Short-term heritable variation cancels hundreds of generations of mutational variance for metabolic traits in *Caenorhabditis elegans*

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11 Heritable Metabolic Variance

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13 Keywords: Epigenetic; Metabolic enzyme; Mutation accumulation; Mutational variance;

14 transgenerational inheritance

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ABSTRACT

Metabolic disorders commonly have a large heritable component, and have increased markedly over the past few generations. Genome-wide association studies of metabolic traits typically find a substantial unexplained fraction of the total heritability, suggesting an important role for the effects of spontaneous mutation. An alternative explanation, considered less likely, is that epigenetic effects contribute significantly to the heritable variation. Here we report a study designed to quantify the cumulative effects of spontaneous mutation on adenosine metabolism in the nematode *Caenorhabditis elegans*, including both the activity and concentration of two metabolic enzymes (ADA and ADK) and the standing pools of their associated metabolites. A previous study with the same set of *C. elegans* mutation accumulation (MA) lines found a large cumulative effect of mutation on adenosine concentration. The only prior study on the effects of mutation on metabolic enzyme activity, in *Drosophila melanogaster*, found that total enzyme activity presents a mutational target similar to that of morphological and life-history traits. However, those experiments were not designed to account for short-term heritable effects. We find that the means of some traits (6/17) change significantly over the course of ~250 generations under MA conditions, consistent with previous findings, but that the short-term heritable variance for all but one trait (total soluble protein concentration) is of the same order of magnitude as the mutational variance. This result has important implications for the design and interpretation of MA studies, and suggests that the potential effects of epigenetic variation in human metabolic disease warrant additional scrutiny.

INTRODUCTION

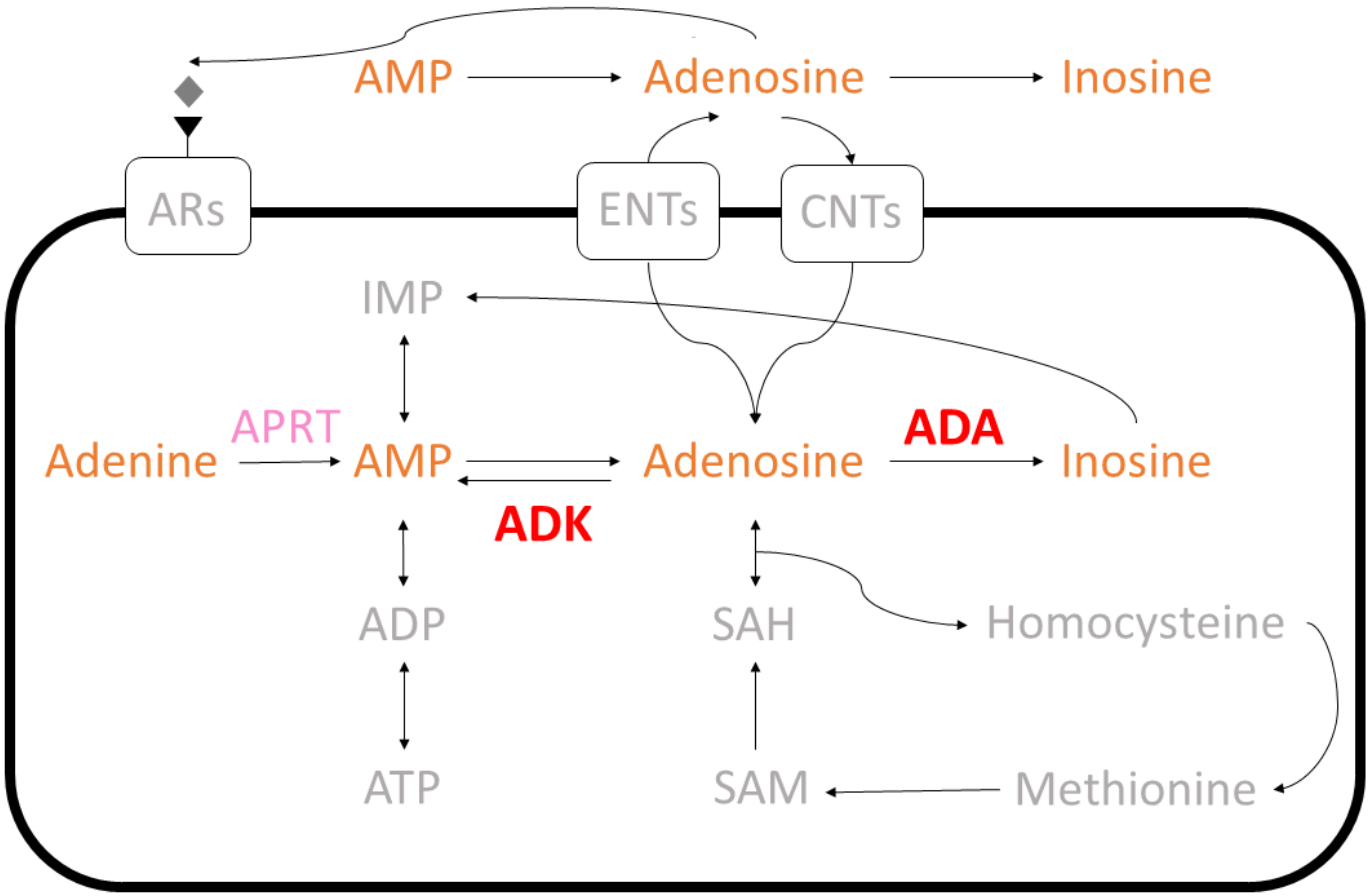
Human metabolic diseases have increased markedly in frequency over the past few generations (SAKLAYEN 2018). Large genome-wide association studies (GWAS) conducted on the human metabolome have shown that metabolic traits are highly heritable, but that a substantial fraction of the heritability of metabolic traits remains unexplained by the cumulative effects of mQTL (RHEE *et al.* 2013; SHIN *et al.* 2014; MAHAJAN *et al.* 2018). This discrepancy indicates that the remainder of the heritable variation is the result of some combination of (1) rare, highly deleterious variants recently arisen in the population; (2) many variants with effects too small to be detected by the typical GWAS (MANOLIO *et al.* 2009; EICHLER *et al.* 2010; BOYLE *et al.* 2017); and/or (3) cross-generational epigenetic effects that are heritable but leave no genetic signature (FURROW *et al.* 2011; RICHARD *et al.* 2017). Scenarios (1) and (2) imply a significant role of spontaneous mutation in the risk of metabolic disease, although the rapid increase in frequency further implies some sort of genotype-environment interaction. A recent onslaught of epigenetic effects is considered less likely as a general explanation for the "missing heritability" of human complex traits (WAINSCHTEIN *et al.* 2019), but specific examples of cross-generational effects are known in humans (PEMBREY *et al.* 2006; CURLEY *et al.* 2011; VEENENDAAL *et al.* 2013; RANDO AND SIMMONS 2015), and are well-documented in other organisms (e.g., plants; MUNIR *et al.* 2001; LUNA *et al.* 2012; RASMANN *et al.* 2012) and *C. elegans*; (GREER *et al.* 2011; RECHAVI *et al.* 2011; ASHE *et al.* 2012; JOBSON *et al.* 2015; MARRÉ *et al.* 2016).

To our knowledge, the cumulative effects of spontaneous mutation on metabolic traits have been investigated in only two experiments. In a groundbreaking study in *Drosophila melanogaster*, CLARK *et al.* (1995) quantified the input of mutational (co)variance in the activity of a set of 12 metabolic enzymes and two metabolites. Mutational heritability (h_M^2, the per-generation increase in genetic variation (V_M) scaled as a fraction of the residual variance, V_E) of enzyme activity was on the order of that of life-history and morphological traits ($h_M^2 \approx 10^{-3}$/generation; HOULE *et al.* (1996)). In several of the mutation accumulation (MA) lines studied,

there were large changes in enzymatic activity relative to the population mean over the course of 44 generations of evolution under minimal selection. Results for the two metabolites studied were analogous, but there was no attempt to assess the relationship between enzyme activity and metabolite concentration in the context of metabolic pathways.

More recently, DAVIES *et al.* (2016) examined the changes in metabolite concentration for 29 metabolites in a set of *C. elegans* MA lines that had undergone ~250 generations of evolution under minimal selection and found that metabolites vary considerably in their response to spontaneous mutation, as quantified by the change in mean metabolite concentration (ΔM) and by the mutational (co)variance. Associations between mutational correlations between pairs of metabolites (r_M, presumably the result of pleiotropy) and proximity of the metabolites in the global metabolic network were, on average, positive but weak (JOHNSON *et al.* 2018). The weakness of the association between mutational pleiotropy and network proximity suggests that pleiotropic effects propagate throughout the metabolic network and are not confined to local modules. However, there was no attempt to link changes in metabolite concentration to the properties of associated metabolic enzymes.

Here we report results of a study designed to investigate the cumulative effects of mutation on the concentration and activity of the enzymes in the adenosine metabolism pathway and their associated metabolites (Figure 1), using the same set of *C. elegans* MA lines as in DAVIES et al. (2016). We chose this particular metabolic pathway for investigation because adenosine was one of the metabolites with the largest mutational variance, indicative of a large mutational target. In addition, adenosine levels are assumed to be tightly regulated due to its role as a critical signaling molecule for energetic homeostasis as a metabolite involved in ATP: AMP, as well has having other critical functions (PARK AND GUPTA 2008; BOISON 2013). Lastly, the adenosine pathway has well-defined network topology and is highly conserved.



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95 **Figure 1.** Adenosine metabolism pathway. Activity and concentration of enzymes Adenosine deaminase (ADA, red) and

96 Adenosine kinase (ADK, red) were measured. We were unable to measure the concentration of APRT (pink). Metabolites

97 in orange had concentrations quantified, those in gray were not measured.

In contrast to the aforementioned studies (CLARK *et al.* 1995; DAVIES *et al.* 2016; JOHNSON *et al.* 2018), our experimental design allows us to infer the relative contributions of both mutation and short-term heritable (i.e., epigenetic) effects on the total heritable variance of metabolic traits.

MATERIALS AND METHODS

Mutation Accumulation:

A detailed description of the construction and propagation of the mutation accumulation (MA) lines is given in BAER *et al.* (2005). Briefly, 100 replicate MA lines were initiated from a nearly isogenic population of N2-strain *C. elegans* and propagated by single-hermaphrodite descent at four-day (one generation) intervals for approximately 250 generations. The common ancestor of the MA lines ("G0") was cryopreserved at the outset of the experiment; MA lines were cryopreserved upon completion of the MA phase of the experiment (Figure 2). Based on extensive whole-genome sequencing (DENVER *et al.* 2012; SAXENA *et al.* 2019), we estimate that the average MA line carries about 60-80 mutant alleles in the homozygous state. In this study we used the same 43 N2-strain MA lines assayed by DAVIES et al (2016).

The ideal design of a phenotypic assay of a MA experiment includes replicates of the (putatively) unmutated common ancestor, which we call "pseudolines" and which are treated identically to MA lines in analyses (LYNCH 1985; LYNCH AND WALSH 1998; TEOTÓNIO *et al.* 2017). The among-pseudoline component of variance includes the effects of residual segregating genetic variation in the ancestor, as well as short-term heritable (epigenetic) effects that are propagated across assay generations and purely environmental effects resulting from (sometimes unavoidable) imperfections of experimental design, such as a temporal correlation between line and assay time. In the absence of a pseudoline control, some fraction of the among-MA line (co)variance will potentially be the result of non-mutational factors, and resulting estimates of V_M and COV_M will be upwardly biased.

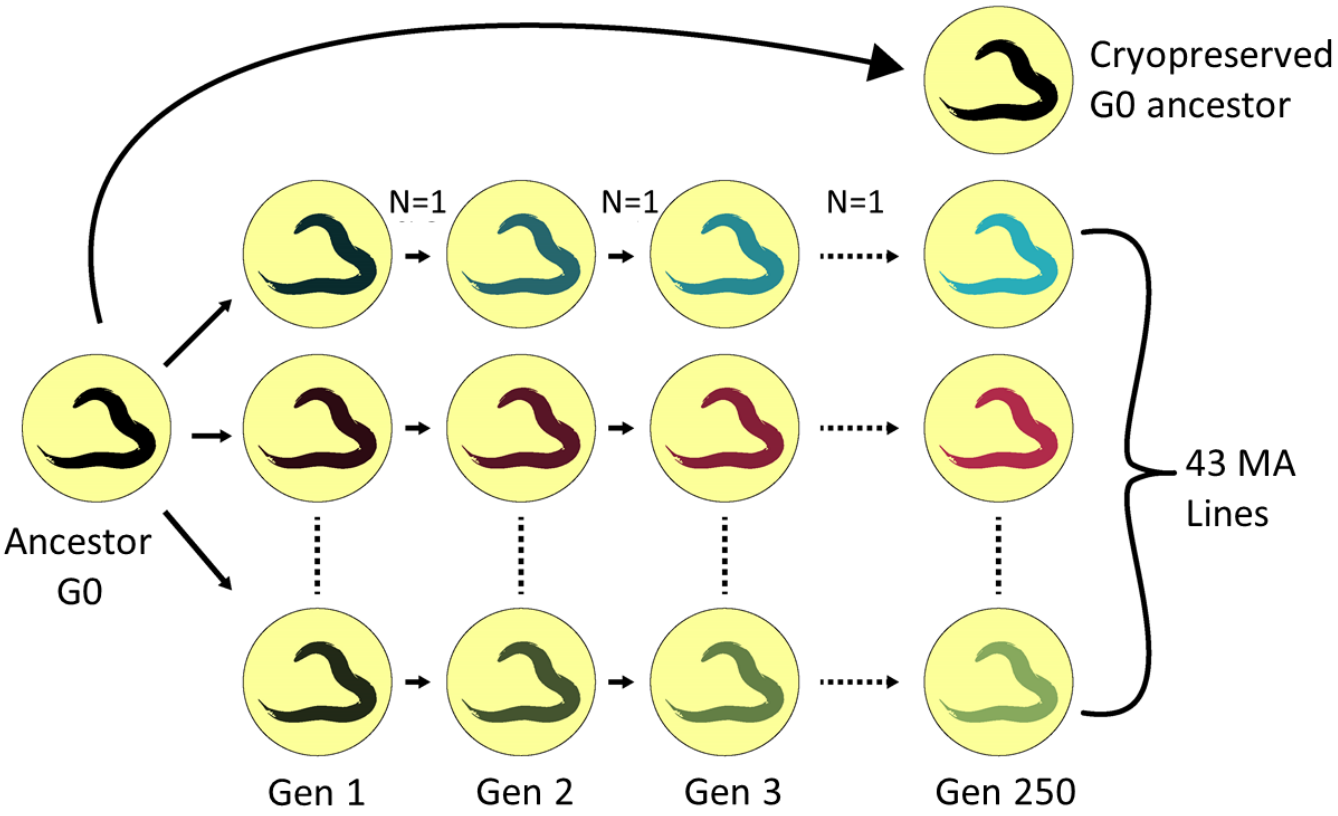

Figure 2. Propagation of mutation accumulation (MA) lines. The G0 ancestor was thawed from a cryopreserved sample and a single hermaphrodite picked onto each of 100 agar plates. MA lines were propagated via single worm descent for ~250 generations. 43 MA lines and the G0 ancestor were included in this experiment.

Here, a set of 15 pseudolines (PS) of the G0 ancestor were included along with the MA lines (Figure 3A). PS lines were generated by thawing a sample of the N2 ancestor and allowing it 24 hours to recover from freezing, at which time 15 hermaphrodites were plated individually onto 60 mm NGM plates seeded with 100 μl of an overnight culture of *E. coli* OP50 (P0 generation in Figure 3A). P0 worms were allowed to reproduce until the bacterial food on the plate was consumed (two generations; F1 and F2), at which time worms were cryopreserved (F2) (HOPE 1999). The demographic features of this protocol mimic those of our standard protocol for cryopreserving MA lines. From this point forward, MA lines and ancestral PS lines are experimentally identical.

Protein Extraction:

This study includes six independent experimental tests: concentration and activity of two enzymes (ADA and ADK), total protein concentration, and mass spectrometry of pooled metabolites (*Note*: we were only able to include two of the three enzymes in the adenosine pathway, ADA and ADK (Figure 1). We were unable to measure the activity of the third enzyme in the pathway, adenosine phosphoribosyltransferase (APRT), because commercially available assay kits require too much material to be practical for application to *C. elegans*. Accordingly, six aliquots of protein (plus metabolites) were extracted and cryopreserved from the same individual sample of each experimental replicate. Protein extraction was performed in five blocks of 10-12 lines per block, to ensure that all samples were handled at the appropriate stage of development (see below). In each protein extraction block, the lines selected were a random mix of MA and PS lines; the experimental design is outlined in Figure 3B. Each line was thawed and transferred onto a 60mm agar plate. The following day, five L4-stage hermaphrodites from each line were transferred individually onto 35mm agar plates (parental generation, P1 in Figure 3B), resulting in a total of 290 samples (five replicates of each of 15 PS lines and 43 MA lines). Four days later, a single offspring (F1 generation) L4 hermaphrodite was transferred from each

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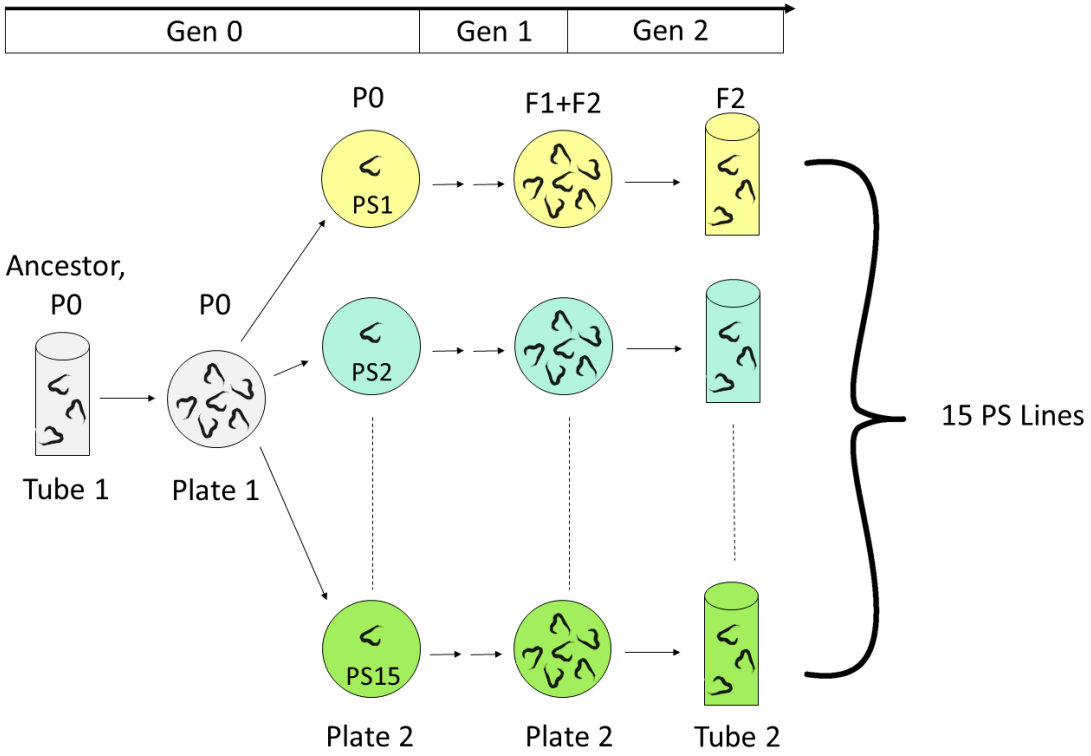

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B)

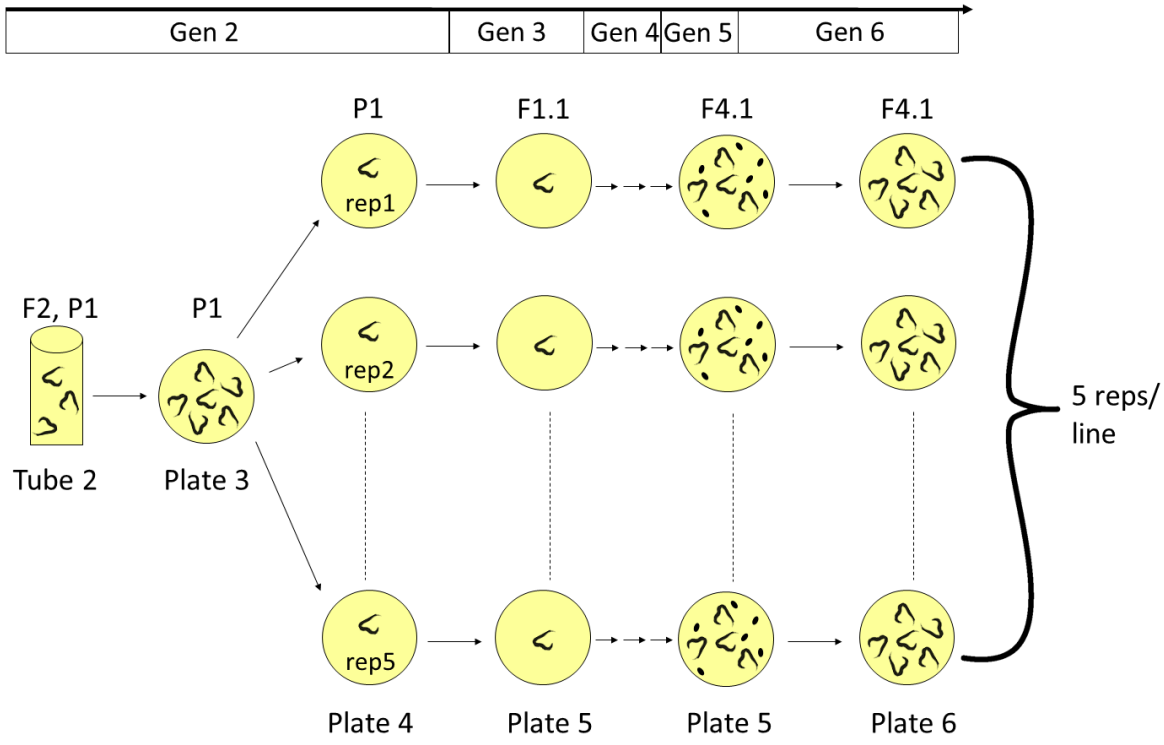

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Figure 3. A) Generation of G0 pseudolines (PS lines). The G0 ancestor was thawed from a cryopreserved sample ("Tube 1", "Plate 1") and 15 individuals were picked onto individual agar plates ("Plate 2"; PS1-PS15) and allowed to reproduce for two generations prior to cryopreservation ("Tube 2"). **B**) Replication of lines for protein/metabolite extraction. Lines (P1, "Tube 2" from [A]) were thawed (plate 3) and five individuals were picked onto individual agar plates ("Plate 4", Rep1-Rep5) and propagated by single-worm descent for another generation (F1.1, "Plate 5"). F1.1 worms were allowed to reproduce for two generations (F2.1, F3.1), and when the plates contained gravid worms (F3.1) and eggs (F4.1) they were bleached. The resulting eggs (F4.1) were transferred to a new plate ("Plate 6") and allowed to hatch and grow to the young adult stage, at which time protein and metabolites were extracted. The timeline at the top represents the number of generations of reproduction of PS lines subsequent to divergence of the lines from the common ancestor.

P1 plate onto a 100mm plate (F1.1 in Figure 3B). The F1 worms were grown for ten days (two generations, F2.1 and F3.1 in Figure 3B) of self-replication to ensure that F3 worms were gravid and there were abundant eggs on the plate (F4.1 in Figure 3B). Worms were washed from the plate and "bleached" in an NaOH and sodium hypochlorite solution (SULSTON AND HODGKIN 1988) .This process kills all hatched worms by breaking down their cuticle and leaves only eggs (F4.1 in Figure 3B), resulting in a population that is closely synchronized in developmental timing. Once F4 worms had been bleached, hatched, and reached the L4 stage, they were washed five times in ion-free NGM buffer, mixed with protease inhibitor cocktail, and homogenized via sonication (TANG AND CHOE 2015). Homogenized samples were centrifuged, and the protein-rich supernatant was distributed equally into six cryovials and stored at -80 C°.

All lines, both MA and PS, were labeled with their true line number until cryopreservation, at which time each replicate was assigned a random number to obscure sample identity. This resulted in six identical sets of 290 samples to be tested. One replicate of one PS line and all replicates of MA line 571 were lost during protein extraction, resulting in a total of 284 samples from 15 PS and 42 MA lines.

Estimating Total Soluble Protein via Bicinchoninic Acid Assay (BCA)

We used total soluble protein as a proxy for the number of individual worms in a sample. To quantify the total soluble protein in each sample we used a bicinchoninic acid assay (BCA) following the protocol from Thermo Scientific (Pierce BCA Protein Assay Kit #23225). Briefly, a set of known concentrations of bovine serum albumin is used to generate a standard curve against which one can estimate the concentrations of unknown protein samples. A total of 13 BC assays were performed, each with its own set of standards.

Enzyme activity assays:

(i) Adenosine kinase (ADK)

Adenosine kinase (ADK) activity was measured using the Novocib PRECISE ADK assay kit (Novocib, Ref #K0507-01). This assay measures ADK activity based on the production on $NADH_2$, which is generated by the dephosphorylation of ATP by ADK. To ensure that ADK activity is not limited by available ATP, an excess of human ATP was added to each sample. Absorbance at 340nm was measured at one-minute intervals for 40 minutes. The slope of the line over the linear phase quantifies the activity of each sample in units of absorption per minute. A set of positive (human ADK, provided in the kit) and negative (no enzyme) controls were included with the unknown samples in each assay plate and used to quantify assay quality, per the manufacturer's instructions. Thirty of the 290 samples were not included in the ADK activity assay. All samples that were run included at least two technical replicates, in which extracts from a sample were split and assayed independently.

(ii) Adenosine deaminase (ADA):

ADA activity was measured using Abcam's Adenosine Deaminase (ADA) Activity Assay Kit (Abcam, ab21193). This kit utilizes an ADA developer and converter which react with inosine formed from the breakdown of adenosine by ADA to produce uric acid. Uric acid concentration is then measured via absorbance at 293nm once a minute for 45 minutes. Each kit is run with a set of known concentration standards that are used to generate a standard curve. The quantity of uric acid was then measured and used to calculate the activity of the ADA in a given sample in units of nmol/min/µg, following the manufacturer's instructions.

ADA activity was assayed in six 96-well plates, each including a positive (manufacturer supplied ADA) and negative (no sample) control. For one assay plate, the highest concentration standard had an unusually low reading; we therefore omitted this point from the standard curve for this assay. Omission of that point had no effect on the interpretation of the data because all unknown samples had absorbance values greater than the second lowest standard. All of the 290 samples had maximum measured activity well below the highest concentration standard. Four samples with erratic absorption readings were omitted from further analyses.

Enzyme concentration:

Enzyme concentrations were estimated by Western blot (WB) (Supplemental Figure S1). Extracted samples were denatured in 2X Laemmli buffer (with β-mercaptoethanol) and boiled at 70° for 10 minutes. Each gel lane was loaded with 7ug of total soluble protein calculated from the BCA data (Bio-Rad 10% polyacrylamide gel, product #4561033). Each blot included eight samples, a DNA-ladder and an internal control standard consisting of a homogenate of *C. elegans*. We used the Trans-Blot Turbo Transfer System (Bio-Rad, #1704156) to transfer proteins separated by gel electrophoresis onto blotting paper. After the primary (enzyme-specific) and secondary (visualization) antibodies were bound (antibodies described below), antibody binding was visualized using the Pierce ECL Western Blotting Substrate (Thermo Fischer Product # 32106). Brightness of each band relative to the internal control was estimated using ImageJ image-analysis software and used as a proxy for enzyme concentration. 246 of the 284 samples contained sufficient protein to be visualized by Western Blot.

The concentration of tubulin in a sample is commonly used as a loading control, and we quantified tubulin in each sample for both enzymes (Tubulin antibody DSHB, E7). However, tubulin concentration was not independent of treatment (MA vs. PS), so we treat it as an experimental trait rather than a control (see Results).

(i) ADK concentration

The antibody used was Abcam's Anti-ADK antibody – C-terminal (Abcam, ab226187), which was designed and tested in mouse and humans and which is homologous with the *C. elegans* ADK protein, R07H5.8 .The assay resulted in multiple binding sites, with distinct bands at ~100kd, ~37kd, ~25kd, and ~18kd (Supplemental Figure S2). To determine which of these binding sites represented the *C. elegans* ADK, samples of each band were extracted from the gel and analyzed using protein mass spectroscopy. Results were then analyzed using Scaffold

4; only the sample at ~37kd contained the worm ADK homolog (R07H5.8, molecular weight = 37.5 kd; Wormbase). 112 of the 246 samples did not contain sufficient ADK to be measured by Western blot. These lines were tested in duplicate and failed to produce ADK bands both times, so the low concentration of ADK is presumably a true property of the sample and not an experimental artifact.

(ii) Adenosine deaminase (ADA) concentration:

The primary anti-body used was Abcam's Anti-ADAT2 antibody (Abcam, ab122280). This antibody is homologous with the *C. elegans* ADA protein ADR-1 which is known to code for ADA in worms (Wormbase). The assay resulted in multiple binding sites, with distinct bands at ~100kd, ~60kd, and ~22kd (Supplemental Figure S3). Samples of each band were extracted from the gel and analyzed using protein mass spectroscopy as for ADK. The band at ~100kd contained the worm ADA homolog ADR-1, isoform D (101.8kd). 202 of the 246 samples contained ADA in sufficient concentration to be quantified by Western blotting.

Metabolomics:

To assess the relationship between enzyme concentration and activity and the concentration of their associated metabolites, we targeted four metabolites in the adenosine metabolic pathway: adenosine, inosine, AMP, and adenine. Several other metabolites not in the adenosine pathway were also measured, including GMP, guanine, guanosine, hypoxanthine, xanthine, and uric acid because they were part of a routine panel that included the metabolites of interest. Metabolite quantification was performed using liquid chromatography/mass spectroscopy (LC-MS), calibrated with known standards at the Southeast Center for Integrated Metabolomics at UF.

Internal standards were prepared as follows: Adenine-$^{15}N_2$ (Cat #A2880477), guanine-4,5-$^{13}C_2$ 7-^{15}N (Cat #G836003), hypoxanthine-$^{13}C_2$ ^{15}N (Cat #H998504) and xanthine-^{13}C $^{15}N_2$ (Cat #X499954) were purchased from Toronto Research Chemicals (Toronto, ON). Adenosine-$^{15}N_5$ 5′-monophosphate (Cat #662658), adenosine-$^{15}N_5$ 5′-triphosphate (Cat

#707783), guanosine-$^{15}N_5$ 5′-monophosphate (Cat #900380) and guanosine-$^{13}C_{10}$ 5′-triphosphate (Cat #710687) were purchased from Sigma-Adrich (St. Louis, MO). The labeled adenosine and guanosine triphosphates were dephosphorylated with alkaline phosphatase (Promega, Madison, WI; Cat #M1821) according to the manufacturer's directions to produce the corresponding labelled nucleosides. Uric acid-^{13}C ^{18}O was synthesized from urea-^{13}C ^{18}O (Cambridge Isotopes, Andover, MA; Cat #COLM-4861) and 5,6-diaminouracil sulfate (Sigma-Aldrich; Cat #D15103) according to methods of Cavalieri et al (CAVALIERI *et al.* 1948).

For the purine assay, internal standard (10µl) was added to 50µl worm homogenate and acetonitrile (100µl) was added to precipitate proteins for LC-MS/MS analysis. Samples were chromatographed on a Waters Cortecs UPLC HILIC column (2.1 x 150 mm, 1.6µm) eluted with an acetonitrile-water gradient: Buffer A) 5 mmol/L ammonium acetate and 0.1% acetic acid in acetonitrile: water (:: 98: 2); Buffer B) 10 mmol/L ammonium formate and 0.5% formic acid in water. Mass spectrometric detection was on a Bruker EvoQ Elite MS/MS in positive ion mode, using heated electrospray ionization.

Stock solutions of the purines analyzed were prepared from authentic standards, and their concentrations determined by absorbance (UMBREIT *et al.* 1960). The stock solutions were then mixed to give an appropriate working standard, which was then serially diluted to produce standard curves. Peak area ratios were calculated by dividing the metabolite peak area by the peak area of its isotopically labeled internal standard. Metabolite concentrations were calculated by comparing these peak area ratios to the standard curves.

Data Analysis:

(i) Estimation of mutational parameters

To quantify the cumulative effects of mutation on individual traits, we calculated the per-generation change in the trait mean (ΔM, the "mutational bias") and the per-generation rate of increase in genetic variance (V_M, the "mutational variance"). Mutational bias is calculated as:

$$\Delta M = \frac{\bar{z}_{MA} - \bar{z}_0}{t\bar{z}_0}$$

where z_{MA} and z_0 are the means of the MA lines and the G0 PS lines, respectively, and t is the number of generations of MA (t=250) (LYNCH AND WALSH 1998).

The mutational variance (V_M) is calculated as:

$$V_M = \frac{V_{L,MA} - V_{L,0}}{2t}$$

where $V_{L,MA}$ is the among-line variance of the MA lines, $V_{L,0}$ is the among-line variance of the PS lines, and t is the number of generations of MA. The among-line variance of the PS lines includes the effects of any residual segregating genetic variance, but also heritable epigenetic effects and the heritable effects of genotype-environment correlation (LYNCH 1985).

The mutational covariance between traits (COV_M) is estimated analogously to V_M, with the among-line components of variance (V_L) replaced with the among-line components of covariance (i.e., the off-diagonal elements in the variance-covariance matrix).

(ii) Statistical analyses

Our primary interest is in the two enzymes, ADA and ADK. The enzyme activity assays measure the composite effects of enzyme activity *per se* (i.e., the inherent kinetic properties of the protein) and the concentration of the enzyme in the sample. For a given sample, the rate at which substrate is converted to product depends on both the amount and the inherent activity of the enzyme present. Because we have an independent measure of the amount of enzyme present in the sample (from the Western blots), we can statistically partition the effects of inherent activity from those of concentration by including enzyme concentration as a covariate in a general linear model (GLM). The concentration of protein measured in the Western blot is standardized by the total protein in the sample, so enzyme activity also needs to be standardized relative to the total protein in the sample, which can be similarly included in a GLM. The ADA activity assay includes total protein in the calculation of activity, so total protein is not included in the GLM. The full GLM can be written as:

$$y_{ijkl} = \mu + c_i + p_i + b_j + a_k + L_{l|j} + \varepsilon_{ijkl},$$

where y_{ijkl} is the measured activity of the enzyme in sample i, μ is the overall mean, c_i is the effect of the concentration of the enzyme in sample i (estimated from the Western blot of the same sample), p_i is the total protein concentration in sample i, b_j is the random effect of assay block j, a_k is the fixed effect of MA group k (MA or PS), $L_{l|j}$ is the random effect of line l given MA group k, and ε_{ijkl} is the residual effect. Variance components (V_L and V_E) and their standard errors were estimated separately for each MA treatment group by restricted maximum likelihood (REML), with degrees of freedom determined by the Kenward-Roger method (KENWARD AND ROGER 1997). Significance of the fixed effect of MA treatment group was tested by F-test on Type III sums of squares. ΔM was calculated from mean values of the two groups estimated by least squares. Model analysis was preformed using the lme4 package in R ((BATES *et al.* 2015).

Protein concentrations (ADA, ADK, tubulin) were calculated relative to a predetermined amount of total protein (see section V above). Mutational statistics for protein concentrations were calculated from the same linear model as above without the covariates. Protein concentration data were log-transformed to meet the assumptions of the GLM, and statistical inferences are based on the transformed data. Mutational statistics are reported on the untransformed scale.

Metabolite concentrations were normalized relative to an internal standard. Mutational statistics were estimated from the same linear model as above, with total protein included as a covariate.

Among-line correlations were estimated from pairwise correlations of line means, using the R package corr.test (REVELLA 2019). Correlations of line means were used rather than among-line components of covariance because some analyses failed to converge. Five of the 57 lines were excluded from the analysis due to missing data. Trait values were standardized relative to the G0 mean across all PS lines.

RESULTS

Per-generation change in trait means (ΔM):

Of the seventeen traits, four declined significantly over the course of 250 generations of MA (i.e., ΔM<0), two increased significantly, and the remainder did not change significantly (Table 1). Of the traits that did not change significantly, the point estimate of ΔM for ADK concentration was the largest of any trait, but ADK concentration also had the largest sampling variance in both MA and PS lines. Mean total protein concentration, by which the other trait values were standardized, was nearly identical in the G0 ancestor and in the MA lines. The close concordance in the average amount of total protein in a sample indicates that the average number of worms in a sample did not differ consistently between ancestor and MA lines. A caveat is in order, however. Although samples were synchronized by bleaching and were cultured to the same qualitative stage of development ("a few" eggs were present on the plate), subtle differences in the distributions of developmental stages may exist at any hierarchical level in the experiment (PS vs. MA; among lines; among replicates within a line). It is known that there are consistent changes in the genome-wide transcriptional profile over the course of a few hours of development (FRANCESCONI AND LEHNER 2014; ZALTS AND YANAI 2017), and there is reason to expect that changes in metabolite levels would change at least as fast. implies, however, that the measured output of the reaction depends on factors other than the enzyme itself, because in the PS lines the protein sequence is presumably identical in all samples (transcriptional and translational errors notwithstanding).

Unexpectedly, the mean (normalized) concentration of tubulin decreased with MA. Tubulin is commonly included as a quantification control in studies of enzyme activity (RYBAK-WOLF *et al.* 2014; DYSHLOVOY *et al.* 2017), although it is notoriously variable (FERGUSON *et al.* 2005; YU *et al.* 2011; EATON *et al.* 2013; LEE *et al.* 2016; MORITZ 2017).Tubulin concentration was separately quantified in the assays of ADA and ADK concentration, and the estimates of the mean decrease were nearly identical (Table 1). There is not an obvious explanation for why

Trait	M_{MA}	M_0	*ΔM*
ADA Activity	0.01 (0.01, 1.20E-02)	0.004 (0.003, 4.3E-03)	**0.0085** (0.007, 0.0102)
ADK Activity	0.02 (0.02, 2.27E-02)	0.02 (0.02, 2.58E-02)	-0.0003 (-0.0009, 0.0005)
ADA Concentration	0.27 (0.19, 0.36)	0.44 (0.25, 0.65)	-0.0014 (-0.0026, 0.0006)
ADK Concentration	3.10 (1.31, 5.32)	0.78 (0.41, 1.16)	**0.0131** (0.0023, 0.032)
Tubulin Conc. (ADA)	0.13 (0.11, 0.15)	0.17 (0.14, 0.19)	**-0.0009** (-0.0014, -0.0002)
Tubulin Conc. (ADK)	0.14 (0.12, 0.17)	0.18 (0.14, 0.19)	-0.001 (-0.0024, 0.001)
Total Protein	0.70 (0.64, 0.76)	0.70 (0.63, 0.77)	0.0000215 (-0.0005, 0.0006)
AMP	14.40 (12.39, 16.50)	21.24 (16.41, 26.19)	**-0.0012** (-0.0019, -0.0004)
Adenine	0.49 (0.38, 0.61)	0.45 (0.32, 0.61)	0.0004 (-0.011, 0.0025)
Adenosine	2.77 (1.25, 4.75)	6.52 (1.70, 12.71)	-0.0018 (-0.0034, 0.0029)
GMP	4.69 (3.71, 5.97)	7.17 (5.56, 9.05)	**-0.0013** (-0.0021, -0.0002)
Guanine	2.59 (2.08, 3.24)	3.47 (2.40, 4.57)	-0.0009 (-0.0019, 0.0006)
Guanosine	3.19 (1.45, 5.66)	4.84 (1.99, 8.44)	-0.001 (-0.003, 0.0033)
Hypoxanthine	6.45 (5.06, 7.91)	7.38 (5.36, 9.56)	-0.0004 (-0.0015, 0.0011)
Inosine	1.73 (1.12, 2.43)	2.59 (1.29, 4.39)	-0.0011 (-0.0027, 0.0018)
Uric Acid	22.98 (17.84, 29.18)	26.06 (19.60, 35.08)	-0.0004 (-0.0016, 0.0011)
Xanthine	4.71 (3.93, 5,59)	6.73 (5.30, 8.42)	**-0.0012** (-0.0019, -0.0003)

377 **Table 1.** Means. Bold values of ΔM are significantly different from zero (P<0.05); empirical 95% bootstrap confidence

378 intervals in parentheses. See Methods for details of the estimation of trait means.

379 the concentration of tubulin should decrease relative to total protein in MA lines. A subtle, but
380 consistent, difference in the distribution of developmental stages, perhaps associated with the
381 cell cycle, seems as likely as any.
382
383 **Mutational variance (or the Lack Thereof):**
384 As mutations accumulate over time, MA lines are expected to diverge in trait values, leading to
385 a consistent, long-term increase in the among-line component of variance (V_L). Scaled per-
386 generation, this increase is the "mutational variance", V_M (LYNCH AND WALSH 1998, p. 330). For
387 various reasons, however, some fraction of the among-line variance may be due to factors other
388 than the accumulation of new mutations. Possible reasons include residual segregating
389 variation in the ancestor of the MA lines, genotype-environment correlations (sometimes
390 unknown or unknowable), and heritable epigenetic effects (RECHAVI AND LEV 2017; PEREZ AND
391 LEHNER 2019). To account for potential non-genetic contributions to the among-line variance, it
392 is necessary to include a set of "pseudolines" (PS) of the ancestor, which are treated both
393 experimentally and statistically as if they were MA lines (LYNCH 1985; TEOTÓNIO *et al.* 2017).
394 To our considerable surprise, for most traits the among-line variance of the PS lines is of
395 the same order of magnitude as that of the MA lines (Table 2). We report two different
396 standardizations of V_M. First, the difference in the among-line variance between the PS and MA
397 lines is divided by the square of the mean of the PS lines ($V_{M,0}$); this is equivalent to the squared
398 coefficient of variation, standardized by the ancestral mean. This quantity is often called the
399 "evolvability" (HOULE 1992), and is the customary way of scaling mutational variance. However,
400 if the trait mean changes over the course of evolution, scaling the MA lines by the ancestral
401 mean will underestimate the true mutational variance if mutational effects are multiplicative (i.e.,
402 the CV is constant; FRY AND HEINSOHN 2002; BAER *et al.* 2006). Because several traits changed
403 significantly, we also report V_M scaled by the group mean ($V_{M,MA}$; i.e., PS lines are scaled by the
404 square of the PS mean and MA lines are scaled by the square of the MA mean). When scaled

Trait	$V_{L,MA}$	$V_{L,PS}$	$V_{M,0}$	$V_{M,MA}$	$V_{E,MA}$	$V_{E,PS}$
ADA Activity	1.41E-07 (0, 5.07E-07)	3.332E-09 (0, 2.18E-08)	2.18E-08 (0, 8.51E-08)	**7.18E-08** (0, 2.64E-07)	3.83E-06 (2.91E-06, 4.85E-06)	6.49E-06 (3.95E-06, 9.16E-06)
ADK Activity	3.86E-10 (0, 4.99 E-09)	6.277 (0, 3.19E-07)	5.99E-09 (0, 0.00E+00)	0 (0, 0.00E+00)	5.95E-07 (4.28E-07, 7.78E-07)	7.66E-07 (4.11E-07, 1.18E-06)
ADA Concentration	**0.053** (0.013, 0.103)	**0.096** (0.007, 0.186)	4.25E-05 (0, 0.0003)	0 (0, 0.0004)	0.05 (0.03, 0.08)	0.05 (0.02, 0.09)
ADK Concentration	3.239 (0, 8.064)	0.0412 (0,0.133)	0.002 (0, 0.006)	0.008 (0, 0,027)	77.94 (4.68, 192.73)	0.97 (0.16, 2.26)
Tubulin Conc. (ADA)	0.0007 (0, 0.002)	0.0004 (0, 0.003)	5.76E-06 (0, 2.97E-05)	3.38E-06 (0, 2.27E-05)	0.007 (0.004, 0.01)	0.008 (0.005, 0.015)
Tubulin Conc. (ADK)	**0.007** (0.0003, 0.021)	0.000003309 (0,0)	**9.63E-05** (5.87E-06, 0.003)	**8.06E-05** (4.33E-06, 0.0002)	0.010 (0.005, 0.015)	0.014 (0.008, 0.021)
Total Protein	**0.023** (0.010, 0.037)	0 (0, 0)	**6.68E-05** (3.02E-05, 0.0001)	**6.74E-05** (2.9E-05, 0.0001)	0.06 (0.04, 0.08)	0.09 (0.05, 0.13)
AMP	**16.11** (7.642, 27.21)	10.89 (0, 24.03)	0.001 (0,0.003)	0.0005 (0, 0.002)	27.76 (17.89, 39.55)	49.29 (30.57, 69.71)
Adenine	**0.013** (0.005, 0.021)	0.006 (0, 0.016)	2.33E-05 (0, 7.19E-05)	2.91E-05 (0, 9.06E-05)	0.04 (0.03, 0.06)	0.08 (0.02, 0.16)
Adenosine	**22.96** (1.043, 47.29)	**63.77** (0.117, 137.8)	0 (0, 0.018)	0 (0, 0.026)	6.84 (0.73, 15.05)	66.03 (9.58, 130.22)
GMP	**3.322** (0.157, 8.804)	1.237 (0, 3.021)	0.001 (0, 0.003)	0.0006 (0, 0,002)	6.88 (3.98, 10.23)	9.25 (4.74, 14,58)
Guanine	**0.792** (0.135, 1.829)	**0.967** (0.291, 1.670)	1.26E-05 (0, 0.001)	0 (0, 0,0007)	0.89 (0.61, 1.21)	1.54 (0.81, 2.48)
Guanosine	**16.54** (2.314, 35.09)	**24.52** (2.771, 53.69)	1.92E-05 (0, 0.008)	0 (0, 0,016)	9.34 (1.21, 20.93)	15.36 (1.98, 30.91)
Hypoxanthine	**5.518** (1.482, 10.79)	4.554 (0, 12.12)	0.0005 (0, 0.003)	0.0003 (0, 0,003)	17.22 (11.45, 23.32)	20.75 (11.35, 31.38)
Inosine	**2.759** (0.546, 4.835)	**5.293** (0.034, 9.856)	0 (0, 0.004)	0 (0, 0.005)	2.68 (0.61, 5.47)	10.28 (0.44, 28.20)
Uric Acid	**38.29** (17.57, 59.97)	**41.94** (5.871**,** 99.22)	0.0003 (0, 0.0035)	0 (0, 0,004)	83.36 (48.54, 126.80)	81.95 (39.29, 132.79)

Xanthine	**2.719** (0.475, 5.329)	2.039 (0, 5.214)	0.0005 (0, 0.002)	0.0002 (0, 0,001)	6.23 (3.61, 9.77)	11.38 (6.09, 17.00)

405

406 **Table 2.** Variances. Column headings are: $V_{L,MA}$, among-line variance of the MA lines; $V_{L,PS}$, among-line variance of the
407 PL lines; $V_{M,0}$, the mutational variance standardized by the ancestral mean; $V_{M,MA}$, the mutational variance standardized
408 by the mean of the group; $V_{E,MA}$, the residual (within-line) variance of the MA lines; $V_{E,PS}$, the residual (within-line) variance
409 of the PS lines. Values of V_L and V_M in bold are significantly greater than zero; empirical 95% bootstrap confidence
410 intervals are shown in parentheses. See Methods for details of the estimation of variance components.

by the ancestral mean ($V_{M,0}$), 3/17 traits have significant mutational variance (tubulin concentration in the ADK assay, total protein, and ADA activity). When scaled by the group mean ($V_{M,MA}$), only tubulin concentration in the ADK assay and total protein remain significant. Importantly, the general lack of mutational variance is not because there is little among-line variance in the MA lines; in 13/17 cases V_L in the MA lines is significantly greater than zero.

Conceivably, technical variance associated with enzyme or metabolite assays could swamp biological variation and lead to a spurious partitioning of variance. However, several lines of evidence suggest this is not the cause of the substantial variance among PS lines. Technical replicates (i.e., samples of extracted material were split and assayed independently) for some of the 290 biological samples were run for ADK activity, ADK concentration, and tubulin concentration (in the ADK assay). In every case, the among-technical replicate variance was much less than the within-line variance (Supplemental Figure S4). Based on previous experience with our metabolomics screen, technical replicate variance for the metabolic pools is expected to be less than 5% for all metabolites except for GMP and uric acid which are expected to be less than 10% (Eoin Quinlivan, Southeast Center for Integrative Metabolomics, personal communication).

It is also extremely unlikely that residual segregating genetic variance could explain the similar magnitudes of the among-line variance in the PS and MA lines. First, any residual genetic variation would be equivalently partitioned among PS lines and MA lines, and would contribute equally (on average, sampling variance notwithstanding). The MA lines were initiated in March, 2001, at which time the G0 ancestor was expanded to large population size (three generations) and cryopreserved. Over the intervening 16 years, the ancestor has been thawed, re-expanded, and re-frozen several times. We do not know exactly how many times the ancestor has been thawed/expanded/re-frozen, but five is a conservative (high) estimate. If we assume that each expansion takes three generations and there have been five such

436 expansions, then any two PS lines will have diverged for 2x5x3=30 generations. In contrast,
437 any two MA lines have diverged for 2x(250+3)≈500 generations.
438 If technical and/or residual genetic variation cannot explain the among-line variance of
439 PS lines, the most likely remaining possibility is heritable epigenetic effects. We cannot rule out
440 a vertically-transmitted pathogen, such as a virus or an intracellular parasite, e.g., microsporidia.
441 However, there is no reason to expect variation in such a pathogen in long-term laboratory lines,
442 whereas there is abundant evidence for heritable epigenetic effects in *C. elegans.* Data were
443 collected on the F4 descendants of the most recent common ancestor of a line (Figure 3B;
444 Supplementary Table 1), which means that any non-genetic short-term heritable effects that are
445 common to a line had to have been maintained for at least four generations, and perhaps since
446 the founder of the PS line six generations back (Figure 3A). Thus, effects common to a line
447 meet the definition of "transgenerational" effects (i.e. passed down to at least the F3, RECHAVI
448 AND LEV 2017). We return to the topic of epigenetic inheritance in the Discussion.
449
450 **Among-line correlations**
451 The absence of significant mutational variance precludes estimation of mutational covariances,
452 which was one of the underlying motivations of this study. However, because there is
453 significant among-line variance for most traits in both the PS and MA lines, it is meaningful to
454 investigate the among-line correlations over the set of all lines (Figure 4). Note that these are
455 not phenotypic correlations in the usual sense. Presumably, the among-line correlations reflect
456 what might be thought of as epi-pleiotropy – the effects of an epigenetic variant (whatever it may
457 be) on multiple traits – as well as the cumulative pleiotropic effects of new mutations.
458 There were significant positive correlations between the concentration of ADA and its
459 substrate (adenosine; (r = 0.57, p<0.0001) and product (inosine; r= 0.63, p<0.0001), and
460 between the pools of adenosine and inosine (r = 0.59, p<0.0001). ADK concentration was
461 positively correlated with the concentration of its substrate (adenosine; r = 0.32, p<0.0002), but

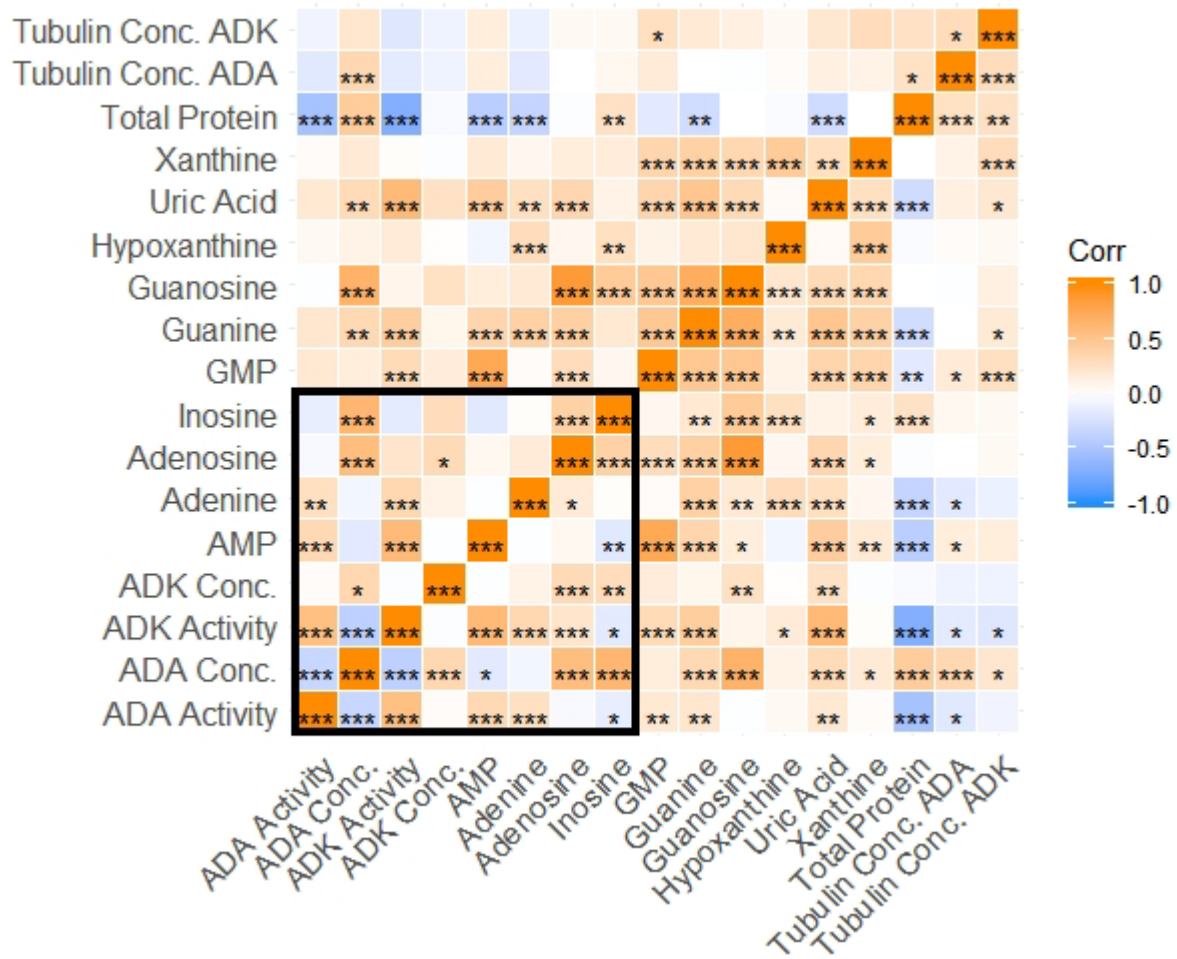

Figure 4. Heatmap of Pearson's correlations between traits. Correlations within the black box are those traits within the adenosine pathway. Significance levels are shown as follows: *** = p<0.001, ** = p<0.01, *=p<0.05.

uncorrelated with its product, AMP. Pools of AMP and adenosine were uncorrelated. We were unable to measure the activity or concentration of adenosine phosphoribosyltransferase (APRT), which converts Adenine to AMP.

DISCUSSION

We are confronted with two results which appear contradictory. First, for some traits the means change significantly over the course of ~250 generations of evolution under MA conditions (4/17 decline, 2/17 increase). However, the mutational variance (V_M) does not differ significantly from zero for any trait. That trait means change with MA is not unexpected, and there is no *a priori* reason to expect every trait to change in the same direction; metabolic traits are not fitness components *per se*. The average absolute ΔM is on the order of 0.1%/generation, consistent with a wide variety of traits in these lines (summarized in Supplemental Table S2 of DAVIES et al. (2016)). Notably, the activity of the two enzymes we investigated remained either unchanged (ADK) or changed only very slightly (ADA), consistent with the coding sequence of an enzyme providing a small mutational target.

Conversely, the general lack of significant mutational variance is unexpected, especially because several traits show clear evidence for the cumulative effects of mutation on the trait mean. The lack of mutational variation is not because there is no variation between MA lines. The cumulative effects of mutation were not swamped by technical or microenvironmental noise (i.e., residual variance; V_E in the parlance of quantitative genetics). For most traits, the among-line variance of the MA lines is significantly greater than zero (Table 2). Rather, the variance among pseudolines of the ancestral control of similar magnitude to the variance among MA lines. Again, enzyme activity is an exception; there is no variance in enzyme activity among MA lines or among PS lines, as expected if enzyme activity is primarily a function of the protein itself and the coding sequence of the gene remains unmutated.

At this point, we must confront an uncomfortable truth. We chose the adenosine metabolism pathway for further scrutiny based on two findings of DAVIES et al. (2016). In that study, mean adenosine concentration *increased* by over 4% per generation – one of the largest values of ΔM reported for any trait in any organism – whereas in this study we found a slight (non-significant) decline in mean adenosine concentration of about 0.2%/generation *in the same set of MA lines*. Similarly, DAVIES et al. reported a mutational heritability (V_M/V_E) for adenosine concentration of about 0.004/generation – toward the high end of mutational heritabilities (HOULE *et al.* 1996) – whereas, we found no significant mutational variance. Clearly, the two studies are at odds: they can't both be right, although they may both be wrong in different ways. Admittedly, the methods of quantifying metabolite concentration were different in the two studies; we used LC-MS in this study, whereas DAVIES et al. used GC-MS, but a poor workman blames his tools.

For economic reasons (metabolomics is expensive), DAVIES et al. did not include pseudolines of the G0 ancestor in their study. As it happens, all but three of the 43 MA lines included in the DAVIES et al. study had mean adenosine concentrations greater than that of the G0 ancestor, which was an order of magnitude less than the mean of the MA lines in normalized units ($\bar{z}_{MA}$ = 22.6±3.4, $\bar{n}$=3.9; z_0= 2.1±0.7,*n*=9; see Figure 1 of DAVIES et al. (2016)). Because ΔM is measured relative to the ancestor, if the mean value of the ancestor is atypically small, ΔM will be atypically large. We have no reason to doubt the accuracy of the estimate of mean adenosine concentration of the G0 ancestor in the DAVIES et al. study. 3/43 MA lines had mean concentrations lower than the ancestor, and another seven MA lines had means less than the largest of the nine replicates of the ancestor. Moreover, the average metabolite concentration of the ancestor was not low relative to the MA lines when all 29 metabolites are considered: the median rank of the ancestor is 34/44 (data from DAVIES et al. (2016) are archived in Dryad, at https://datadryad.org/stash/dataset/doi:10.5061/dryad.2dn09).

It is important to carefully consider the differences between the ways the ancestral controls were treated in the two studies. At the outset of the DAVIES et al. study, in 2009, a single cryopreserved sample of the ancestor was thawed in the Baer lab (Florida) and plated. From that plate, a "chunk" containing hundreds of worms was transferred onto another plate and sent to the Leroi lab in England, at which time worms were washed from the plate and cryopreserved at -80° C. Later, one tube of the ancestor was thawed and plated onto a 100 mm plate. When the population on that plate reached high density (2-3 generations), worms were washed from the plate and "bleached" (SULSTON AND HODGKIN 1988), and surviving L1 larvae were chunked onto a new plate. From that plate, nine replicate plates were initiated from a single individual, and the populations grown to high density (2-3 generations) and synchronized by bleaching. Surviving L1s were plated and grown until worms reached young adulthood, at which time worms were collected for extraction of metabolites. In this design, the nine replicate plates are conceptually identical to the five replicates of each MA line, and the among-replicate (=within-line) variance is the residual variance, V_E.

In this study (depicted in Figure 3A), 15 replicate plates were initiated from a single individual, grown to high density (two generations), and cryopreserved. These are the 15 ancestral pseudolines (PS). Subsequent to thawing (depicted in Figure 3B), the PS lines were treated identically to MA lines, with five replicate plates per PS line initiated from a single individual worm taken from the thawed plate. The replicates then were then propagated to the F3 descendants of the original founder of the replicate, and their offspring (F4) collected for analysis. The variation among replicates is the residual variance, V_E. Any effects that are common to a PS line (i.e., which contribute to V_L) must necessarily have been maintained at least since the replicates diverged from their most recent common ancestor four generations previously, and potentially for as many as the six generations subsequent to the founding of the PS lines.

We believe the source of the discrepancy in ΔM between the two studies is likely the same as the source of the discrepancy in V_M: short-term heritable, epigenetic variation. For example, there is a ~120X difference between the mean adenosine concentrations between the two most extreme of the 43 MA lines in the DAVIES et al. study. The conventional interpretation is (and was) that spontaneous mutations accumulated over a couple of hundred generations can lead to huge differences in metabolite concentrations (and presumably in the concentrations of other biological molecules). However, there is a ~100X difference in the mean adenosine concentration between the two most extreme of the PS lines in this study, lines that have diverged for only a few generations. If the one aliquot of the ancestor sampled in the DAVIES et al. study just happened by chance to fall in the lower tail of the distribution, voilà: ΔM "among the largest reported for any trait" (quoting DAVIES et al. 2016, p. 2243).

Given that the short-term heritability observed here is in fact epigenetic, what might be the cause(s), both proximate (i.e., mechanistic) and ultimate (e.g., environmental)? There is a burgeoning literature on heritable epigenetic effects in *C. elegans*, which can have a number of mechanistic causes, including several varieties of small RNA (RECHAVI AND LEV 2017), histone modifications (FURUHASHI *et al.* 2010; RECHTSTEINER *et al.* 2010; TABUCHI *et al.* 2018), and possibly 6-methyl adenine in DNA (GREER *et al.* 2015). Heritable epigenetic effects have been shown to affect a wide variety of traits (SCHOTT *et al.* 2014; DEMOINET *et al.* 2017; HAN *et al.* 2017; KISHIMOTO *et al.* 2017), and in some cases have been shown to last for tens of generations (ASHE *et al.* 2012; RECHAVI AND LEV 2017). Parental age (PEREZ *et al.* 2017) and nutrition status (MIERSCH AND DORING 2012; TAUFFENBERGER AND PARKER 2014; JOBSON *et al.* 2015) are especially well-documented drivers of epigenetic variation and are obvious potential sources of variation in the experiments reported here.

Nailing down the mechanistic cause(s) responsible for the epigenetic variation inferred here would be both very interesting and very challenging, but it is beyond the scope of this study. To do so would involve a multi-omics study, including whole-genome transcriptomics,

568 metabolomics, proteomics, and ChIP-seq with a smorgasbord of histone-tag antibodies to
569 determine the underlying chromatin status. However, while we do not know the mechanistic
570 underpinning(s) of the epigenetic variation among the ancestral pseudolines, the fact that we
571 detected so much epigenetic variation suggests that it is an important consideration in mutation
572 accumulation studies, and more generally, in any quantitative genetic study in which phenotypic
573 variance is partitioned within and among genotypes.

574 In the only study comparable to this one, CLARK *et al.* (1995, Table 3) found significant
575 mutational heritability for the activity of 8/12 metabolic enzymes in a set of *Drosophila*
576 *melanogaster* MA lines that had evolved under MA conditions for 44 generations. However, their
577 assay conflates variation in enzyme activity *per se* and variation in enzyme concentration into
578 the composite category "enzyme activity" (normalized by body weight and total protein
579 concentration), without correcting for enzyme concentration. The *Drosophila melanogaster*
580 genomic mutation rate is perhaps 3X greater than that of *C. elegans* (SHARP AND AGRAWAL
581 2012; SCHRIDER *et al.* 2013), which suggests that after 44 generations of MA, a Drosophila MA
582 line would have accumulated approximately half as many mutations as one of our *C. elegans*
583 MA lines. Contrary to our expectation based on the preceding evidence, neither of the two
584 metabolic enzymes we assayed (ADA and ADK) exhibited among-line variance for activity *per*
585 *se* in either the MA lines or the PS lines. Thus, for those traits, we cannot attribute the absence
586 of V_M to the confounding effects of among-line variance in the ancestor. It is interesting that the
587 activity of these two enzymes is similarly unperturbed by both mutation and epigenetic factors.
588 However, neither ADA nor ADK was included in the CLARK et al. study; it is certainly possible
589 that had those enzymes been included in that study, they would have fallen in the group of
590 enzymes without significant V_M.

591 We conclude with two thoughts. First, for this set of metabolic traits (enzyme activity
592 notwithstanding), a few generations of short-term heritable (presumably) epigenetic effects
593 swamp the signal of ~250 generations of accumulated mutations. Perhaps that should not be

surprising: it is simply phenotypic plasticity, albeit of a different sort than evolutionary biologists are used to thinking about. It does strongly suggest, however, that investigators doing MA experiments need to be especially mindful of how the ancestor is treated. But also, second: these findings cast the recent increase in human metabolic complex disease in a different light. Although we remain skeptical of epigenetic variation as a general cause of "missing heritability", it may be that metabolic traits are particularly susceptible to epigenetic regulation and are worthy of closer scrutiny in that regard.

Data Availability

Raw data are included in online supplemental file Supplemental Data and are deposited in Dryad (URL XXX).

Acknowledgments

We thank Leigh Boardman, Chao Chen, and Clancy Short for help troubleshooting western blots, Cody Howard for figure editing, and Joanna Dembek, Stephanie Kowalski, Shannon Sawtell and Michael Snyder for worm husbandry. We thank Ayush Saxena and Marta Wayne for helpful discussions. Support was provided by a University of Florida Genetics Institute seed grant to CFB and DAH, NIH award GM107227 to CFB and E. C. Andersen, and NSF award DEB 1639005 to DAH. OJM was partially supported by NIH training grant R25GM115298.

References

Ashe, A., A. Sapetschnig, E. M. Weick, J. Mitchell, M. P. Bagijn *et al.*, 2012 piRNAs Can Trigger a Multigenerational Epigenetic Memory in the Germline of *C. elegans*. Cell 150**:** 88-99.

Baer, C. F., N. Phillips, D. Ostrow, A. Avalos, D. Blanton *et al.*, 2006 Cumulative effects of spontaneous mutations for fitness in Caenorhabditis: Role of genotype, environment and stress. Genetics 174**:** 1387-1395.

Baer, C. F., F. Shaw, C. Steding, M. Baumgartner, A. Hawkins *et al.*, 2005 Comparative evolutionary genetics of spontaneous mutations affecting fitness in rhabditid nematodes. Proceedings of the National Academy of Sciences 102**:** 5785-5790.

Bates, D., M. Maechler, B. Bolker and S. Walker, 2015 Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software 67**:** 1-48.

Boison, D., 2013 Adenosine kinase: Exploitation for therapeutic gain. Pharmacological Reviews 65**:** 906-943.

Boyle, E. A., Y. I. Li and J. K. Pritchard, 2017 An Expanded View of Complex Traits: From Polygenic to Omnigenic. Cell 169**:** 1177-1186.

Cavalieri, L. F., V. E. Blair and G. B. Brown, 1948 The synthesis of uric acid containing isotopic nitrogen. Journal of the American Chemical Society**:** 1240.

Clark, A. G., L. Wang and T. Hulleberg, 1995 Spontaneous mutation rate of modifiers of metabolism in Drosophila. Genetics 139**:** 767-779.

Curley, J. P., R. Mashoodh and F. A. Champagne, 2011 Epigenetics and the origins of paternal effects. Horm Behav 59**:** 306-314.

Davies, S. K., A. Leroi, A. Burt, J. G. Bundy and C. F. Baer, 2016 The mutational structure of metabolism in Caenorhabditis elegans. 70**:** 2239-2246.

Demoinet, E., S. Li and R. Roy, 2017 AMPK blocks starvation-inducible transgenerational defects in. Proc Natl Acad Sci U S A 114**:** E2689-E2698.

Denver, D. R., L. J. Wilhelm, D. K. Howe, K. Gafner, P. C. Dolan *et al.*, 2012 Variation in base-substitution mutation in experimental and natural lineages of caenorhabditis nematodes. Genome Biology and Evolution 4**:** 513-522.

Dyshlovoy, S. A., K. Otte, S. Venz, J. Hauschild, H. Junker *et al.*, 2017 Proteomic-based investigations on the mode of action of the marine anticancer compound rhizochalinin. Proteomics 17**:** 170048.

Eaton, S. L., S. L. Roche, M. Llavero Hurtado, K. J. Oldknow, C. Farquharson *et al.*, 2013 Total Protein Analysis as a Reliable Loading Control for Quantitative Fluorescent Western Blotting. PLoS ONE 8**:** e72457.

Eichler, E. E., J. Flint, G. Gibson, A. Kong, S. M. Leal *et al.*, 2010 Missing heritability and strategies for finding the underlying causes of complex disease. Nature Reviews Genertics 11**:** 446-450.

Ferguson, R. E., H. P. Carroll, A. Harris, E. R. Maher, P. J. Selby *et al.*, 2005 Housekeeping proteins: A preliminary study illustrating some limitations as useful references in protein expression studies. Proteomics 5**:** 566-571.

Francesconi, M., and B. Lehner, 2014 The effects of genetic variation on gene expression dynamics during development. Nature 505**:** 208-211.

Fry, J. D., and S. L. Heinsohn, 2002 Environment dependence of mutational parameters for viability in *Drosophila melanogaster*. Genetics 161**:** 1155-1167.

Furrow, R. E., F. B. Christiansen and M. W. Feldman, 2011 Environment-sensitive epigenetics and the heritability of complex diseases. Genetics 189**:** 1377-1387.

Furuhashi, H., T. Takasaki, A. Rechtsteiner, T. Li, H. Kimura *et al.*, 2010 Trans-generational epigenetic regulation of C. elegans primordial germ cells. Epigenetics Chromatin 3**:** 15.

Greer, E. L., M. A. Blanco, L. Gu, E. Sendinc, J. Liu *et al.*, 2015 DNA Methylation on N6-Adenine in C. elegans. Cell 161**:** 868-878.

Greer, E. L., T. J. Maures, D. Ucar, A. G. Hauswirth, E. Mancini *et al.*, 2011 Transgenerational epigenetic inheritance of longevity in Caenorhabditis elegans. Nature 479**:** 365-371.

Han, S., E. A. Schroeder, C. G. Silva-García, K. Hebestreit, W. B. Mair *et al.*, 2017 Mono-unsaturated fatty acids link H3K4me3 modifiers to C. elegans lifespan. Nature 544**:** 185-190.

Hope, I. A., 1999 *C. elegans - A Practical Approach*. Oxford University Press, New York.

Houle, D., 1992 Comparing evolvability and variability of quantitative traits. Genetics 130**:** 195-204.

Houle, D., B. Morikawa and M. Lynch, 1996 Comparing mutational variabilities. Genetics 143**:** 1467-1483.

Jobson, M. A., J. M. Jordan, M. A. Sandrof, J. D. Hibshman, A. L. Lennox *et al.*, 2015 Transgenerational Effects of Early Life Starvation on Growth, Reproduction, and Stress Resistance in Caenorhabditis elegans. Genetics 201**:** 201-212.

Johnson, L. M., L. M. Chandler, S. K. Davies and C. F. Baer, 2018 Network Architecture and Mutational Sensitivity of the C. elegans Metabolome. Frontiers in Molecular Biosciences 5**:** 69.

Kenward, M. G., and J. H. Roger, 1997 Small sample inference for fixed effects from restricted maximum likelihood. Biometrics 53**:** 983-997.

Kishimoto, S., M. Uno, E. Okabe, M. Nono and E. Nishida, 2017 Environmental stresses induce transgenerationally inheritable survival advantages via germline-to-soma communication in Caenorhabditis elegans. Nat Commun 8**:** 14031.

Lee, H. G., J. Jo, H. H. Hong, K. K. Kim, J. K. Park *et al.*, 2016 State-of-the-art housekeeping proteins for quantitative western blotting: Revisiting the first draft of the human proteome. Proteomics 16**:** 1863-1867.

Luna, E., T. J. Bruce, M. R. Roberts, V. Flors and J. Ton, 2012 Next-generation systemic acquired resistance. Plant Physiol 158**:** 844-853.

Lynch, M., 1985 Spontaneous Mutations for Life-History Characters in an Obligate Parthenogen. Evolution 39**:** 804-818.

Lynch, M., and B. Walsh, 1998 *Genetics and Analysis of Quantitative Traits*. Sinauer, Sunderland, MA.

Mahajan, A., D. Taliun, M. Thurner, N. R. Robertson, J. M. Torres *et al.*, 2018 Fine-mapping type 2 diabetes loci to single-variant resolution using high-density imputation and islet-specific epigenome maps. Nat Genet 50**:** 1505-1513.

Manolio, T. A., F. S. Collins, N. J. Cox, D. B. Goldstein, L. A. Hindorff *et al.*, 2009 Finding the missing heritability of complex diseases. Nature 461**:** 747-753.

Marré, J., E. C. Traver and A. M. Jose, 2016 Extracellular RNA is transported from one generation to the next in Caenorhabditis elegans. Proc Natl Acad Sci U S A 113**:** 12496-12501.

Miersch, C., and F. Doring, 2012 Paternal dietary restriction affects progeny fat content in Caenorhabditis elegans. Iubmb Life 64**:** 644-648.

Moritz, C. P., 2017 Tubulin or Not Tubulin: Heading Toward Total Protein Staining as Loading Control in Western Blots. Proteomics 17**:** 1600189–1600200.

Munir, J., L. A. Dorn, K. Donohue and J. Schmitt, 2001 The effect of maternal photoperiod on seasonal dormancy in Arabidopsis thaliana (Brassicaceae). Am J Bot 88**:** 1240-1249.

Park, J., and R. S. Gupta, 2008 Adenosine kinase and ribokinase - the RK family of proteins. Cellular and Molecular Life Sciences 65**:** 2875-2896.

Pembrey, M. E., L. O. Bygren, G. Kaati, S. Edvinsson, K. Northstone *et al.*, 2006 Sex-specific, male-line transgenerational responses in humans. Eur J Hum Genet 14**:** 159-166.

Perez, M. F., M. Francesconi, C. Hidalgo-Carcedo and B. Lehner, 2017 Maternal age generates phenotypic variation in Caenorhabditis elegans. Nature 552**:** 106-109.

Perez, M. F., and B. Lehner, 2019 Intergenerational and transgenerational epigenetic inheritance in animals. Nature Cell Biology 21**:** 143-151.

Rando, O. J., and R. A. Simmons, 2015 I'm eating for two: parental dietary effects on offspring metabolism. Cell 161**:** 93-105.

Rasmann, S., M. De Vos, C. L. Casteel, D. Tian, R. Halitschke *et al.*, 2012 Herbivory in the previous generation primes plants for enhanced insect resistance. Plant Physiol 158**:** 854-863.

714 Rechavi, O., and I. Lev, 2017 Principles of Transgenerational Small RNA Inheritance in Caenorhabditis
715 elegans. Curr Biol 27**:** R720-R730.

716 Rechavi, O., G. Minevich and O. Hobert, 2011 Transgenerational Inheritance of an acquired small RNA-
717 based antiviral response in *C. elegans*. Cell 147**:** 1248-1256.

718 Rechtsteiner, A., S. Ercan, T. Takasaki, T. M. Phippen, T. A. Egelhofer *et al.*, 2010 The histone H3K36
719 methyltransferase MES-4 acts epigenetically to transmit the memory of germline gene
720 expression to progeny. PLoS Genet 6**:** e1001091.

721 Revella, W., 2019 psych: Procedures for Psychological, Psychometric, and Personality Research, pp.,
722 Northwestern University, Evanston, Illinois.

723 Rhee, E. P., J. E. Ho, M.-h. Chen, D. Shen, S. Cheng *et al.*, 2013 Resource A Genome-wide Association
724 Study of the Human Metabolome in a Community-Based Cohort. Cell Metabolism 18**:** 130-143.

725 Richard, M. A., T. Huan, S. Ligthart, R. Gondalia, M. A. Jhun *et al.*, 2017 DNA Methylation Analysis
726 Identifies Loci for Blood Pressure Regulation. American Journal of Human Genetics 101**:** 888-
727 902.

728 Rybak-Wolf, A., M. Jens, Y. Murakawa, M. Herzog, M. Landthaler *et al.*, 2014 A variety of dicer substrates
729 in human and C. elegans. Cell 159**:** 1153-1167.

730 Saklayen, M. G., 2018 The Global Epidemic of the Metabolic Syndrome. Current Hypertension Reports
731 20**:** 12.

732 Saxena, A. S., M. P. Salomon, C. Matsuba, S. D. Yeh and C. F. Baer, 2019 Evolution of the Mutational
733 Process under Relaxed Selection in Caenorhabditis elegans. Molecular biology and evolution 36**:**
734 239-251.

735 Schott, D., I. Yanai and C. P. Hunter, 2014 Natural RNA interference directs a heritable response to the
736 environment. Sci Rep 4**:** 7387.

737 Schrider, D. R., D. Houle, M. Lynch and M. W. Hahn, 2013 Rates and genomic consequences of
738 spontaneous mutational events in Drosophila melanogaster. Genetics 194**:** 937-954.

739 Sharp, N. P., and A. F. Agrawal, 2012 Evidence for elevated mutation rates in low-quality genotypes. Proc
740 Natl Acad Sci U S A 109**:** 6142-6146.

741 Shin, S.-y., E. B. Fauman, A.-k. Petersen, J. Krumsiek, R. Santos *et al.*, 2014 An atlas of genetic influences
742 on human blood metabolites. 46**:** 543-550.

743 Sulston, J., and J. Hodgkin, 1988 The Nematode Caenorhabditis elegansns, pp. 587-606. Cold Spring
744 Harbor Labratory, Cold Springs Harbor.

745 Tabuchi, T. M., A. Rechtsteiner, T. E. Jeffers, T. A. Egelhofer, C. T. Murphy *et al.*, 2018 Caenorhabditis
746 elegans sperm carry a histone-based epigenetic memory of both spermatogenesis and
747 oogenesis. Nat Commun 9**:** 4310.

748 Tang, L., and K. P. Choe, 2015 Characterization of skn-1/wdr-23 phenotypes in Caenorhabditis elegans;
749 pleiotrophy, aging, glutathione, and interactions with other longevity pathways. Mechanisms of
750 Ageing and Development 149**:** 88-98.

751 Tauffenberger, A., and J. A. Parker, 2014 Heritable transmission of stress resistance by high dietary
752 glucose in Caenorhabditis elegans. PLoS Genet 10**:** e1004346.

753 Teotónio, H., S. Estes, P. C. Phillips and C. F. Baer, 2017 Experimental evolution with Caenorhabditis
754 nematodes. Genetics 206**:** 691-716.

755 Umbreit, W. W., R. M. C. Dawson, D. C. Elliott and K. M. Jones, 1960 Data for Biochemical Research. AIBS
756 Bulletin.

757 Veenendaal, M. V., R. C. Painter, S. R. de Rooij, P. M. Bossuyt, J. A. van der Post *et al.*, 2013
758 Transgenerational effects of prenatal exposure to the 1944-45 Dutch famine. BJOG 120**:** 548-
759 553.

760 Wainschtein, P., D. P. Jain, L. Yengo, Z. Zheng, L. A. Cupples *et al.*, 2019 Recovery of trait heritability from
761 whole genome sequence data. bioRxiv.

762 Yu, H. R., H. C. Kuo, H. C. Huang, L. T. Huang, Y. L. Tain *et al.*, 2011 Glyceraldehyde-3-phosphate
763 dehydrogenase is a reliable internal control in Western blot analysis of leukocyte subpopulations
764 from children. Analytical Biochemistry 413**:** 24-29.

765 Zalts, H., and I. Yanai, 2017 Developmental constraints shape the evolution of the nematode mid-
766 developmental transition. Nature Ecology & Evolution 1**:** 0113.

767