Dauer diapause has transgenerational effects on starvation survival and gene expression plasticity

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ABSTRACT

Phenotypic plasticity is facilitated by epigenetic regulation, and remnants of such regulation may persist after plasticity-inducing cues are gone. However, the relationship between plasticity and transgenerational epigenetic memory is not understood. Dauer diapause in *Caenorhabditis elegans* provides an opportunity to determine how a plastic response to the early-life environment affects traits later in life and in subsequent generations. We report that after extended diapause, post-dauer worms initially exhibit reduced reproductive success and greater inter-individual variation. In contrast, F3 progeny of post-dauers display increased starvation resistance and lifespan, revealing potentially adaptive transgenerational effects. Transgenerational effects are dependent on the duration of diapause, indicating an effect of extended starvation. In agreement, RNA-seq demonstrates a transgenerational effect on nutrient-responsive genes. Further, post-dauer F3 progeny exhibit reduced gene expression plasticity, suggesting a trade-off between plasticity and epigenetic memory. This work reveals complex effects of nutrient stress over different time scales in an animal that evolved to thrive in feast and famine.

Epigenetic regulation mediates phenotypic plasticity, such that a single genotype can produce different phenotypes in response to environmental conditions (1). Most epigenetic modifications are reset at the beginning of each generation, but those that persist have the potential to impact environmental adaptation and evolution independent of DNA sequence (2-4). Consequently, there is substantial interest in identifying environmental perturbations that elicit transgenerational epigenetic effects. Epigenetic inheritance has been reported in the roundworm *Caenorhabditis elegans*, but these studies typically focus on an aphysiological stimulus, such as a mutation or exogenous RNAi trigger (5, 6), or they assay a molecular rather than organismal phenotype, such as gene expression (7-10). Consequently, the importance of epigenetic inheritance to environmental adaptation is unclear. Identification and characterization of plasticity-induced transgenerational effects manifest at the organismal level can address important questions. Do epigenetic changes in gene regulation affect organismal traits? Does epigenetic memory provide a predictive adaptive response that improves fitness? Alternatively, does epigenetic memory arise due to a failure to reset the epigenetic program between generations, potentially posing a fitness cost?

C. elegans has a variety of developmental responses to nutrient availability (11-14). In particular, larvae respond to specific environmental cues (high population density, low food availability, and high temperature) and undergo an alternative developmental program resulting in dauer arrest (15). Dauer arrest is a classic example of diapause since dauer larvae develop in response to cues perceived in advance of arrest (16). Dramatic morphological and gene regulatory changes occur during dauer development (17-19) under the regulation of highly conserved signaling pathways (20-24). Dauers have a thicker cuticle and plugged pharynx, supporting increased stress resistance but preventing feeding. Dauers also have an altered nervous system, gut, and muscles compared to non-dauers (25-27). They are provisioned with fat stores that aid survival during prolonged starvation (28, 29). If environmental conditions improve, larvae exit dauer arrest and ultimately become reproductive adults.

62 *C. elegans* collected from the wild are mostly in the dauer stage, providing evidence that
63 starvation stress during dauer diapause is a common feature of their life history (30). Time spent
64 in dauer does not alter the length of the adult lifespan, suggesting an "ageless" state (31).
65 Nonetheless, dauer larvae do eventually die, with about one-third failing to recover at 60 days
66 (31). Further, germline defects increase in frequency as the duration of dauer diapause
67 increases (32), suggesting that at least some consequences of starvation incurred during
68 diapause persist in post-dauers. It is currently unknown if effects of long-term dauer diapause
69 persist upon recovery to affect important life-history traits, including progeny quality and
70 starvation resistance. The consequences of long-term dauer diapause beyond one generation
71 are, to our knowledge, completely unexplored.
72 Here, we report the consequences of long-term dauer diapause over multiple
73 generations. We found that long-term dauers recovered to produce fewer, smaller and
74 starvation-sensitive F1 progeny that exhibited greater inter-individual variation, suggesting
75 proximal fitness costs. In contrast, great-grandprogeny (F3 generation) of long-term dauers
76 exhibited increased starvation resistance and lifespan, consistent with potentially adaptive
77 transgenerational effects. Increased starvation resistance was dependent on the time spent in
78 the ancestral dauer diapause, suggesting that it is starvation during dauer, rather than dauer
79 development itself, leading to these transgenerational effects. Consistent with this, we identified
80 epigenetic differences in nutrient-responsive gene expression in the F3 generation including a
81 dampened response to nutrient availability. These results indicate that ancestral environment
82 can influence how worms respond to their current environment, and they suggest a trade-off
83 between epigenetic memory and plasticity.
84
85

RESULTS

We carefully controlled worm population density and bacterial food concentration in a liquid culture system to cause wild-type worms to either enter dauer diapause or to bypass it (18) (Fig 1). Ancestors of dauer and control worms were well fed for multiple generations (>3) to control for multigenerational effects of starvation or dietary restriction. Long-term dauers remained dauers for 36-45 days, and then were put on plates with food to recover. In parallel, control worms that did not experience dauer were plated from liquid culture so that post-dauer and control worms were paired for experiments.

Long-term dauer diapause reduces reproductive success in the P0 generation

We determined the proximal consequences of long-term dauer diapause on reproductive success. Following diapause, post-dauer worms recovered to produce fewer F1 progeny (Fig 2A and Supplementary Fig 1A). Post-dauer adults also produced smaller embryos (Fig 2B and Supplementary Fig 1B). *C . elegans* embryos hatch as L1 larvae. In the absence of food, L1 larvae survive starvation by remaining arrested as L1s, and this is reversible upon feeding. Survival during L1 arrest is a measure of starvation resistance (12). We found that post-dauers produced F1 progeny that were relatively sensitive to starvation by two metrics: L1 starvation survival (Fig 2C) and growth rate during recovery from L1 starvation (Fig 2D and Supplementary Fig 1C). Thus, long-term dauer arrest incurs costs to average fecundity as well as to progeny quality and starvation resistance, indicating reduced reproductive success.

Because we identified differences in *mean* trait values for post-dauer and control worms upon recovery and in the F1 generation, we also considered the possibility that experiencing long-term dauer diapause could alter the inter-individual *variation* of key traits within a worm population. We found that post-dauer worms exhibited greater variation in brood size than controls, and that F1 embryos were more variable in length (Supplementary Fig 2A-B). Consistent with these observations, once these embryos hatched as L1s, they appeared to

exhibit greater variation in body length after recovery from 8 days of starvation, but not 1 day of starvation (Supplementary Fig 2C). This suggests that increased inter-individual variation is uncovered by the stress of extended L1 starvation. Together our results suggest that the proximal consequences of long-term diapause include decreased reproductive success and increased phenotypic variation.

Long-term dauer diapause increases starvation resistance and lifespan transgenerationally

We asked whether effects of long-term dauer diapause persisted beyond the F1 generation to the F3 generation. Persistence to the F3 generation suggests transgenerational epigenetic inheritance, while effects in the F1 and F2 generations could be due to maternal effects (33). We measured starvation resistance in three ways: L1 starvation survival, body length following 48 hours of recovery from L1 starvation, and brood size following recovery from L1 starvation. In contrast to F1 L1 larvae, we found that F3 progeny of long-term dauers exhibited increased L1 starvation survival (Fig 3A). Likewise, F3 progeny of long-term dauers allowed to recover for 48 hr from 8 days of L1 starvation were larger than controls recovering from 8 days of L1 starvation, demonstrating increased growth rate (Fig 3B and Supplementary Fig 3A). In addition, the broods of F3 progeny of long-term dauers following 8 days of L1 starvation were larger than the broods of F3 controls following 8 days of L1 starvation (Fig 3C and Supplementary Fig 3B). We also assayed growth rate and brood size in the F3 post-dauers with just 1 day of L1 starvation and found no significant differences in these traits (Fig 3B-C and Supplementary Fig 3A-B). In addition, we did not find evidence of differences in inter-individual variation in the F3 generation (Supplementary Fig 3F-G), suggesting that the differences in mean trait values in the F3 generation were not due to a difference in the shape of the underlying trait distribution. Together these data support the conclusion that long-term dauer diapause leads to epigenetic inheritance of increased starvation resistance in F3 progeny.

We found transgenerational effects of long-term dauer arrest that were present beyond the L1 stage and into adulthood in F3 worms that were never starved. F3 progeny of long-term dauers lived longer as adults than controls (Fig 4D, Supplementary Fig 3C-E), demonstrating that there are transgenerational consequences of long-term dauer diapause outside the context of starvation.

We next wanted to determine whether the transgenerational increase in starvation resistance depends on the duration of dauer diapause. When worms were dauers for just six days, F3 progeny of these short-term dauers did not exhibit differences in starvation survival or brood size following starvation (Supplementary Fig 4). We had the statistical power to detect differences in starvation survival of less than a day (Supplementary Table 1; power analysis not shown). This suggests that the transgenerational effects of experiencing long-term dauer arrest are dependent on the length of starvation during arrest as opposed to the dauer-inducing culture conditions, dauer formation, or dauer recovery.

Epigenetic memory of dauer diapause affects gene expression globally

Transgenerational effects of long-term dauer diapause on important organismal traits suggest epigenetic regulation of gene expression. We used mRNA-seq to determine if gene expression patterns in both fed and starved F3 progeny of controls, short-term dauers, and long-term dauers differed (Fig 4A). This two-factor design allowed us to analyze both transgenerational and instantaneous effects of nutrient availability on gene expression. We performed principal component analysis (PCA) on the normalized mean counts per million (CPM) values for six conditions as well as on individual biological replicates (Fig 4B, Supplementary Fig 5). PC1 separated condition means depending on whether the worms were fed or starved as F3 L1s, while PC2 separated them according to length of time the initial generation spent in dauer diapause. Notably, instantaneous effects of nutrient availability (PC1) appeared to explain substantially greater variance in gene expression than epigenetic effects

(PC2). Nonetheless, these results suggest that ancestral environmental conditions play a small, but detectable, role in shaping gene expression in the F3 generation.

We compared gene expression plasticity in F3 long-term post-dauers to controls. That is, we assessed gene expression differences between starved and fed F3 worms with long-term dauer ancestors to starved and fed F3 worms with control ancestors (Fig 5A). Regardless of ancestral background, starved and fed worms exhibited dramatic gene expression differences (Fig 5A, Supplementary File 1), consistent with PC1. Gene expression responses to nutrient availability were also highly correlated. Notably, virtually no genes had large differences in one comparison and not the other. However, the slope of the linear regression was significantly less than 1, suggesting that gene expression plasticity in response to nutrient availability in the F3 generation following long-term dauer diapause is dampened relative to controls.

Dampened plasticity could occur if fed F3 progeny of long-term dauers had a transcriptional profile that appeared “less fed” compared to fed controls or if starved F3 progeny of long-term dauers appeared transcriptionally “less starved” compared to starved controls. First, we found fed F3 progeny of long-term dauers appeared less fed (or, equivalently, more starved) relative to fed controls by comparing the effect of plasticity in controls to the epigenetic effect in fed larvae (Fig 5B,C). We further tested this effect using the most differentially expressed nutrient-responsive genes. These genes, highlighted in Fig 5B, showed that genes that were significantly up- or down-regulated in starved worms compared to fed worms tend to also be up- or down-regulated, respectively, in fed F3 progeny of long-term dauers relative to fed controls (Figs 5B,C). We also found that the regulatory targets of the transcription factor *daf-16* (34), which promotes starvation survival (12), overlap with the nutrient-responsive genes and display a similar epigenetic effect of long-term dauer diapause (Fig 5D, Supplementary Fig 6A). This result corroborates the epigenetic effect on nutrient-responsive genes using gene lists defined outside the context of this study. These results provide evidence that dampened gene

189 expression plasticity is driven at least in part by differences in nutrient-responsive gene
190 expression in fed F3 progeny of long-term dauers.
191 We wanted to determine whether there are differences in nutrient-responsive gene
192 expression in the *starved* F3 progeny of long-term dauers compared to controls. In contrast to
193 the fed F3 progeny, the starved F3 progeny of long-term dauers exhibited a negative correlation
194 with the control nutrient response (Fig 5E,F). This was corroborated by the fact that *daf-16*
195 target genes displayed similar behavior (Fig 5G). Together these results suggest that there are
196 transgenerational effects on gene expression in both fed and starved F3 larvae that collectively
197 contribute to reduced gene expression plasticity.
198 F3 progeny of short-term dauers did not display detectable epigenetic effects on
199 starvation resistance, but we did detect effects on gene expression. We found that F3 progeny
200 of short-term dauers showed similar transcriptional changes as identified in the F3 progeny of
201 long-term dauers, including dampening of nutrient-responsive gene expression (Fig 5H,
202 Supplementary Fig 6B-D). However, transgenerational changes in gene expression following
203 short-term dauer appear to be less consistent and possibly of a smaller magnitude than those
204 elicited by long-term dauer. PC2 aligns means of short-term dauer samples between control and
205 long-term dauer samples (Fig 4A). The slope of the dampening response of F3 progeny of
206 short-term dauers compared to controls was closer to 1 than F3 progeny of long-term dauers,
207 and F3 progeny of long-term dauers exhibit dampening relative to short-term dauers (Fig 5H,
208 Supplementary Fig 6B). In addition, we examined gene expression shifts within individual paired
209 replicates (replicates in which an F3 dauer sample was collected in parallel with a control). All
210 paired replicates for starved F3 progeny of long-term dauers compared to starved controls
211 showed shifts in nutrient-responsive genes in the expected direction (Supplementary Fig 6E).
212 Paired replicates for F3 progeny of short-term dauers exhibited shifts in gene expression for the
213 majority, but not all, pairs (Supplementary Fig 6F). Collectively, these results suggest that
214 transgenerational epigenetic effects of short-term dauer are detectable and qualitatively similar

215 to those of long-term dauer, but that they are less consistent, likely accounting for lack of
216 detectable effects on starvation resistance.
217
218 **DISCUSSION**
219 We utilized a well-studied model of phenotypic plasticity, dauer diapause, to interrogate
220 the proximal and transgenerational consequences of early-life environment on organismal traits
221 and gene expression. Post-dauer worms displayed reduced reproductive success after long-
222 term diapause, but their F3 progeny exhibited increased starvation resistance and lifespan. F3
223 progeny of both short- and long-term dauers exhibited changes in gene expression in nutrient-
224 responsive genes, consistent with a reduction in gene expression plasticity in response to
225 nutrient availability. This work has broad implications for understanding the consequences of
226 starvation, including the potential for epigenetic inheritance in response to nutrient availability
227 and potentially adaptive responses across generations.
228 Long-term dauers recovered to produce fewer, smaller F1 progeny that are starvation
229 sensitive compared to worms that do not enter dauer. We also found that long-term post-dauers
230 exhibit increased inter-individual variation, consistent with developmental decanalization (35)
231 stemming from deleterious effects of extended starvation (See Supplementary Discussion).
232 Dauer diapause is evolutionarily adaptive, as it allows worms to survive conditions that would
233 otherwise be lethal. Our results suggest that while it is adaptive to be able to enter dauer to
234 survive adverse conditions, there is a cost to extended dauer diapause. The proximal costs of
235 long-term dauer diapause are consistent with our lab's previous work identifying costs to eight
236 days of L1 starvation, as worms recovered from L1 starvation are more sensitive to subsequent
237 starvation and produce smaller broods with decreased progeny quality (36). Both extended L1
238 starvation and dauer diapause appear costly, but this is likely due to the costs of starvation
239 experienced during these states, rather than simply arresting development. Post-dauer worms
240 that experienced dauer for just one day live longer and produce larger broods (37). Together

241 these observations suggest that hormesis, in which a mild stress can increase fitness (38), may
242 occur when starvation is brief, but that extended starvation is costly as the buffering capacity of
243 the organism is overwhelmed.

244 We found that the F3 progeny of long-term dauers exhibit increased starvation
245 resistance as larvae and live longer as fed adults, indicating that transgenerational effects
246 manifest in both fed and starved animals and that they persist through the life cycle.
247 Multigenerational consequences of L1 starvation have been previously examined and exhibit
248 some similarities to those of long-term dauer diapause. Following 8 days of L1 starvation, we
249 previously observed increased starvation resistance in the F2 but not F3 generation (36). We
250 did not detect a reproducible effect on lifespan following L1 starvation in any generation, though
251 increased lifespan in the F3 generation following L1 starvation has been reported in another
252 study (39). Notably, transmission of increased starvation resistance to F1 and F2 progeny of
253 worms that experienced L1 starvation required sorting larvae by size after 2 days of recovery
254 from starvation (36), and in the present study such sorting was not required. Still, both L1 and
255 dauer starvation paradigms reveal apparent proximal fitness costs of extended starvation
256 followed by potentially adaptive increases in starvation resistance in subsequent generations.
257 The switch from proximal starvation sensitivity to heritable starvation resistance in both
258 paradigms is of particular interest.

259 In addition to life-history trait differences, we identified transgenerational effects of dauer
260 diapause on gene expression plasticity in response to nutrient availability. Specifically, we found
261 that F3 progeny of both short-term and long-term dauers exhibited reduced gene expression
262 plasticity, with starved F3 worms appearing less starved and fed F3 worms appearing less fed.
263 Theory suggests that there should be costs and limits to plasticity, though there are relatively
264 few empirical examples of such costs (40, 41). While we emphasize that the transgenerational
265 effect on gene expression plasticity does not necessarily indicate plasticity at other levels of
266 regulation, one interpretation of this result is that maintaining an epigenetic memory of ancestral

267 environmental conditions limits plastic responses to current environmental conditions. In other
268 words, there is hypothetically a fixed capacity to respond to environmental conditions, but this
269 capacity can be exhausted by instantaneous and epigenetic regulation.

270 It is possible that subtle changes in starvation resistance and gene expression reflect
271 physiological fine-tuning in anticipation of future conditions based on ancestral history. In
272 contrast, it is possible that epigenetic memory of ancestral conditions occurs but is not
273 necessarily adaptive, as a potentially neutral or even costly by-product of some of other
274 selected trait. Epigenetic stability from parent to offspring is favorable given that four conditions
275 are met: 1) the environment is variable; 2) parental environment has some predictive power
276 over offspring environment; 3) transgenerational effects increase fitness of parents and/or
277 offspring; and 4) costs associated with the transgenerational response are low (42). Though it is
278 clear that *C. elegans* experience variable nutrient conditions in the wild (43), it is currently
279 unknown how predictive parental environment is of offspring environment over multiple
280 generations. Here, we find that F3 progeny of long-term dauers are more starvation resistant,
281 and this is not accompanied by detectable costs in growth or fecundity, consistent with the
282 possibility that this transgenerational effect is adaptive. Recognizing that it is ultimately difficult
283 to determine if such a complex trait as epigenetic inheritance is evolutionarily adaptive, we hope
284 that future studies in the context of the natural ecology of *C. elegans* will shed light on this
285 question.
286

287 **MATERIALS AND METHODS**

288 ***Dauer and control cultures***

289 The wild-type strain N2 was used for all experiments. N2 was obtained from the
290 Sternberg collection at the California Institute of Technology, originally from the CGC in 1987.
291 Worms were maintained for at least 3 generations in standard laboratory conditions without
292 starving prior to beginning experiments. ~10 adults were picked onto each of 4-5 10 cm NGM

plates seeded with *E. coli* OP50 and maintained at 20ºC. Embryos were obtained by standard hypochlorite treatment after four days in culture. For dauer-forming conditions, embryos were suspended in S-complete at a density of 5 per microliter with 1 mg/mL *E. coli* HB101(18). HB101 was prepared as described previously (44). For control conditions, embryos were suspended at 1 per microliter with 38 mg/ml HB101. Worms were cultured at 180 rpm and 20ºC in 25 mL glass Erlenmeyer flasks in a volume of 5 mL (25,000 worms for dauer conditions and 5,000 for control conditions). For experiments that required greater than 5,000 control worms, a 20 mL volume in a 250 mL Erlenmeyer flask with the same density and food concentration was used. Dauer formation occurs in approximately 4 days with nearly 100% penetrance. Short-term dauers remained in culture for 10 days, being arrested as dauers for 6 days. Long-term dauers remained in culture for 40-49 days, being arrested as dauers for 36-45 days. Survival was 90 - 100% in long-term dauer cultures. Control worms were in culture for 40-44 hours and were plated as L4 larvae. The L4 stage was chosen because dauers recover to become L4 larvae.

Dauer recovery and maintenance

Dauer and control conditions were paired such that control cultures were set up to be recovered at the same time as the dauer culture. Thus, recovery, maintenance, sampling and assaying were done in parallel. Worms were taken from liquid cultures and plated on 10 cm NGM plates seeded with OP50 and incubated at 20ºC. To obtain F1 progeny, approximately 1000 P0 worms were plated per seeded plate, and these were hypochlorite treated 2 days later to obtain F1 progeny for analysis. To obtain F3 progeny, 10 P0 worms were plated per seeded plate. These worms laid F1 embryos, which grew to become gravid adults on the same plates (~1000 F1 worms per plate). Five days after plating, the F1 worms on these plates were hypochlorite treated to obtain F2 embryos. Approximately 1000 F2 embryos were plated per new seeded plate, and these worms were hypochlorite treated 3 days later to obtain F3 progeny. In all cases, hypochlorite treatment was performed prior to worms starving on plates.

L1 starvation survival

Embryos were suspended following hypochlorite treatment in virgin S-basal (no cholesterol or ethanol) at a density of 1 embryo/µL in a volume of 5 mL in a 16 mm glass test tube and placed on a tissue culture roller drum at approximately 25 rpm and 21-22ºC. Beginning at day 1 (24 hr after hypochlorite treatment) and continuing every other day, a 100 µL sample was taken and plated on a 6 cm NGM plate with a spot of OP50 in the center. The sample was plated to the side of the OP50. The number of worms plated was scored (total plated = T_P). Two days later, the number of worms that were alive on the plate were scored (total alive = T_A). The proportion alive at each time point was calculated as T_A / T_P.

Brood size

Worms were singled onto 6 cm NGM plates with OP50 as L4 larvae. Worms were transferred to new plates each day until they stopped producing progeny. The number of progeny per plate was scored 2 days after removal of the mother. The total brood size per worm was determined by summing the progeny per plate across all plates for a single worm. Worms were censored if they died during egg laying. This affected 11 worms total in P0 brood size (5 post-dauers, 6 controls). In the F3 generation following long-term dauer, for 0 days of arrest, number of worms that died during egg laying included 0 controls and 1 post-dauer. For 8 days of arrest, number of worms that died during egg laying include 16 controls and 14 post-dauers; 2 controls and 2 post-dauers were sterile. In the F3 generation following short-term dauer, 9 control worms and 9 post-dauer worms died during egg laying; 1 post-dauer worm was sterile.

Embryo length

Embryos were plated onto unseeded 10 cm NGM plates. Embryos were imaged with a Zeiss Discovery. V20 stereomicroscope with a 10x objective (KSC 190-975). The images were analyzed with FIJI as described previously (44). Lengths of embryos were measured by

thresholding embryos and calculating the long axis from ellipse fitting. The background was subtracted, images were thresholded, converted to binary, holes were filled, and particles were analyzed. Analysis was done in batch and the results were manually curated to ensure only quality embryo images were included.

Worm length

L1 larvae that had been starved for 1 or 8 days were recovered by plating on 10 cm NGM plates with OP50 as previously described (44). After 48 hours of recovery, worms were washed off the plates with virgin S-basal and plated on unseeded 10 cm NGM plates for imaging. Images were taken on a ZeissDiscovery.V20 stereomicroscope with automated zoom. Images were analyzed with the WormSizer plugin for FIJI to determine worm length and manually passed or failed (45).

Lifespan

For each condition and replicate, 150 L1 larvae arrested for 1 day in virgin S-basal were plated on 6 cm NGM plates seeded with OP50. After two days, 72 worms were randomly picked onto new seeded plates in groups of 12. Adults were picked away from their progeny onto fresh plates every day until egg laying ceased. Worms that responded to gentle prodding with a platinum wire were transferred to fresh plates every 2 - 3 days. Worms were considered dead when they failed to respond to prodding. Worms that crawled off the agar were considered lost and subtracted from the total *n*. No other animals were censored. Lifespan curves were analyzed to determine mean survival using OASIS (46).

Statistical analysis

Statistics were calculated in R or Microsoft Excel. To test for differences in means across groups, linear mixed effect models were fit to the data using the “nlme” package in R.

371 The summary function was used to calculate p-values for the models, which implements the
372 Wald test. Fixed effects included condition (post-dauer vs. control) and, where applicable, length
373 of starvation (0 or 1 day vs. 8 days). An interaction term was included for experiments with both
374 types of fixed effects. A random effect of biological replicate was included for all models. To test
375 for differences in inter-individual variation across conditions, data were mean normalized within
376 each biological replicate and condition, individual worms across all replicates were pooled, and
377 Levene's test was used to assess homogeneity of variance across conditions. For starvation
378 survival (Figs 2,4, and 5), logistic curves were fit to survival data and median survival times
379 were calculated (47). Paired t-tests were performed on median survival times. Statistical tests
380 and significance are indicated in figure legends. Plots were generated using ggplot2 in R.
381
382 ***Sample collection for RNA-seq***
383 F3 embryos were suspended at 1 embryo/µL in S-complete either with or without 25
384 mg/mL HB101 to obtain fed or starved L1s, respectively. At least 10,000 embryos were used
385 per condition per replicate. Fed larvae were collected 18 hr after hypochlorite treatment as
386 early-stage developing L1 larvae. Starved larvae were collected 24 hr after hypochlorite
387 treatment as arrested L1 larvae that had hatched approximately 12 hr earlier. To collect starved
388 samples, cultures were transferred to 15 mL conical tubes and spun for 1 minute at 3000 rpm.
389 Liquid was aspirated to < 100 uL containing the worm pellet. Worms were washed 0-1X with S-
390 basal. The worm pellet was transferred to a 1.5 mL microcentrifuge tube, flash frozen in liquid
391 nitrogen, and stored at -80ºC until RNA isolation. To collect fed samples, cultures were
392 transferred to 15 mL conical tubes and spun at 3000 rpm for 10 seconds. Liquid was aspirated
393 to < 100 uL containing the worm pellet. The pellet was quickly washed 3-4X with 10 mL S-basal,
394 visually inspected to ensure removal of the vast majority of bacteria, transferred to a 1.5 mL
395 microcentrifuge tube, flash frozen, and stored at -80ºC until RNA isolation.
396

397 ***RNA isolation and library preparation***

398 RNA was isolated using TRIzol (Invitrogen) using the manufacturer's protocol with minor
399 modifications. 1 mL of TRIzol was used per sample along with 100 uL of acid-washed sand.
400 mRNA-seq libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina
401 (E7530) in two batches, utilizing either 500 or 100 ng of starting RNA per library and 12 or 15
402 PCR cycles, respectively. Libraries were sequenced using Illumina HiSeq 4000 to obtain single-
403 end 50 bp reads.

404

405 ***RNA-seq analysis***

406 Bowtie was used to map reads to the WS210 genome (48). We also included transcripts
407 annotated in WS220 mapped back to the WS210 genome coordinates, as described previously
408 (49). Mapping efficiencies ranged from 81-86% for all libraries. HTSeq was used to generate
409 count tables for each library (50). Count tables were analyzed for differential expression using
410 the edgeR package in R, which utilizes a negative binomial model to estimate dispersions (51).
411 Detected genes were considered those expressed at a level of at least 4 counts per million
412 (CPM) across all libraries for all conditions, reducing the number of genes included in the
413 analysis to 8649. The "calcNormFactors" function was used to normalize for RNA composition
414 and the tagwise dispersion estimate was used for differential expression. The exact test was
415 used for pairwise comparisons. Log2 fold change estimates from differential expression analysis
416 in edgeR were used for generating plots in Fig 7 and Supplementary Fig 4. Kolmogorov-
417 Smirnov tests were used to determine differences in cumulative distributions of log2 fold
418 changes.

419

420 ***Data availability***

421 Raw and processed RNA-seq data is available through the GEO NCBI database with accession
422 number GSE113500.

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FIGURES

Figure 1. Schematic of experimental design

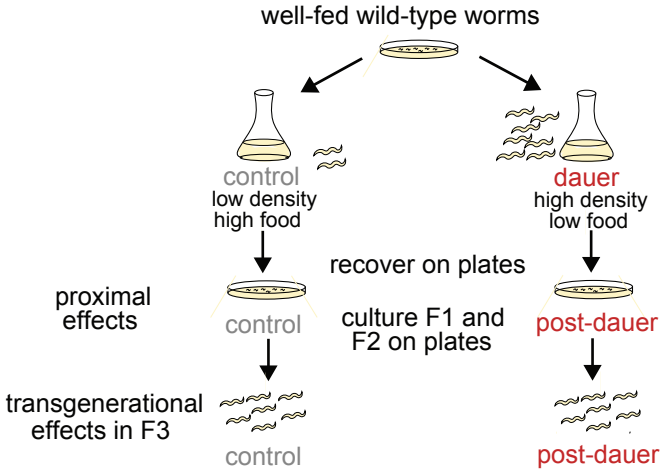

Fig 1: Schematic of experimental design. Well-fed, wild-type worms were hypochlorite treated as gravid adults to obtain embryos, which were placed in dauer or control conditions. For long-term dauer conditions, worms remained in culture for 40-49 days and were dauers for 36-45 days. Worms from dauer and control cultures were plated on food in parallel. These lay F1 progeny, some of which were used for experiments of proximal effects of long-term dauer diapause. Other F1 adults were hypochlorite treated to obtain F2 embryos. F2 adults were hypochlorite treated to obtain F3 embryos. F3 post-dauer and control worms were used for experiments.

Figure 2: Long-term dauers recover to produce fewer, smaller F1 progeny that are starvation sensitive

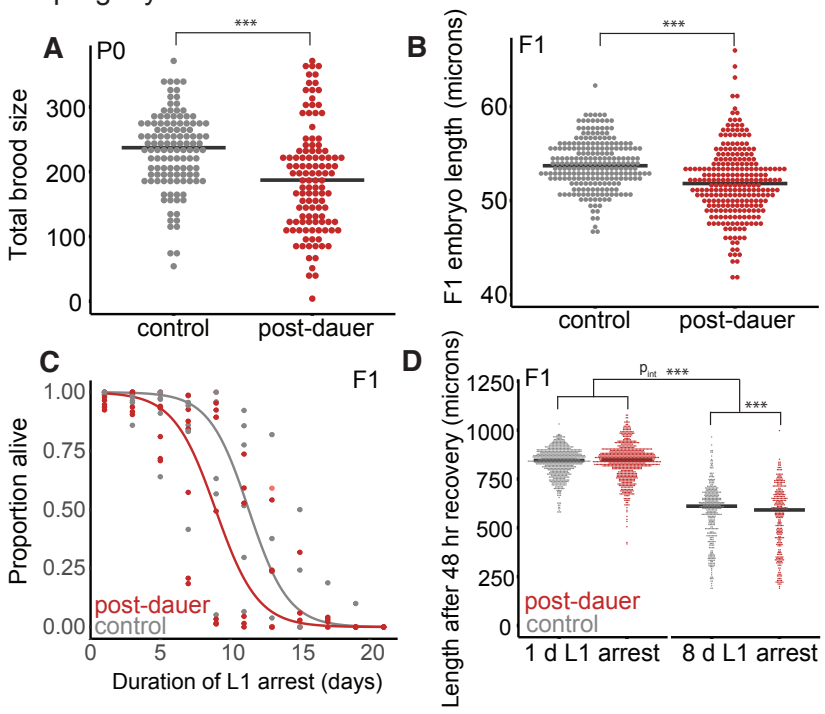

Fig 2: Long-term dauers recover to produce fewer, smaller F1 progeny that are starvation sensitive.
A. Brood sizes following long-term dauer diapause or control conditions. 7 biological replicates were scored with 13-18 individuals per biological replicate per condition. Effect of condition, $p = 4.46 \times 10^{-7}$.
B. Embryo length in the F1 progeny of post-dauers and controls. 6 biological replicates were scored with 23-70 individual embryos scored per biological replicate. Effect of condition, $p = 9.0 \times 10^{-13}$.
C. L1 starvation survival for 7 biological replicates of F1 progeny of post-dauers and controls. Logistic regression curves were fit, and median survival was determined for each replicate. Paired t-test on median survival, $p = 0.10$.
D. Worm length after 48 hr of recovery from 1 or 8 days of L1 arrest was scored in the F1 progeny of post-dauers and controls. 8 biological replicates were scored. For 1 day, 50-335 worms were scored per biological replicate; following 8 days, 7-118 worms scored per replicate. Effect of condition for 1 day, $p = 0.95$; effect of condition for 8 days, $p < 2.0 \times 10^{-16}$. A linear mixed-effect model was fit with condition and length of starvation as fixed effects and biological replicate as a random effect. An interaction term was included for fixed effects. Effect of the interaction of condition and length of starvation, $p = 5.1 \times 10^{-14}$; effect of starvation, $p < 2.0 \times 10^{-16}$; effect of condition, $p = 0.20$.
A,B,D. Linear mixed-effect models were fit with condition (post-dauer vs. control) as a fixed effect and biological replicate as a random effect. P-values were calculated using the Wald test.
Horizontal black lines represent medians. For means of biological replicates and grand mean, see Supplementary Fig 1.
***p<0.001

Figure 3: F3 progeny of long-term dauers exhibit increased starvation resistance and lifespan

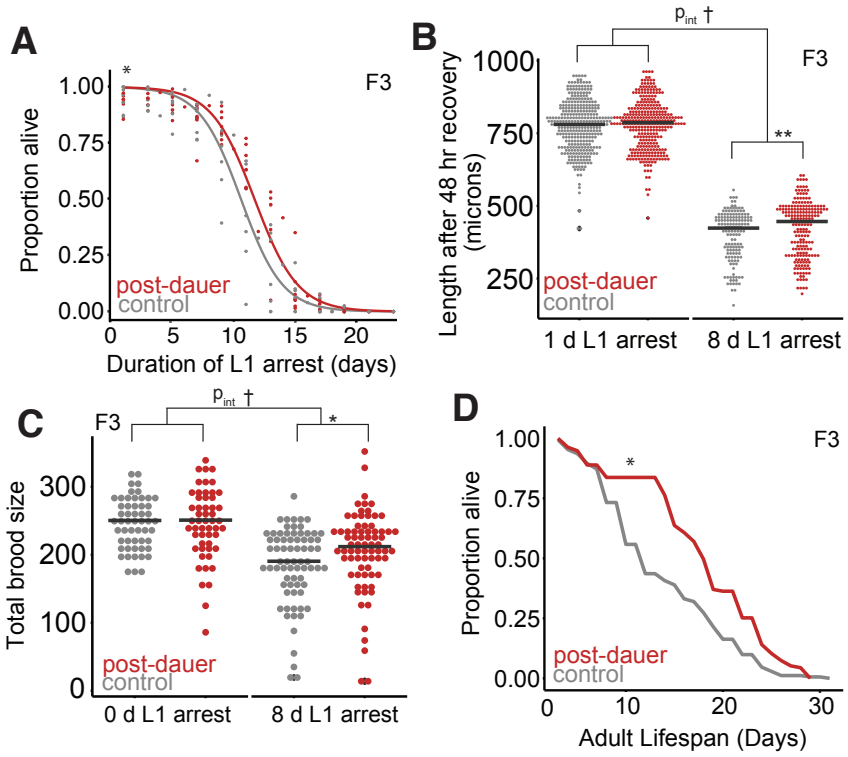

Fig 3: F3 progeny of long-term dauers exhibit increased starvation resistance and lifespan. A. L1 starvation survival was scored in the F3 progeny of post-dauers and controls. 8 biological replicates were scored, logistic curves were fit, and median survival times were determined. Paired t-test on median survival, p=0.01.

B. Worm body length following 48 hours of recovery from either 1 or 8 days of L1 arrest. 4 biological replicates were scored, consisting of 342 controls starved 1 day, 323 post-dauers starved 1 day, 148 controls starved 8 days, and 204 post-dauers starved 8 days. Effect of condition for 1 day, p = 0.25; effect of condition for 8 days, p = 0.0048. Effect of the interaction between condition and length of starvation, p = 0.066; effect of the length of starvation, $p < 2.0 \times 10^{-16}$; effect of condition, p = 0.93.

C. Brood size was scored for F3 progeny of controls and post-dauers that experienced 0 or 8 days of L1 arrest. 3 biological replicates for 0 days; 5 biological replicates for 8 days. For 0 d L1 arrest, 54 controls and 53 post-dauers; for 8 d of L1 arrest, 72 controls and 74 post-dauers. Effect of condition for 0 days, p = 0.82; effect of condition for 8 days, p = 0.03. Effect of the interaction between condition and length of starvation, p = 0.087; effect of the length of starvation, $p < 1.0 \times 10^{-4}$; effect of condition, p = 0.87.

D. Lifespans of at least 135 F3 progeny of post-dauers and controls from 3 biological replicates (See Supplementary Fig 3C-E for individual replicates). Paired t-test on the means of biological replicates, p = 0.026.

B,C. Linear mixed-effect models were fit with condition (post-dauer vs. control) as a fixed effect and biological replicate as a random effect. Next, a linear mixed-effect model was fit with condition (postdauer vs. control) and length of starvation (0 or 1 vs. 8 days) as fixed effects and biological replicate as a random effect. An interaction term was included for fixed effects. P-values were calculated using the Wald test.

*p<0.05, **p<0.01, ***p<0.001; † interaction p<0.1; n.s. not significant. Horizontal black lines represent medians. For means of biological replicates, see Supplementary Fig 2.

Figure 4: mRNA-seq reveals relative contributions of current environment and ancestral environment in shaping gene expression variation in the F3 generation

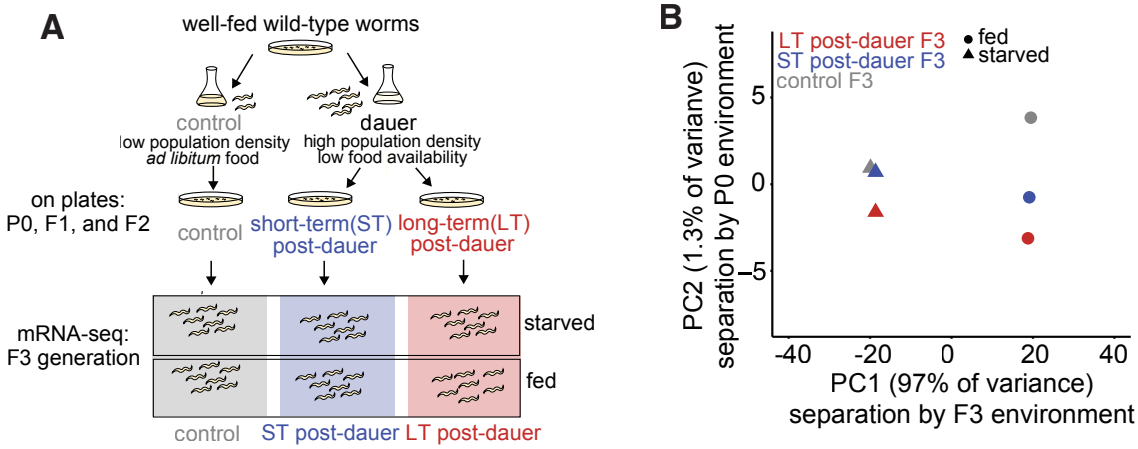

Fig 4: mRNA-seq reveals relative contributions of current environment and ancestral environment in shaping gene expression variation. A. Schematic of experimental set-up for collection of RNA-seq samples.
B. Principal component analysis (PCA) of 6 conditions including all 8649 reliably detected genes. 97% of variance explained by whether worms were fed or starved at collection (PC1). 1.3% of variance explained by whether ancestors experienced control, short-term dauer, or long-term dauer conditions (PC2).

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Figure 5: F3 progeny of dauers exhibit reduced gene expression plasticity driven by differences in both fed and starved worms

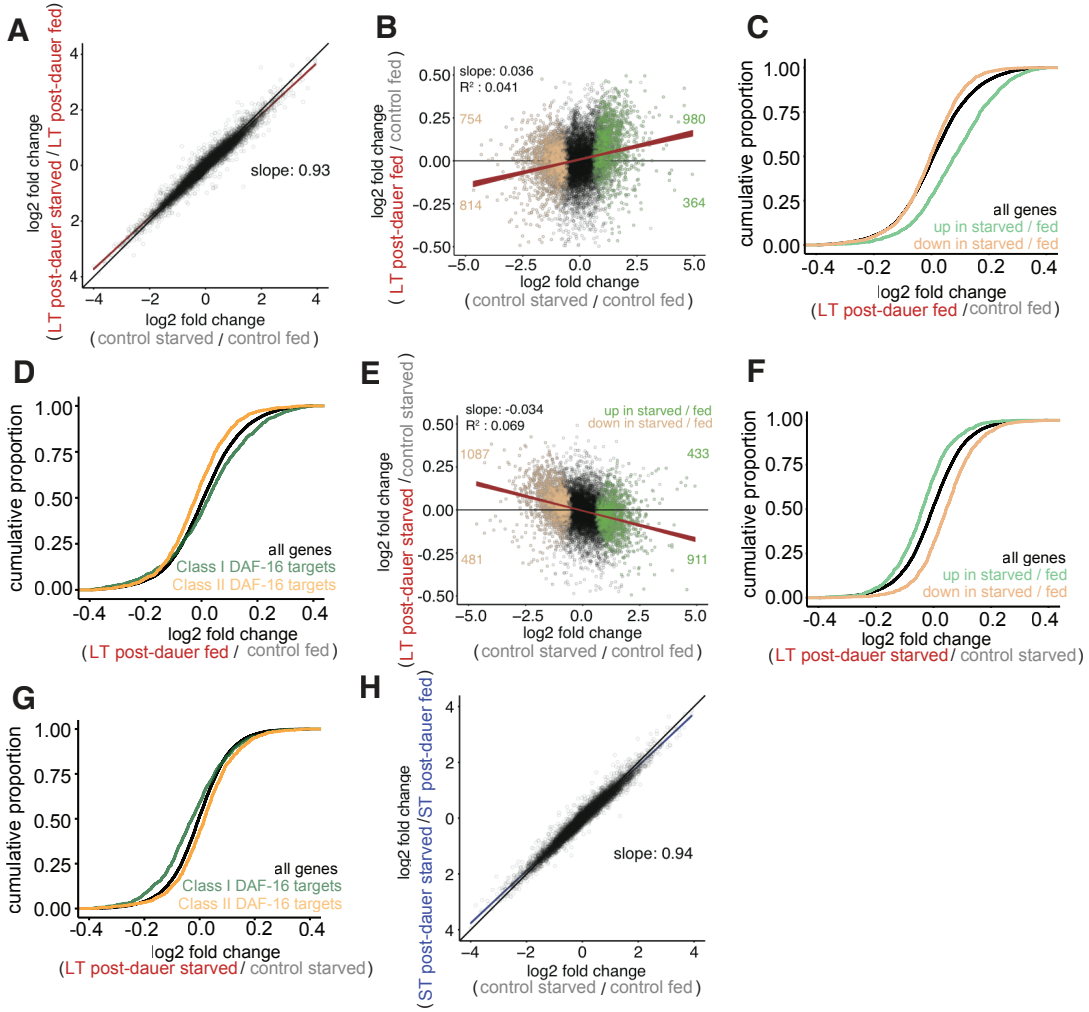


Fig 5: F3 progeny of dauers exhibit reduced gene expression plasticity driven by differences in both fed and starved worms.

A. Log2 fold changes of all genes in control starved / control fed plotted against LT post-dauer starved / LT post-dauer fed. Red line is linear regression through all points and width indicates 95% confidence interval (CI). Black line is the line y = x. Slope is 0.93, with CI from 0.926 to 0.934.

B. Log2 fold changes of all genes in control starved / control fed plotted against log2 fold changes of LT post-dauer fed F3 / control fed plotted in black. Slope is significantly different from 0, $p < 2.2 \times 10^{-16}$

C. Genes up in starved / fed: p = 0; to genes down in starved / fed: $p = 3.3 \times 10^{-8}$.

D. Class I and Class II targets defined in Tepper et al. Class I targets: $p = 1.6 \times 10^{-5}$; to Class II targets: $p = 2.3 \times 10^{-11}$.

E. Log2 fold changes of all genes in control starved / control fed plotted against log2 fold changes of LT post-dauer starved F3 / control starved plotted in black. Slope is significantly different from 0, $p < 2.2 \times 10^{-16}$.

F. Genes up in starved / fed: p = 0; to genes down in starved / fed: p = 0

G. Class I targets: $p = 1.0 \times 10^{-12}$; to Class II targets: $p = 4.0 \times 10^{-7}$.

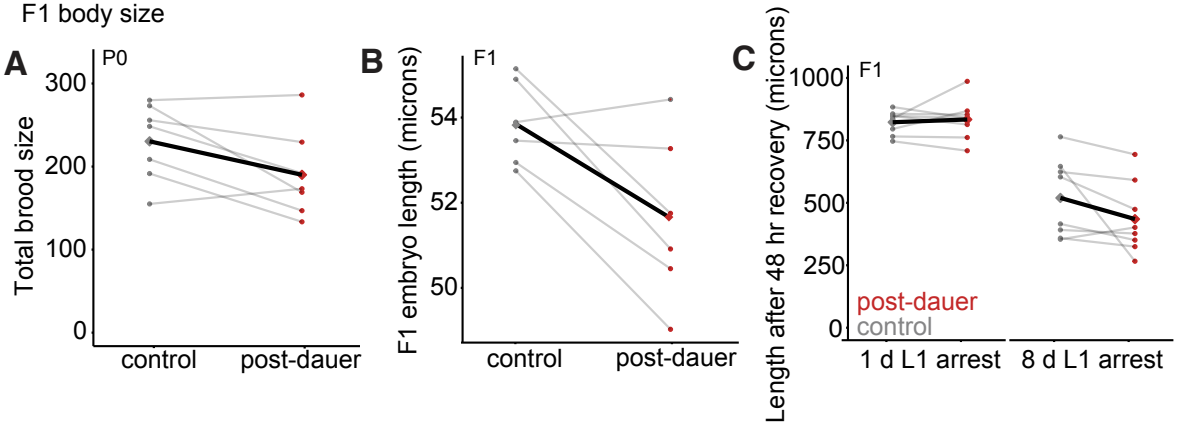
H. Log2 fold change of all genes in control starved / control fed compared to log2 fold change in ST post-dauer starved / ST post-dauer fed. Blue line indicates simple linear regression through all points, and thickness of line indicates 95% CI. Slope is 0.94, with CI from 0.936 to 0.943.

B,E. Genes up in control starved / control fed using FDR 1×10^{-10} are plotted in green. Genes down in control starved / control fed are plotted in tan. Red line is a simple linear regression through all points, and thickness of line indicates 95% confidence interval. Number of genes differentially expressed in control starved / control fed in each quadrant is indicated.

C,D,F,G. Within the indicated comparison, cumulative distribution functions (CDFs) of indicated gene lists are plotted. Kolmogorov-Smirnov test used to assess significance of gene list distribution compared to all genes.

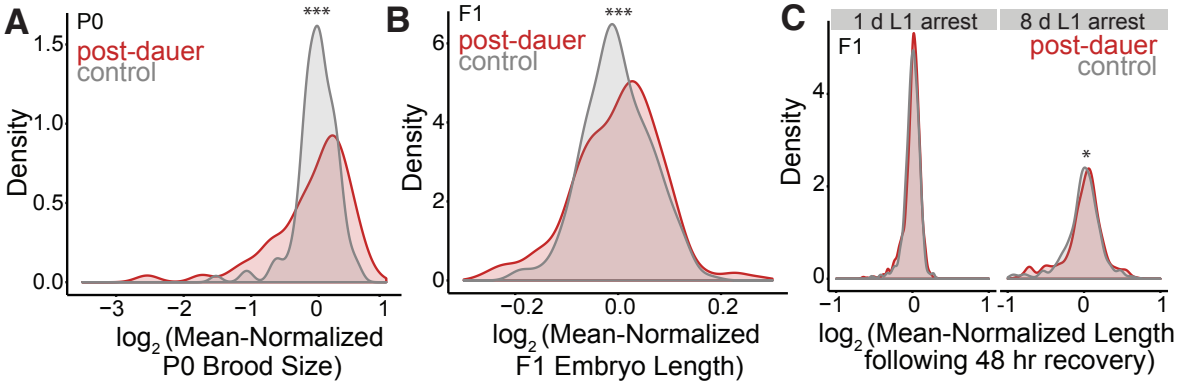
SUPPORTING INFORMATION

Supplementary Fig 1: Proximal effects of long-term dauer on P0 brood size, F1 embryo length, and F1 body size



Supplementary Fig 1: Proximal effects of long-term dauer on brood size, embryo length, and body size. A-C. Each point represents the mean of individual worms for the indicated trait within a biological replicate. Paired replicates are connected with a gray line. Grand means are connected with a black line.

Supplementary Fig 2: Long-term dauer diapause promotes variability in F1 progeny number and size



Supplementary Fig 2: Long-term dauer diapause promotes variability in F1 progeny number and size. A. Brood size data were pooled across 7 biological replicates including 115 post-dauer worms and 120 control worms, $p = 2.2 \times 10^{-8.}$
B. Embryo length data were pooled across 6 replicates including 256 post-dauer embryos and 235 control embryos, p = 0.00033.
C. Worm body lengths following recovery following 1 or 8 days of L1 arrest in F1 progeny of post-dauers and controls were pooled from 8 biological replicates including 748 controls starved for 1 day, 897 post-dauers starved for 1 day, 421 controls starved for 8 days, and 337 post-dauers starved for 8 days. For 1 day, p = 0.13; for 8 days, p = 0.02.
A-C. Trait values for individual post-dauer and control worms were mean-normalized to the mean trait value for the corresponding condition and replicate. Data were pooled across biological replicates. Levene's test was used to assess differences in variance.
*p<0.05, ***p<0.001

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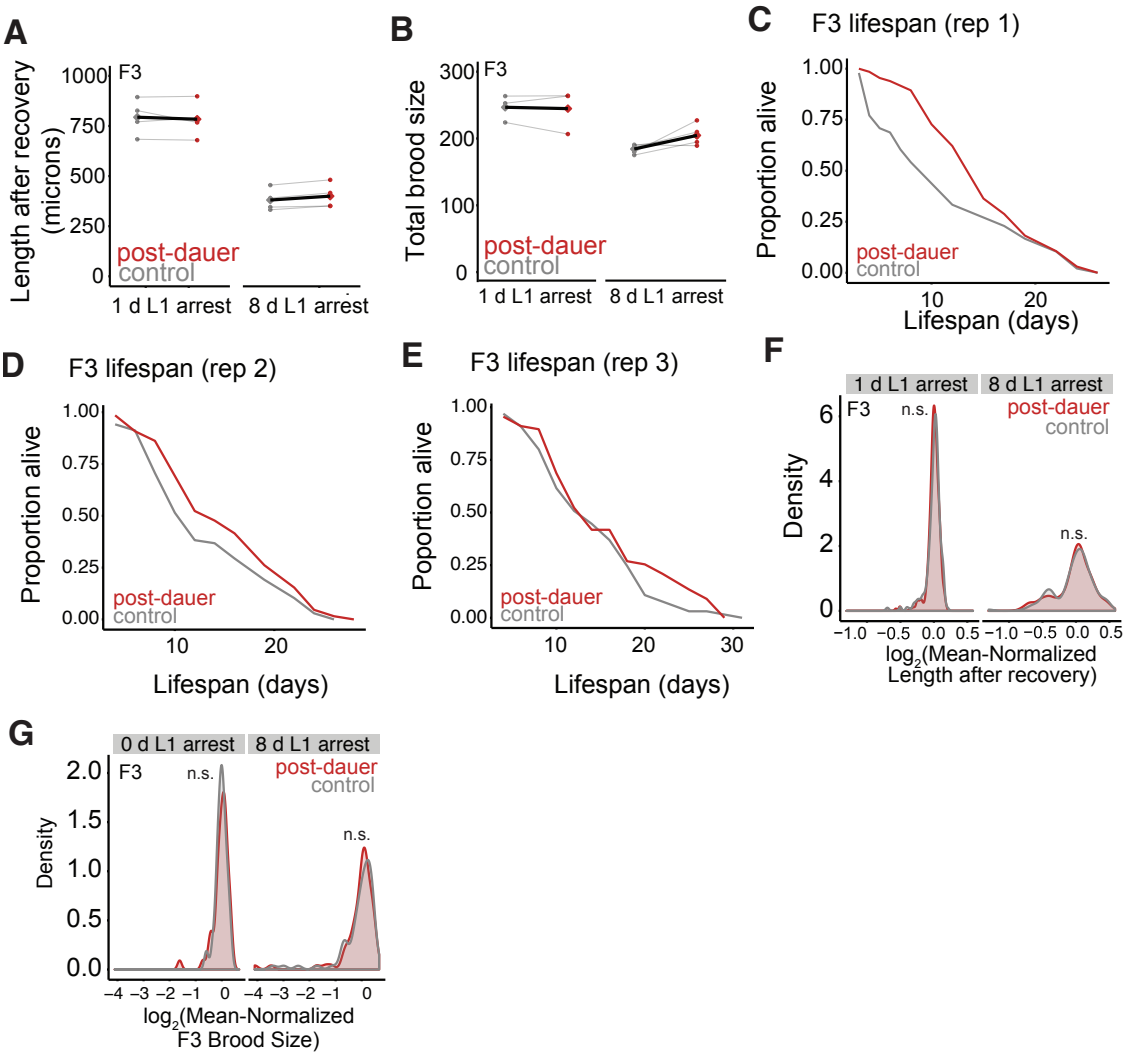
Supplementary Fig 3: Transgenerational effects of long-term dauer diapause in the F3 generation



Supplementary Fig 3: Transgenerational effects of long-term dauer diapause in the F3 generation. A-B. Each point represents the mean of individual worms in the F3 generation for the indicated trait within a biological replicate. Paired replicates are connected with a gray line. Grand means are connected with a black line.
C. Replicate 1 of post-dauer and control F3 lifespan. 47 control and 66 post-dauer F3 worms assayed. Control mean: 12.1 days; post-dauer mean: 15 days
D. Replicate 2 of post-dauer and control F3 lifespan. 64 control and 64 post-dauer F3 worms assayed. Control mean: 13.6 days; post-dauer mean: 15.5 days
E. Replicate 3 of post-dauer and control F3 lifespan. 63 control and 64 post-dauer F3 worms assayed. Control mean: 14.4 days; post-dauer mean: 16.2 days
F. Worm body length measurements were pooled across replicates to test for differences in variance (same data as 3B), For 1 day, p = 0.089; for 8 days, p = 0.82
G. Brood sizes data were pooled across replicates to test for differences in variance (same data as Fig 3C). For 0 d: p = 0.18; for 8 d: p = 0.48.
F-G. Trait values for individual post-dauer and control worms were mean-normalized to the mean trait value for the corresponding condition and replicate. Data were pooled across biological replicates. Levene's test was used to assess differences in variance.
n.s. not significant

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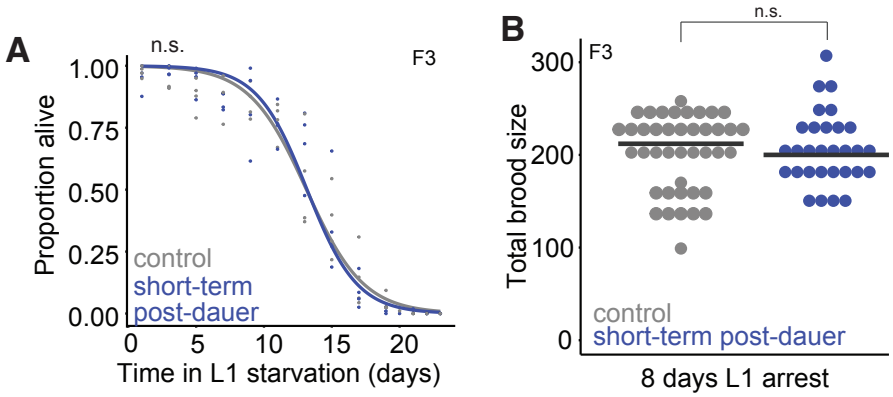
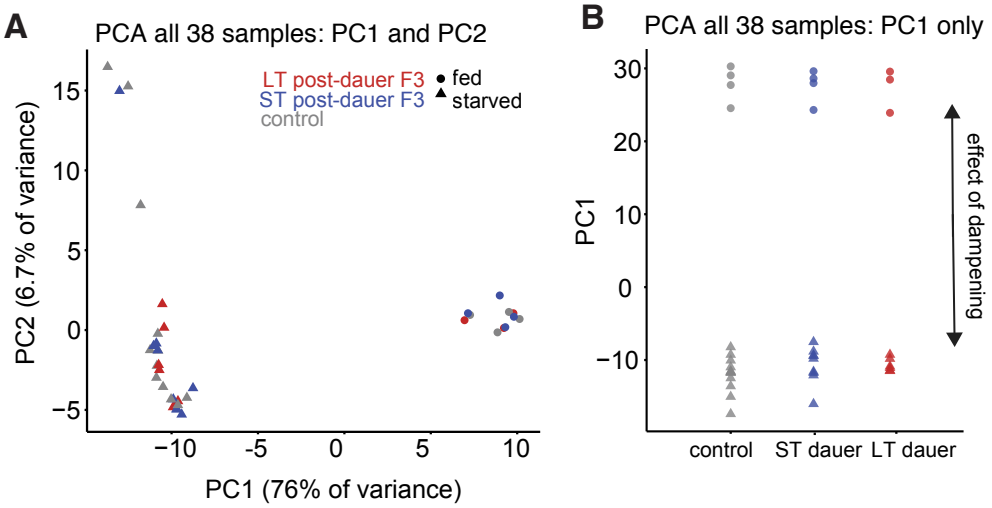
Supplementary Fig 4: Transgenerational starvation resistance is not apparent in the F3 generation following short-term dauer diapause



Supplementary Fig 4: Transgenerational starvation resistance is not apparent in the F3 generation following short-term dauer diapause. A. Starvation survival in the F3 progeny of controls and short-term dauers (6 days as a dauer). 4 biological replicates were scored, logistic curves were fit to data, and median survival times were determined. Paired t-test on median half-lives, p = 0.73.
B. Brood size of F3 progeny of controls and short-term dauers was scored after worms experienced 8 days of L1 arrest. 3 biological replicates of 5-18 individual worms per condition were scored. Grand mean for control: 200; for post-dauers: 201. A linear mixed-effect model was fit to brood size data with condition (post-dauer vs. control) as a fixed effect and biological replicate as a random effect. P-values were calculated using the Wald test. Effect of condition, p = 0.75.
n.s.: not significant. Horizontal black lines represent median.

Supplementary Figure 5: Individual replicates separate on PCA



Supplementary Fig 5: Individual replicates separate on PCA. A. Principal component analysis (PC1 and PC2) of all biological replicates included in differential expression analysis.
B. Biological replicates plotted in PC1 only. Considering both current environment (fed vs. starved) and ancestral environment (control, short-term dauer, and long-term dauer) as factors, both effects are significant in a multivariate ANOVA using Wilks' Lambda for PC1. Effect of current environment, $p < 2.2 \times 10^{-16}$; effect of ancestral environment, p = 0.00022.

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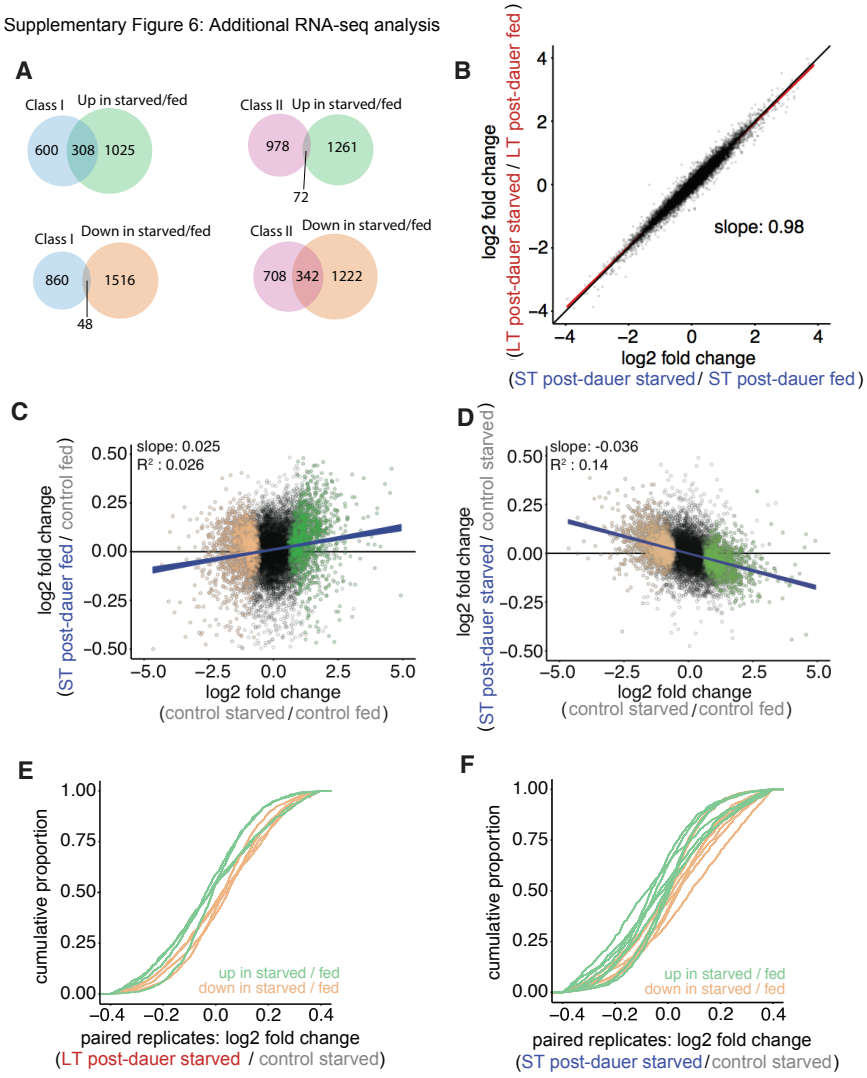

Supplementary Fig 6: Additional RNA-seq analysis. A. Class I and Class II targets defined as genes positively or negatively regulated by DAF-16. Class I and Class II targets were filtered to include those with FDR < 0.05 that were also in the background set of 8649 genes included in starved / fed differential expression analysis. Class I targets are over-enriched in genes up in starved / fed (hypergeometric $p = 1 \times 10^{-49}$). Class II targets are over-enriched among genes in starved / fed (hypergeometric $p = 2.6 \times 10^{-34}$). Class I targets can explain 23.1% of starvation upregulation; class II targets can explain 21.9% of starvation downregulation.

B. Log2 fold change of all genes in ST post-dauer starved / ST post-dauer fed compared to log2 fold change in LT post-dauer starved / LT post-dauer fed. Red line indicates simple linear regression through all points, and thickness of line indicates 95% confidence interval. Slope is 0.98, with confidence interval ranging from 0.973 to 0.981.

C-D. Genes up in control starved / control fed using FDR < 1×10^{-10} are plotted in green. Genes down in control starved / control fed are plotted in tan. Blue line is a simple linear regression through all points, and thickness of line indicates confidence interval.

C. Log2 fold changes of all genes in control starved / control fed plotted against log2 fold changes of ST post-dauer fed F3 / control fed plotted in black.

D. Log2 fold changes of all genes in control starved / control fed plotted against log2 fold changes of ST post-dauer starved F3 / control starved plotted in black.

E-F. Cumulative distribution function plots for individual paired replicates using the same groups of genes defined as up- and down-regulated in control starved / control fed comparison. Paired t-test on median fold changes for these two gene groups.

E. LT post-dauer starved / control starved comparison, $p = 0.0083$.

F. ST post-dauer starved / control starved comparison, $p = 0.088$.

Supplementary Table 1: Starvation survival and lifespan statistics

Starvation survival

F1 progeny of long-term dauer and control	Avg median survival (days)	StDev (days)	n	Avg delta compared to control (days)	StDev of deltas compared to control (days)	p-value vs. control
Control	11	3	7	N/A	N/A	N/A
Long-term dauer	9.1	3.1	7	-1.9	2.6	0.11

F3 progeny of long-term dauer and control	Avg median survival (days)	StDev (days)	n	Avg delta compared to control (days)	StDev of deltas compared to control (days)	p-value vs. control
Control	10.5	1.1	8	N/A	N/A	N/A
Long-term dauer	11.9	0.6	8	1.4	1.1	0.01

F3 progeny of short-term dauer and control	Avg median survival (days)	StDev (days)	n	Avg delta compared to control (days)	StDev of deltas compared to control (days)	p-value vs. control
Control	13.3	1.6	4	N/A	N/A	N/A
Short-term dauer	13.3	1.5	4	0.08	0.4	0.73

Lifespan

F3 progeny of long-term dauer and control	Avg median lifespan (days)	StDev (days)	n	Avg delta compared to control (days)	StDev of deltas compared to control (days)	p-value vs. control
Control	13.4	1.2	3	N/A	N/A	N/A
Long-term dauer	15.6	0.6	3	2.2	0.6	0.02

Supplementary File 1: edgeR output for pairwise RNA-seq comparisons (.xlsx file)

SUPPLEMENTARY DISCUSSION

Increased phenotypic variation after long-term dauer diapause

In addition to long-term post-dauer worms producing fewer, smaller, starvation-sensitive F1 progeny, there is also greater inter-individual variation in P0 brood size and F1 progeny size. Greater inter-individual variability could occur as a result of 1) decanalization or 2) an evolutionary bet-hedging strategy (52). Decanalization occurs when phenotypic robustness is compromised, as a consequence of, for example, conditions that result in pathology. We believe the observed decreases in average P0 brood size and F1 progeny size and starvation resistance following long-term dauer arrest reflect pathological consequences of extended starvation exceeding the buffering capacity of the organism. Consistent with this interpretation, appreciable lethality and developmental abnormalities affecting the reproductive system have been reported after 60 and 25 days of dauer arrest, respectively (31, 32). Alternatively, bet hedging is thought to be evolutionarily adaptive in unpredictable environments (53). In a bet-hedging scenario, an isogenic population exhibits inter-individual variability such that it is suited for variable environmental conditions. In a particular condition, the bet-hedging population may be less fit than a highly specialized population. However, the bet-hedging population reduces variation in fitness across environments. If post-dauer worms display a bet-hedging strategy, then a subpopulation of post-dauers would be expected to perform better than controls in at least some environmental condition. Our data do not provide compelling evidence for a sub-population of post-dauers or their progeny that performs better than controls for any of the proximal traits assayed, despite increased variability. However, we cannot formally exclude the possibility that there are other environmental conditions that would reveal an advantage to increased phenotypic variation as a consequence of extended dauer diapause. We also emphasize that this consideration of bet hedging is in the context of proximal traits. Since we found that the great-grandprogeny of long-term dauers survive starvation better, it is possible

637 that any advantage to a bet-hedging strategy is only apparent on the scale of multiple

638 generations.

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