 2015). Hence, on average, we observe a greater decline in fitness of N_e = 1 lines as measured by these two traits under conditions of osmotic stress. It should be reiterated that the fitness of the ancestral control in these osmotic stress assays was normalized to 1; hence the observed greater decline in fitness of the experimental lines under osmotic stress relative to benign laboratory conditions signifies a greater sensitivity of the former in detecting the fitness decline associated with an accumulated mutation load.

Discussion

We conducted a long-term spontaneous MA experiment in *C. elegans* to determine the rate of fitness decline, if any, in populations of varying size and quantify the extent to which smaller populations are compromised due to genetic stochastic events occurring under conditions of strong genetic drift relative to their larger counterparts. We also aimed to directly test if small to moderate populations (N = 10–100 individuals) are vulnerable to a buildup of mutational load and subsequent fitness decline as predicted by the "mutational meltdown" hypothesis (Lynch and Gabriel 1990; Lande 1994; Lynch et al. 1995). In an earlier study conducted under benign conditions, we observed significant fitness decline in N_e = 1 populations but not in the larger populations (Katju et al. 2015). Productivity and survivorship of the N_e = 1 lines declined by 55% and 19%, respectively, under osmotic stress compared to 44% (0.12% per generation) and 12% under benign conditions. A greater decline in fitness of MA lines in putative harsher environments relative to benign conditions has previously been observed in *Drosophila* (Kondrashov and Houle 1994). Because our fitness data in the MA lines was standardized by that of the ancestral control, the decline in these fitness components in the N_e = 1 lines is over and above the lowered fitness of the ancestor in the hypersaline environment. In other words, the overall deleterious fitness

effect of mutations is exacerbated under osmotic stress. Several hypotheses might explain the observed greater fitness decline in the $N_e = 1$ lines. First, the osmotic stress may more sensitively reveal the effects of mutations with large selection coefficients that are expected to be eradicated by purifying selection in large population sizes. Alternatively, harsh environmental conditions may unmask the cumulative fitness effects of a load of cryptic mutations with slightly deleterious fitness coefficients that were undetected under benign assay conditions (Baer 2008; Andrew et al. 2015). However, this load of slightly deleterious mutations ($s < 0.01$) is also predicted to accumulate in a neutral fashion in each of our three experimental population size treatments (Fig. S2), and should therefore engender a fitness decline in the $N_e = 5$ and $N_e = 50$ lines as well. Our osmotic stress fitness could not distinguish between the mean fitness of $N_e = 5$ and $N_e = 50$ treatment lines relative to the ancestral controls. Therefore, our results are consistent with the hypothesis that (i) fitness decline is primarily due to a few mutations of large effect (Keightley and Caballero 1997; Halligan et al. 2003; Estes et al. 2004; Sanjuán et al. 2004; Halligan and Keightley 2009; Heilbron et al. 2014) that are further exacerbated under environmental stress, and (ii) these large-effect deleterious mutations are eradicated via purifying selection at even small to moderate population sizes of $N_e = 5$ to 50 individuals. Our estimates of the average mutational effects, *E(a)*, for both traits are consistent with this interpretation. *E(a)* for both productivity and survivorship under osmotic stress (–0.215 and –0.143, respectively) were more negative compared to benign conditions (–0.164 and –0.118, respectively; Katju et al. 2015). Conversely, our estimates of the genomic mutation rate tend to be lower under these hyperosmotic conditions relative to those estimated in a benign environment (Katju et al. 2015). These differences in mutation parameter estimates among different environments represents changes in the distribution of fitness effects of mutations as the number of accumulated mutations is constant within each genetic background (Halligan and Keightley 2009).

Small populations are vulnerable to a suite of stochastic threats, including demographic and environmental ones. In addition, genetic stochasticity is deemed a serious threat to the viability of small populations. First, under the Fundamental Theorem of Natural Selection (Fisher 1930), the rate of evolutionary change in a population is proportional to the level of standing genetic variation in a population. Diminished genetic diversity within a population reduces the potential for evolutionary change in response to environmental perturbances or challenges. Second, a sustained low effective population size leads to higher levels of inbreeding and to lowered genetic variance. Inbreeding in turn may reduce the fitness of a population due to inbreeding depression (Charlesworth and Charlesworth 1987). Furthermore, it has been posited that deleterious mutations of small to moderate effects can accumulate at low effective population size via genetic drift, leading to a gradual build-up of the mutation load which in turn undermines the long-term persistence of a population. This "mutational meltdown" hypothesis predicts an increased likelihood of extinction of small populations due to a synergistic interaction between an increase in both the mutation load and the magnitude of genetic drift (Lynch and Gabriel 1990; Lande 1994; Lynch et al. 1995). A minimum $N_e = 500$ was initially proposed by Soulé (1980) and Franklin (1980) for natural populations to enable maintenance of sufficient genetic variation for long-term adaptive evolution. However, mathematical simulations and genetic models investigating the rate of fitness decline in small populations due to input of deleterious mutations under an evolutionary regime dominated by genetic drift led to the conclusion that an $N_{e,crit}$ of 5000–10,000 is required to mitigate the risk of mutational meltdown (Lynch and Gabriel 1990; Lande 1995; Lynch et al. 1995; Lynch and Lande 1998). In particular, Lynch et al. (1995) predicted that populations with $N_e < 100$ are highly vulnerable to extinction via mutational meltdown on timescales as short as 100 generations.

One of the major goals of our MA experiment with varying N_e was to quantify the degree to which the persistence of small populations is compromised by the erosion of genetic integrity due to the accumulation of a spontaneous mutation load, with important implications for the conservation and management of small, threatened populations in the wild. Our conclusions add to other studies (Estes et al. 2004; Silander et al. 2007; Katju et al. 2015) that have failed to observe a fitness decline in small to moderately sized populations subjected to an extensive regime of bottlenecks. At face value, our results do not provide experimental support for the "mutational meltdown" hypothesis (Lynch and Gabriel 1990; Lande 1995, 1998; Lynch et al. 1995; Lynch and Lande 1998) that predicts genetic stochasticity to be a major factor compromising the persistence of populations maintained at sizes of $N_e < 100$ (Lynch et al. 1995). However, certain important caveats are in order. Thus far, the generalizability of these results from a viral and a nematode system to other species is still an open question. Second, it is possible that our simplistic *osmotic stress* assay fails to pose a sufficient biological challenge akin to a more complex environment of abiotic and biotic challenges faced by natural populations in the wild. Hence, it is still possible that the larger populations in our study harbored a cryptic mutational load that remained obscured under the conditions of *osmotic stress* imposed in this study. Thirdly, even if genetic stochasticity may not pose a substantial threat to small populations, demographic, and environmental stochasticity remain serious challenges that small populations have to contend with. Lastly, a population's ability to adapt in the face of rapid environmental change is directly proportional to the level of genetic variation it harbors (Fisher 1930). Small to moderate population sizes (N_e of 10–100) may not, on average, harbor sufficient genetic diversity to render a

population immune from extinction and with the ability to adapt to rapid environmental change. In conclusion, while we argue that genetic stochasticity may not be as potent a threat to the persistence of small population as other demographic and environmental stochastic factors, the persistence of natural populations is best assured by maintaining large population sizes to provide species the needed genetic armor to tackle rapid environmental change.

AUTHOR CONTRIBUTIONS

V.K. conceived and designed the experiments and coordinated the study; V.K. and L.B.P. created the MA lines and performed fitness assays; V.K. collected the data; P.D.K. contributed to M.L. analyses; V.K. and P.D.K. analyzed the data; V.K. wrote the paper; all authors edited and approved the final draft.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA ARCHIVING

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.d6441s7.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Schematic of *C. elegans* spontaneous MA experiment with three population size treatments.

Figure S2. Subdivision of the spectrum of mutational effects into a wide range of successively narrower classes across the three population size treatments of the MA experiment.

Table S1. Two-level nested Model I ANOVA for productivity and survivorship to adulthood of ancestral control and MA lines of *C. elegans* under *osmotic stress* assay conditions following 409 successive generations of bottlenecking.

Table S2. Multiple pair-wise mean comparisons in a producitvity assay under *osmotic stress* of ancestral controls and three sets of *C. elegans* experimental lines following 409 MA generations.

Table S3. Multiple pair-wise mean comparisons in a survivorship to adulthood assay under *osmotic stress* of ancestral controls and three sets of *C. elegans* experimental lines following 409 MA generations.

Table S4. Two-level nested Model I ANOVA for productivity and survivorship to adulthood of ancestral control and MA lines of *C. elegans* under *osmotic stress* assay conditions following 100 successive generations of bottlenecking.

Table S5. Two-level nested Model I ANOVA for productivity and survivorship to adulthood of ancestral control and MA lines of *C. elegans* under *osmotic stress* assay conditions following 172 successive generations of bottlenecking.

Table S6. Two-level nested Model I ANOVA for productivity and survivorship to adulthood of ancestral control and MA lines of *C. elegans* under *osmotic stress* assay conditions following 300 successive generations of bottlenecking.