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SHORT COMMUNICATION

Mutation accumulation in populations of varying size: large effect mutations cause most mutational decline in the rotifer *Brachionus calyciflorus* under UV-C radiation

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Abstract

Theory predicts that fitness decline via mutation accumulation will depend on population size, but there are only a few direct tests of this key idea. To gain a qualitative understanding of the fitness effect of new mutations, we performed a mutation accumulation experiment with the facultative sexual rotifer *Brachionus calyciflorus* at six different population sizes under UV-C radiation. Lifetime reproduction assays conducted after ten and sixteen UV-C radiations showed that while small populations lost fitness, fitness losses diminished rapidly with increasing population size. Populations kept as low as 10 individuals were able to maintain fitness close to the nonmutagenized populations throughout the experiment indicating that selection was able to remove the majority of large effect mutations in small populations. Although our results also seem to imply that small populations are effectively immune to mutational decay, we caution against this interpretation. Given sufficient time, populations of moderate to large size can experience declines in fitness from accumulating weakly deleterious mutations as demonstrated by fitness estimates from simulations and, tentatively, from a long-term experiment with populations of moderate size. There is mounting evidence to suggest that mutational distributions contain a heavier tail of large effects. Our results suggest that this is also true when the mutational spectrum is altered by UV radiation.

Introduction

The majority of new mutations are deleterious (Keightley & Lynch, 2003) and these mutations are major constituents of genetical theories for various topics including the evolution of sex and recombination (reviewed in Otto, 2009), the maintenance of genetic variation (Lynch *et al.*, 1998), inbreeding depression (Charlesworth & Charlesworth, 1987) and the evolution of ploidy (Otto & Goldstein, 1992). Mutation pressure has also generated concerns for the persistence of small populations (Lynch & Gabriel, 1990; Lynch *et al.*, 1995) and human health (Lynch, 2010). Although there is a constant influx of new deleterious mutations into populations, their ultimate fates depend on their effect sizes (s, i.e. the extent that fitness is reduced), and the effective population size (N_e), which influences the probability of fixation. It is well established in theory that mutations with effect size $|s| \ll 1/N_e$ will undergo nearly the same dynamics as neutral mutations, but those with larger effect are quickly removed from the population (Kimura, 1962). This key theoretical result leads to the prediction that deleterious mutation accumulation should cause more rapid fitness declines in smaller populations compared to larger ones. This prediction is important for conservation biology and underlies many inferences made in molecular

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population genomics. However, there are few direct empirical tests of this prediction (see below).

A classical tool to study mutations is to perform mutation accumulation (MA) experiments whereby organisms are maintained in multiple lines and bottlenecked to a few individuals (typically one or two), to reduce the efficacy of selection, over successive generations (Mukai, 1964). Over time, these lines accumulate mutations at approximately the rate they occur, except (near) lethal mutations, and the observed reduction in fitness and increase in fitness variance among lines can be used to estimate rate and effect of mutations (Halligan & Keightley, 2009). Estes *et al.* (2004) was the first to extend this method by maintaining MA lines under different population sizes. Increasing population size (N) should increase the efficacy of selection, thereby preventing stronger mutations from accumulating. MA lines with very low N should accumulate most types of mutations, but lines with larger N would mostly accumulate small effect deleterious mutations (i.e. with higher N, selection effectively prevents the fixation of larger effect mutations). Contrasting fitness reduction between MA lines with different population sizes can give us qualitative and quantitative insights into the distribution of selection strengths. Currently, this type of MA experiment has been performed in only three studies of two species (Estes *et al.*, 2004; Silander *et al.*, 2007; Katju *et al.*, 2015). In the nematode, *Caenorhabditis elegans* (Estes *et al.*, 2004; Katju *et al.*, 2015), and the bacteriophage, Φ174 (Silander *et al.*, 2007), an N of < 5 and 30, respectively, caused significant fitness loss relative to controls. At moderate population sizes (N larger than 10 and 30, respectively), the fitness of MA lines remained similar to controls. This suggests that mutations of large effect occur commonly but only accumulate in populations with very low N_e. Selection thus seems to be surprisingly efficient even at low population sizes, but the generality of these results remains uncertain. Here, we perform an MA experiment at six different populations sizes ($N = 1$, 2, 4, 10, 20, 100) with the facultatively sexual rotifer *Brachionus calyciflorus*. *Brachionus calyciflorus* possess fast generation times (1–1.5 days) making them well suited for MA experiments. *Brachionus calyciflorus* is maintained here as an asexual diploid so typical mutations are expected to be expressed in heterozygous state even when 'fixed'. This is unlike the previous studies on *C. elegans* and the bacteriophage where mutations are fully expressed due to high selfing and haploidy, respectively. Throughout the experiment, we artificially increase mutation rates using UV-C radiation. As a result, we cannot estimate the mutation rate or effect size of mutations that occur in natural populations of *B. calyciflorus*. Strong UV-C (and UV-B) radiation is likely to shift the mutational spectrum because it tends to cause C to T transitions and double-strand breaks (Dunker & Kaina, 2002; Pfeifer *et al.*, 2005; Rastogi *et al.*, 2010; Brash, 2015). Our main interest is to study patterns of fitness decline in lines maintained under different population sizes but experiencing the same mutation pressure.

Despite the differences in how mutations are induced, breeding system, habitat and numerous other life-history traits, our results were surprisingly similar to the results from *C. elegans* (Estes *et al.*, 2004; Katju *et al.*, 2015), populations kept as low as 10 individuals were able to maintain fitness close to the control throughout the experiment (16 radiations, 60–80 generations). Together, these studies show that large effect mutations underlie most of the fitness decline in small populations even when mutations are caused by very different sources. However, we caution against interpreting these results as evidence that small populations ($N \geq 10$) can maintain high fitness indefinitely.

Materials and methods

Experimental overview

We performed a mutation accumulation experiment with the rotifer *Brachionus calciflorus* at six population sizes ($N = 1$, 2, 4, 10, 20 and 100). We elevated the mutation rate using UV-C radiation which causes hydrolytic damage and produces radical oxygen species resulting in base changes and strand breaks (Rastogi *et al.*, 2010); this is also likely to change the mutational spectrum relative to that in nature. To reduce unintended selection for UV resistance, the time between radiations was randomly varied between four and 14 days. Lifetime reproductive success was measured after 0, 10 and 16 exposures to UV-C radiation.

Creation and maintenance of experimental lines

The experiment started with a single rotifer from a laboratory stock population that was hatched from resting eggs 4 months prior (80 generations). These resting eggs originated from a laboratory population originally collected from Lake Onondaga, New York, in spring 2009. Both the laboratory population that produced the resting eggs and stock population used to initiate the experiment were well adapted to laboratory conditions. The selected rotifer was clonally propagated and used to initiate 205 mutagenized MA lines that varied in population size from one to one hundred individuals (40 replicate lines for population sizes of 1, 2, 4, 10 and 20; five replicates for populations of 100). Forty additional lines with a population size of 1 and five lines with a population size of 100 were initiated to serve as a nonmutagenized reference. Populations were maintained in six-well plate filled with 10 mL artificial freshwater medium (see Luijckx *et al.*, 2017), containing 400 000 algae mL^{-1} (chemostat cultured algae, *Monoraphidium minutum*, SAG 278-3, Algae Collection University of Goettingen). To ensure

asexual reproduction, rotifers were transferred every other day to new plates containing fresh medium (this prevents the accumulation of the mixis inducing protein and thus prevents sexual reproduction). However, pilots revealed that larger population sizes (20 and 100) showed occasional sexual reproduction if kept in a single container. Consequently, these were kept in larger volumes separated into multiple wells but were fully mixed each transfer (two wells for population of size 20, six wells for populations of size 100). Lines were allowed to grow up to four times their population size prior to each bottleneck. Remaining rotifers were used to initiate backup populations to prevent stochastic extinction in small populations or extinction due to stress associated with radiation treatment (two backups for $N = 1$, 2 and 4 and one for $N = 10$; $N = 20$ and $N = 100$ had no backups). New backups were created after each radiation event and previous backups were maintained as a secondary backup until the next radiation. Lines that needed to be restarted from secondary backups more than four times, where all backups failed to survive or where no fitness measures were obtained due to mortality of al replicates during fitness assays, were considered extinct. All experimental populations were kept at 22 ± 1 °C and permanent light (Phillips cool-white), and their position in the incubator was randomized every other day.

Mutagen treatment

Radiation treatments occurred, on average, every 8 days (Table S1 for specific dates). Before each radiation, focal and backup populations were bottlenecked to their assigned population size by transferring the appropriate amount of juvenile rotifers to six-well plates filled with 2 mL of sterile medium to optimize penetration and minimize differences in mutagenicity through absorption of UV radiation in the media. Plates were exposed to 450 μw cm^{-3} UV-C radiation (Cole-Parmer) for 1 min. Directly after radiation, plates were filled up to 10 mL of medium containing 400 000 algae mL^{-1} and stored in the dark for at least 12 h to prevent potential photograph repair (Grad *et al.*, 2003). Nonmutagenized lines were handled in the same way except that rotifers were added after radiation took place.

Fitness assays

Fitness was measured at the start of the experiment and after 10 and 16 radiation events. We estimated fitness using the lifetime reproduction of a single rotifer for each line (mutagenized and nonmutagenized) with five replicates per line. Fitness assays after 10 radiations were split into two blocks: the first block consisted of 20 lines for each population size, excluding the $N = 100$ lines, while the second block contained the other half of the lines and the mutagenized $N = 100$ lines (nonmutagenized $N = 100$ lines were not measured at this time point). Fitness assays following radiation 16 were all performed in a single block for all lines. To ensure that we obtained a fitness measurement, up to four rotifers were sampled from each line at each time point, but only one was used for fitness assays. Sampled rotifers were placed singly in a well of a 24-well plate containing 2 mL of medium and transferred daily for at least three asexual generations to help remove any maternal effects. Five juvenile offspring were then each transferred to an individual well containing 1 mL of medium and transferred every 12 h until death; eight juveniles were used for each $N = 100$ line in the fitness assay after 16 radiations. During each transfer, the number of offspring was recorded and the average lifetime reproductive success of the five replicates was used as an estimate of fitness.

Data analysis

All statistical analyses were performed in R (R Core Team, 2013). Blocked measurements after 10 radiations were combined as no significance difference between blocks was detected ($F_{1,214} = 1.21$, $P = 0.27$). All analyses on lifetime reproductive success, juvenile mortality (death before reproduction) and fecundity (lifetime reproduction for the subset of individuals that survive to reproduce) were performed excluding extinct lines. The extinct lines are depicted in Fig. 1, and their inclusion strengthens the main patterns that we report excluding these lines. We tested whether our UV-C radiations were successful in elevating the mutation rate by comparing the number of extinctions and measurements of fitness between the mutagenized and nonmutagenized $N = 1$ lines at the end of the experiment. We compared the number of extinct lines using a Fisher exact test, lifetime reproduction and fecundity using a *t*-test, and juvenile mortality using a Wilcox signed-rank test, as assumptions of normality were violated (mutagenized: $W = 0.76$, $P < 0.001$, nonmutagenized: $W = 0.68$, $P < 0.001$). To understand the effect of population size on the performance of the mutagenized lines, we compared the number of extinctions and the loss in relative fitness per radiation event. To compare extinction probability among different population size treatments for the mutagenized lines, we examined the generalized linear model 'number of extinctions ~ population size', where population size was treated as a categorical variable. The loss in relative fitness per radiation event was determined separately for each mutagenized line by standardizing fitness measurements (for both time points) to the mean of the nonmutagenized $N = 1$ lines at that time point and fitting a linear regression with a fixed intercept at a relative fitness of one. The slopes

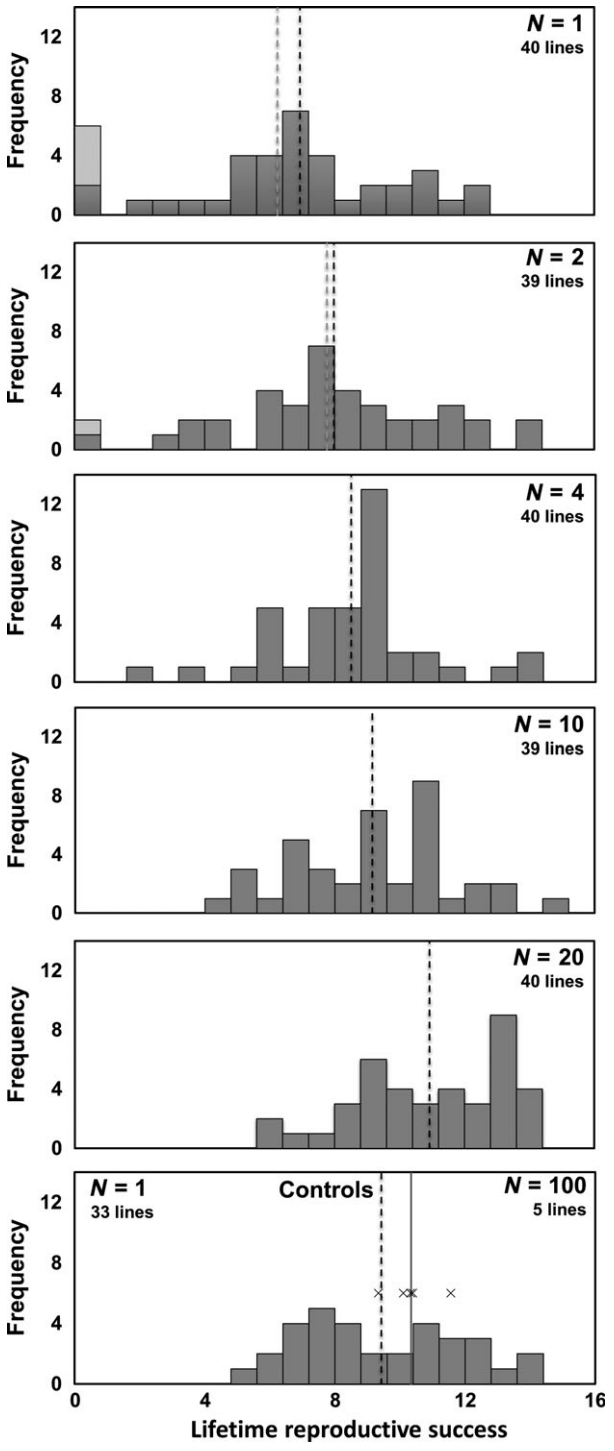

Fig. 1 Distribution of fitness among MA lines. Measures of lifetime reproduction of 232 MA lines (bars in dark grey), following 16 radiation events, showed increased variance with a decrease in population size. Lines kept at population sizes of 10 individuals or greater showed similar variance and mean to nonmutagenized lines kept at a population size of 1. Greater variance in the smaller populations was mostly caused by a few lines with very low fitness. In the two smallest population sizes ($N = 1$, 2), some lines had to be restored from secondary backups four or more times at which point we considered these lines to be extinct. The number of extinct lines is depicted by a light grey bar above the number of extant lines measured has having zero fitness in the fitness assay. Mean fitness of extant lines only is shown as a black dashed vertical line in each panel; mean fitness including both extant and extinct lines is depicted by a light grey dashed line. The bottom panel shows values for nonmutagenized lines; dark grey bars show the distribution of 33 $N = 1$ nonmutagenized lines (mean shown as dashed black line) and the five $N = 100$ are each shown by an 'x' (mean shown as solid grey line).

from these regressions were used as the line-specific estimates of fitness decline per radiation event. Only lines for which we had data for both time points were included in this analysis. Lines with missing data at either time point were excluded. The $N = 100$ lines were not used in this model because of low replication (only five replicates per line) and because fitness was only measured at the last time point for these lines (i.e. after 16 radiations); see Fig. S1 for results that included these lines with fitness standardized relative to the nonmutagenized $N = 100$ lines instead of the nonmutagenized $N = 1$ lines. To compare fitness decline among different population size treatments for the mutagenized lines, we examined the linear model 'fitness decline ~ population size', where population size was treated as a categorical variable. Given significant heterogeneity, this was followed by a *post hoc* Tukey's HSD. These analyses were performed for lifetime reproductive success, juvenile mortality and fecundity. However, for juvenile mortality, the intercept of the linear regression, used to determine the increase in juvenile mortality per radiation event, was fixed to the initial mean survival (0.97) and a nonparametric test was used to test for statistical significance (Kruskal–Wallis followed by multiple comparison) as assumptions of normality (Shapiro–Wilk, $W = 0.9207$, $P < 0.001$) and homogeneity of variance (Fligner-Killeen, $\chi^2_4 = 19.62$, < 0.001) were violated. Besides a reduction in mean fitness, mutation accumulation lines also often show increased variance among lines although this can be harder to estimate because of the high uncertainty associated with variance estimates (Halligan & Keightley, 2009). We attempted to obtain estimates of among-line variance in fitness using MCMCglmm (Hadfield, 2010) but did not detect significant differences in fitness variance among lines, potentially due to high fitness variance within lines (results not shown).

Result and discussion

For accurate estimates of changes in fitness, mutation accumulation experiments require a reference that does not accumulate mutations. However, rotifers cannot be

frozen or otherwise kept in stasis, so we approximated the ancestral state of the population using two different nonmutagenized treatments. First, a set of forty nonmutagenized lines was kept at a population size of 1 and may have slightly lower fitness than the ancestral state due to the accumulation of naturally occurring mutations. Second, a set of five nonmutagenized lines was kept at a population size of 100, which are unlikely to fix deleterious mutations over the time period of the experiment (60–80 generations) but may gain fitness if rare beneficial mutations occurred. Thus, the true ancestral state is likely to have fitness in between our nonmutagenized $N = 1$ and $N = 100$ treatments. Measures of lifetime reproduction, taken by serial passaging a focal rotifer and counting their total number of offspring until death, show that both nonmutagenized treatments have high fitness at the end of the experiment (Figs 1–3). However, as expected, the $N = 1$ lines had slightly but nonsignificantly lower fitness than the $N = 100$ lines ($t_{19} = -1.6448$, $P = 0.12$). The small and nonsignificant difference in fitness between the two nonmutagenized treatments is consistent with a low natural mutation rate, suggesting that the nonmutagenized treatments serve as a reasonably proxy for the ancestor (relative to the mutagenized populations).

UV-C exposure is expected to drastically elevate the mutation rate and thereby reduce fitness when selection is weak relative to drift (i.e. low N). The predicted effects of UV-C are most easily observed by comparing the mutagenized and nonmutagenized lines that were maintained at $N = 1$. None of the 33 nonmutagenized lines went 'extinct' (according to our criteria; see Methods, however seven lines were lost due to human error), whereas 6/40 of the mutagenized lines did (Fisher exact test $P = 0.029$). Of the $N = 1$ lines that did not go extinct, the mutagenized lines had significantly lower lifetime reproduction ($t_{65} = 3.35$, $P = 0.0014$), lower fecundity ($t_{65} = 2.90$, $P = 0.0051$) and a wider fitness distribution than the nonmutagenized lines (Figs 1 and 2). Juvenile mortality was higher in mutagenized lines but not significantly ($W = 625$, $P = 0.37$). These results are consistent with the expectation that UV-C exposure elevates the mutation rate and that most mutations affecting fitness are deleterious.

As population size increases, selection should become more efficient at preventing the accumulation of deleterious mutations. We observe significant variation in probability of extinction among mutagenized populations maintained at different population sizes ($\chi^2_4 = 17.42$, $P = 0.0016$) with more extinction at smaller populations sizes (Fig. 1), although it is impossible to infer the extent to which this variation is caused directly by demographic differences rather than mutation accumulation. More importantly, we observe significant variation in fitness components of mutagenized populations that did not go extinct (lifetime reproduction: $F_{4,175} = 14.95$, $P < 0.001$, fecundity: $F_{4,175} = 11.87$, $P < 0.001$, juvenile mortality: Kruskal–Wallis, $\chi^2_4 = 13.7807$, $P = 0.0080$). This followed the expected pattern with populations maintained at higher population size having better fitness

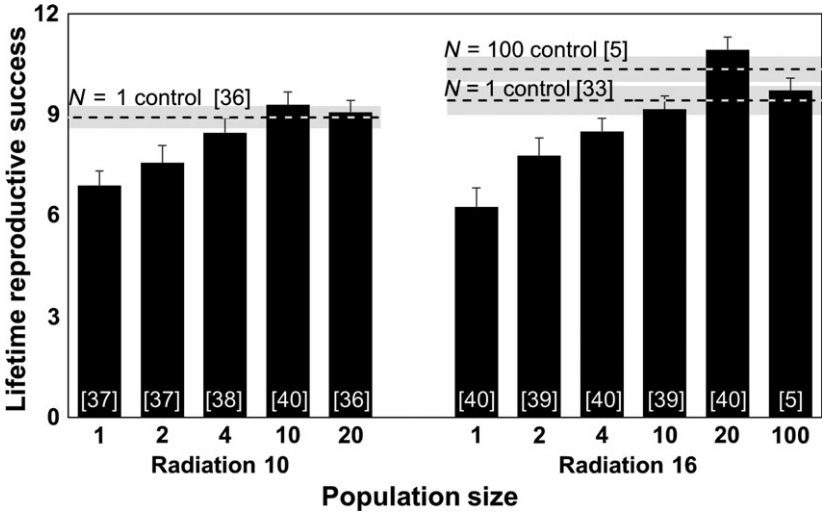

Fig. 2 Average lifetime reproduction, including extinct lines, after 10 and 16 radiation events for mutation accumulation lines kept at different population sizes (black bars; error bars represent one standard error). Number of lines (in brackets on black bars) is slightly different between both time points due failure of some fitness assays. Mutagenized lines kept at population sizes of four or lower had reduced lifetime reproduction compared to the nonmutagenized lines (average of each nonmutagenized treatments shown as a dashed line with the associated standard error shown as grey shading, due to time limitations the nonmutagenized $N = 100$ lines were not measured at after 10 radiations). Populations as small as 10 individuals maintained fitness similar to nonmutagenized lines kept at a population size of 1.

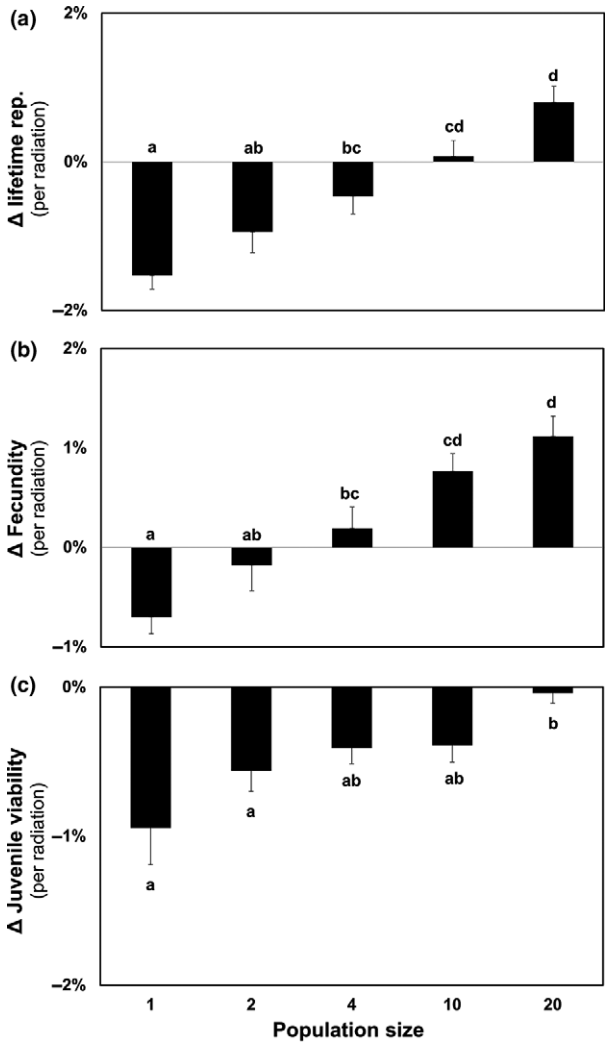

Fig. 3 Change in fitness traits per radiation cycle (standardized to the nonmutagenized $N = 1$ lines, see Fig. S1 for figure standardized to the nonmutagenized $N = 100$ lines). Lifetime reproduction (panel a), fecundity (panel b) and juvenile mortality per radiation cycle (Panel c). While small populations lost close to 2% ($N = 1$) of lifetime reproductive success per radiation event, larger populations showed little change or even had slightly increased fitness ($N = 20$; Panel a). Most of the decline was caused by an increase in juvenile mortality, which was high in populations of small size (Panel c), although a loss in fecundity also contributed to the overall decline (Panel b). Population sizes with different letter codes are significantly different at the $P < 0.05$ (based on Tukey HSD for lifetime reproduction and fecundity and multiple comparison following Kruskal–Wallis for juvenile mortality). Error bars represent one standard error.

(Fig. 3). We did not detect significant differences in among-line variance, likely due to the higher uncertainty associated with variance estimates. Qualitatively we observed that variance in fitness among lines is higher in small than that in larger populations (Fig. 1). The inability to detect statistically significant among-line variance, even with large sample sizes, has been reported in other mutation accumulation studies (Shabalina *et al.*, 1997).

Visual inspection of the data for mean life reproduction (Fig. 2) shows that mutagenized populations kept at $N = 1$ and 2 (and to a lesser extent, $N = 4$) experienced notable fitness declines relative to the nonmutagenized populations whereas the mutagenized populations at $N = 10$ or 20 did not. The fact that populations as small as 10 individuals maintained fitness similar to the nonmutagenized lines suggests that most of the fitness decline in small populations was caused by few mutations of large effect (i.e. selection in populations as small as $N = 10$ was effective in removing the majority of the large effect mutations).

The $N = 20$ populations have a slightly, but non-significantly ($t_{16} = 1.1251$, $P = 0.2775$), higher mean fitness than the $N = 100$ nonmutagenized lines (Fig. 2). This might suggest that a few of the $N = 20$ lines have accumulated rare beneficial mutations, but there is no strong evidence for this interpretation. Other studies have found more compelling evidence for the presence of beneficial mutations. For example, in *Saccharomyces cerevisiae* it was estimated that 6–24% of mutations are beneficial (Joseph & Hall, 2004; Hall & Joseph, 2010). While other studies have inferred the frequency of beneficial mutations to be between 0% and 18% (e.g. 4% in a RNA virus, up to 18% bacteriophage Φ ×174; Sanjuan *et al.*, 2004; Silander *et al.*, 2007).

Our experiment represents the first MA study in asexual diploids where population size was manipulated at more than two levels. Mutational effects in asexually reproducing rotifers are only partially expressed when there is partial dominance, unlike experiments that use haploid or highly inbred systems. Despite this and differences in the source of mutations (see below), our results are similar to those in the highly selfing *C. elegans*, where mutations are, for the most part, fully expressed. A population size of at least 10 individuals was sufficient for MA lines to maintain fitness at levels similar to nonmutagenized lines in *B. calyciflorus* (Fig. 2) and ancestral populations in *C. elegans* (Estes *et al.*, 2004; Katju *et al.*, 2015). In the bacteriophage, Φ174, a slightly larger population size of 30 was required to maintain high fitness (Silander *et al.*, 2007). Results from these three highly divergent taxa suggest that deleterious mutations of detectable effects (> ~1%) can be common in some environments but only accumulate in very small populations sizes.

Because of the relationship between s, N and fixation probability (Kimura, 1962), the rate of fitness decline for a given N will depend upon the distribution of mutational effects (Estes *et al.*, 2004). The pattern reported here and elsewhere (Estes *et al.*, 2004; Silander *et al.*, 2007; Katju *et al.*, 2015) in which the rate of fitness decline diminishes rapidly with small increases in

N likely results from a mutational distribution with a heavy tail of large effect mutations. Although we do not have the power to estimate this distribution directly, this interpretation is consistent with inferences made using other approaches (Avila & Garcio-Dorado, 2002; Eyre-Walker & Keightley, 2007; Halligan & Keightley, 2009; Kousathana & Keightley, 2013).

Our experiment differs from similar MA studies (Estes *et al.*, 2004; Silander *et al.*, 2007; Katju *et al.*, 2015) with regard to the source of mutation input. Increasing UV-C radiation not only elevates mutation rates but also shifts the mutation spectrum as UV-C biases towards C to T transitions and double-strand breaks (Dunker & Kaina, 2002; Pfeifer *et al.*, 2005; Rastogi *et al.*, 2010; Brash, 2015). Therefore, the distribution of fitness effects we observe among our MA lines may differ from that observed in nature. This is not an issue unique to our study. MA experiments that use lines deficient of repair pathways, such as the MMR-deficient lines in *C. elegans* (Estes *et al.*, 2004), can also change the mutational spectrum (Denver *et al.*, 2006; Lang *et al.*, 2013). Laboratory conditions may alter the mutational spectrum relative to that in nature. For example, differences in the levels of stress can alter the rate and types of mutations that occur (Bjedov *et al.*, 2003; Wang & Agrawal, 2012; Jiang *et al.*, 2014; Sharp & Agrawal, 2016). Lastly, it is likely that fitness effects measured under laboratory conditions are different than in nature (Agrawal & Whitlock, 2010; Rutter *et al.*, 2012; Yun & Agrawal, 2014; Latta *et al.*, 2015; Stearns & Fenster, 2016). Bearing these caveats in mind, it remains an interesting observation that mutational decline is drastically reduced as population size goes from $N = 1$ to 20 and that similar patterns have been seen in other systems, and this is consistent with inferences of heavy-tailed distributions made from different approaches.

Our results, and others (Estes *et al.*, 2004; Silander *et al.*, 2007; Katju *et al.*, 2015), found that small populations ($N \geq 10$) did not suffer from fitness decline. However, given sufficient time, populations of moderate to large size should experience declines in fitness from accumulating weakly deleterious mutations (Kimura, 1962). We show this by simulating a haploid population that receives two classes of deleterious mutations: common but weak effect and rare but strong effect (Appendix S1; simulation program can be downloaded at https://github.com/EddieKHHo/AsexualMutAccum). Our simulations qualitatively replicated the trends in our data when we assumed weak and strong mutations occur 87% and 13% of the time, respectively (many other parameters sets could also recreate this pattern; Appendix S1). After 16 radiation events, simulations show that lines with small population size ($N = 1, 2, 4$) suffer significant fitness decline due to accumulating a few large effect mutations while moderately sized populations ($N \geq 10$) maintained high fitness, similar to our data. However, after 100 radiations the simulations suggest that we would observe fitness loss in populations of moderate size ($N \geq 10$) from accumulating many weak effect mutations (Fig. S2).

In a separate supplementary experiment, we performed a longer MA experiment with *B. calyciflorus*. Over 9 months, we exposed 54 lines of asexually maintained *B. calyciflorus* ($N \sim 200$) to UV-C radiation at a higher dose than in our main experiment (Appendix S2). After 34 weekly radiations (~ 150 generations), MA lines showed a 32% and 26% decline in population growth and lifetime reproduction, respectively, compared to the ancestral line maintained at large population size ($N \sim 5000$; Fig. S3). However, we note that the fitness difference between the MA and ancestral line may not be entirely due to deleterious mutations because MA and ancestral lines were housed in different types of containers in this supplementary experiment (i.e. selection may have differed) and the possibility of beneficial mutations in the ancestral population (see Appendix S2 for further discussion). Given these caveats, these results offer tepid support for the claim that longer MA studies can reveal the accumulation of small effect mutations. Estes *et al.* (2004) also found support for mutational decline in larger populations by showing that MA lines of *C. elegans* with $N = 10$, 25 had lower fitness than expected given their estimates of mutation rate and selection strength. However, Katju *et al.* (2015) found no fitness decline in *C. elegans* with $N = 10$ and 100 under natural mutation rates (over 400 generations). Lastly, we note that fitness measures alone in short-term MA experiments are insufficient to determine the ultimate fate of small to moderately sized populations. Fitness decline on short time scales may simply reflect populations approaching drift-mutation-selection balance (Crow & Kimura, 1970). Of conservation concern is whether accumulation continues indefinitely (Lande, 1994; Lynch *et al.*, 1995) and the contribution of segregating and fixed mutations to the genetic load (Paland & Schmid, 2003; Willi *et al.*, 2013).

Currently, there are two powerful and complementary approaches to estimating the distribution of mutational effects. Experiments that measure the fitness effects of spontaneous mutations, such as MA experiments, and analyses of DNA sequences that contrast patterns of polymorphisms between neutral and selected sites (Eyre-Walker & Keightley, 2007; Halligan & Keightley, 2009). Sequence analyses have strong power in estimating the effects of weakly deleterious mutations but are unlikely to capture large effects mutations, which are inherently less likely to be segregating within populations (Keightely & Eyre-Walker, 2010). This limitation is complemented by MA experiments where the fitness effects of moderate and strongly, but nonlethal, deleterious mutations can be estimated. MA experiments that vary population sizes, and thereby the

efficacy of selection, provide additional information about the contribution of mutations with different effect sizes to fitness decline. Our study in *B. calyciflorus* provides evidence that mutations with strong effects are reasonably common, at least under UV-C radiation. There is mounting evidence that distribution of mutational effects may be multimodal (Halligan & Keightley, 2009; Kousathana & Keightley, 2013). Our results suggest that this may be true even when the mutational spectra differ from that in standard laboratory MA experiments. Although these results and those of others (Estes *et al.*, 2004; Silander *et al.*, 2007; Katju *et al.*, 2015) inform us of minimum population sizes for avoiding fitness decline in the short term, it is limited in assessing long-term consequences (see above). In addition, measures of fitness in benign laboratory conditions likely underestimate the rate and effect of deleterious mutations in nature (Agrawal & Whitlock, 2010; Rutter *et al.*, 2012; Wang & Agrawal, 2012; Jiang *et al.*, 2014; Latta *et al.*, 2015; Stearns & Fenster, 2016). Understanding the consequences of mutational pressure and assessing conservation concerns awaits estimates of mutational parameters for many more species under different (especially natural) environments.

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Author contributions

PL EKHH AFA designed the experiment, performed analysis and wrote the manuscript. PL, EKHH and AS conducted research.

Conflict of interests

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Table S1 UV radiation schedule for main MA experiment.

Figure S1 Change in fitness traits per radiation cycle (relative to the non-mutagenized $N = 100$ lines).

Figure S2 Simulation results.

Figure S3 Results from long-term MA experiment.

Appendix S1 Description of mutation accumulation simulation.

Appendix S2 Description of the long-term MA experiment.

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