No Evidence of Elevated Germline Mutation Accumulation Under Oxidative Stress in *Caenorhabditis elegans*

Joanna Joyner-Matos,^{*,1} Laura C. Bean,^{*} Heidi L. Richardson,^{*} Tammy Sammeli,^{*} and Charles F. Baer[†]

^{*}Department of Biology, Eastern Washington University, Cheney, Washington 99004, and [†]Department of Biology and University of Florida Genetics Institute, University of Florida, Gainesville, Florida 32611

ABSTRACT Variation in rates of molecular evolution has been attributed to numerous, interrelated causes, including metabolic rate, body size, and generation time. Speculation concerning the influence of metabolic rate on rates of evolution often invokes the putative mutagenic effects of oxidative stress. To isolate the effects of oxidative stress on the germline from the effects of metabolic rate, generation time, and other factors, we allowed mutations to accumulate under relaxed selection for 125 generations in two strains of the nematode *Caenorhabditis elegans*, the canonical wild-type strain (N2) and a mutant strain with elevated steady-state oxidative stress (*mev-1*). Contrary to our expectation, the mutational decline in fitness did not differ between N2 and *mev-1*. This result suggests that the mutagenic effects of oxidative stress in *C. elegans* are minor relative to the effects of other types of mutations, such as errors during DNA replication. However, *mev-1* MA lines did go extinct more frequently than wild-type lines; some possible explanations for the difference in extinction rate are discussed.

VARIATION in metabolic rate is often invoked as playing a causal role in variation in rates of molecular evolution (Martin and Palumbi 1993; Martin 1999; Gillooly *et al.* 2005; Gillooly *et al.* 2007; but see Lanfear *et al.* 2007; and Galtier *et al.* 2009). Usually, it is supposed that organisms with high metabolic rates have higher rates of mutation, and thus faster rates of evolution, than do organisms with low metabolic rates. There are two (not mutually exclusive) ways by which metabolic rate might affect mutation rate. First, organisms with high metabolic rates have shorter generation times (Savage *et al.* 2004; Bromham 2009) and thus undergo relatively more rounds of DNA replication per generation (Laird *et al.* 1969; Ellegren 2007; Nikolaev *et al.* 2007; Thomas *et al.* 2010) than do organisms with low metabolic rates. If most mutations occur during DNA replication, a positive relationship between metabolic rate and mutation rate, and thus rates of evolution, is expected. Second, the oxygen-centered free radicals that are by-products

of aerobic metabolism (Boveris and Chance 1973; Fridovich 2004) can cause oxidative damage to DNA (Hsie *et al.* 1986; Gille and Van Berkel 1994; Halliwell and Gutteridge 1999; Guetens *et al.* 2002; Evans and Cooke 2004) that can be mutagenic (Cheng *et al.* 1992; Dollé *et al.* 2000; Busuttill *et al.* 2003, 2005, 2007). Thus, organisms with elevated metabolic rates are predicted to have elevated mutation rates (Martin and Palumbi 1993; Martin 1999; Stoltzfus 2008).

However, the relative importance of free radicals to the relationship between metabolic rate and mutation rate is unclear for several reasons. First, metabolic rates do not always correlate with free radical production since mitochondria can become more efficient when metabolism increases (Loschen *et al.* 1971; Speakman *et al.* 2004; Balaban *et al.* 2005; Barja 2007). Second, much of the experimental evidence linking free radicals to mutation has been collected in the soma, where mutational processes differ from those of the germline (Jeffreys and Neumann 1997; Drake *et al.* 1998; Fortune *et al.* 2000; Martorell *et al.* 2000; Hill *et al.* 2005; Crabbe and Hill 2010; Lynch 2010). Third, many studies that link mutations with free radicals do so within the context of aging biology (e.g., Dollé *et al.* 2000; Busuttill *et al.* 2003; 2005; Hill *et al.* 2005; Busuttill *et al.* 2007; Crabbe and Hill 2010), potentially confounding age-related increases in oxidative stress with age-related changes in DNA repair

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¹Corresponding author: Department of Biology, Eastern Washington University, 258 Science Bldg., Cheney, WA 99004. E-mail: jmatos@ewu.edu

pathways (e.g., Preston *et al.* 2006; Chen *et al.* 2007; Simon *et al.* 2009).

To separate the mutagenic effects of oxidative stress from other causes, it is necessary to allow organisms to experience differing levels of oxidative stress while maintaining the same background level of mutations resulting from all other causes. The standard approach to inducing oxidative stress is to culture organisms in elevated O₂ or in media with free radical-inducing chemicals (Ishii *et al.* 1990; Mockett *et al.* 2001; Yanase *et al.* 2002; Vermeulen *et al.* 2005; Crabbe and Hill 2010). However, these approaches typically are used for short-term challenges and are not appropriate for a long-term study of heritable mutation rates because they may induce artifactual oxidative stress during periods of handling and may alter generation time (e.g., Feng *et al.* 2001; Frazier *et al.* 2001) or metabolic rate (e.g., Allen *et al.* 1984).

Here we report results of an experiment in which mutations were allowed to accumulate for 125 generations in the relative absence of natural selection in two strains of the nematode *Caenorhabditis elegans* that differ in steady-state free radical metabolism but not in generation times or (apparently) in metabolic rate. The N2 strain is the canonical lab strain, for which the mutational properties are well characterized (e.g., Keightley and Caballero 1997; Vassilieva and Lynch 1999; Baer *et al.* 2005). The *mev-1(kn-1)* mutation (Ishii *et al.* 1990) severely decreases activity in complex II of the mitochondrial electron transport chain (Ishii *et al.* 1998). *mev-1* nematodes have increased mitochondrial free radical production (Senoo-Matsuda *et al.* 2001), elevated oxidative stress, and apoptosis (for review, Ishii *et al.* 2007), and reduced life span and fecundity (Ishii *et al.* 1990) in comparison to N2. The oxidative stress accompanying the *mev-1* mutation is considered to be hypermutagenic (Hartman *et al.* 2001; 2004; Ishii *et al.* 2005). However, age-matched *mev-1* and N2 nematodes appear to have comparable metabolic rates as indicated by ATP content (Senoo-Matsuda *et al.* 2001) and O₂ consumption rates (Yasuda *et al.* 2010). Additionally, DNA repair capabilities of UV-induced mutations in *mev-1* and N2 are indistinguishable when the worms are maintained at atmospheric O₂ levels (N. Ishii and T. Ohnishi, unpublished data).

We used the mutation accumulation (MA) protocol to characterize the cumulative effects of mutations on relative fitness. The rate of change of the trait mean (ΔM) in a MA experiment is the product of the genome-wide mutation rate (U) and the average effect of a mutation ($E[a]$) (i.e., $\Delta M = UE[a]$, Lynch and Walsh 1998, Chap. 12). This protocol does not separate differences in mutation rate from differences in mutational effects, but if strains differ in ΔM , one or both of the factors must necessarily differ. Therefore, comparing the cumulative effects of mutations in a strain carrying the *mev-1* mutation to that of the wild type in a common environment and genetic background (N2) isolates the effects of oxidative stress from the effects of confounding factors like metabolic rate, generation time, and the environment

(Bromham 2009). If free radical-generated mutations in the germline contribute significantly to the overall mutational load, as they do in the soma, then hypotheses that invoke associations between mutation and oxidative damage are supported. Conversely, failure to observe a relationship between oxidative stress and germline mutation load suggests that the effects of free radicals on the germline may be minor relative to other causes of mutation.

Materials and Methods

Generation of *mev-1* mutant

All worms were cultured on NGM agar plates seeded with 100 μ l of the OP50 strain of *Escherichia coli* and were incubated at 20°. The *mev-1(kn1)* allele was backcrossed into the canonical Baer lab N2 (*C. elegans*) genomic background (Baer *et al.* 2005) for 12 generations, followed by 6 generations of selfing by single-worm transfer to render the introgressed strain homozygous and to allow it to reach mutation-drift equilibrium (Lynch and Hill 1986), at which time the strain was cryopreserved using standard methods (Wood 1988). Genotyping at each generation was conducted using standard techniques of single-worm DNA extraction, PCR with primers matching those reported by Ishii *et al.* (1998), and restriction digest with the *MroI* enzyme (marketed as *BspEI* by New England BioLabs). The restriction site for this enzyme distinguishes the single nucleotide difference of the *mev-1(kn1)* mutant from the wild-type sequence. Expression of this mutant allele in a variety of wild-type backgrounds consistently results in elevated oxidative stress (Guo and Lemire 2003; Ishii *et al.* 2005; Tsuda *et al.* 2007; Ishii *et al.* 2011).

Mutation accumulation

We followed standard *C. elegans* MA procedures (Vassilieva and Lynch 1999; Baer *et al.* 2005). Briefly, starting strains of N2 and *mev-1(kn1)* (introgressed into N2) were thawed, carried through 6 generations of single-worm descent (Lynch 1985), expanded to large population size, and cryopreserved (our “ancestral control”). We initiated 72 replicate lines of *mev-1* and 48 lines of N2 by randomly selecting single L4-stage hermaphrodites from the large populations and then carried MA lines through 125 “bottlenecks” ($G_{max} = 125$) at 4-day intervals by transferring a single L4 hermaphrodite per line. We maintained two backup generations so we could repeat a bottleneck event if a worm did not reproduce (“going to backup,” GTB); lines were considered “extinct” when they failed four successive generations. *mev-1* lines were cryopreserved at generations 25, 50, 90, and 125, and N2 lines were cryopreserved at generations 50 and 125 (Baer *et al.*, unpublished data).

Fitness assay

We conducted a single fitness assay (at 20°) of ancestral control strains, all MA lines that survived to G_{max} 125,

and three *mev-1* MA lines that went extinct between generations 90 and 125 using established techniques (Baer *et al.* 2005). Ancestral controls from each strain were replicated into 10 “pseudolines”; fitness was assayed for five replicates of each MA line and control pseudoline. Focal worms at the L4 stage were put on randomly numbered plates, transferred daily to new plates until they stopped laying eggs (≤ 6 days), and monitored until they died. Plates were incubated overnight at 20° to allow eggs to hatch and then stored at 4° until stained with 0.075% toluidine blue and counted (supporting information, File S1).

Data analysis

Absolute fitness (W) is the total reproductive output of an individual (referred to as “total fitness” in previous reports). Relative fitness (w) reflects the reproductive output and the timing of reproduction and is calculated as $w = \sum_x e^{-r_0 x} l_x m_x$, where m_x is fecundity at day x , l_x is survivorship to day x , and r_0 is the expected intrinsic rate of increase of ancestral control strains, calculated by solving $\bar{w}_0 = \sum_x e^{-r_0 x} \bar{l}_x \bar{m}_x = 1$, using the average l_x and m_x values of all control lines (Charlesworth 1994; Peters *et al.* 2003). We made one estimate of r per strain. We define w as 0 for individuals that fail to reproduce and set $x = 4.75$ for the first day's count (Vassilieva *et al.* 2000) and added 1 to each x for each successive day of reproduction.

Per-generation change in mean fitness (ΔM): In this study, relative fitness of MA lines is scaled relative to the ancestral control mean of 1 (measures of the ancestral control are indicated with a subscripted 0 and MA strains with a subscripted MA). The difference between the MA mean and the control mean relative fitness is scaled as a fraction of the control mean, $(\bar{w}_{MA} - \bar{w}_0)/\bar{w}_0 = (\bar{w}_{MA} - 1)/1$, so that the per-generation rate of change of mean relative fitness is calculated as $\Delta M_w = (\bar{w}_{MA} - 1)/t$, where t is the number of generations of MA. Because the ancestral control mean relative fitness is defined as 1, a difference in ΔM between strains will be apparent as a difference in the slope of the regression of w against generations of MA in the different strains.

We compared ΔM_w between groups using restricted maximum likelihood (REML) with the MIXED procedure of SAS (v. 9.21). The independent variables Gmax and Strain are fixed effects while Line and Replicate(Line) are random effects. MA Treatment (ancestral control, MA) is a categorical dummy variable equivalent to Gmax and is included to permit grouping of the random effects. Since w of the ancestral control is (defined to be) the same in both strains, the information in the main effect of Strain is identical to the information in the Gmax * Strain interaction; for that reason, the main effect of Strain is not included in the model. We analyzed the model $w = Gmax + Gmax * Strain + Line (Strain * Treatment) + Replicate(Line * Strain * Treatment)$; the last term is the within-line (residual) variance. Among-line and within-line components of variance were estimated separately for each Strain * Treatment combination.

We considered the probability that an individual worm initially present in the fitness assay survived to reproduce. Survivorship (s) is a Bernoulli random variable given value 1 (it survived to reproduce) or 0 (it did not). Data were analyzed by generalized linear mixed model (GLIMMIX procedure, SAS), assuming a binomial distribution with a logit link function. Strain, MA treatment, and their interaction are fixed effects; line (or pseudoline) is a random effect. A significant strain * treatment interaction indicates that ΔM_s differs between strains.

Per-generation change in the among-line variance (V_M):

The per-generation change in genetic variance resulting from accumulated mutations, the mutational variance V_M , is calculated as the difference in the among-line component of variance between ancestral control and MA lines divided by $2 * Gmax$ (Lynch and Walsh 1998, Chap. 12). Because mean fitness declines with MA, the relative fitness (w) of each individual is divided by the relevant group mean (control or MA mean of each strain) such that $w_0^* = w_i/\bar{w}_0$ and $w_{MA}^* = w_i/\bar{w}_{MA}$. The variance of the rescaled data, $Var(w^*)$ is I , the “opportunity for selection” (Crow 1958; Houle 1992; Wade 2006), calculated as the variance of the raw values divided by the square of the mean. In the remainder of the article we refer to the quantity $(I_{MA} - I_0)/2t$ as V_M .

Variance components were calculated for each strain/treatment combination using REML (MIXED procedure, SAS). To test whether there is significant mutational variance (V_M) within a strain, we compared the likelihoods of the model $w^* = Treatment + Line(Treatment) + Rep (Line * Treatment)$ with the among-line component of variance estimated separately and then jointly for the control and the MA treatments. The two models differ by one parameter (differentiating ancestral controls from MA lines), so the difference between the $(2 \times)$ log-likelihood ratio scores is χ^2 distributed with 1 d.f.

Results

Change in mean fitness

Results are presented in Table 1. Averaged over the two strains, w is significantly lower in MA worms than in the ancestral controls (*i.e.*, a significant main effect of Gmax, $P = 0.0225$), although the decline does not reach significance ($P > 0.05$) in either strain individually. The per-generation change in mean relative fitness, ΔM_w , does not differ significantly between the two strains (*i.e.*, the interaction between Gmax and Strain is not significant, $P > 0.16$), and in fact the point estimate of ΔM_w is ~50% lower ($-0.123\%/generation$) in *mev-1* than in N2 ($-0.195\%/generation$).

Both MA and control fitness influence ΔM_w ; if fitness of either treatment is atypical, then ΔM_w will be misleading. Considerable experience with MA experiments in *Caenorhabditis* has taught us that if one treatment is atypical, it

Table 1 Means and variances in fitness

Strain	Treatment	<i>N</i>	<i>W</i>	<i>w</i>	$\Delta M_w (\times 10^3)$	V_L	V_E	$V_M (\times 10^4)$
<i>mev-1</i>	Control	10	112.6 (7.7)	1.006 (0.102)		0.0047 (0.053)	0.474 (0.108)	
	MA	60	88.9 (5.2)	0.819 (0.056)	−1.23 (0.88)	0.162 (0.053)	0.563 (0.053)	6.292
N2	Control	10	165.8 (15.6)	0.999 (0.131)		0.067 (0.084)	0.518 (0.12)	
	MA	47	121.4 (7.8)	0.741 (0.055)	−1.95 (0.92)	0.123 (0.056)	0.649 (0.068)	2.24

All values in parentheses are standard error of the mean (SEM). Abbreviations: *N* is number of lines (MA) or pseudolines (AC); *W* is absolute fitness or total reproductive output per worm; *w* is relative fitness; $\Delta M_w (\times 10^3)$ is per-generation change in mean relative fitness; V_L is among-line component of variance; V_E is within-line (environmental) variance; V_M is mutational variance. See *Materials and Methods* for details of the calculations.

is almost always the control (e.g., Vassilieva *et al.* 2000; Baer *et al.* 2005) and that atypical fitness is always too low. In principle, fitness is analogous to standardized test scores in that environmental effects can cause an individual to perform worse than it is capable of, but not better. There is abundant evidence that fecundity, survivorship, and timing of development in *Caenorhabditis* are sensitive to demographic and environmental effects (e.g., Andux and Ellis 2008).

The absolute fitness (*W*) of N2 worms is significantly greater than that of *mev-1* worms ($P = 0.0003$) and *W* in both strains declines with MA ($P = 0.0022$), but there are no significant interactions between MA treatment and strain. Absolute fitness of both the N2 and *mev-1* ancestral controls is well within the range of what is typical. Our estimate of *W* of N2 is slightly low (compare to Vassilieva *et al.* 2000; Baer *et al.* 2005), which would tend to reduce ΔM_w . In fact, ΔM_w for N2 is somewhat higher than is typical; it is usually around 0.1%/generation, although values of ΔM_w approaching 0.2% have occasionally been observed in N2 (Bégin and Schoen 2006). Since the absolute fitness of the *mev-1* ancestral controls is consistent with previous estimates (average 77 offspring, Ishii *et al.* 1990; average 120 offspring, Lin *et al.* 2006; average 117 offspring, Cai *et al.* 2008), there is no evidence that ΔM_w in *mev-1* is reduced due to artificially low fitness of the ancestral control.

We tested for differences in relative fitness between the strains in two additional ways. First, we asked: Is the fraction of MA lines with a point estimate of *w* greater than 1 different between the two strains? It is not (Figure 1). For *mev-1*, 18 lines went up ($w > 1$) and 42 went down (30% up); for N2, 11 lines went up and 36 went down (23% up); the pattern is not different from the random expectation (2×2 contingency table likelihood-ratio test, $P > 0.44$). Second, we asked: Given that *w* does not equal 1, does the average magnitude of change differ between the strains? For MA lines that went up ($w > 1$), $\bar{w}$ is 1.35 for *mev-1* and 1.24 for N2. For lines that went down ($w < 1$), $\bar{w}$ is 0.58 for *mev-1* and 0.59 for N2. Neither of these analyses suggests that the two strains differ in some meaningful way that is not reflected in ΔM_w .

L4-to-adult survivorship of ancestral controls is slightly but not significantly lower in *mev-1* ($s = 0.958$) than in N2 ($s = 1$; data not shown). The rate of change of survivorship does not differ between strains (N2, $\Delta M_s = 0.49 \times 10^{-3}$ /gen; *mev-1* $\Delta M_s = -0.59 \times 10^{-3}$ /gen, $P > 0.93$).

Per-generation increase in genetic variance

Variances are shown in Table 1. In neither strain does the among-line component of variance of the MA lines differ from zero after accounting for the small (but nonzero) among-(pseudo)line variance in the controls. The point estimate of the mutational variance is greater in *mev-1* than in N2, but the difference in V_M between strains is not significantly different from zero. This result is not entirely surprising; changes in variances are much more difficult to measure with precision than changes in means (Shabalina *et al.* 1997).

Other considerations: Line extinction, number of bottlenecks, and life span and bagging during fitness assay

Additional variables that are informative but not usually considered in MA experiments (except as nuisance variables) are the fraction of lines extinct, the frequency of failed bottlenecks, and mortality patterns. Fifteen of the 72 *mev-1* lines (21%) and one of the 48 N2 lines (2%) went extinct by Gmax 125 (data not shown), a difference both highly significant (2×2 contingency table LRT, $P < 0.002$) and

Figure 1 Frequency histogram of relative fitnesses (*w*) of the *mev-1* and N2 MA lines. Data are presented as the proportion of *mev-1* and N2 MA lines in each of 10 bins from 0.1 to 1.9 (*mev-1* $n = 60$ lines, N2 $n = 47$ lines); *w* of the ancestral control in each strain is defined to equal 1. The proportion of lines above or below $w = 1$ does not differ between strains. Calculations for *w* are in *Materials and Methods*.

substantially greater than the difference in absolute fitness between the two strains. A 2% extinction rate is typical for N2 MA experiments of 100 or more generations.

There was a subtle but highly significant difference in the frequency of failed bottlenecks, or GTB between the strains, measured as the ratio of the number of bottlenecks (Gbot) to Gmax. Gbot/Gmax was 87.5% in *mev-1* and 92.9% in N2 (one-tailed *t*-test assuming unequal variances, $t = -4.290$, $P < 0.0001$; data not shown). GTB has two roles in a MA experiment, as a mediator of effective population size, N_e , and as an indicator of fitness. In a population in which census size (N) fluctuates over time, N_e is established by the harmonic mean N , which is essentially established by the small(est) values of N (Crow and Kimura 1970, p. 109). Assuming that a successful bottleneck resulted in $N = 1$ and GTB resulted in $N = 200$ (typical N2 ancestral control fitness, e.g., Baer *et al.* 2005), N_e was 1.15 for *mev-1* and 1.09 for N2, a trivial difference with respect to the demarcation of effective neutrality. Both line extinction and GTB are associated with low fecundity and low survivorship. L4 to adult survivorship is only slightly lower (~5%) in the *mev-1* ancestral control than N2. We do not have data for egg-to-adult survivorship.

N2 worms lived significantly longer (ancestral control lines, 12.4 ± 0.7 days; MA lines, 12.8 ± 0.4 days) than *mev-1* worms (ancestral control lines, 10.3 ± 0.7 days; MA lines, 9.9 ± 0.2 days; N2 vs. *mev-1*, $P = 0.0001$), but there was no effect of MA treatment on life span, nor significant among-line variation in life span (data not shown). When each worm was confirmed dead, we noted whether the worm was “bagging,” a phenomenon in which eggs hatch and feed inside the adult worm (Chen and Caswell-Chen 2004). A significantly higher proportion of *mev-1* worms were bagging when they died (proportion from ancestral control lines, 0.25; from MA lines, 0.23) than were N2 worms (proportion from ancestral control lines, 0.14; from MA lines, 0.14; N2 vs. *mev-1*, 2×2 contingency table, $P < 0.0061$).

Discussion

The fitness data (ΔM_w) do not support the hypothesis that oxidative stress constitutes a significant source of deleterious germline mutations. This does not imply that free radicals never generate mutations in the germline, but rather that the cumulative mutational effects of oxidative stress, if any, are relatively minor compared to the cumulative effects of mutations from all other sources. Our results can be profitably compared with other MA experiments in which the *a priori* reasons to expect worm strains to accumulate mutations faster than wild type were supported. MA lines that carried transposable-element mutators (Bégin and Schoen 2006) and mismatch-repair-deficient MA lines (Estes *et al.* 2004; Denver *et al.* 2005; D. R. Denver, personal communication) all declined in fitness much faster than did N2. Given that mutational declines in fitness were indistinguishable

between *mev-1* and N2, the most parsimonious conclusion is that the mutation rate in *mev-1* does not differ from that of N2. If the mutation rate of *mev-1* is elevated over that of N2, it necessarily means that the average effects of mutations resulting from oxidative damage are minor relative to the cumulative effects of mutations arising from other causes such as those associated with DNA replication. Distinguishing between these conclusions would require whole-genome sequencing of MA lines to determine mutation rates and spectra.

Our extinction and backup (GTB) data complicate the picture. We can envision four general classes of explanation for why the *mev-1* extinction rate is higher than that of N2, all of which are unsatisfying. First, there may be a class of mutations of large effect that occurs more frequently in *mev-1* than in N2 but that is still relatively rare. For example, the post-mitotic tissues of aging mice (Dollé *et al.* 2000) and fruit flies (Garcia *et al.* 2010) have high incidences of large genome rearrangements and relatively few 1-bp deletions and GC-AT mutations, spectra that may be consistent with elevated oxidative stress (Gille and Van Berkel 1994; Melov *et al.* 1995; Busuttill *et al.* 2007). It is possible that large genome rearrangements could have contributed to the high extinction rates in the *mev-1* MA lines. This explanation is problematic, however, because it seems unlikely that a highly deleterious mutation could reach high frequency on a backup plate and lines were declared extinct only after they failed four successive bottlenecks. We can envision a scenario of the sequential fixation of multiple mutations of medium effect. However, if such mutations were common, presumably they would have contributed to a larger decline in fitness of surviving lines that carried only one or two such mutations. Second, it may be that epistasis is strongly negative (synergistic), and mutational effects are therefore larger on the low fitness background. If that were the case, we would predict a more rapid decay in fitness of *mev-1*, contrary to the observed result. Third, *mev-1* might be substantially more sensitive to random environmental effects than N2; increased environmental variance is extremely common in organisms carrying mutations of large effect (Scharloo 1991; Baer 2008). This was not the case; $V(E)$ in the *mev-1* ancestral control is essentially the same as in N2 (Table 1). Finally, a fitness of zero is an absorbing boundary, and all lines will eventually go extinct. Worm (C. F. Baer, personal observation) and fly (N. Sharp and A. Agrawal, personal communication) strains that start MA experiments with low fitness tend to be more likely to go extinct and often decline in fitness more rapidly than do high-fitness strains. Although *mev-1* started off substantially closer to zero than N2, the magnitude of the difference in extinction between the strains is much greater than that predicted by the initial difference in absolute fitness and this increase in extinctions was not accompanied by increased ΔM_w .

If we provisionally accept the conclusion that the cumulative mutational effects on fitness in *mev-1* do not differ from wild type, we must consider the possibility that

our introgressed *mev-1* lines did not have elevated steady-state oxidative stress. The complex suite of traits associated with the *mev-1(kn1)* mutation in worms is very well characterized (for review, see Ishii *et al.* 2007) and is replicated when the mutation is inserted into mammals and mammalian cell lines (e.g., Ishii *et al.* 2005, 2011), yeast (Guo and Lemire 2003), and *Drosophila* (Tsuda *et al.* 2007). Several lines of evidence suggest that our introgressed strains of *mev-1(kn1)* differ from our N2. First, we confirmed that the five *mev-1* MA lines with the highest absolute fitness were homozygous for the *mev-1(kn1)* allele by pooling the DNA of 20+ worms within each MA line and confirming the restriction fragment profiles. Second, the absolute fitness of *mev-1* worms (pooled across MA treatment) was significantly lower than that of N2 worms and the absolute fitness of the *mev-1* ancestral controls was consistent with previous reports (Ishii *et al.* 1990; Lin *et al.* 2006; Cai *et al.* 2008). Third, the average life span of the *mev-1* worms was significantly lower than that of N2. The proportional decrease in life span from *mev-1* to N2 ancestral control worms (~17%) was slightly lower than reported values, which vary from 20 to 49% (Ishii *et al.* 1990; Adachi *et al.* 1998; Hartman *et al.* 2001; Yanase *et al.* 2002; Cai *et al.* 2008), likely in part because of our very small sample size ($N = 5/\text{replicate}$). Additionally, *mev-1* worms tended to develop more slowly, as documented by others (e.g., Ishii *et al.* 1990; Yasuda *et al.* 1999), although the development rates of the *mev-1* worms were not different enough from the N2 that we had to lengthen the intervals between MA bottlenecks, as we have in previous studies (e.g., Baer *et al.* 2010). It is possible that we tended to pick offspring born earlier in the parent's life in *mev-1* than in N2; if so, this would perhaps bias toward elevated mutation rates in N2 if offspring born later in a parent's life have a higher mutation rate than offspring born earlier. Fourth, substantially more *mev-1* worms than N2 worms bagged during the fitness assay. Bagging is typically viewed as an indication of stress, particularly in food-limited environments (Chen and Caswell-Chen 2004; Angelo and Van Gilst 2009). The worms in our assay were never food limited and the proportion of worms that bagged was not related to worm life span nor the individual who handled the worm. To our knowledge, bagging has not been associated with oxidative stress, but bagging may indicate overall decreased physiological condition. Taken together, these results would suggest that the *mev-1* phenotype was present in our introgressed strains. Although we have not directly measured steady-state free radical production (e.g., Senoo-Matsuda *et al.* 2001) or oxidative damage (e.g., Hartman *et al.* 2004) in our introgressed *mev-1* lines, such findings are consistently accompanied by reduced life span and/or reduced fecundity (e.g., Ishii *et al.* 1990; Adachi *et al.* 1998; Yanase *et al.* 2002).

If we accept that the introgressed *mev-1* strain exhibited traits consistent with elevated oxidative stress, why did the decline in fitness not differ between *mev-1* and N2? The consequences of oxidative stress on DNA structure and repair pathways for DNA oxidative damage are well charac-

terized (Hsie *et al.* 1986; Demple and Harrison 1994; Gille and Van Berkel 1994; Halliwell and Gutteridge 1999; Guetens *et al.* 2002; Evans and Cooke 2004) as are the links between oxidative stress and mutation in the soma (e.g., Dollé *et al.* 2000; Busuttill *et al.* 2003, 2005; Hill *et al.* 2005; Busuttill *et al.* 2007; Crabbe and Hill 2010). The oxidative stress that accompanies the *mev-1(kn1)* mutation is considered to be mutagenic, as indicated by increased transformation rates in a transgenic mouse cell line with the equivalent mutation (Ishii *et al.* 2005) and elevated mutation rates in the *fem-3* reporter system in *C. elegans* (Hartman *et al.* 2001, 2004). The higher nuclear mutation rates reported in *mev-1* nematodes relative to wild type (Hartman *et al.* 2001, 2004) relied on scoring visible revertants of a temperature-dependent sterile mutant at the *fem-3* locus. There is no reason to suspect an interaction between *mev-1* and *fem-3* that would atypically inflate the mutation rate at *fem-3*. However, the *fem-3* reporter system requires that strains be kept at 15°, rather than at 20° (as in this study), and it is conceivable that the effects of the *mev-1* mutation differ between the two temperatures. In *C. elegans* (N2), the spontaneous mutation process does not appear to differ between 18° and 26°, but it does in *C. briggsae* (C. F. Baer, personal observation). Moreover, small differences in temperature (~1°) can result in large differences in absolute fitness in *C. elegans* (Anderson *et al.* 2011; C. F. Baer, unpublished results), which suggests that at least some alleles must have very sharp temperature thresholds.

We can conceive of several potential reasons why our fitness assay did not detect the expected mutagenic effect in *mev-1* worms. Since mutational effects on fitness are dependent on the environment in which fitness is measured (e.g., Rutter *et al.* 2010), differences between *mev-1* and N2 might have been more apparent if we had measured fitness when the worms were challenged by an exogenous oxidative stress or an alternate stress like high temperature. In a similar fashion, successfully discriminating between *mev-1* and N2 worms on the basis of oxidative damage markers requires assaying aging (>10-day-old) individuals, in which steady-state oxidative stress is elevated (Adachi *et al.* 1998; Yasuda *et al.* 1999). Additionally, it is possible that the elevated steady-state oxidative stress in the *mev-1* worms was of such a magnitude (presumably minor) that it had a hormetic effect, enhancing the DNA repair capability (Calabrese and Baldwin 2003; Le Bourg 2009; Ristow and Zarse 2010) and thereby reducing the mutation rate in *mev-1* relative to that of N2. Enhanced protection against and/or repair of oxidative damage is common in organisms adapted to chronic oxidative stress (e.g., Storey 1996; Hermes-Lima *et al.* 1998, 2001). Comparisons of DNA oxidative damage, DNA repair enzyme activities, and mutational spectra between *mev-1* and N2 lines may shed some light on this possibility. Finally, we know that mutational processes in general (Jeffreys and Neumann 1997; Drake *et al.* 1998; Fortune *et al.* 2000; Martorell *et al.* 2000; Lynch 2010) differ in the germline and the soma, particularly the apparent lack of an age-specific pattern of mutation in the testis (Hill *et al.*

2005; Uehara *et al.* 2009; Crabbe and Hill 2010). Part of the motivation for this study was to determine whether the proposed free radical → DNA damage → mutation cycle established for somatic tissues applies to the germline. Our results suggest that at least in *C. elegans*, conditions of elevated oxidative stress do not substantially contribute to mutational declines in fitness.

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GENETICS

Supporting Information

<http://www.genetics.org/content/suppl/2011/10/06/genetics.111.133660.DC1>

No Evidence of Elevated Germline Mutation Accumulation Under Oxidative Stress in *Caenorhabditis elegans*

Joanna Joyner-Matos, Laura C. Bean, Heidi L. Richardson, Tammy Sammeli, and Charles F. Baer

File S1

Supporting Data

File S1 is available for download at <http://www.genetics.org/content/suppl/2011/10/06/genetics.111.133660.DC1> as an Excel file. It provides the following information for each replicate of each mutation accumulation (MA) line and ancestral control pseudoline: random plate number, strain (N2 or *mev-1*), treatment (MA or ancestral), maximum MA generation (0, 90, 125), Line ID, replicate ID, brooding (1 = died while brooding, blank cell = not brooding), each reproductive day (R day) reproductive output, total productivity (sum of R day output, W), intrinsic rate of increase (r_c), and relative fitness (w). See Methods section for details about calculations.