Genetics and Genomics

Gene expression variation across genetically identical individuals predicts reproductive traits

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eLife Assessment

This **important** study addresses the role of non-genetic factors in individual differences in phenotype. Using C. elegans, the study finds that non-genetic differences in gene expression, partly influenced by the environment, correlate with individual differences in two reproductive traits. This supports the use of gene expression data as a key intermediate for understanding complex traits. The clever study design makes for **compelling** evidence.

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Abstract

In recent decades, genome-wide association studies (GWAS) have been the major approach to understand the biological basis of individual differences in traits and diseases. However, GWAS approaches have limited predictive power to explain individual differences, particularly for complex traits and diseases in which environmental factors play a substantial role in their etiology. Indeed, individual differences persist even in genetically identical individuals, although fully separating genetic and environmental causation is difficult in most organisms. To understand the basis of individual differences in the absence of genetic differences, we measured two quantitative reproductive traits in 180 genetically identical young adult *Caenorhabditis elegans* roundworms in a shared environment and performed single-individual transcriptomics on each worm. We identified hundreds of genes for which expression variation was strongly associated with reproductive traits, some of which depended on individuals' historical environments and some of which was random. Multiple small sets of genes together were highly predictive of reproductive traits, explaining on average over half and over a quarter of variation in the two traits. We manipulated mRNA levels of predictive genes to identify a set of causal genes, demonstrating the utility of this approach for both prediction and understanding underlying biology. Finally, we found that the chromatin environment of predictive genes was enriched for H3K27 trimethylation, suggesting that gene expression variation may be driven in part by chromatin structure. Together, this work shows that individual, non-genetic differences in gene expression are both highly predictive and causal in shaping reproductive traits.

Introduction

Over the past two decades, genome-wide association studies (GWAS) have sought to identify the genetic basis of individual differences driving traits and diseases. While there have been some notable successes[1,2], GWAS analyses often require hundreds of thousands of individuals to identify genetic loci that typically explain a small fraction of variation in traits and diseases[3], and the clinical utility of such loci is often unclear[4,5]. Complex traits and diseases that are driven by a strong environmental component are particularly problematic for GWAS, as these environmental inputs cannot be experimentally controlled in humans[6,7]. This lack of control means that some loci may be spuriously identified and others left undiscovered. More recent approaches, including transcriptome-wide association studies[8] (TWAS) and epigenome-wide association studies[9] (EWAS) recognize the intermediate role of epigenetic regulation and gene expression in driving complex traits and diseases. However, TWAS approaches focus specifically on predicted genetic effects on expression to prioritize GWAS candidates and thus do not account for potentially causal environmentally induced changes in gene expression. EWAS approaches, like GWAS and TWAS, are performed in individuals for which genetic and environmental information are not experimentally controlled, and thus it is unclear if identified epigenomic changes are driven by a genetic difference or an environmental effect[10]. Overall, the major approaches used to uncover the etiology of complex traits and diseases are limited in their ability to separate genetic and environmental effects. Here, we show that even stochastic differences among individuals can be strongly predictive of complex reproductive traits when analyzed through the lens of differences in gene expression.

Complete control of genetic background, especially as layered across different environment inputs, is impossible within human populations. Model organism studies have therefore been invaluable in building our understanding of environmentally induced effects on epigenetic regulation, gene expression, and traits of interest in a controlled genetic background[11–17]. However, such analyses generally focus on bulk populations (typically either to ensure ample material or to average differences across individuals within a replicate for model organisms such as *Drosophila* and *C. elegans*[18,19]), or they use very few individuals (typically due to difficulty sampling many larger organisms such as mice[20]), precluding knowledge of how individual life trajectories may influence gene expression, epigenetic information, and traits of interest. In recent years, it has become possible to generate individual transcriptomic profiles in *C. elegans* and *Drosophila,* and methods to profile individuals in high throughput have improved[21–27]. This has revealed that genetically identical individuals in a shared environment indeed differ in their gene expression profiles. However, the effects that gene expression differences across genetically identical individuals may have on complex fitness-related phenotypes of these same individuals — which also vary substantially across genetically identical individuals – remain largely unknown. To fully separate genetic and environmental causation, we use an isogenic population of *C. elegans* to show that individuals in a common environment at the same developmental stage exhibit substantial differences in two fitness-related reproductive traits. By performing mRNA-seq on each individual worm, we identify hundreds of genes for which variation in expression is strongly associated with reproductive trait variation, determine the extent to which expression variation is driven by noise versus known environmental history, identify small sets of genes that are highly predictive of both reproductive traits, and show experimentally that reducing mRNA levels of individual genes causally affects progeny production. Finally, we show that predictive gene sets for reproductive traits are enriched for genes with the chromatin modification H3K27me3, revealing that chromatin environment defines which loci are subject to the individual variation in gene expression that drives variation in complex traits.

Results

To determine if differences in gene expression levels correspond to differences in complex traits, we measured reproductive traits and whole-animal mRNA transcriptomes for each of 180 isogenic *C. elegans* in a shared environment at the same stage of early adulthood (**Fig 1A**). Two reproductive traits – egg-laying onset (ELO) and the number of progeny produced in the first 24 hours of egg-laying (early brood) – were measured for each individual. To generate additional phenotypic variation and allow us to distinguish between gene expression noise and known environmental sources of variation, worms experienced controlled environmental perturbations early in development in a two-factor design: they were either born to a day 1 or day 3 adult parent (corresponding to the beginning and middle of egg-laying in *C. elegans*, respectively), and, once hatched, they were subject to either a constant 20°C temperature or an eight-hour shift to 25°C (both corresponding to typical rearing temperatures for *C. elegans*). This design is ideal for at least two reasons. First, in standard worm rearing, worms that at the same developmental stage in a shared environment have experienced some differences in their environmental histories that are typically unknown to the observer because they appear physiologically identical. Some of these differences in environmental history include factors that we directly controlled in this experiment, including the age of each worm's parent and small fluctuations in rearing temperature. By explicitly incorporating this *historical* environmental heterogeneity, we can distinguish between gene expression variability induced by environmental history and gene expression variability driven by 'noise'. Noise encompasses both unknown extrinsic factors (e.g., microenvironmental differences in food availability) and intrinsic factors (e.g., stochasticity in biochemical reactions). Second, with this approach we can ask whether sequencing adult worms in a shared environment is a viable approach for phenotyping-by-transcriptomics and whether such an approach depends on knowledge of the historical environment. In other words, if an adult worm is picked off a plate and sequenced, can we determine when that worm began reproducing and how many progeny it produced, without regard to its previous environment or its ancestors' environment? In our experiment, isogenic adult worms in a common environment (with distinct historical environments) exhibited a range of both ELO and early brood trait values (Fig. S1A), and variation in these traits was partially driven by environmental history, consistent with prior work on parental age and rearing temperature in *C. elegans*[27–29] (Fig S1B). To determine if gene expression differences identified in single-worm mRNA-seq were directly associated with reproductive traits measured in the same worms, we used a linear mixed modeling approach and, remarkably, identified significant genes corresponding to each trait (**Fig 1B-C**, Fig S2). Expression of 448 genes was strongly associated with early brood (**Fig 1B-C**, Bonferroni p-value of 0.05, nominal p-value of 5.7×10^{-6}), while expression of 11 genes was strongly associated with ELO (Fig S2). This model was agnostic to the environmental history of the worm, but accounted for biological replicate, and we confirmed that these effects were not driven by mutations arising in the population over the course of the experiment (see Methods). This reveals many genes, particularly for early brood, for which expression differences across genetically identical individuals strongly associate with the reproductive traits of these individuals. Importantly, this indicates that expression levels of hundreds of genes provide useful information about the reproductive status of individual worms.

We next quantified the effect sizes on reproductive traits explained by the individual genes that were significant in the previous analysis. Further, because of our experimental design, we also determined the effect size and significance of the association after accounting for environmental history in our model, allowing us to distinguish how much of the effect of gene expression on reproductive traits is gene expression 'noise' and not driven by known environmental history. At the phenotypic level, differences in early brood were partially driven by parental age but not affected by early-life temperature (**Fig 1C** and Fig S1B). Differences in the timing of egg-laying onset were partially driven by both parental age and temperature. Because temperature and

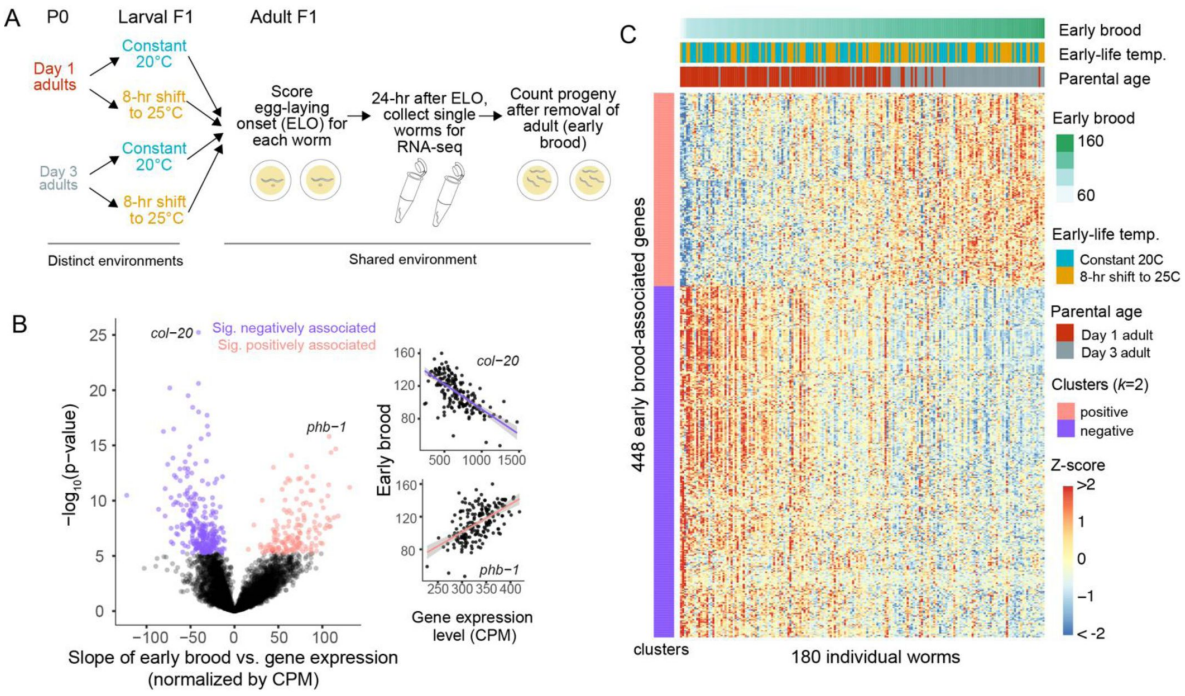


Figure 1.

Differences in early brood across isogenic individuals are associated with expression levels of hundreds of genes.

A. Experimental design for single-individual mRNA-seq. P0 worms are allowed to lay progeny as Day 1 or Day 3 adults. F1 progeny are either subject to a constant temperature or an eight-hour shift to 25°C. Beginning in mid-larval stages all F1 worms experience the same environment. For each F1 worm, time to egg-laying onset is scored. 24 hours after egg-laying onset, each F1 worm is collected for single-individual mRNA-seq. F2 progeny laid on plates in the first 24 hours of egg-laying are counted to determine the early brood of each F1 worm.

B. Each of 8824 expressed genes was analyzed in a mixed model to assess the strength of its association with early brood. Significant genes are in pink (positively associated) or purple (negatively associated), non-significant genes are in black. Significance determined by a Bonferroni-corrected p-value cutoff of 0.05 (nominal p-value of 5.67×10^{-6}). Phenotypic and expression data for each worm is shown for the top two genes, *col-20* and *phb-1* with linear model fits in black and 95% confidence intervals in gray.

C. Heatmap of 448 brood-associated genes for all 180 individuals. Worms are sorted from left to right from lowest to highest early brood. Early brood data and environmental information is shown in the bars at the top. Genes are sorted into two clusters based on whether the association was positive or negative.

parental age have effects on progeny phenotypes, we wanted to test the extent to which the association between gene expression and reproductive traits was driven by environmental history (parental age or early-life temperature) for the identified genes. Previous work on parental age found that maternal age affects vitellogenin gene expression to impact progeny phenotypes[27], but how other genes may be affected is not yet known. To determine effect sizes, we calculated the standardized beta coefficient for each gene using a linear mixed model without environmental history, in line with the analysis done in **Figure 1** (β_1 in **Fig. 2A-B**). We then generated a linear mixed model that incorporates environmental history and calculated the standardized beta for the expression component of the model, effectively isolating gene expression 'noise' that is not explained by environmental history (β_2 in **Fig. 2A-B**). These two approaches to calculating standardized beta values for expression are illustrated as path analyses in **Fig. 2A-B**. Model 1 considers expression alone and its association with brood, while Model 2 is a causal model that incorporates environmental history and its effects on gene expression. Equations for these models, including the coefficients for $\beta_3 - \beta_6$, can be found in the methods. Values for all coefficients can be found in Supplementary File 1. Here we primarily focus on standardized beta values β_1 and β_2, because these respectively represent 1) the total effect of expression on each trait and 2) the effect of expression on each trait after controlling for historical environment. In **Figure 2A**, *puf-5* is shown as an example gene that is part of the group of 448 genes significantly associated with brood. The β_1 value of 0.39 for *puf-5* is significant and indicates that a 1 standard deviation change in gene expression is associated with a 0.39 standard deviation change in early brood. The β_2 value of 0.26 for *puf-5* is also significant, meaning that after accounting for historical environment, a 1 standard deviation change in gene expression is associated with a 0.26 standard deviation change in early brood. The standard deviation in early brood is 20.3, so this indicates that these gene expression changes associated with *puf-5* account for approximately 5-8 progeny produced in the first day of egg-laying, which is a sizable effect on fitness. Similarly, in **Figure 2B**, *grsp-4* is shown as an example gene with a significant association with egg-laying onset both before and after accounting for environmental history. The absolute values of β_1 and β_2 for all significant genes are shown in **Figure 2C-D**. Early brood genes had a median absolute value of β_1 of 0.38 and a median absolute value of β_2 of 0.23, indicating that for a 1 standard deviation change in gene expression for a typical significant gene, there was a corresponding 0.38 or 0.23 standard deviation change, respectively, in early brood. Egg-laying onset genes had a median β_1 value of 0.35 and β_2 value of 0.31. Thus, while we are able to identify many more genes for early brood compared to egg-laying onset, genes for both traits have largely comparable effect sizes. Among significant genes for both traits, β_2 values were consistently lower than β_1 (**Fig. 2C-D**), suggesting some of the total effect size was driven by environmental history rather than pure noise. Nonetheless, β_2 values were often still highly significant with substantial explanatory power, indicating that gene expression noise does indeed explain differences in reproductive traits. For egg-laying onset, 5 of 11 genes have highly significant values of β_2 (Bonferroni corrected p-value of less than 0.05), though all are nominally significant. Of the 448 genes associated with early brood, 157 genes have highly significant values of β_2, indicating that gene expression noise independent of historical environment is driving associations for these genes.

In our previous analysis, we identified genes for which variation is associated with early brood or egg-laying onset and determined which of these genes had expression variation that was not explained by the historical environment (i.e., the 157 noise genes). Because of the unique structure of our dataset, we can also directly ask whether the historical environment has effects on gene expression, regardless of whether these expression changes lead to changes in our reproductive traits. To address this, we used a negative binomial mixed model to identify genes that are differentially expressed due to parental age or early-life temperature. We identified 186 genes with expression changes significantly affected by parental age, and just 2 genes significantly affected by early-life temperature. 140 of the 186 genes differentially expressed due to parental age in the previous generation overlapped with the original 448 genes for which expression variation is associated with early brood, suggesting that the historical environment, i.e. parental age, at least partially accounts for the gene expression changes that are associated with early

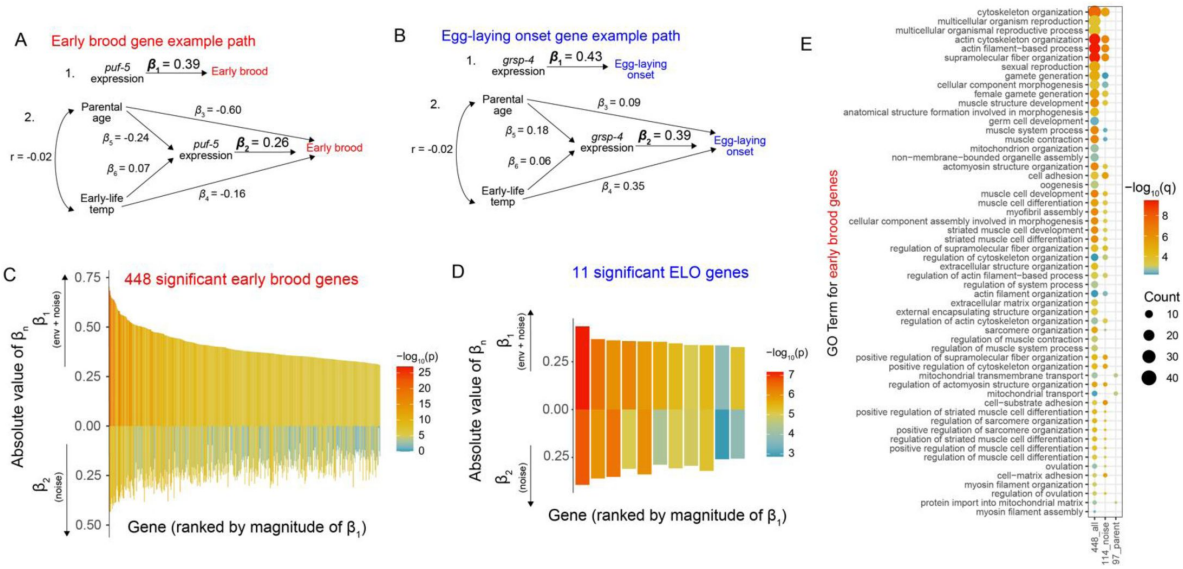


Figure 2

Associations between gene expression and reproductive traits are partially driven by both noise and historical environment.

A. Two example path analyses showing the relationship between gene expression and early brood. In Model 1, ene expression of a particular gene, *puf-5*, drives differences in early brood and β_1 is the coefficient of the linear mixed model. β_1 represents the total effect of gene expression on early brood. In Model 2, β_2 represents the effect f *puf-5* on early brood that is independent of the historical environment.

B. Two example path analyses showing the relationship between gene expression and egg-laying onset, nalogous to A. In Model 1, β_1 is the total effect of *grsp-4* on egg-laying onset, while in Model 2, β_2 represents the ffect of *grsp-4* that is independent of historical environment.

C. The 448 genes with for which expression variation is significantly associated with early brood, ordered by the magnitude of their β_1 effect sizes. The absolute values of β_1 and β_2 are plotted upward and downward, espectively, for each gene.

D. The 11 genes for which expression variation is significantly associated with egg-laying onset across ndividuals, ordered by the magnitude of their β_1 effect sizes. The absolute values of β_1 and β_2 are plotted upward nd downward, respectively, for each gene.

C-D. Legend indicates the $-\log_{10}$(p-value) of β_1 and β_2 for each gene.

E. GO Terms for the 448 genes associated with early brood with a q-value < 0.01 are shown. GO Terms for the wo subsets of the 448 genes with overlapping GO Terms are also shown: 114 genes that have significant values f β_2 but parental age does not have a significant effect on expression ('noise' genes), and 97 genes that do not ave significant values of β_2 but parental age does have a significant effect on expression (parental age genes).

brood. 43 of the 186 genes also overlap with our 'noise' genes, meaning they exhibit gene expression variability significantly explained by *both* historical environment and by noise; that is, these two sources of variation operate together for these genes. Thus, among genes for which expression variation is associated with early brood, we can gain insight on whether this variability in primarily driven by expression 'noise', historical environmental perturbations, or both.

We next assessed Gene Ontology (GO) enrichments for genes associated with reproductive trait variation. We focused on early brood genes because hundreds of genes were identified, giving us power to detect enriched terms. We evaluated GO terms for all 448 genes as well as two subsets of the 448 genes: 1) genes that have a significant value of β_2 and a non-significant effect of parental age on gene expression (114 genes, the 'noise' group) and 2) genes that have a non-significant value of β_2 and a significant value of parental age on gene expression (97 genes, the 'parental age' group). Enrichments for all three gene groups are shown in **Figure 2E**. We find that the majority of enriched terms for the 448 genes are also enriched in either the noise group or parental age group, in a mutually exclusive manner (i.e. no term is enriched in both subsets), suggesting that these two sources of gene expression variation may, to some extent, be regulated by distinct processes. Indeed, the enriched parental age terms all impinge on mitochondrial regulation, consistent with parental age affecting mitochondria-related gene expression in progeny. Notable categories among enriched noise terms include cytoskeleton organization and female gamete generation, with the latter category particularly relevant for affecting progeny production.

The analysis shown in **Figure 2** provides a framework to understand individual gene expression as useful both for prediction and understanding causal mechanisms. Toward the goal of using gene expression profiles to predict phenotypes, we next asked if we could use *aggregate* gene expression profiles for combinations of genes to predict the reproductive traits of individuals. For example, the β_1 value of a single gene provides an indication of to what extent the phenotype is associated by that single gene. If instead of generating individual regressions for each gene, we combined genes into a single model, would we be able to better predict phenotypes? Because we are focused on prediction rather than the distinction between expression noise and the historical environment in this analysis, we focus on an analysis that is agnostic to environmental history, but we emphasize that all worms are genetically identical and collected while in a shared environment at the same developmental stage. Principal component analysis (PCA) provides one such aggregate profile which simplifies gene expression data to linearly independent factors (PCs) that together explain the variation in the gene expression data and are ordered by how much variation they explain (*i.e.*, PC1 explains the most variation, PC2 explains the second-most, etc.). We therefore first asked whether ordered PCs together in a multiple regression explain more variation than expected by chance. Indeed, gene expression PCs explained both ELO and early brood data substantially better than randomized ELO and early brood data, indicating that individual gene expression profiles provide a clear signal of phenotypic information (**Fig. 3A**). Because we have phenotypic and transcriptomic data for 180 worms, and therefore up to 180 linearly independent PCs that can be added a multiple regression model, the total R^2 eventually rises to 1 even for randomized data, as shown in **Fig. 3A**. Therefore, the optimal number of PCs occurs at the inflection points of the graph, which is after only 7 PCs for early brood (R^2 of 0.55) but 28 PCs for ELO (R^2 of 0.56). Thus, for early brood in particular, very few PCs explain over half of the variation in the data and explain substantially more variation than for shuffled phenotypic data. Therefore, whole transcriptomes are a viable approach to trait prediction in isogenic *C. elegans*.

Because PCA incorporates data from all expressed genes, we next wanted to determine if a smaller subset of genes could be similarly predictive of reproductive traits. We again used a multiple regression approach in which we successively selected each gene by identifying the gene that explained the most additional variance compared to the previous model (**Fig. 3B**). Genes that exhibit highly correlated expression patterns, even if they are each individually associated with traits of interest, would not explain much additional variance in a multiple regression approach.

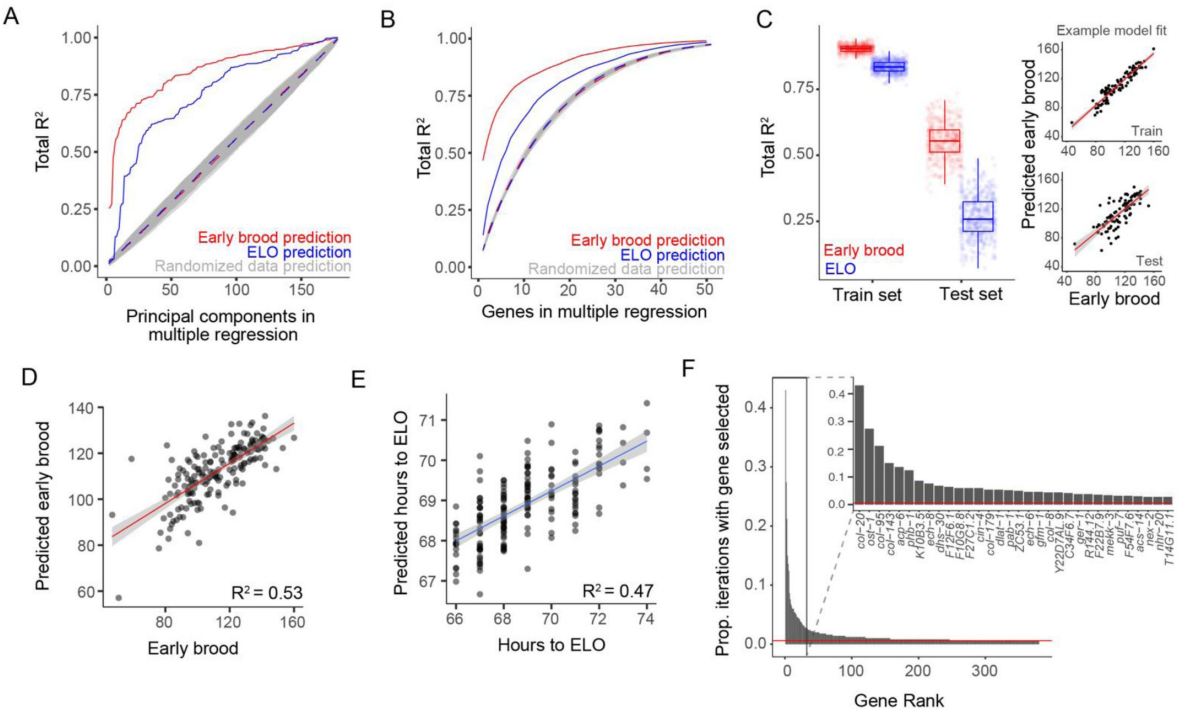


Figure 3.

Multi-gene models are highly predictive of early brood and egg-laying onset at an individual level.

A. Total R^2 of gene expression principal components (PCs) in a multiple regression. PCs explain early brood (red), ELO (blue), and randomized data (dotted lines and gray shading).
B. Total R^2 of top genes in a multiple regression. Gene sets explain early brood (red), ELO (blue), and randomized data (dotted lines and gray shading).
A-B. Dotted lines represent average of randomized data iterations and gray shading indicates standard deviation.
C. Total R^2 of top 10 genes in a multiple regression identified in a train set and total R^2 of the same 10 genes used in a test set. Train and test sets randomly selected 500 times and top 10 genes identified in each iteration. Box plot center lines indicate median and box limits indicate upper and lower quartiles. An example of one iteration of train and test sets and corresponding model fits are shown for early brood.
D-E. Machine learning model elastic net regression and leave-one-out cross validation used to identify a predicted trait for each worm given transcriptomic profile and compared to true trait data. Linear fit used to determine R2. Lines indicate linear fit, and gray indicates 95% confidence intervals.
D. Early brood model, E. ELO model
F. Proportion of 500 iterations from C in which a given gene is selected as one of the top 10 predictive genes for early brood, ordered by those selected from most to least often, and an inset showing the genes that are most frequently selected as predictive.

Therefore, this approach prioritizes identifying genes that are most predictive in conjunction with other genes. We found relatively small sets of genes predict ELO and early brood phenotypes very well, and do so significantly better than randomized trait data, with diminishing returns for variance explained as the number of genes increases (**Fig. 3B**). Maximum differences in the proportion of variance explained between trait data and randomized data occur in a model with only 4 genes for the early brood analysis (R^2 difference of 0.433) and with 7 genes in the egg-laying onset analysis (R^2 difference of 0.195). While less powerful than utilizing the entire transcriptome, this result highlights that individual worm gene expression profiles consisting of only a handful of key selected genes are highly informative for predicting the individual's traits.

We next wanted to develop a quantitative estimate of how much trait variation is explained by aggregate gene expression profiles using two distinct approaches that prevent model over-fitting and would therefore be more feasible to use for trait prediction in a new dataset. First, as an extension of the multiple regression analysis identifying predictive genes, we randomly split the full dataset (consisting of trait data and gene expression profiles) into a training set and test set. In the training set, we used the multiple regression approach to identify the set of 10 genes that explained the most variance in the trait data. We then asked how well this set of 10 genes explained variance in the test set and repeated this process 500 times, each time randomly splitting the data into training and test sets (**Fig. 3C**). We found that median proportion of variance explained in the test sets was 0.55 (range 0.36 to 0.73) for early brood and 0.26 (range 0.07 to 0.49) for ELO, and an example of the fit of one of these iterations is also shown (**Fig. 3C**).

Thus, sets of only 10 genes explain over half of trait variation in early brood and over a quarter of variation in ELO using test sets of data that were not used to select predictive genes. All 500 sets of 10 predictive genes are available as part of Supplementary File 1. As a second approach, we used elastic net regression and leave-one-out cross validation on the full set of expressed genes. In other words, we used a machine learning approach designed to prevent overfitting to develop a model for all individuals except one, used the model to predict the trait for the remaining individual, then repeated for all individuals. Consistent with the multiple regression approach, the proportion of variance explained was 0.53 for early brood and 0.47 for ELO (**Fig. 3D-E**). Aggregate gene expression profiles identified via multiple approaches are thus highly predictive of reproductive traits among isogenic individuals in a shared environment, reliably explaining about half of reproductive trait variation among genetically identical individuals.

A key remaining question is whether predictive genes are causal to affect phenotypes and whether similar underlying regulation underlies expression variation in predictive genes. To address causality, we focused on the early brood trait because predictive genes explained a higher proportion of variance for this trait compared to ELO. We used RNA interference (RNAi) to reduce the level of gene expression of predictive genes and prioritized genes that were also in the 448 brood-associated genes, either highly independent of parental age or dependent on parental age, and have an ortholog in humans. Within this curated set, genes causally affected early brood in 5 of 7 cases compared to empty vector (**Fig. 4A**). We followed up on one pair of genes from this screen, *puf-5* and *puf-7*, to ask whether perturbing the dose of RNAi shows a dose response effect on brood. These genes have previously been shown to act redundantly in fertility in *C. elegans*[30,31] and when knocked down simultaneously in our assay have a large effect on brood without causing sterility (**Fig. 4A**). Consistent with the idea that differences in the level of mRNA affect the early brood trait, the dose of RNAi corresponds to the effect size on brood (**Fig. 4B**). Thus, this analysis reveals that the predictive genes also make excellent candidates as causal effectors of complex traits and can affect these traits dependent on the amount of mRNA available.

Expression of predictive genes can be causally related to reproductive traits (**Fig. 4A-B**), but do these genes share underlying regulation? The majority of *C. elegans* genes fall into one of two chromatin domains with distinct levels of gene activity[32–34]. Regulated domains include genes

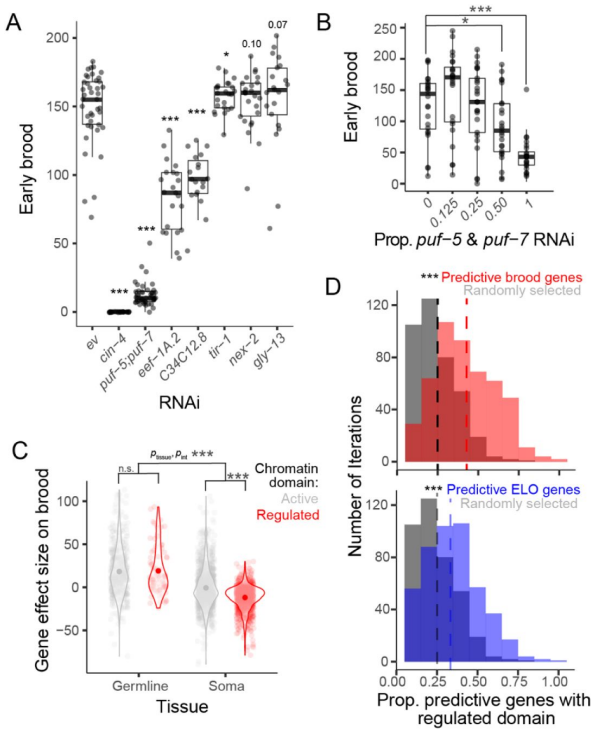


Figure 4.

Genes predictive of reproductive traits causally affect early brood and are enriched for H3K27me3

A. RNAi knockdown of selected predictive genes compared to empty vector (ev) and effects on early brood.
B. Dose response of *puf-5* and *puf-7* RNAi together with empty vector and effects on early brood.
A-B. Linear mixed model with RNAi as fixed effect and biological replicate as a random effect. Center lines, median; box limits, upper and lower quartiles. *p<0.05, ***p<0.001
C. Effect sizes on brood for genes categorized by tissue (soma or germline) and chromatin domain (active or regulated). Two-way ANOVA showed significant interaction between tissue and chromatin domain ($p = 1.58 \times 10^{-6}$) and a significant main effect of tissue ($p < 2 \times 10^{-16}$). Post-hoc Tukey tests corrected for multiple testing showed a significant difference between chromatin domains within somatic tissues (adjusted p = 0) but not germline tissues (adjusted p = 0.98). Within somatic genes, active somatic genes effects on average do not differ from 0 (p = 0.3, one-sample t-test with μ = 0), while regulated somatic genes on average have a significant negative association with brood ($p < 2.2 \times 10^{-16}$, one-sample t-test with μ = 0). ***p<0.001
D. Histogram of all iterations identifying predictive gene sets and the proportion of each set in a regulated chromatin domain marked with H3K27me3 compared to randomly selected sets of genes from the same background set. Early brood iterations are shown in pink, ELO in blue, and the same gray control is shown for both histograms. The vertical lines are color-coded to indicate the median proportion of genes in regulated domains in the corresponding condition. Kolmogorov-Smirnov test used to determine statistical significance. ***p<0.001

that are expressed at specific stages of development and are marked by the facultative heterochromatin modification H3K27me3. In contrast, active domains include genes that are expressed broadly across cell types, including in the germline, and marked by the euchromatic modification H3K36me3[32,34,35]. These two marks are generally mutually exclusive, and marks are robust across several stages of development in *C. elegans*[34] despite major changes in gene expression that occur during this time. Importantly, the robustness of these chromatin marks throughout development means that we can use existing data to determine whether genes in active or regulated domains are differentially associated with gene expression variation linked to reproductive traits in our study. We therefore used genes previously classified as regulated or active to gain insights into the epigenetic status of genes associated with and predictive of reproductive traits.

Because active genes are enriched for being expressed in the germline, we performed a two-factor analysis that takes into account both tissue (soma or germline) and chromatin domain (regulated and active). Among early brood-associated genes, germline genes are strongly positively associated with brood, regardless of chromatin environment, but chromatin environment and tissue also exhibit a significant interaction (**Fig. 4C**). Post-hoc pairwise comparisons reveal that this interaction is driven by differences in the chromatin environment in the soma. In particular, while active somatic genes have on average no effect on brood, regulated somatic genes are strongly negatively associated. Because regulated genes are typically expressed in a temporally controlled or tissue-specific manner in the soma, this may indicate that aberrant expression of these genes has negative consequences for reproduction, or similarly, that appropriate regulation and silencing of these genes promotes reproduction. To determine if epigenetic regulation may underlie variation in predictive genes for both traits overall, without regard to the direction of association, we used all gene sets identified from iterations of predictive sets (**Fig. 3C,F**) to determine whether these genes are more likely to be found in regulated domains. For both reproductive traits, predictive genes were strongly over-enriched for being in a regulated domain compared to randomly selected gene sets from the same background set (**Fig. 4D**). Genes in regulated domains are more likely to be variable across developmental stages and cell types[34], and we find in our data that genes with the most expression variation across individuals unexplained by experimental factors are also more likely to be found in regulated domains (Fig. S3). Taken together, these results suggest that regulated domains are subject to gene expression variation in genetically identical individuals and that this variation is an important substrate underlying complex trait variation.

Discussion

Individuals differ from each other for a variety of traits over the course of their lives, but being able to reliably predict these differences and understand their causal basis is a challenge. Gene expression is a critical intermediate functional layer between genotype and phenotype, but the role that gene expression plays in shaping individual organismal traits independently of genetic variation and environmental factors has remained elusive. Here we effectively screened for genes that exhibit expression variation between isogenic *C. elegans* individuals and for which this variation corresponds to two critical reproductive traits – time to egg-laying onset and early brood. We identified hundreds of genes associated with these traits, differentiated between expression variation that is dependent and independent of historical environmental factors, and defined sets of highly predictive and causal genes. Predictive genes for both traits are enriched for a particular chromatin modification – H3K27me3 – highlighting the possibility that chromatin structure may underlie the potential for gene expression to vary between individuals. These findings build upon past research and suggest key areas for future work.

With our framework, we identify individual differences in gene expression that are associated with reproductive traits, and we parse whether these effects are primarily driven by noise or by known historical environmental factors. For example, *puf-5* and *puf-7* expression is highly associated with early brood after accounting for the known historical environment, and knockdown of these genes causally affects early brood in a dose-dependent manner. On the other hand, genes like C34C12.8 exhibit an overall association with early brood that is primarily driven by the effect of parental age on gene expression; nonetheless, C34C12.8 is also causally linked to early brood. Thus, by experimentally manipulating and measuring *individuals*, our approach is highly efficient in dissecting multiple sources of environmental variation — including noise and known environmental perturbations – underlying multiple traits that would otherwise be obscured by bulk experiments. In both cases, the mechanism(s) generating gene expression differences across individuals remains unknown. For 'noise' genes in particular, whether differences in gene expression are driven by extrinsic or intrinsic factors is unclear. While every effort was made to control the environment for our experiment, isogenic individuals nonetheless exhibit some extrinsic differences in their lives, such as how they choose to wander on a plate of food, that may to some extent impact gene expression and reproduction. We further expect that some of the variation observed is intrinsic, based on literature exploring the stochastic nature of transcription[36], as well as examples that suggest the amount of phenotypic variation present among individuals of a given genetic background is itself genetically controlled[37]. Continued experimentation to determine the extent to which intrinsic or extrinsic factors regulate gene expression variability in this context — where such variability is directly related to complex phenotypes — is of high interest for future work.

When genes expression differences are identified across individuals by single-worm mRNA-seq, such differences could occur because individuals have different numbers of cells of a given type (e.g., germ cells) but each cell express genes at consistent levels, or alternatively, that individuals differ because within each cell, additional copies of a gene are being transcribed while cell numbers themselves remain constant. Of course, a combination of these scenarios is possible as well. *C. elegans* uniquely possess an invariant somatic cell lineage[38], but can exhibit differences in cell number in the adult germline, including in the primordial zone[39] or in the uterus [40]. Our results show that, in general, genes expressed in the germline tend to be positively associated with early brood, while somatic genes tend to be negatively associated with brood. Of somatic genes, genes marked with H3K27me3 are particularly predictive of early brood. Somatic genes marked with H3K27me3 are also more likely to exhibit gene expression variability in general. Given that the somatic lineage is invariant and somatic genes marked with H3K27me3 are the most variable in expression and most predictive of early brood, it is unlikely that cell number differences are the major driver of gene expression differences. While single-cell RNAseq has been performed in *C. elegans*, such analysis requires thousands of individuals to be pooled to obtain single cells and is not technically possible at the level of single individuals. Thus, to fully understand cell-type specific variation in single *C. elegans*, and how these relate to individuals' traits, further innovation on this front is required.

We observed that differences in chromatin domains are associated with differences in gene expression and traits across individuals. Regulated genes are defined by the histone modification H3K27me3, a mark that is robust across developmental stages despite differences in gene expression, while active genes are marked with H3K36me3 and are typically broadly expressed, including in the germline. In our data, regulated genes are enriched both among genes with the most unexplained variation and among genes for which variation is predictive of reproductive traits. In contrast, germline genes that predominantly consist of genes in active domains are specifically positively associated with brood. Taken together, one possible model is that regulated genes marked with H3K27me3 are more subject to inter-individual variation than genes marked with H3K36me3, which may lead to differences in gene expression without changing the levels of these marks. This is consistent with observations that regulated genes exhibit a higher coefficient of variation across conditions and that increased expression of genes marked with H3K27me3 is

not associated with a change in histone post-translational modifications (*e.g.*, to an active mark like H3K36me3)[41]. An alternative hypothesis is that the levels of the marks themselves vary and that this leads to changes in gene expression across individuals. While it is clear that regulated genes are more subject to inter-individual variability in expression, at this stage it is unknown whether this variability is driven by differences in the level of chromatin marks. Performing analysis on histone modifications genome-wide in individual worms, which is still technically challenging, will be a key next step to determine whether differences in chromatin structure correlate with gene expression changes at an individual level.

Individual gene expression differences underlying reproductive traits suggest that gene expression differences may be important for a variety of other complex traits. Gene expression differences among isogenic individuals in a shared environment have been previously documented as predictive of lifespan[42–44]. In these studies, reporter gene expression for specific candidate genes predicted individuals' later lifespan. Our results extend on such studies by identifying predictive genes in the context of reproductive traits in a high-throughput manner, rather than relying on specific reporters. Our approach can in theory be used directly in the context of other traits that occur throughout the life cycle, such as those related to development, but must be used indirectly for time-to-death traits like lifespan or stress resistance. Because a worm, once collected for RNA, cannot be subsequently phenotyped for traits like lifespan, using predictive reporters for time-to-death traits in conjunction with transcriptomics will likely be a useful approach for identifying novel regulators for a broad swath of traits.

Genetically identical individuals can have large differences in phenotype if gene expression varies across those individuals. Our work illustrates that differences in gene expression can be causally linked to, and are highly predictive of, important reproductive phenotypes among genetically identical individuals.

Assaying isogenic individuals for both organismal and molecular phenotypes enables us to uniquely understand the basis of non-genetic variation underlying individual differences and provides an avenue for high-throughput phenotyping by transcriptomics[17]. Given the major role of non-genetic factors in regulating complex traits and diseases in humans, identification of these molecular drivers of variation may be a powerful next step for advancing precision medicine.

Supplementary figures

Methods

Strains used

Strain N2-PD1073 was used for all experiments. N2-PD1073 is a subclone of the N2-derivative strain VC2010 that was generated in the process of assembling a new N2 reference. Worms were passaged on *E. coli* OP50 except during RNAi experiments, when worms were fed HT115 bacteria expressing dsRNA for genes of interest (see 'RNAi' section).

Experimental set-up for single-worm RNA-seq

Worms were well-fed at 20°C for several generations prior to beginning experiments. The experiment takes place over the course of 10 days. On day 1, for each of 5 biological replicates, P0 embryos and L4s were picked to separate 5 cm NGM plates with OP50 and kept at 20°C. Approximately 10-20 individuals were placed on each plate and several plates were picked for each stage, ensuring that worms do not experience dietary restriction. On day 4, P0 worms were either in the first day of adulthood ('young adult') or third day of adulthood ('older adult'). P0

Figure S1.

Isogenic worms at the same developmental stage exhibit variability in egg-laying onset and early brood that partially depends on previous environmental conditions

A. Univariate histograms of early brood and egg-laying onset trait values for all worms
B. Bivariate scatterplots of early brood and egg-laying onset trait for all worms. The same data is plotted twice but color-coded according to two previous environmental perturbations (age of parent and early-life temperature). Center lines for box plots indicate median; box limits indicate upper and lower quartiles. Significance determined using a linear mixed model with environmental perturbation as a fixed effect, ***p<0.001, *p<0.05

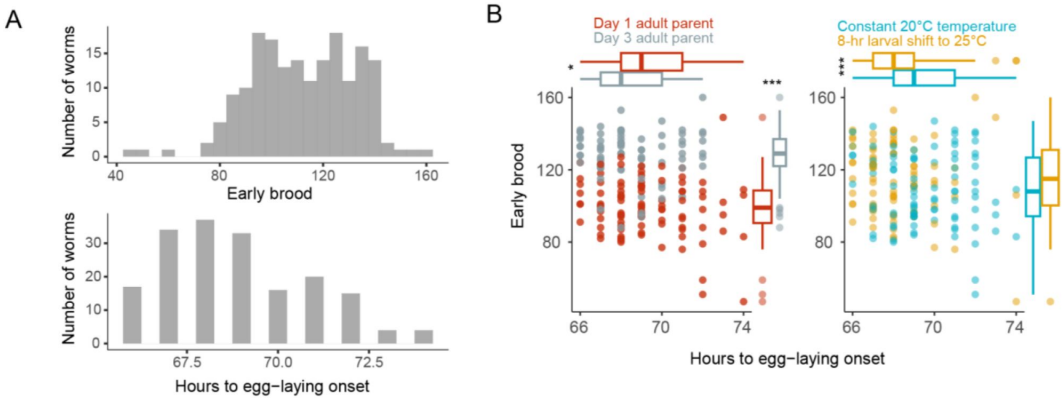
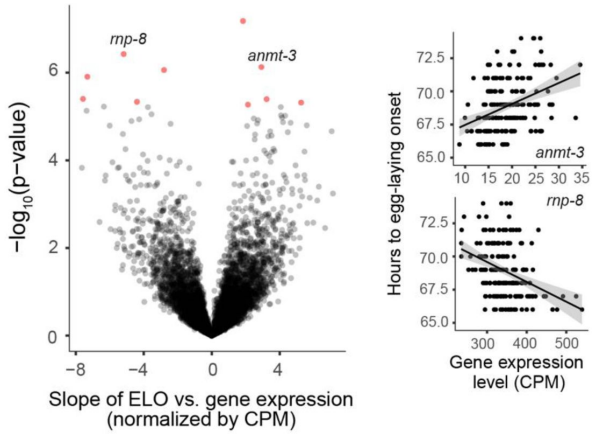


Figure S2.

Variation in 11 genes is significantly associated with egg-laying onset.

Each of 8824 expressed genes was analyzed in a mixed model to assess the strength of its association with egg-laying onset. Significant genes are in red, non-significant genes are in black. Significance determined by a Bonferroni-corrected p-value cutoff of 0.05 (nominal p-value of 5.67×10^{-6}). Phenotypic and expression data for each worm is shown for two exemplar genes, *anmt-3* and *rnp-8*. Black lines from linear model fit and gray shading indicates 95% confidence intervals.



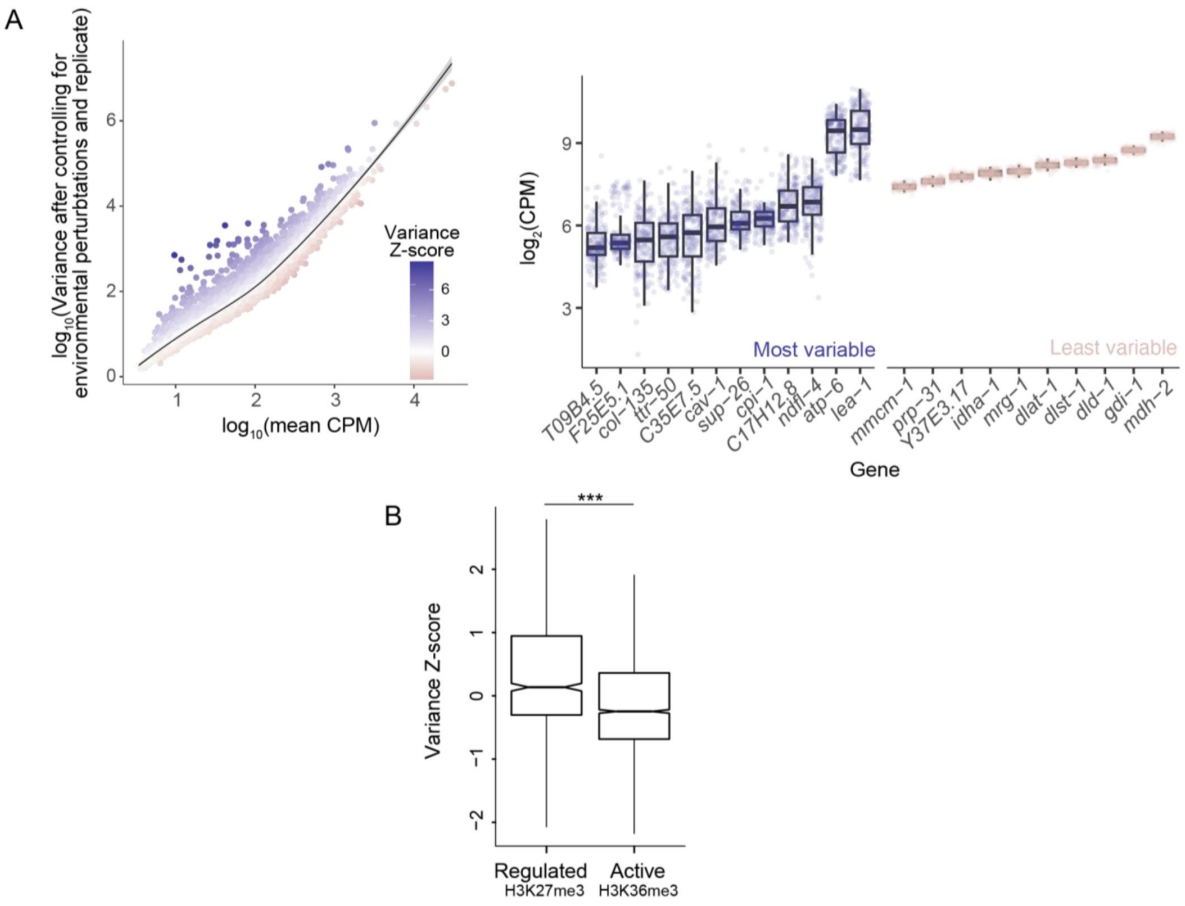


Figure S3.

Single-individual mRNA-seq reveals most and least variable genes after controlling for environmental perturbations and biological replicate.

A. Log_{10}-transformed unexplained variance in mRNA-seq after controlling for environmental factors and biological replicate is plotted against log_{10}-transformed average CPM across all worms and a loess fit line is shown in black with a 95% confidence interval in gray. Each point is a different gene and genes are color-coded according to their distance from the fit line. Genes in purple are most variable given their level of expression, while genes in pink are the least variable. Exemplar genes with comparable expression levels are shown on the right. Center lines for box plots indicate median; box limits indicate upper and lower quartiles.

B. Variance Z-scores for genes with regulated domains (H3K27me3) compared to variance Z-scores for genes with active domains (H3K36me3). Wilcoxon test, ***$p < 2 \times 10^{-16}$

young adults and older adults were singled to new plates, allowed to lay progeny for two hours at 20°C, then removed from the plate. Hour 0 of development for F1 worms is considered the end of this egg-laying window. F1 worms were then incubated at 20°C until day 5 of the experiment (undergoing approximately 18 hours of development as L1/L2 larvae). On day 5, approximately half of these larvae remained at 20°C while the other half were shifted to 25°C for 8 hours then shifted back to 20°C. On day 6 all worms remained at 20°C and were singled to individual NGM plates with OP50 to ensure they can be individually phenotyped for reproductive traits. On day 7, from 66-74 hours of development, egg-laying onset was assessed every hour by checking for embryos on each plate. Worms that died, bagged, or otherwise did not reach egg-laying onset in this time interval were not included in subsequent analysis, and all together this affected 6% of worms across all biological replicates. Time to egg-laying onset was noted for each worm. 24 hours after egg-laying onset, on day 8, each worm was collected for cell lysate generation for mRNA-seq (see 'Cell lysate generation' section of Materials and Methods). On day 10, the number of progeny laid on each plate during the 24 hours between egg-laying onset and collection were counted as late larvae/young adults to ensure all progeny were counted. All together, each F1 worm collected experienced two environmental perturbations (young adult or older adult parent, and early-life constant temperature or temperature shift) and was measured for two traits (egg-laying onset time and number of progeny produced within 24 hours) prior to mRNA-seq.

Cell lysate generation

Individual worms were collected for chemical lysis 24 hours after egg-laying onset (that is, worms that reached egg-laying onset at 72 hours were collected at 96 hours, worms reaching egg-laying onset at 73 hours were collected at 97 hours, etc. for each hour). 200 µL of lysis mix, consisting of 187.2 µL Elution buffer (10 mM Tris, 0.1 mM EDTA) and 12.8 µL Proteinase K, was made fresh every 2-3 hours on ice. 6 µL of lysis mix was pipetted into each PCR tube according to how many worms needed to be collected in a given hour. Each worm was picked into a 10 µL drop of dH2O for a quick wash to remove any excess bacteria, then transferred to a second drop of dH2O. After this, each worm was picked into an individual PCR tube with lysis mix. Immediately after worms are placed into tubes with lysis mix, we proceeded with the lysis protocol (typically no more than a dozen worms were prepared at once). The lysis protocol is 65°C for 10 min, 85°C for 1 min, and 4°C hold and is based on a previously published protocol[22]. After the lysis protocol, worms were moved to ice and then stored at –80°C until mRNA-seq library preparation.

Preparation and sequencing of mRNA-seq libraries

The KAPA mRNA-seq preparation kit (KK8580, Roche Sequencing Solutions) was used to prepare libraries. 5 µL of each cell lysate preparation described above, which contains total RNA, was used for each sample. Reagents were used at 0.25X and kit instructions were followed to generate libraries. Library concentrations were quantified using Qubit, and samples were pooled together in equimolar ratios in two batches of 96 for sequencing. Custom dual-indexed adapters were designed by the University of Oregon Genomics and Cell Characterization Core Facility and sequencing data was generated with the Illumina NovaSeq 6000 at the same facility.

Analysis of single-worm mRNA-seq data

FASTQ files were aligned to WBcel235 version of the *C. elegans* genome using subread-align[45] with flag –t 0. Bam files were counted using featureCounts with flag –T 5. 12 of 192 samples were filtered out at this stage due to particularly low read counts (less than 1 million reads) and/or poor mapping quality (less than 70% of reads both mapped to the genome and assigned to a genomic feature). The remaining 180 samples had an average of 5.8 million reads (SD 3.4 million), and an average of 85.8% (SD 3.1%) of reads both mapping to the genome and assigned to a genomic feature. Resulting count data from these 180 samples was CPM-normalized in R using the package edgeR[46] and only protein-coding genes were included. Depending on the downstream analysis, genes were filtered in one of two ways for further analysis: 1) genes expressed at a level of CPM >

10 in at least one library (8824 genes) or 2) genes expressed at a level of CPM > 1 across all libraries (7938 genes). While these two sets are largely overlapping, the first way of filtering allows identification of genes that are not expressed at all in some samples but moderately expressed in others. However, as models become more complex, having some samples with 0 expression causes problems for model fitting and thus having a more stringently-defined set is more appropriate. To identify genes associated with early brood and egg-laying onset, we looped through the set of 8824 genes and tested two linear mixed models using the 'nlme' package[47] in R to test the effect of each gene on each of two reproductive phenotypes. In both cases the fixed effect was the normalized gene expression level in each worm and the random effect was biological replicate. For the first model, the dependent variable was early brood and for the second model the dependent variable was hours to egg-laying onset. To determine the effect of environmental perturbations on gene expression, we looped through each gene and fit a negative binomial mixed model to each gene. In this case, for each gene, fixed effects included environmental perturbations (parental age and early-life temperature), the random effect included biological replicate, and the dependent variable was gene expression level. For both sets of models, the summary() function was used to generate p-values, and a Bonferroni correction was used to determine genes with significant effects. Gene expression variation that was unexplained by the negative binomial (residual variance compared to the model) was used to determine the most and least variable genes in the data after controlling for environmental effects and biological replicate. Because variance increases with higher expression, we identified most and least variable genes by fitting a loess model to $\log_{10}$(unexplained variance) vs. $\log_{10}$(mean CPM) for the 7938 genes. Z-scores were calculated for each gene relative to the loess fit to determine genes that were most and least variable given their level of gene expression.

Path analysis and effect size calculations

Using the R package 'lme4'[48], for each individual *i* and biological replicate *j*, the following linear mixed models were fit for each gene *k*:

$$(\text{Early brood})_{ij} = \beta_0 + \beta_1(\text{Expression level of gene } k) + u_j + e_{ij}$$

$$(\text{Early brood})_{ij} = \beta_0 + \beta_2(\text{Expression level of gene } k) + \beta_3(\text{Parental age}) + \beta_4(\text{Early-life temperature}) + u_j + e_{ij}$$

$$(\text{Egg-laying onset})_{ij} = \beta_0 + \beta_1(\text{Expression level of gene } k) + u_j + e_{ij}$$

$$(\text{Egg-laying onset})_{ij} = \beta_0 + \beta_2(\text{Expression level of gene } k) + \beta_3(\text{Parental age}) + \beta_4(\text{Early-life temperature}) + u_j + e_{ij}$$

$$(\text{Expression level of gene } k) = \beta_0 + \beta_5(\text{Parental age}) + \beta_6(\text{Early-life temperature}) + u_j + e_{ij}$$

Parental age was binarized such that progeny of day 1 adults were given the value of 1 and progeny of day 3 adults were given the value of 0. Early-life temperature was binarized such that individuals experiencing a constant 20°C temperature throughout their lives were given the value of 1 and individuals that experienced a 25°C temperature shift were given the value of 0. All values of β_n were standardized such that a 1 standard deviation change in the independent variable represents a shift in the dependent variable corresponding to the value of β_n. u_j represents the random intercept for each biological replicate, and e_{ij} is the error term. Coefficients β_1 through β_6 are shown in a path analysis format in **Figure 2A-B**, and coefficients β_1 and β_2 are shown graphically in **Figures 2C-D** for all significant genes from **Figure 1** and Figure S2. The values of all coefficients for all significant genes are in Supplementary File 1.

Gene Ontology (GO) analysis

GO Term analysis was performed using the R package 'clusterProfiler'[49]. The enrichGO function was used for each gene list with the following parameters: OrgDb = org.Ce.eg.db, keyType = "SYMBOL", ont = "BP", pAdjustMethod = "BH", qvalueCutoff = 0.05. Output for all three gene lists is included in Supplementary File 1. **Figure 2E** shows all enriched terms with a q-value < 0.01 for the list of 448 genes and any enriched terms that overlap with this set from that are enriched from the subsets of 97 parental age genes or 114 noise genes. The set of 97 genes was generated by identifying genes in the set of 448 that were significantly affected by parental age and did not have a significant value of β_2. The set of 114 genes was generated by identifying genes in the set of 448 that were not significantly affected by parental age and had a significant value of β_2.

Prediction analysis

Principal component analysis was performed on 7938 CPM-normalized genes (without regard to reproductive phenotype data). CPM values for each gene were mean-normalized and log2-transformed prior to use in the prcomp function in R. To determine whether principal components explained variance in the reproductive traits, each principal component was successively added to a multiple linear regression model, beginning with the principal component explaining the most variance in the gene expression data (*i.e.,* PC1), to determine the total variance explained by cumulative principal components. As a control comparison, phenotypic data was shuffled repeatedly (100x) and the same multiple regression analysis was performed. This analysis was performed for both the early brood trait and egg-laying onset trait.

To identify a set of genes that explains variance in each trait, all 7938 genes were looped through in a linear regression model to identify the single gene explaining the most variance. Then the remaining 7937 genes were looped to identify which gene explained the most additional variance when the first gene is already included in the model. This was repeated until the total variance explained was close to 1. This analysis was repeated for shuffled phenotypic data as a control comparison.

To determine how well sets of genes would be likely to work in a new dataset, we used two approaches. First, we randomly split the data (including matched phenotypic data and gene expression data) into two equal groups, the train set and test set, 500 times. Each time, for the train set, the protocol described above was used to identify the top 10 genes that together explain the most variance in the data using a multiple regression. This set of 10 genes was then used on the test set to determine how much variation is explained when the genes were not selected because of how well they work in this set. The typical variance explained in the test set provides an estimate of how well the genes should explain variance in a new dataset. In a second machine learning approach, we used elastic net regression using the glmnet package[50] in R with alpha = 0.5 and leave-one-out cross validation. That is, we trained the model for all data points except for one, then used the model to predict that remaining data point, and repeated for all data points.

Enrichment analysis

To determine whether brood-associated genes and variable gene sets were enriched for particular chromatin environments and tissue specificities we used our previously generated annotation for *C. elegans* transcriptional units[51]. Briefly, in this approach we used published data[32,35] to obtain tissue specificity and chromatin environment for *C. elegans* regulatory elements genome wide and then used bedtools2[52] to annotate regulatory elements to the nearest gene using the coordinates of transcriptional units extracted from the Wormbase gff3 (version 279).

To determine if there was an interaction between tissue and chromatin environment for effects on early brood (**Fig. 4C**), we restricted genes to those expressed in the soma or germline, and in either an active or regulated chromatin domain. We then performed a two-way ANOVA to determine if these factors affected the effect sizes for each gene on brood (the same effect sizes plotted in **Fig. 1B**), followed by post-doc Tukey's tests for pairwise comparisons and one-sample t-tests. To determine if gene expression variation differed by chromatin domain (Fig. S3B), we performed a Wilcoxon test comparing the variance z-scores for regulated genes to the variance z-scores for active genes.

To determine whether predictive genes were enriched for regulated or active genes as shown in **Figure 4D**, we used the 500 sets of 10 genes for each trait described in the 'Prediction analysis' section of the methods that were used to predict reproductive phenotypes (**Figure 3C**). We filtered the chromatin dataset described above to include only genes that were classified as either active or regulated. We then merged this with the background of 7938 expressed genes from our gene expression analysis. Of 10 predictive genes, a median of 7 genes were either active or regulated for each of the 500 iterations and included in subsequent analysis. For each iteration, the proportion of regulated genes was calculated. As a control, 500 random sets of 10 genes were selected from our background set, merged with the filtered chromatin data, and the proportion of regulated and active genes was determined. The distribution of the proportion of predictive regulated genes for each trait was compared to the proportion of regulated genes from random selected sets of genes using a Kolmogorov-Smirnov statistical test.

RNAi

RNAi was performed by feeding. Colonies of RNAi bacteria (*E. coli* HT115 containing a specific RNAi construct) were individually grown in 5 mL LB with carbenicillin overnight (~16-18 hours) at 37°C with shaking. RNAi bacteria was obtained from the Ahringer library[53] for empty vector (ev) and for genes of interest: ZK1127.7 (*cin-4*), F54C9.8 (*puf-5*), B0273.2 (*puf-6/7),* R03G5.1 (*eef-1A.2*), C34C12.8, F13B10.1 (*tir-1*), T07C4.9 (*nex-2*), and B0416.6 (*gly-13*). Plates used for RNAi were made with NGM, carbenicillin, and IPTG as described previously[54]. These plates were seeded with RNAi bacteria and allowed to dry and grow for ~30 hours. L4 larvae were plated in groups of ~10 on these plates, then singled the next day (~16-18 hours later) onto new RNAi plates prepared at the same time as the original batch of plates. Adult worms were allowed to lay progeny for 24 hours, then adults were removed from the plates. If a worm died during this time it was censored. The number of progeny laid by each adult was counted 2 days later, resulting in an early brood measurement for these experiments. For the *puf-5* and *puf-7* dose response, *puf-5* and *puf-7* RNAi constructs were grown separately as described then pooled together 1:1. This mixed RNAi was considered the full dose of *puf-5* and *puf-7* RNAi and was pooled with ev in the ratios shown in **Fig. 4C** for the varying doses. Statistical analysis on early brood data was performed by comparing RNAi treatment targeting a particular gene to empty vector for biological replicates within a batch. Linear mixed effects models were used in the R package 'nlme'[55] with RNAi treatment as a fixed effect and biological replicate as a random effect. The summary() function was used to generate p-values.

Validation of isogenic worms

We used the mRNA-seq data to validate that the frequency of genetic variants among the essentially isogenic individual worms was very low. First, we used samtools[56] to generate coverage files from the sorted bam files resulting from mRNA-seq data processing. We filtered coverage files for each worm to include only nucleotides that had at least 20 reads mapping to that nucleotide. For each worm and nucleotide, we determined whether the base call of that nucleotide was unambiguous (all 20+ reads have the same call), ambiguous with two genotypes (exactly two different bases were called), or ambiguous with more than two genotypes called. All worms with sufficient depth at a nucleotide were used to determine putative homozygous or heterozygous variants. A particular nucleotide was included in subsequent analysis if at least 10 worms had

sufficient coverage at the site. This resulted in 8,361,705 unique sites and a total of 768,738,255 sites across all worms. Candidate homozygous variants included nucleotides that were called as one base unambiguously in at least one worm and called as a different base unambiguously in other worms. Candidate heterozygous variants were identified by filtering ambiguous nucleotides with two genotypes in which the frequency of each genotype was present at a frequency of between 0.3 and 0.7 and for which at least 10 other worms were unambiguously called a single genotype. These criteria reduce the possibility that a heterozygous mutant is erroneously called if a site was merely subject to rare sequencing errors. This resulted in a homozygous variant rate of 2.21×10^{-8} and a heterozygous variant rate of 9.86×10^{-7}. While already quite low, these rates likely represent over-estimates because visual inspection of bam files in some cases revealed ambiguous base calls. Over three quarters of sites (78.5%) with a putative heterozygous variant were found only in a single worm, meaning that these rare variants cannot account for an association with expression across 180 worms.

Statistical analysis

Statistical tests are described in detail in figure legends and in the corresponding section of the methods. All individual data points are plotted in figures whenever possible.

Data availability statement

All raw mRNA-seq data is available at NCBI GEO accession GSE244875. All data used to generate figures are available as part of Supplementary File 1.

Code availability statement

Scripts are available at *https://github.com/amykwebster/SingleWormRNAseq* and have been preserved on Zenodo at time of publication at *https://doi.org/10.5281/zenodo.15489998*.

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Additional files

Supplemental File 1

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Reviewer #1 (Public review):

Summary:

Genome-wide association studies have been an important approach to identifying the genetic basis of human traits and diseases. Despite their successes, for many traits, a substantial amount of variation cannot be explained by genetic factors, indicating that environmental variation and individual 'noise' (stochastic differences as well as unaccounted for environmental variation) also play important roles. The authors' goal was to address how gene expression variation in genetically identical individuals, driven by historical environmental differences and 'noise', could be used to predict reproductive trait differences.

Strengths:

To address this question, the authors took advantage of genetically identical C. elegans individuals to transcriptionally profile 180 adult hermaphrodite individuals that were also measured for two reproductive traits. A major strength of the paper is in its experimental design. While experimenters aim to control the environment that each worm experiences, it is known that there are small differences even when worms are grown together on the same agar plate - e.g., the age of their mother, their temperature, the amount of food they eat, and the oxygen and carbon dioxide levels depending on where they roam on the plate. Instead of neglecting this unknown variation, the authors design the experiment up front to create two differences in the historical environment experienced by each worm: 1) the age of its mother and 2) 8 8-hour temperature difference, either 20 or 25 C. This helped the authors interpret the gene expression differences and trait expression differences that they observed.

Using two statistical models, the authors measured the association of gene expression for 8824 genes with the two reproductive traits, considering both the level of expression and the historical environment experienced by each worm. Their data supports several conclusions. They convincingly show that gene expression differences are useful for predicting reproductive trait differences, predicting ~25-50% of the trait differences depending on the trait. Using RNAi, they also show that the genes they identify play a causal role in trait differences. Finally, they demonstrate an association with trait variation and the H3K27 trimethylation mark, suggesting that chromatin structure can be an important causal determinant of gene expression and trait variation.

Overall, this work supports the use of gene expression data as an important intermediate for understanding complex traits. This approach is also useful as a starting point for other labs in studying their trait of interest.

Weaknesses:

There are no major weaknesses that I have noted. Some important limitations of their work are worth highlighting, though (and I believe the authors would agree with these points):

(1) A large remaining question in the field of complex traits remains in splitting the role of non-genetic factors between environmental variation and stochastic noise. It is still an open question which role each of these factors plays in controlling the gene expression differences they measured between the individual worms.

(2) The ability of the authors to use gene expression to predict trait variation was strikingly different between the two traits they measured. For the early brood trait, 448 genes were statistically linked to the trait difference, while for egg-laying onset, only 11 genes were found. Similarly, the total R2 in the test set was ~50% vs. 25%. It is unclear why the differences occur, but this somewhat limits the generalizability of this approach to other traits.

(3) For technical reasons, this approach was limited to whole worm transcription. The role of tissue and cell-type expression differences is important to the field, so this limitation is relevant.

Comments on revisions: The authors have addressed my previous comments to my satisfaction.

https://doi.org/10.7554/eLife.106525.2.sa3

Reviewer #2 (Public review):

This paper measures associations between RNA transcript levels and important reproductive traits in the model organism C. elegans. The authors go beyond determining which gene expression differences underlie reproductive traits, but also (1) build a model that predicts these traits based on gene expression and (2) perform experiments to confirm that some transcript levels indeed affect reproductive traits. The clever study design allows the authors to determine which transcript levels impact reproductive traits, and also which transcriptional differences are driven by stochastic vs environmental differences. In sum, this is a comprehensive study that highlights the power of gene expression as a driver of phenotype, and also teases apart the various factors that affect the expression levels of important genes.

Overall, this study has many strengths, is very clearly communicated, and has no substantial weaknesses that I can point to.

One question that emerges for me is whether these findings apply broadly. In other words, I wonder whether gene expression levels are predictive of other phenotypes in other organisms. I think this question has largely been explored in microbes, where some studies (PMID: 17959824) but not others (PMID: 38895328) found that differences in gene expression were predictive of phenotypes like growth rate. Microbes are not the focus here, and instead, the discussion is mainly focused on using gene expression to predict health and disease phenotypes in humans. This feels a little complicated since humans have so many different tissues. Perhaps an area where this approach might be useful is in examining infectious single-cell populations (bacteria, tumors, fungi). But I suppose this idea might still work in humans, assuming the authors are thinking about targeting specific tissues for RNAseq.

In sum, this is a great paper that really got me thinking about the predictive power of gene expression and where/when it could inform about (health-related) phenotypes.

Comments on revisions: No additional comments

https://doi.org/10.7554/eLife.106525.2.sa2

Reviewer #3 (Public review):

Summary:

Webster et al. sought to understand if phenotypic variation in the absence of genetic variation can be predicted by variation in gene expression. To this end they quantified two reproductive traits, the onset of egg laying and early brood size in cohorts of genetically identical nematodes exposed to alternative ancestral (two maternal ages) and same generation life histories (either constant 20 ºC temperature or 8-hour temperature shift to 25 ºC upon hatching) in a two-factor design; then, they profiled genome-wide gene expression in each individual.

Using multiple statistical and machine learning approaches, they showed that, at least for early brood size, phenotypic variation can be quite well predicted by molecular variation, beyond what can be predicted by life history alone.
Moreover, they provide some evidence that expression variation in some genes might be causally linked to phenotypic variation.

Strengths:

Cleverly designed and carefully performed experiments that provide high-quality datasets useful for the community.

Good evidence that phenotypic variation can be predicted by molecular variation.

Weaknesses:

What drives the molecular variation that impacts phenotypic variation remains unknown. While the authors show that variation in expression of some genes might indeed be causal, it is still not clear how much of the molecular variation is a cause rather than a consequence of phenotypic variation.

Comments on revisions: I have no more comments for the authors

https://doi.org/10.7554/eLife.106525.2.sa1

Author Response:

The following is the authors' response to the original reviews

> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *Genome-wide association studies have been an important approach to identifying the genetic basis of human traits and diseases. Despite their successes, for many traits, a substantial amount of variation cannot be explained by genetic factors, indicating that environmental variation and individual 'noise' (stochastic differences as well as unaccounted for environmental variation) also play important roles. The authors' goal was to address whether gene expression variation in genetically identical individuals, driven by historical environmental differences and 'noise', could be used to predict reproductive trait differences.*
>
> *Strengths:*
>
> *To address this question, the authors took advantage of genetically identical C. elegans individuals to transcriptionally profile 180 adult hermaphrodite individuals that were*

> *also measured for two reproductive traits. A major strength of the paper is its experimental design. While experimenters aim to control the environment that each worm experiences, it is known that there are small differences that each worm experiences even when they are grown together on the same agar plate - e.g. the age of their mother, their temperature, the amount of food they eat, and the oxygen and carbon dioxide levels depending on where they roam on the plate. Instead of neglecting this unknown variation, the authors design the experiment up front to create two differences in the historical environment experienced by each worm: 1) the age of its mother and 2) 8 8-hour temperature difference, either 20 or 25 {degree sign}C. This helped the authors interpret the gene expression differences and trait expression differences that they observed.*
>
> *Using two statistical models, the authors measured the association of gene expression for 8824 genes with the two reproductive traits, considering both the level of expression and the historical environment experienced by each worm. Their data supports several conclusions. They convincingly show that gene expression differences are useful for predicting reproductive trait differences, predicting ~25-50% of the trait differences depending on the trait. Using RNAi, they also show that the genes they identify play a causal role in trait differences. Finally, they demonstrate an association with trait variation and the H3K27 trimethylation mark, suggesting that chromatin structure can be an important causal determinant of gene expression and trait variation.*
>
> *Overall, this work supports the use of gene expression data as an important intermediate for understanding complex traits. This approach is also useful as a starting point for other labs in studying their trait of interest.*

We thank the reviewer for their thorough articulation of the strengths of our study.

> *Weaknesses:*
>
> *There are no major weaknesses that I have noted. Some important limitations of the work (that I believe the authors would agree with) are worth highlighting, however:*
>
> *(1) A large remaining question in the field of complex traits remains in splitting the role of non-genetic factors between environmental variation and stochastic noise. It is still an open question which role each of these factors plays in controlling the gene expression differences they measured between the individual worms.*

Yes, we agree that this is a major question in the field. In our study, we parse out differences driven between known historical environmental factors and unknown factors, but the 'unknown factors' could encompass both unknown environmental factors and stochastic noise.

> *(2) The ability of the authors to use gene expression to predict trait variation was strikingly different between the two traits they measured. For the early brood trait, 448 genes were statistically linked to the trait difference, while for egg-laying onset, only 11 genes were found. Similarly, the total R2 in the test set was ~50% vs. 25%. It is unclear why the differences occur, but this somewhat limits the generalizability of this approach to other traits.*

We agree that the difference in predictability between the two traits is interesting. A previous study from the Phillips lab measured developmental rate and fertility across *Caenorhabditis* species and parsed sources of variation (1). Results indicated that 83.3% of variation in developmental rate was explained by genetic variation, while only 4.8% was explained by individual variation. In contrast, for fertility, 63.3% of variation was driven by genetic

variation and 23.3% was explained by individual variation. Our results, of course, focus only on predicting the individual differences, but not genetic differences, for these two traits using gene expression data. Considering both sets of results, one hypothesis is that we have more power to explain nongenetic phenotypic differences with molecular data if the trait is less heritable, which is something that could be formally interrogated with more traits across more strains.

> *(3) For technical reasons, this approach was limited to whole worm transcription. The role of tissue and celltype expression differences is important to the field, so this limitation is important.*

We agree with this assessment, and it is something we hope to address with future work.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *This paper measures associations between RNA transcript levels and important reproductive traits in the model organism C. elegans. The authors go beyond determining which gene expression differences underlie reproductive traits, but also (1) build a model that predicts these traits based on gene expression and (2) perform experiments to confirm that some transcript levels indeed affect reproductive traits. The clever study design allows the authors to determine which transcript levels impact reproductive traits, and also which transcriptional differences are driven by stochastic vs environmental differences. In sum, this is a rather comprehensive study that highlights the power of gene expression as a driver of phenotype, and also teases apart the various factors that affect the expression levels of important genes.*
>
> *Strengths:*
>
> *Overall, this study has many strengths, is very clearly communicated, and has no substantial weaknesses that I can point to. One question that emerges for me is about the extent to which these findings apply broadly. In other words, I wonder whether gene expression levels are predictive of other phenotypes in other organisms. I*
>
> *think this question has largely been explored in microbes, where some studies (PMID: 17959824) but not others (PMID: 38895328) find that differences in gene expression are predictive of phenotypes like growth rate. Microbes are not the primary focus here, and instead, the discussion is mainly focused on using gene expression to predict health and disease phenotypes in humans. This feels a little complicated since humans have so many different tissues. Perhaps an area where this approach might be useful is in examining infectious single-cell populations (bacteria, tumors, fungi). But I suppose this idea might still work in humans, assuming the authors are thinking about targeting specific tissues for RNAseq.*
>
> *In sum, this is a great paper that really got me thinking about the predictive power of gene expression and where/when it could inform about (health-related) phenotypes.*

We thank the reviewer for recognizing the strengths of our study. We are also interested in determining the extent to which predictive gene expression differences operate in specific tissues.

> ***Reviewer #3 (Public review):***
>
> *Summary:*

> *Webster et al. sought to understand if phenotypic variation in the absence of genetic variation can be predicted by variation in gene expression. To this end they quantified two reproductive traits, the onset of egg laying and early brood size in cohorts of genetically identical nematodes exposed to alternative ancestral (two maternal ages) and same generation life histories (either constant 20C temperature or 8-hour temperature shift to 25C upon hatching) in a two-factor design; then they profiled genome-wide gene expression in each individual.*
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> *Using multiple statistical and machine learning approaches, they showed that, at least for early brood size, phenotypic variation can be quite well predicted by molecular variation, beyond what can be predicted by life history alone.*
>
> *Moreover, they provide some evidence that expression variation in some genes might be causally linked to phenotypic variation.*
>
> *Strengths:*
>
> *(1) Cleverly designed and carefully performed experiments that provide high-quality datasets useful for the community.*
>
> *(2) Good evidence that phenotypic variation can be predicted by molecular variation.*

We thank the reviewer for recognizing the strengths of our study.

> *Weaknesses:*
>
> *What drives the molecular variation that impacts phenotypic variation remains unknown. While the authors show that variation in expression of some genes might indeed be causal, it is still not clear how much of the molecular variation is a cause rather than a consequence of phenotypic variation.*

We agree that the drivers of molecular variation remain unknown. While we addressed one potential candidate (histone modifications), there is much to be done in this area of research. We agree that, while some gene expression differences cause phenotypic changes, other gene expression differences could in principle be downstream of phenotypic differences.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *I have a number of suggestions that I believe will improve the Methods section.*
>
> *(1) Strain N2-PD1073 will probably be confusing to some readers. I recommend spelling out that this is the Phillips lab version of N2.*

Thank you for this suggestion; we have added additional explanation of this strain in the Methods.

> *(2) I found the details of the experimental design confusing, and I believe a supplemental figure will help. I have listed the following points that could be clarified:*
>
> *a. What were the biological replicates? How many worms per replicate?*

Biological replicates were defined as experiments set up on different days (in this case, all biological replicates were at least a week apart), and the biological replicate of each worm can be found in Supplementary File 1 on the Phenotypic Data tab.

> *b. I believe that embryos and L4s were picked to create different aged P0s, and eggs and L4s were picked to separate plates? Is this correct?*

Yes, this is correct.

> *c. What was the spread in the embryo age?*

We assume this is asking about the age of the F1 embryos, and these were laid over the course of a 2-hour window.

> *d. While the age of the parents is different, there are also features about their growth plates that will be impacted by the experimental design. For example, their pheromone exposure is different due to the role that age plays in the combination of ascarosides that are released. It is worth noting as my reading of the paper makes it seem that parental age is the only thing that matters.*

The parents (P0) of different ages likely have differential ascaroside exposure because they are in the vicinity of other similarly aged worms, but the F1 progeny were exposed to their parents for only the 2-hour egg-laying window, in an attempt to minimize this type of effect as much as possible.

> *e. Were incubators used for each temperature?*

Yes.

> *f. In line 443, why approximately for the 18 hours? How much spread?*

The approximation was based on the time interval between the 2-hour egg-laying window on Day 4 and the temperature shift on Day 5 the following morning. The timing was within 30 minutes of 18 hours either direction.

> *g. In line 444, "continually left" is confusing. Does this mean left in the original incubator?*

Yes, this means left in the incubator while the worms shifted to 25°C were moved. To avoid confusion, we re-worded this to state they “remained at 20°C while the other half were shifted to 25°C”.

> *h. In line 445, "all worms remained at 20 {degree sign}C" was confusing to me as to what it indicated. I assume, unless otherwise noted, the animals would not be moved to a new temperature.*

This was an attempt to avoid confusion and emphasize that all worms were experiencing the same conditions for this part of the experiment.

> *i. What size plates were the worms singled onto?*

They were singled onto 6-cm plates.

> *j. If a figure were to be made, having two timelines (with respect to the P0 and F1) might be useful.*

We believe the methods should be sufficient for someone who hopes to repeat the experiment, and we believe the schematic in Figure 1A labeling P0 and F1 generations is sufficient to illustrate the key features of the experimental design.

> *k. Not all eggs that are laid end up hatching. Are these censored from the number of progeny calculations?*

Yes, only progeny that hatched and developed were counted for early brood.

> *(3) For the lysis, was the second transfer to dH20 also a wash step?*

Yes.

> *(4) What was used for the Elution buffer?*

We used elution buffer consisting of 10 mM Tris, 0.1 mM EDTA. We have added this to the "Cell lysate generation" section of the methods

> *(5) The company that produced the KAPA mRNA-seq prep kit should be listed.*

We added that the kit was from Roche Sequencing Solutions.

> *(6) For the GO analysis - one potential issue is that the set of 8824 genes might also be restricted to specific GO categories. Was this controlled for?*

We originally did not explicitly control for this and used the default enrichGO settings with OrgDB = org.Ce.eg.db as the background set for *C. elegans*. We have now repeated the analysis with the "universe" set to the 8824-gene background set. This did not qualitatively change the significant GO terms, though some have slightly higher or lower p-values. For comparison purposes, we have added the background-corrected sets to the GO_Terms tab of Supplementary File 1 with each of the three main gene groups appended with "BackgroundOf8824".

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) The abstract, introduction, and experimental design are well thought through and very clear.*

Thank you.

> *(2) Figure 1B could use a clearer or more intuitive label on the horizontal axis. The two examples help. Maybe the genes (points) on the left side should be blue to match Figure 1C, where the genes with a negative correlation are in the blue cluster.*

Thank you for these suggestions. We re-labeled the x-axis as "Slope of early brood vs. gene expression (normalized by CPM)", which we hope gives readers a better intuition of what the coefficient from the model is measuring. We also re-colored the points previously colored red in Figure 1B to be color-coded depending on the direction of association to match Figure 1C, so these points are now color-coded as pink and purple.

> *(3) If red/blue are pos/neg correlated genes in 1C, perhaps different colors should be used to label ELO and brood in Figures 2 and 3. Green/purple?*

We appreciate this point, but since we ended up using the cluster colors of pink and purple in Figure 1, we opted to leave Figures 2 and 3 alone with the early brood and ELO colorcoding of red and blue.

> *(4) I am unfamiliar with this type of beta values, but I thought the explanation and figure were very clear. It could be helpful to bold beta1 and beta2 in the top panels of Figure 2, so the readers are not searching around for those among all the other betas. It could also be helpful to add an English phrase to the vertical axes inFigures 2C and 2D, in addition to the beta1 and beta2. Something like "overall effect (beta1)" and"environment-controlled effect (beta2)". Or maybe "effect of environment + stochastic expression differences*
>
> *(beta1)" and "effect of stochastic expression differences alone (beta2)". I guess those are probably too big to fit on the figure, but it might be nice to have a label somewhere on this figure connecting them to the key thing you are trying to measure - the effect of gene expression and environment.*

Thank you for these suggestions. We increased the font sizes and bolded β1 and β2 in Figure 2A-B. In Figure 2C-D, we added a parenthetical under β1 to say “(env + noise)” and β2 to say “(noise)”. We agree that this should give the reader more intuition about what the β values are measuring.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *The authors collected individuals 24 hours after the onset of egg laying for transcriptomic profiling. This is a well-designed experiment to control for the physiological age of the germline. However, this does not properly control for somatic physiological age. Somatic age can be partially uncoupled from germline age across individuals, and indeed, this can be due to differences in maternal age (Perez et al, 2017). This is because maternal age is associated with increased pheromone exposure (unless you properly controlled for it by moving worms to fresh plates), which causes a germline-specific developmental delay in the progeny, resulting in a delayed onset of egg production compared to somatic development (Perez et al. 2021). You control for germline age, therefore, it is likely that the progeny of day 1 mothers are actually somatically older than the progeny of day 3 mothers. This would predict that many genes identified in these analyses might just be somatic genes that increase or decrease their expression during the young adult stage.*
>
> *For example, the abundance of collagen genes among the genes negatively associated (including col-20, which is the gene most significantly associated with early brood) is a big red flag, as collagen genes are known to be changing dynamically with age. If variation in somatic vs germline age is indeed what is driving the expression variation of these genes, then the expectation is that their expression should decrease with age. Vice versa, genes positively associated with early brood that are simply explained by age should be increasing. So I would suggest that the authors first check this using time series transcriptomic data covering the young adult stage they profiled. If this is indeed the case, I would then suggest using RAPToR (https://github.com/LBMC/RAPToR), a method that, using reference time series data, can estimate physiological age (including tissue-specific one) from gene expression. Using this method they can estimate the somatic physiological age of their samples, quantify the extent of variation in somatic age across individuals, quantify how much of the observed differences in expressions are explained just by differences in somatic age and correct for them during their transcriptomic analysis using the estimated soma age as a covariate (https://github.com/LBMC/RAPToR/blob/master/vignettes/RAPToR-DEcorrection-pdf.pdf).*

> *This should help enrich a molecular variation that is not simply driven by hidden differences between somatic and germline age.*

To first address some of the experimental details mentioned for our paper, parents were indeed moved to fresh plates where they were allowed to lay embryos for two hours and then removed. Thus, we believe this minimizes the effects of ascarosides as much as possible within our design. As shown in the paper, we also identified genes that were not driven by parental age and for all genes quantified to what extent each gene's association was driven by parental age. Thus, it is unlikely that differences in somatic and germline age is the sole explanatory factor, even if it plays some role. We also note that we accounted for egg-laying onset timing in our experimental design, and early brood was calculated as the number of progeny laid in the first 24 hours of egg-laying, where egg-laying onset was scored for each individual worm to the hour. The plot of each worm's ELO and early brood traits is in Figure S1. Nonetheless, we read the RAPToR paper with interest, as we highlighted in the paper that germline genes tend to be positively associated with early brood while somatic genes tend to be negatively associated. While the RAPToR paper discusses using tissue-specific gene sets to stage genetically diverse *C. elegans* RILs, the RAPToR reference itself was not built using gene expression data acquired from different *C. elegans* tissues and is based on whole worms, typically collected in bulk. I.e., age estimates in RILs differ depending on whether germline or somatic gene sets are used to estimate age when the the aging clock is based on N2 samples. Thus, it is unclear whether such an approach would work similarly to estimate age in single worm N2 samples. In addition, from what we can tell, the RAPToR R package appears to implement the overall age estimate, rather than using the tissue-specific gene sets used for RILs in the paper. Because RAPToR would be estimating the overall age of our samples using a reference that is based on fewer samples than we collected here, and because we already know the overall age of our samples measured using standard approaches, we believe that estimating the age with the package would not give very much additional insight.

> *Bonferroni correction:*
>
> *First, I think there is some confusion in how the author report their p-values: I don't think the authors are using a cut-off of Bonferroni corrected p-value of 5.7 x 10-6 (it wouldn't make sense). It's more likely that they are using a Bonferroni corrected p of 0.05 or 0.1, which corresponds to a nominal p value of 5.7 x 10-6, am I right?*

Yes, we used a nominal p-value of 5.7 x 10-6 to correspond to a Bonferroni-corrected p-value of 0.05, calculated as 0.05/8824. We have re-worded this wherever Bonferroni correction was mentioned.

> *Second, Bonferroni is an overly stringent correction method that has now been substituted by the more powerful Benjamini Hochberg method to control the false discovery rate. Using this might help find more genes and better characterize the molecular variation, especially the one associated with ELO?*

We agree that Bonferroni is quite stringent and because we were focused on identifying true positives, we may have some false negatives. Because all nominal p-values are included in the supplement, it is straightforward for an interested reader to search the data to determine if a gene is significant at any other threshold.

> *Minor comments:*
>
> *(1) "In our experiment, isogenic adult worms in a common environment (with distinct historical environments) exhibited a range of both ELO and early brood trait values (Fig S1A)" I think this and the figure is not really needed, Figure S1B is already enough to*

> *show the range of the phenotypes and how much variation is driven by the life history traits.*

We agree that the information in S1A is also included in S1B, but we think it is a little more straightforward if one is primarily interested in viewing the distribution for a single trait.

> *(2) Line 105 It should be Figure S2, not S3.*

Thank you for catching this mistake.

> *(3) Gene Ontology on positive and negatively associated genes together: what about splitting the positive and negative?*

We have added a split of positive and negative GO terms to the GO_Terms tab of Supplement File 1. Broadly speaking, the most enriched positively associated genes have many of the same GO terms found on the combined list that are germline related (e.g., involved in oogenesis and gamete generation), whereas the most enriched negatively associated genes have GO terms found on the combined list that are related to somatic tissues (e.g., actin cytoskeleton organization, muscle cell development). This is consistent with the pattern we see for somatic and germline genes shown in Figure 4.

> *(4) A lot of muscle-related GOs, can you elaborate on that?*

Yes, there are several muscle-related GOs in addition to germline and epidermis. While we do not know exactly why from a mechanistic perspective these muscle-related terms are enriched, it may be important to note that many of these terms have highly overlapping sets of genes which are listed in Supplementary File 1. For example, “muscle system process” and “muscle contraction” have the exact same set of 15 genes causing the term to be significantly enriched. Thus, we tend to not interpret having many GO terms on a given tissue as indicating that the tissue is more important than others for a given biological process. While it is clear there are genes related to muscle that are associated with early brood, it is not yet clear that the tissue is more important than others.

> *(5) "consistent with maternal age affecting mitochondrial gene expression in progeny " - has this been previously reported?*

We do not believe this particular observation has been reported. It is important to note that these genes are involved in mitochondrial processes, but are expressed from the nuclear rather than mitochondrial genome. We re-worded the quoted portion of the sentence to say “consistent with parental age affecting mitochondria-related gene expression in progeny”.

> *(6) PCA: "Therefore, the optimal number of PCs occurs at the inflection points of the graph, which is after only7 PCs for early brood (R2 of 0.55) but 28 PCs for ELO (R2 of 0.56)."*
>
> *Not clear how this is determined: just graphically? If yes, there are several inflection points in the plot. How did you choose which one to consider? Also, a smaller component is not necessarily less predictive of phenotypic variation (as you can see from the graph), so instead of subsequently adding components based on the variance, they explain the transcriptomic data, you might add them based on the variance they explain in the phenotypic data? To this end, have you tried partial least square regression instead of PCA? This should give gene expression components that are ranked based on how much phenotypic variance they explain.*

Thank you for this thoughtful comment. We agree that, unlike for Figure 3B, there is some interpretation involved on how many PCs is optimal because additional variance explained with each PC is not strictly decreasing beyond a certain number of PCs. Our assessment was therefore made both graphically and by looking at the additional variance explained with each additional PC. For example, for early brood, there was no PC after PC7 that added more than 0.04 to the R2. We could also have plotted early brood and ELO separately and had a different ordering of PCs on the x-axis. By plotting the data this way, we emphasized that the factors that explain the most variation in the gene expression data typically explain most variation in the phenotypic data.

> *(7) The fact that there are 7 PC of molecular variation that explain early brood is interesting. I think the authors can analyze this further. For example, could you perform separate GO enrichment for each component that explains a sizable amount of phenotypic variance? Same for the ELO.*

Because each gene has a PC loading in for each PC, and each PC lacks the explanatory power of combined PCs, we believe doing GO Terms on the list of genes that contribute most to each PC is of minimal utility. The power of the PCA prediction approach is that it uses the entire transcriptome, but the other side of the coin is that it is perhaps less useful to do a gene-bygene based analysis with PCA. This is why we separately performed individual gene associations and 10-gene predictive analyses. However, we have added the PC loadings for all genes and all PCs to Supplementary File 1.

> *(8) Avoid acronyms when possible (i.e. ELO in figures and figure legends could be spelled out to improve readability).*

We appreciate this point, but because we introduced the acronym both in Figure 1 and the text and use it frequently, we believe the reader will understand this acronym. Because it is sometimes needed (especially in dense figures), we think it is best to use it consistently throughout the paper.

> *(9) Multiple regression: I see the most selected gene is col-20, which is also the most significantly differentially expressed from the linear mixed model (LMM). But what is the overlap between the top 300 genes in Figure 3F and the 448 identified by the LMM? And how much is the overlap in GO enrichment?*

Genes that showed up in at least 4 out of 500 iterations were selected more often than expected by chance, which includes 246 genes (as indicated by the red line in Figure 3F). Of these genes, 66 genes (27%) are found in the set of 448 early brood genes. The proportion of overlap increases as the number of iterations required to consider a gene predictive increases, e.g., 34% of genes found in 5 of 500 iterations and 59% of genes found in 10 of 500 iterations overlap with the 448 early brood genes. However, likely because of the approach to identify groups of 10 genes that are predictive, we do not find significant GO terms among the 246 genes identified with this approach after multiple test correction. We think this makes sense because the LMM identifies genes that are individually associated with early brood, whereas each subsequent gene included in multiple regression affects early brood after controlling for all previous genes. These additional genes added to the multiple regression are unlikely to have similar patterns as genes that are individually correlated with early brood.

> *(10) Elastic nets: prediction power is similar or better than multiple regression, but what is the overlap between genes selected by the elastic net (not presented if I am not mistaken) and multiple regression and the linear mixed model?*

For the elastic net models, we used a leave-one-out cross validation approach, meaning there were separate models fit by leaving out the trait data for each worm, training a model using the trait data and transcriptomic data for the other worms, and using the transcriptomic data of the remaining worm to predict the trait data. By repeating this for each worm, the regressions shown in the paper were obtained. Each of these models therefore has its own set of genes. Of the 180 models for early brood, the median model selects 83 genes (range from 72 to 114 genes). Across all models, 217 genes were selected at least once. Interestingly, there was a clear bimodal distribution in terms of how many models a given gene was selected for: 68 genes were selected in over 160 out of 180 models, while 114 genes were selected in fewer than 20 models (and 45 genes were selected only once). Therefore, we consider the set of 68 genes as highly robustly selected, since they were selected in the vast majority of models. This set of 68 exhibits substantial overlap with both the set of 448 early brood-associated genes (43 genes or 63% overlap) and the multiple regression set of 246 genes (54 genes or 79% overlap). For ELO, the median model selected 136 genes (range of 96 to 249 genes) and a total of 514 genes were selected at least once. The distribution for ELO was also bimodal with 78 genes selected over 160 times and 255 genes selected fewer than 20 times. This set of 78 included 6 of the 11 significant ELO genes identified in the LMM. We have added tabs to Supplementary File 1 that include the list of genes selected for the elastic net models as well as a count of how many times they were selected out of 180 models.

> *(11) In other words, do these different approaches yield similar sets of genes, or are there some differences?*

In the end, which approach is actually giving the best predictive power? From the perspective of R2, both the multiple regression and elastic net models are similarly predictive for early brood, but elastic net is more predictive for ELO. However, in presenting multiple approaches, part of our goal was identifying predictive genes that could be considered the 'best' in different contexts. The multiple regression was set to identify exactly 10 genes, whereas the elastic net model determined the optimal number of genes to include, which was always over 70 genes. Thus, the elastic net model is likely better if one has gene expression data for the entire transcriptome, whereas the multiple regression genes are likely more useful if one were to use reporters or qRTPCR to measure a more limited number of genes.

> *(12) Line 252: "Within this curated set, genes causally affected early brood in 5 of 7 cases compared to empty vector (Figure 4A).*

" It seems to me 4 out of 7 from Figure 4A. In Figure 4A the five genes are (1) *cin-4*, (2) *puf5; puf-7*, (3) *eef-1A.2*, (4) C34C12.8, and (5) *tir-1*. We did not count *nex-2* (p = 0.10) or *gly-13* (p = 0.07), and empty vector is the control.

> *(13) Do puf-5 and -7 affect total brood size or only early brood size? Not clear. What's the effect of single puf-5 and puf-7 RNAi on brood?*

We only measured early brood in this paper, but a previous report found that *puf-5* and *puf-7* act redundantly to affect oogenesis, and RNAi is only effective if both are knocked down together(2). We performed pilot experiments to confirm that this was the case in our hands as well.

> *(14) To truly understand if the noise in expression of Puf-5 and /or -7 really causes some of the observed difference in early brood, could the author use a reporter and dose response RNAi to reduce the level of puf-5/7 to match the lower physiological noise range and observe if the magnitude of the reduction of early brood by the right amount of RNAi indeed matches the observed physiological "noise" effect of puf-5/7 on early brood?*

We agree that it would be interesting to do the dose response of RNAi, measure early brood, and get a readout of mRNA levels to determine the true extent of gene knockdown in each worm (since RNAi can be noisy) and whether this corresponds to early brood when the knockdown is at physiological levels. While we believe we have shown that a dose response of gene knockdown results in a dose response of early brood, this additional analysis would be of interest for future experiments.

> *(15) Regulated soma genes (enriched in H3K27me3) are negatively correlated with early brood. What would be the mechanism there? As mentioned before, it is more likely that these genes are just indicative of variation in somatic vs germline age (maybe due to latent differences in parental perception of pheromone).*

We can think of a few potential mechanisms/explanations, but at this point we do not have a decisive answer. Regulated somatic genes marked with H3K27me3 (facultative heterochromatin) are expressed in particular tissues and/or at particular times in development. In this study and others, genes marked with H3K27me3 exhibit more gene expression noise than genes with other marks. This could suggest that there are negative consequences for the animal if genes are expressed at higher levels at the wrong time or place, and one interpretation of the negative association is that higher expressed somatic genes results in lower fitness (where early brood is a proxy for fitness). Another related interpretation is that there are tradeoffs between somatic and germline development and each individual animal lands somewhere on a continuum between prioritizing germline or somatic development, where prioritizing somatic integrity (e.g. higher expression of somatic genes) comes at a cost to the germline resulting in fewer progeny. Additional experiments, including measurements of histone marks in worms measured for the early brood trait, would likely be required to more decisively answer this question.

> *(16) Line 151: "Among significant genes for both traits, β2 values were consistently lower than β1 (Figures 2CD), suggesting some of the total effect size was driven by environmental history rather than pure noise".*

We are interpreting this quote as part of point 17 below.

> *(17) It looks like most of the genes associated with phenotypes from the univariate model have a decreased effect once you account for life history, but have you checked for cases where the life history actually masks the effect of a gene? In other words, do you have cases where the effect of gene expression on a phenotype is only (or more) significant after you account for the effect of life history (β2 values higher than β1)?*

This is a good question and one that we did not explicitly address in the paper because we focused on beta values for genes that were significant in the univariate analysis. Indeed, for the sets of 448 early brood genes ad 11 ELO genes, there are no genes for which β2 is larger than β1. In looking at the larger dataset of 8824 genes, with a Bonferroni-corrected p-value of 0.05, there are 306 genes with a significant β2 for early brood. The majority (157 genes) overlap with the 448 genes significant in the univariate analysis and do not have a higher β2 than β1. Of the remaining genes, 72 of these have a larger β2 than β1. However, in most cases, this difference is relatively small (median difference of 0.025) and likely insignificant. There are only three genes in which β1 is not nominally significant, and these are the three genes with the largest difference between β1 and β2 with β2 being larger (differences of 0.166, 0.155, and 0.12). In contrast, the *median* difference between β1 and β2 the 448 genes (in which β1 is larger) is 0.17, highlighting the most extreme examples of β2 > β1 are smaller in magnitude than the typical case of β1 > β2. For ELO, there are no notable cases where β2 > β1. There are eight genes with a significant β2 value, and all of these have a β1 value that is

nominally significant. Therefore, while this phenomenon does occur, we find it to be relatively rare overall. For completeness, we have added the β1 and β2 values for all 8824 genes as a tab in Supplementary File 1.

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