

Transgenerational Effects of Early Life Starvation on Growth, Reproduction, and Stress Resistance in *Caenorhabditis elegans*

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ABSTRACT Starvation during early development can have lasting effects that influence organismal fitness and disease risk. We characterized the long-term phenotypic consequences of starvation during early larval development in *Caenorhabditis elegans* to determine potential fitness effects and develop it as a model for mechanistic studies. We varied the amount of time that larvae were developmentally arrested by starvation after hatching (“L1 arrest”). Worms recovering from extended starvation grew slowly, taking longer to become reproductive, and were smaller as adults. Fecundity was also reduced, with the smallest individuals most severely affected. Feeding behavior was impaired, possibly contributing to deficits in growth and reproduction. Previously starved larvae were more sensitive to subsequent starvation, suggesting decreased fitness even in poor conditions. We discovered that smaller larvae are more resistant to heat, but this correlation does not require passage through L1 arrest. The progeny of starved animals were also adversely affected: Embryo quality was diminished, incidence of males was increased, progeny were smaller, and their brood size was reduced. However, the progeny and grandprogeny of starved larvae were more resistant to starvation. In addition, the progeny, grandprogeny, and great-grandprogeny were more resistant to heat, suggesting epigenetic inheritance of acquired resistance to starvation and heat. Notably, such resistance was inherited exclusively from individuals most severely affected by starvation in the first generation, suggesting an evolutionary bet-hedging strategy. In summary, our results demonstrate that starvation affects a variety of life-history traits in the exposed animals and their descendants, some presumably reflecting fitness costs but others potentially adaptive.

KEYWORDS L1 arrest; L1 diapause; starvation; bet hedging; transgenerational; fitness trade-off

ANIMALS often experience periods of starvation and must adapt or else perish. In humans, malnutrition *in utero* increases adult risk of type 2 diabetes, obesity, cardiovascular disease, and cancer (Roseboom *et al.* 2006). There is also evidence that diet can influence human disease risk transgenerationally (Pembrey *et al.* 2006), and rodent models reveal diet-dependent epigenetic effects on gene expression in progeny (Carone *et al.* 2010; Ng *et al.* 2010). It has been hypothesized that increased disease risk in adults following

nutrient stress during early development stems from environmental mismatch between early and late life. This model posits that experience during early development is used to anticipate environmental conditions during adulthood through epigenetic or other mechanisms (Hales and Barker 2001). The resulting “thrifty phenotype” is characterized by nutrient rationing and increased fat storage. In theory, such adaptation conveys a fitness advantage in poor conditions but increased disease risk in rich conditions. Relatively simple developmental models are needed to explore the adaptive potential of long-term organismal responses to nutrient stress and to determine their mechanistic basis.

The nematode *Caenorhabditis elegans* has evolved to survive adverse environmental conditions, including nutrient scarcity. For example, dauer larvae arrest development during the third larval stage in response to high population density, limited food, and high temperature (Riddle and Albert 1997; Hu 2007). Passage through dauer arrest affects adult

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gene regulation, brood size, and life span, but it is unclear whether these effects are due to starvation as dauer larvae or developmental alterations made during dauer formation and recovery (Hall *et al.* 2010). Starvation can also cause developmental arrest at other stages (Johnson *et al.* 1984; Angelo and Van Gilst 2009; Seidel and Kimble 2011; Schindler *et al.* 2014). L1 arrest (or L1 diapause) occurs in the first larval stage and is the best understood example of nondauer developmental arrest (Baugh 2013). In contrast to dauer arrest, there is no morphological modification in L1 arrest, allowing the effects of starvation to be studied independent of an alternative developmental program.

Long-term phenotypic consequences of larval starvation have not been systematically characterized in *C. elegans*, and they are not well understood in any animal. Development is delayed following L1 arrest, and AMP-activated protein kinase (AMPK) mutants are sterile only after L1 arrest (Fukuyama *et al.* 2012; Lee *et al.* 2012). These observations suggest that L1 arrest compromises organismal fitness, but effects on stress resistance, which could suggest a fitness trade-off, have not been characterized. It has been reported that the great-grandprogeny (F_3) of animals subjected to L1 arrest have increased life span (Rechavi *et al.* 2014), suggesting epigenetic inheritance of potentially adaptive effects, but other life history traits were not examined. Aside from these isolated observations, the long-term phenotypic consequences of L1 arrest have not been systematically characterized. Such characterization is significant given the importance of *C. elegans* as a model for developmental studies and since it bears on the ecology and evolution of it and other nematodes. L1 arrest is commonly used to synchronize cultures, maintain stocks, and freeze strains, providing additional, practical reasons to characterize its long-term effects.

We examined how extended starvation during L1 arrest affects various life history traits. By measuring growth rate, fecundity, and stress resistance we infer how fitness may be affected in favorable and unfavorable environments while establishing a foundation for mechanistic studies of specific phenotypic effects. Our results show that L1 arrest reduces growth rate, fertility, and starvation resistance in exposed animals but that resistance to starvation and heat is increased in their immediate descendants. These transgenerational effects suggest germline epigenetic inheritance of ecologically relevant environmental consequences on organismal phenotype. Together these observations suggest a combination of fitness costs and benefits that unfolds over generations, consistent with the animal drawing from experience to anticipate the future and maximize survival in adverse conditions.

Materials and Methods

Nematode strains

This work used the wild-type Bristol N2 strain. Worms were maintained on agar plates containing standard nematode growth media (NGM) seeded with *Escherichia coli* OP50 at

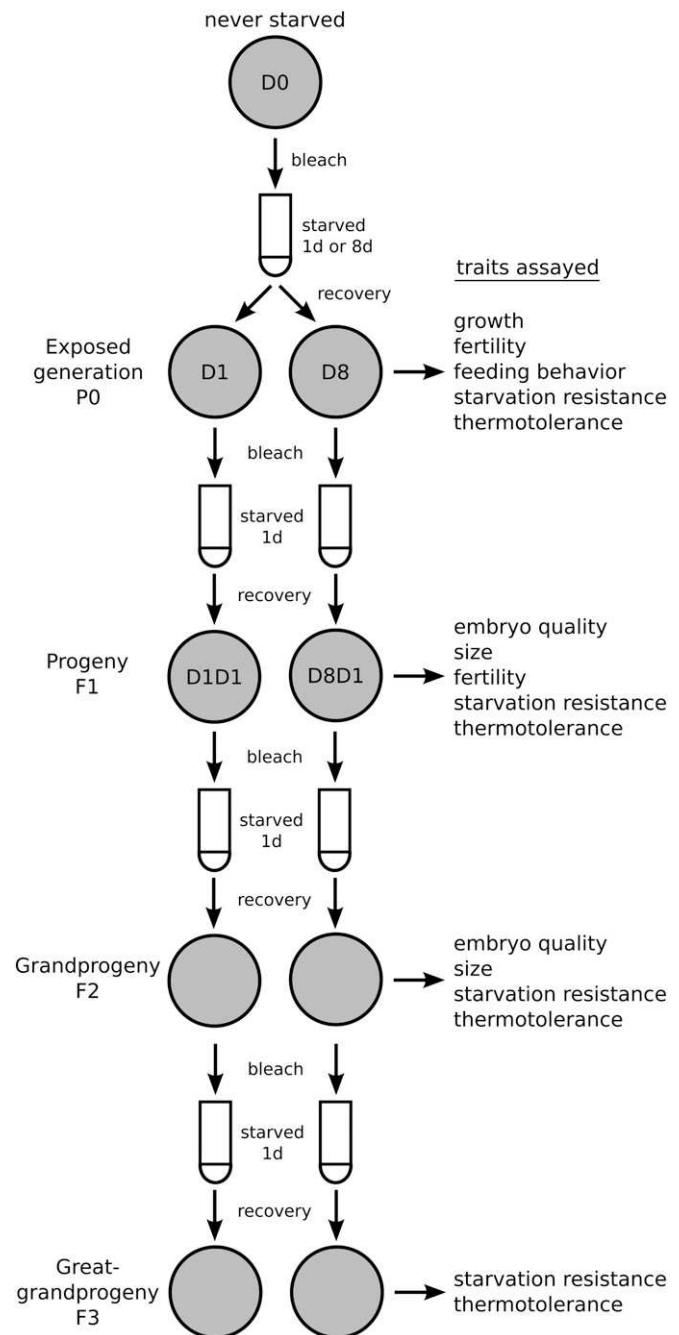


Figure 1 A schematic of the experimental design is presented. Worms were maintained on plates without starvation for several generations prior to initiation of an experiment. To initiate an experiment, worms were bleached and incubated in S-basal buffer so they hatch and enter L1 arrest for either 1 or 8 days. In some cases 4 days L1 arrest was used (Figure 2E). Arrested larvae were plated with food to initiate recovery synchronously. Worms were bleached in subsequent generations and arrested for 1 day to synchronize development. Traits assayed after arrest recovery are indicated for each generation. In some cases larvae were subjected to 8 days L1 arrest two generations in a row (Table 1). In several cases D8 animals were manually sorted based on developmental progression, producing two subpopulations ($D8^{\text{delayed}}$ and $D8^{\text{normal}}$) that were independently assessed in the P_0 and subsequent generations. In some cases D8D1 animals were sorted based on developmental progression, producing two subpopulations for separate analysis (Figure 6D).

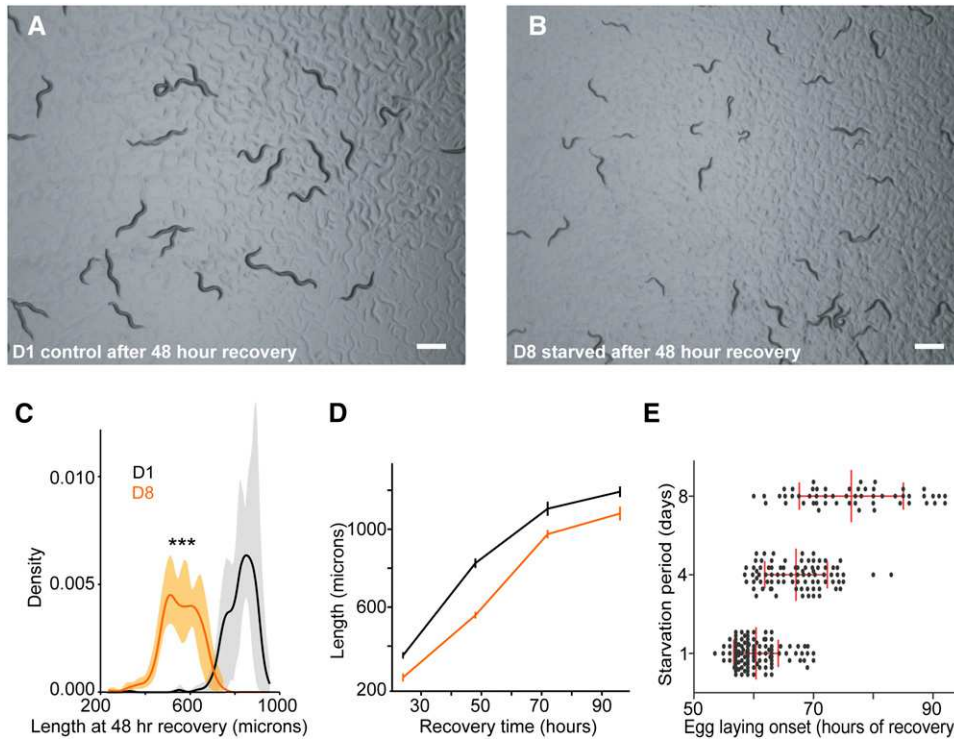


Figure 2 Extended L1 arrest reduces growth rate and delays reproductive onset. (A and B) Images of D1 controls (A) and D8 starved animals (B) after 48 hr recovery from L1 arrest are shown. Bar in A and B, 500 μ m. (C) Density plots of length after 48 hr recovery are shown. Shading indicates 95% confidence intervals after bootstrapping with four biological replicates. *** P -value < 0.001, two-tailed t -test. (D) Quantification of length over time during recovery from L1 arrest is plotted. Error bars correspond to the standard error of the mean (SEM) of biological replicates. (E) Egg-laying onset of individual D1 control, D4, and D8 animals is shown.

20°. Animals were passaged frequently to avoid starvation and overcrowding during routine maintenance.

Bleaching and L1 arrest

Mixed-stage cultures were maintained without starvation. To prepare animals for experiments, 15 L4/young adults were transferred to a new plate and cultured for 3 days, and eggs were prepared by hypochlorite (bleach) treatment. Animals were washed from the plates and dissolved in a bleach solution [7:2:1 ddH₂O, sodium hypochlorite (Sigma, St. Louis), 5 M KOH]. Worms were spun down after 1.5–2 min in the bleach solution and the solution was refreshed. Total time in bleach solution was 8–10 min, ensuring dissolution of worms. Embryos were washed three times in virgin S-basal buffer (no cholesterol or ethanol) and suspended in virgin S-basal at a density of 1/ μ l. Embryos were cultured in a 25-mm glass test tube on a tissue culture roller drum at 25 rpm at 21°–22°. Hatching was scored 24 hr after bleaching to confirm viability. For recovery, 1000 arrested L1s were plated per 10-cm NGM OP50 plate.

Image analysis of worm size

At the appropriate time of recovery, animals were gently washed off plates with virgin S-basal, washed twice, plated in a small volume on room temperature plates with no *E. coli*, and imaged. Arrested L1s were directly plated from buffer at room temperature and imaged. Magnification was 77 \times for arrested L1 larvae, 40 \times for 24 hr recovery, and 20 \times for 48, 72, and 96 hr recovery. A Zeiss (Thornwood, NY) Discovery V2 stereomicroscope equipped with a Zeiss AxioCam camera was used. Images were captured using Axiograph software (Zeiss

v. 4.8.2) and saved as TIFF files. The Fiji plug-in WormSizer was used to analyze the images, producing length estimates (Moore *et al.* 2013). Biological replicates typically included 75–125 individuals. Density plots of the length distribution were plotted in R. Biological replicates were used for bootstrapping (with subsampling to use the same number of individuals per replicate) to determine 95% confidence intervals. The means of biological replicates were used for t -tests.

Egg laying and brood size

At 48 hr recovery, day 1 (D1), D4, and D8 starved animals were singled onto 6-cm seeded plates. D8 animals were manually classified as developmentally delayed or normal at 48 hr. Plates were scored every 2–6 hr to determine egg-laying onset. To measure egg-laying dynamics and brood size, single animals were transferred every 24 hr to a new plate until egg laying ended. The total number of progeny was tracked for each individual on each plate.

Induction of egg laying

At 96–100 hr recovery, animals that had not laid eggs were individually treated with 30 mM serotonin (5-HT, Sigma) to induce vulval contractions. After 1 hr, animals were scored for egg laying and then mounted on a 5% agarose pad for microscopy. The anatomy of the germline and somatic reproductive system was observed at 100 \times with Nomarski optics and anatomical abnormalities were recorded.

Frequency and measurement of abnormal embryos

Animals were bleached after 72 hr recovery. Following bleach treatment, the frequency of abnormally shaped embryos was

Table 1 Manual classification of late larvae as developmentally delayed or normal after 48 hr recovery from L1 arrest

Gen	Treatment group, abbreviation	Normal	Delayed	Delayed (%)	Delayed range (%)
1	Starved 1 day, D1	20,796	39	<1	NR
1	Starved 8 days, D8	11,226	2,925	21	19–23
2	Starved 1 day, D1D1	5,961	18	<1	NR
2	Starved 1 day, D8D1	6,085	673	10	6–14
2	Starved 8 days, D8D8	5,521	2,308	30	25–35

Size was scored by eye at a stereomicroscope. Results of multiple trials by two investigators were pooled. Abbreviations: Gen, generation scored; NR, not reported, all replicates <1%.

visually scored under a stereomicroscope for ~100 individuals. Abnormal embryos are rounder than normal. Independent investigators produced consistent estimates of the frequency of abnormal embryos. Embryonic dimensions were determined by imaging embryos on NGM plates at high magnification on a Zeiss Discovery V2 stereomicroscope equipped with a Zeiss AxioCam camera. Images were captured using Axiograph software (Zeiss v. 4.8.2) and saved as TIFF files. Pixels were calibrated to microns with a micrometer, and length and width of embryos were quantified using Fiji.

Embryonic lethality

Gravid adults were bleached after 72 hr recovery and the embryos were plated on OP50 seeded plates. Either the normal or the abnormal embryos were removed by picking, leaving a population of only one or the other. Hatching frequency was scored after 48 hr. Biological replicates included ~100 individuals.

Analysis of feeding behavior

The pharyngeal grinding assay was performed as described with minor modifications (Kang *et al.* 2007). Animals were recovered from starvation on NGM OP50 plates. Twelve hours before scoring, animals were washed onto plates seeded with GFP-expressing OP50. Animals were subsequently examined for presence of GFP in the gut under a compound fluorescent microscope at 100×. For the ingestion assay, 0.1-μm fluorescent beads (Polysciences, Warrington, PA; no. 16662) were added to a stationary OP50 culture in liquid at a dilution of 1:100 and seeded onto NGM agar plates. Animals were recovered from starvation on regular OP50 plates before being transferred to fluorescent bead-containing plates 5 hr before scoring. The number of beads per animal was counted under a compound fluorescent microscope at 100×. Pharyngeal pumping rate was determined as described (Avery and You 2012). Pumping rate was scored on plates and it was calculated as the average of two 20-sec time periods separated by 5 min.

D8 sorting for preparation of progeny

D1 and D8 animals were recovered on NGM OP50 plates at 1000 per plate for 66–68 hr. D1 control animals and less severely affected D8 animals had clearly begun egg laying, allowing for easy identification of reproductively delayed (D8^{delayed}) animals. D8^{delayed} animals were all manually transferred to a different NGM OP50 plate. The animals

remaining on the plate from which they were transferred were considered D8^{normal}. D1 control plates, D8^{normal} plates, and unsorted D8 (D8^{unsorted}) plates were bleached after 72 hr of recovery, and D8^{delayed} plates were given an additional 16 hr of recovery and bleached at 88 hr. Embryos were incubated in virgin S-basal for 24 hr at a density of 1/μl so they hatch and enter L1 arrest and then they were plated for imaging or recovery.

Starvation survival

For starvation survival during L1 arrest in the F₁ or F₂ generation, P₀ (D8 animals sorted as described above) or F₁ animals were bleached and their embryos were suspended at 1/μl in virgin S-basal in a 16-mm glass test tube on a tissue culture roller drum at 21°–22°. For starvation survival of P₀ larvae recovered from L1 arrest, D1 and D8 animals were plated on several 10-cm NGM OP50 plates at 1000 per plate for recovery. After 27 hr recovery, the animals were washed off the plates with virgin S-basal and washed three times. Larvae were suspended at a density of 1/μl in 16-mm glass test tubes. In both cases, 100-μl aliquots were pipetted around the edge of an *E. coli* OP50 lawn and the total number of plated animals was counted (*T_p*). After 2 days, the number of surviving animals was counted (*T_s*). Survival was determined by calculating *T_s*/*T_p*. Starvation survival analysis was performed on 50% survival times (*t_{half}*) for groups of biological replicates, which were obtained by fitting survival data for each trial with the function

$$S = 100 - \frac{100}{1 + e^{(t_{\text{half}} - t)/\text{rate}}},$$

which we have modified slightly from Artyukhin *et al.* (2013).

Thermotolerance in P₀ generation

D1 and D8 animals were plated on NGM OP50 plates for 48 hr recovery. Plates were wrapped in parafilm and submerged in a 35.5° water bath for 7 hr. Survival was scored by movement in response to prodding after a 16- to 24-hr recovery period at 20°.

To measure thermotolerance in conjunction with size, D0, D1, and D8 animals were plated on NGM OP50 plates for 24 or 48 hr recovery. D0 animals were never starved; that is, they were plated as embryos immediately after being bleached. At 24 and 48 hr, one plate was wrapped in parafilm and submerged in a 35.5° water bath for 5.5 hr. Survival was scored

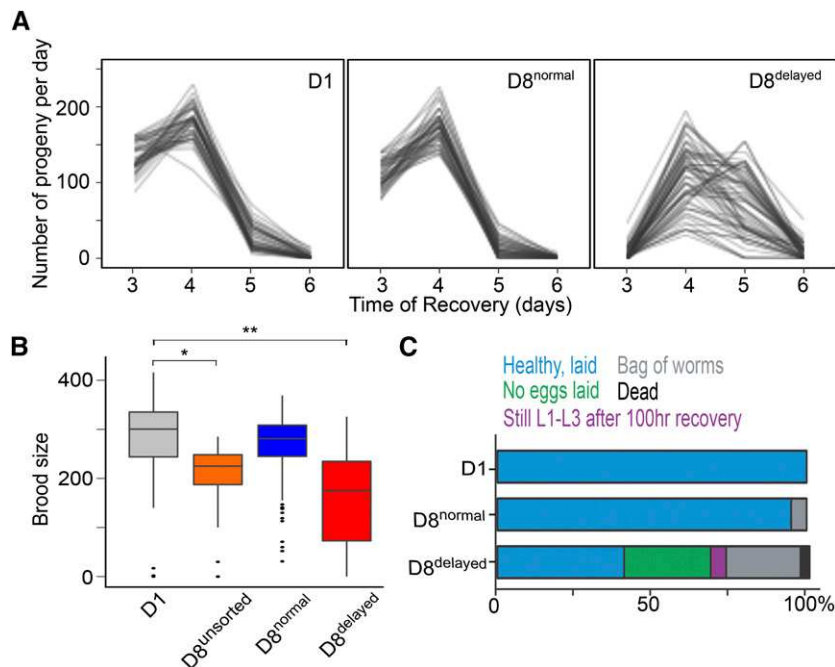


Figure 3 Extended L1 arrest reduces reproductive potential. (A) Egg-laying dynamics of individual animals scored in 24-hr intervals are plotted for D1, D8^{normal}, and D8^{delayed}. (B) Total brood size distributions are plotted for D1, D8^{unsorted}, D8^{normal}, and D8^{delayed} (two to eight biological replicates are pooled). Box and whiskers plots show minimum, 25%, median, 75%, and maximum with outliers as dots. **P*-value < 0.05, ***P*-value < 0.01; two-tailed *t*-test. (C) Proportion of individuals that were healthy and had laid eggs, had formed a bag of worms due to internal hatching, had laid no eggs, were dead, or were still L1–L3 larvae after 100 hr recovery is plotted (D1, *n* = 193; D8^{normal}, *n* = 91; D8^{delayed}, *n* = 274).

by movement in response to prodding after a 16- to 24-hr recovery period at 20°. At the time of submersion, a replicate plate was imaged and analyzed with WormSizer. Biological replicates included ~100 individuals.

Thermotolerance in F₁, F₂, and F₃ generations

P₀ (D8 animals sorted as described above), F₁, or F₂ animals were bleached and their embryos were suspended at 1/μl in virgin S-basal in a 16-mm glass test tube on a tissue culture roller drum at 21°–22°. A total of 400–500 arrested L1 larvae (F₁, F₂, or F₃) were recovered on 6-cm NGM OP50 plates for 48 hr. These plates were then air incubated at 35.5° for 5.5 hr. Survival was scored by movement in response to prodding after a 16- to 24-hr recovery period at 20°. Biological replicates included 100–300 individuals each. Thermotolerance in F₃ was scored blind.

Data analysis and statistics

Data were handled in R, and graphs were plotted in the R package ggplot2 or Excel. Statistical tests used are mentioned in the text and were performed in Excel or R.

Data availability

Figure S1 contains supplemental data in support of Figure 6A.

Results

We used L1 arrest to determine the long-term effects of extended starvation during early larval development. Gravid adults were bleached to collect embryos, which were cultured without food in phosphate-buffered saline (S-basal) so they hatch and enter L1 arrest (Figure 1). Experimental animals were plated with *E. coli* strain OP50 as food after variable periods of starvation (denoted “DX”, where X indicates the

number of days). Control animals (D1) were plated 24 hr after bleaching to synchronously initiate postembryonic development after a brief period of starvation (~12 hr). Synchronized populations were subjected to a variety of phenotypic assays, and their descendants were also examined.

Extended L1 arrest reduces growth

Growth rate was reduced in larvae that were subjected to 8 days L1 arrest (D8). D1 control animals developed synchronously, but D8 animals displayed reduced size and increased variation after 48 hr recovery (Figure 2, A and B). We quantified larval size with image-based analysis, using WormSizer (Moore *et al.* 2013). This analysis revealed that D8 larvae were two-thirds as long as D1 controls at 48 hr recovery (Figure 2C). The proportional difference in size decreased as larvae matured into adults and growth rate declined (Figure 2D). Not only did D8 animals grow slowly, but also they took 16 hr longer on average to reach the onset of egg laying (Figure 2E), indicating that they developed slowly as well and suggesting that they are smaller as adults.

Growth rate was more variable in D8 animals, and we hypothesized that the slowest-growing larvae display the most severe effects of extended starvation. We manually classified D8 individuals as developmentally “delayed” or “normal” at 48 hr recovery based on visual inspection. Twenty-one percent of D8 animals were considered delayed (D8^{delayed}), compared to <1% of D1 control animals (Table 1). Nearly all D8^{delayed} animals reached adulthood within 2 additional days (8% and 5% remain larvae after 72 hr and 100 hr recovery, respectively), indicating that they were viable. It is important to note that while only 21% of D8 animals were classified as delayed the majority of D8 animals were smaller than the average of the D1 control based on quantitative

Table 2 Exogenous serotonin can rescue egg laying in D8^{delayed} nonlaying animals

Animals examined	<i>n</i>	Laid in 30 mM serotonin (%)	No germline (%)	Anatomical disruption (%)	Anatomy intact but nonresponsive (%)
D1 control forced Egl	29	86	0	14	0
D8 ^{delayed} Egl	42	32	16	42	10

At 100 hr recovery, animals that had not laid eggs were individually treated with 30 mM serotonin to induce vulval contractions. After 1 hr, animals were scored for egg laying and then mounted for microscopy. Anatomy of the germline and somatic reproductive system was observed with Nomarski optics. D1 control animals were forced Egl by brief starvation as adults. Abbreviations: Egl, egg-laying defective; D, day.

analysis (Figure 1C), indicating that most of the animals show an effect of extended starvation.

Extended L1 arrest reduces fecundity

Reproduction was compromised in larvae that were subjected to 8 days L1 arrest. In addition to a delayed onset of egg laying (Figure 2E), egg-laying dynamics were also affected (Figure 3A). Given the variable effect of starvation on growth rate, we manually sorted D8 individuals based on size at 48 hr recovery and analyzed the developmentally delayed and normal subpopulations separately. The effect on egg-laying dynamics was clear among the D8^{delayed} individuals, which often required an extra day to reach their peak egg-laying rate. Total brood size of D8 animals was significantly reduced compared to D1 controls without sorting by size (Figure 3B). However, sorting D8 individuals as developmentally delayed or normal showed that the slower-growing animals (D8^{delayed}; ~21% of the population) displayed the greatest reduction in fertility. The effects on egg-laying dynamics and brood size show that these reproductive traits are more severely affected in the developmentally delayed individuals.

Developmental abnormalities were present but relatively rare among larvae that were subjected to 8 days L1 arrest, but egg-laying behavior was broadly affected. Approximately one-quarter of the D8^{delayed} individuals (~5% of the entire D8 population) did not lay any eggs (Figure 3C). We examined these nonlaying individuals more closely to distinguish between sterility, developmental defects, and otherwise healthy animals that hold rather than lay their eggs. We found that 16% (*n* = 42) had no visible germline, as if sterile, and 42% had clear anatomical disruption, including protruded and everted vulvae among other abnormalities (Table 2). The remaining 42% of the nonlaying subpopulation represents ~10% of the D8^{delayed} population and ~2% of the entire D8 population. Egg laying is regulated by serotonin-based neurotransmission at the vulval muscles, and it is sensitive to environmental conditions and stress (Schafer 2005). We reasoned that if these remaining animals were capable of laying eggs, then they should do so in response to exogenous serotonin. Three-quarters of the nonlaying D8^{delayed} subpopulation with an intact germline and no evident anatomical abnormalities laid eggs in response to 30 mM exogenous serotonin (Table 2). This result indicates that these animals developed appropriately and were capable of laying eggs, although they held them all, as if due to altered physiological regulation. These individuals represent the most extreme

example of holding rather than laying eggs in that they never laid a single egg prior to serotonin treatment. In support of our interpretation that regulation of egg laying was affected, approximately one-quarter of D8^{delayed} individuals died within 100 hr recovery due to internal hatching (“bag of worms”) (Figure 3C). The effect on egg laying in these animals was less severe in that they laid some eggs prior to death, but this observation shows that egg-laying behavior is more broadly affected. Furthermore, a small fraction of D8^{normal} individuals also died due to internal hatching within 100 hr. Likewise, the majority of D8^{normal} animals were visibly holding excess eggs (Egl), although they had not progressed to internal hatching (data not shown), providing additional evidence that egg-laying behavior was broadly affected. These observations suggest that regulation of egg-laying behavior is affected by extended starvation, independent of developmental abnormalities, and they suggest that the most severe effects are in the developmentally delayed individuals.

Extended L1 arrest causes defective feeding behavior

Autophagy during L1 arrest can damage the pharynx, the muscular organ used for ingestion of food, hindering recovery after feeding (Kang *et al.* 2007). We hypothesized that slow growth of D8 animals stems from defective feeding. We scored the rate of pharyngeal pumping to assay feeding behavior. D8 animals had a significantly lower average pumping rate than D1 controls during L1 arrest (0 hr recovery) and after 24 hr recovery (Figure 4A). We also used fluorescent beads mixed with *E. coli* OP50 to analyze ingestion and GFP-expressing *E. coli* OP50 to analyze pharyngeal grinding. At 24 hr recovery, D8 animals ingested significantly fewer beads on average than D1, suggesting a reduced ingestion rate (Figure 4B). Also at 24 hr recovery, 60% of D8 animals had intact GFP-expressing bacteria in their intestine compared to <20% of the D1 control animals (Figure 4C). This defect was not as severe as that of a feeding-defective *eat-2* mutant, which displays slow growth, but is consistent with impaired feeding. These results suggest that defective feeding behavior could contribute to the observed slow growth and decreased brood size.

Extended L1 arrest subsequently reduces starvation resistance

Since extended starvation reduces growth and fertility, we wondered whether it increases stress resistance, possibly offsetting those fitness costs. We hypothesized that if early-life

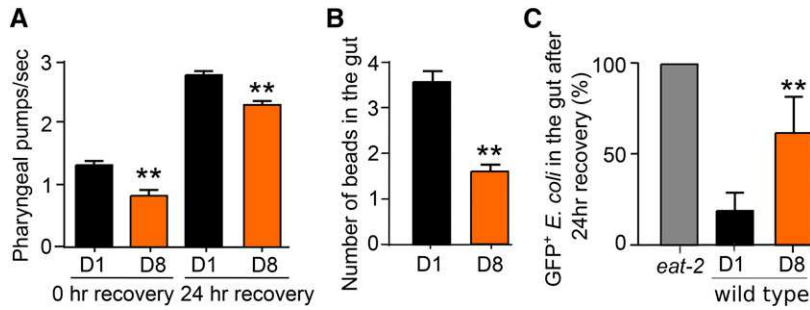


Figure 4 Extended L1 arrest causes defective feeding behavior. (A) The average and SEM of pharyngeal pumping rate are plotted at 0 and 24 hr recovery from L1 arrest. Fifty animals were measured for each bar. (B) The average number of beads ingested per animal in a 5-hr window after 24 hr recovery from L1 arrest is plotted along with the SEM. One hundred animals were measured for each bar. (C) Average and standard deviation of the percentage of larvae with unground GFP-expressing bacteria in the gut after 24 hr recovery from L1 arrest are plotted. Three independent trials are included. The grinding-defective mutant *eat-2(ad465)* is included as a control. (A–C) ***P*-value < 0.01, two-tailed *t*-test.

experience is used to anticipate future conditions, as postulated by the thrifty phenotype hypothesis (Hales and Barker 2001), then previously starved animals could have increased resistance to subsequent starvation. We recovered D1 and D8 animals on plates with food for 27 hr before subjecting them to starvation again. In contrast to our hypothesis, D8 animals were significantly more sensitive to starvation, surviving only ~64% as long as D1 controls (Figure 5A). We also observed a decrease in Oil Red O lipid staining in D8 animals, suggesting reduced fat stores (data not shown). In contrast to the thrifty phenotype hypothesis, these observations suggest that larvae that have experienced extended starvation are not better prepared for subsequent starvation. Rather, these results suggest that prolonged starvation has an immediate fitness cost after resumption of growth, even in adverse conditions.

Smaller larvae are more resistant to heat stress without passage through L1 arrest

Although D8 animals were not able to survive starvation better than worms that had not experienced extended starvation, it could be that they are more resistant to other stressors. We tested whether extended starvation increases heat resistance. D8 animals were more resistant to heat (Figure 5B), seemingly suggesting that extended starvation leads to increased heat resistance. However, among the D8 survivors, 63% appeared developmentally delayed and 38% appeared normal based on visual inspection. Given the smaller average size of the D8 animals, this observation suggests that increased thermotolerance could be due to decreased size. We controlled for size and found that there is an inverse relationship between heat resistance and size, even in animals that were never starved (D0) (Figure 5C). In conclusion, D8 animals are no more heat resistant than expected based on size.

Extended L1 arrest reduces embryo quality as well as progeny size and fertility

Extended starvation during L1 arrest decreased the quality of embryos produced after recovery. Approximately 20% of embryos produced by D8 animals appeared morphologically abnormal, and D8^{delayed} animals produced more abnormal embryos than D8^{unsorted} animals (Figure 6A, insets). These

abnormal embryos were more round than normal, with an ~18% increase in their aspect ratio (width/length; supporting information, Figure S1) stemming from decreased length. Given this abnormality, we were surprised that most of them hatched, but hatching efficiency was lower than for the normal-looking members of their cohort as well as embryos from D1 parents (90–94%, 99%, and 99%, respectively). Reduced hatching efficiency suggests that embryo quality was compromised. Embryonic lethality could result from chromosomal abnormalities. Meiotic X chromosome nondisjunction produces males (Hodgkin *et al.* 1979), suggesting that the incidence of males should be increased if aneuploidy due to chromosomal nondisjunction is involved. The D8^{delayed} individuals produced male progeny at an elevated frequency (3–4% compared to <1% for D1). This observation is consistent with aneuploidy due to chromosomal nondisjunction.

Animals recovered from extended L1 arrest produced small progeny. We analyzed the progeny of D8 animals after arresting them overnight for synchronization (referred to as D8D1). We found that D8D1 animals were smaller upon hatching as L1 larvae (Figure 6B). Visual inspection at 48 hr recovery after L1 arrest in these D8D1 animals revealed that 10% appeared developmentally delayed (Table 1). Furthermore, exposure to extended L1 arrest two generations in a row (D8D8) increased the apparent frequency of delayed animals to 30%, suggesting a compound effect. Quantitative analysis revealed a modest but reproducible, statistically significant reduction in length of D8D1 animals after 48 hr recovery (4%; Figure 6C). These results show that the progeny of animals recovered from extended L1 arrest are smaller upon hatching and after 48 hr of larval development.

Effects on embryo morphology and progeny size did not persist in the grandprogeny of exposed individuals. The frequency of abnormal embryos was not increased among grandprogeny of D8 animals, including grandprogeny of D8^{delayed} individuals (data not shown). There was also no effect on grandprogeny size after 48 hr larval development (data not shown). These observations suggest these traits are due to maternal effects as opposed to epigenetic inheritance.

Fertility is modestly affected in progeny of animals subjected to extended L1 arrest. We measured brood size in D8D1 animals and found no effect in the unsorted population. However, manual sorting of the D8D1 animals that appeared

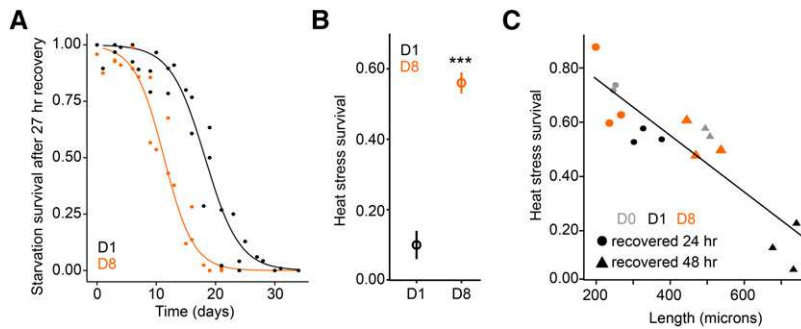


Figure 5 Extended L1 arrest does not increase stress resistance independent of effects on growth rate in recovered animals. (A) Starvation survival after 27 hr recovery from L1 arrest is plotted for D1 and D8 animals. Logistic regression of mean survival from three biological replicates is shown. Half-lives for D1 and D8 were 17.8 and 11.4 days, respectively. $P = 0.01$, two-tailed t -test. (B) Heat stress survival (35.5° for 7 hr) is plotted for D1 and D8. The mean and SEM of three biological replicates is included. *** $P < 0.001$, two-tailed t -test. (C) Heat stress survival (35.5° for 5.5 hr) is plotted against length for D0, D1, and D8 animals at 24 and 48 hr recovery. D0 animals were never starved but instead plated with food as embryos. $R^2 = 0.80$; two or three biological replicates plotted.

developmentally delayed (D8D1^{delayed}; ~10% of the population) revealed a significant reduction in brood size (Figure 6D). Despite affecting only a subpopulation, this result shows that fertility is compromised in progeny of animals subjected to extended L1 arrest.

Extended L1 arrest increases resistance to starvation and heat in immediate descendants

Immediate descendants of D8^{delayed} animals displayed increased resistance to starvation and heat. We sorted animals exposed to extended starvation based on whether they were developmentally delayed or apparently normal to examine stress resistance in their descendants. In contrast to the sorting done at 48 hr recovery in other contexts, we sorted at 66–68 hr recovery. This later time point allowed us to assess whether each individual had become gravid (normal) or not (delayed). D8^{delayed} animals were also given 16 hr additional time prior to bleaching so that embryos were prepared from mothers of approximately the same developmental age (see *Materials and Methods*). The progeny (F_1) of D8^{delayed} animals survive starvation during L1 arrest significantly longer than the progeny of D1 control animals (Figure 7A). Likewise, the grandprogeny (F_2) of D8^{delayed} animals survive L1 arrest significantly longer than the grandprogeny of D1 control animals, although the effect appears weaker than in the F_1 generation (Figure 7B). The progeny and grandprogeny of D8^{delayed} animals also survive heat shock significantly longer than the descendants of D1 control animals (Figure 7C). Furthermore, a reproducible increase in heat resistance was observed in great-grandprogeny of D8^{delayed} animals. Despite a diminished effect in the F_3 generation compared to earlier generations, this result suggests epigenetic inheritance of acquired stress resistance. Notably, this heritable effect is not seen in the descendants of unsorted D8 animals, similar to effects on starvation survival. This pattern of transmission suggests that the individuals most severely affected by extended L1 arrest are also most prone to producing progeny adapted to future adverse conditions.

Discussion

The theory of hormesis asserts that exposure to stress can engage a response that endows the organism with increased

stress resistance after exposure (Gems and Partridge 2008). The thrifty phenotype hypothesis is conceptually related to hormesis, in that it asserts that nutrient stress during early development leads to an altered physiological state that increases fitness in conditions of nutrient scarcity (Hales and Barker 2001). We characterized the long-term phenotypic effects of extended starvation during *C. elegans* L1 arrest with these ideas as motivation. This animal is a particularly appropriate model given its ecology and robust responses to nutrient stress (Felix and Braendle 2010). By quantitatively analyzing growth, reproduction, and stress resistance in the exposed animals and their descendants, we show that extended L1 arrest affects a variety of life-history traits across generations. This work establishes a foundation for future mechanistic studies of specific phenotypic effects of prolonged starvation. This work also demonstrates that researchers should be cautious in using previously starved animals for experiments, because exposure to starvation early in development may have consequences on later developmental processes.

Our results suggest a variety of fitness costs of extended starvation during L1 arrest. Larvae recovering from 8 days L1 arrest grew slowly, such that the effect was clearly visible, and at variable rates, as if decanalized (Waddington 1942). They also produced fewer progeny, with the smallest animals producing the least, consistent with a systemic effect. Notably, passage through dauer arrest increases brood size (Hall *et al.* 2010). This difference suggests that L1 arrest and dauer arrest affect reproductive potential in different ways, possibly due to the alternative developmental program associated with dauer formation and recovery. A very small fraction of animals recovered from extended L1 arrest were sterile, and a similar fraction displayed observable developmental defects (*e.g.*, protruded and everted vulvae), further compromising reproductive potential. Defective feeding behavior was also evident, which may contribute to slow growth and reduced fertility. Defective feeding suggests irreversible damage to the pharynx (Kang *et al.* 2007), and other tissues may also suffer. AMPK mutants are sterile after recovery from L1 arrest, and visible damage in the primordial germ cells of L1 stage larvae is predictive of sterility in adults (Fukuyama *et al.* 2012; Lee *et al.* 2012). These observations suggest that AMPK

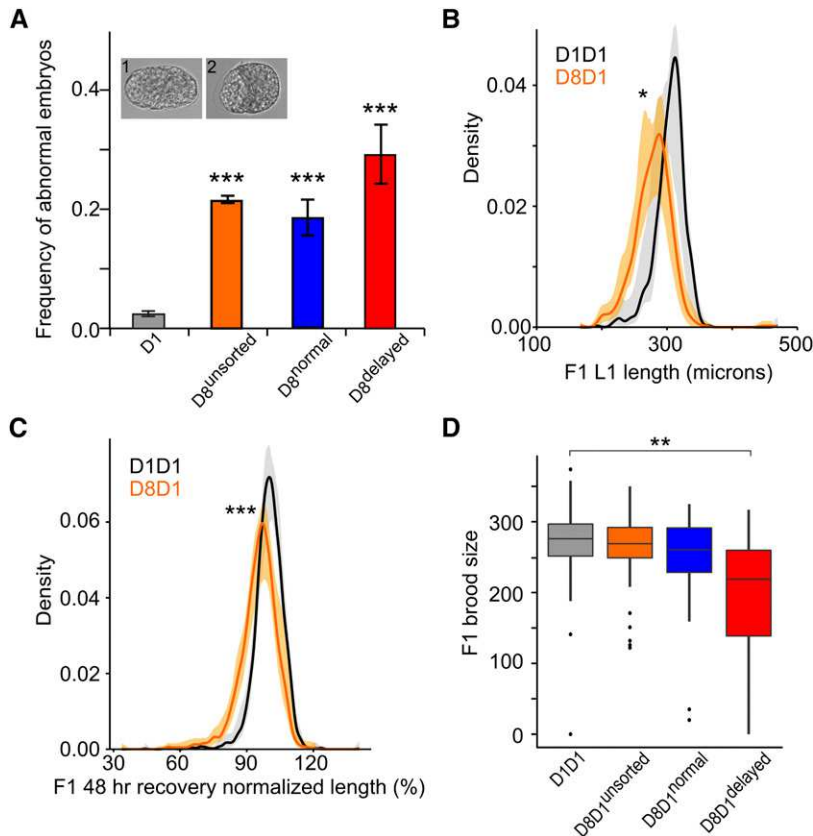


Figure 6 Extended L1 arrest affects progeny size and fecundity. (A) The frequency of abnormally shaped embryos is plotted. Error bars correspond to the SEM. Insets 1 and 2 are images of a D1 control embryo and a D8 abnormal embryo, respectively. ****P*-value < 0.001; two-tailed *t*-test. (B) Density plots of length for arrested L1 larvae are shown. Shading indicates 95% confidence intervals after bootstrapping with four biological replicates. **P*-value < 0.05, two-tailed *t*-test. (C) Normalized density plots of length after 48 hr recovery from L1 arrest are shown. Shading indicates 95% confidence intervals after bootstrapping with nine biological replicates. For each trial individual lengths were divided by the D1D1 control average length for that trial for normalization. ****P*-value < 0.001; paired-sample, two-tailed *t*-test without normalization. (D) Total brood size distributions for D1D1, D8D1^{unsorted}, D8D1^{normal}, and D8D1^{delayed} are plotted. Three to six biological replicates are pooled. Box and whiskers plots show minimum, 25%, median, 75%, and maximum with outliers as dots. ***P*-value < 0.01, two-tailed *t*-test.

mutants enhance the reduced fertility we describe for wild type, and they support the idea that the most severe effects of extended L1 arrest stem in part from irreversible cellular damage.

Extended starvation also affects embryo quality as well as progeny size and fertility, although the effects on progeny size and fertility are relatively subtle compared to the effects in the directly exposed animals. Animals recovered from extended starvation produced a significant fraction of embryos with an abnormally round appearance. These abnormal embryos had reduced hatching efficiency, suggesting they were of diminished quality, possibly due to chromosomal abnormalities. The smallest of the exposed animals (D8^{delayed}) produced male progeny at an elevated frequency (3–4%). Production of males suggests meiotic X chromosome nondisjunction (Hodgkin *et al.* 1979), consistent with diminished embryo quality stemming from chromosomal nondisjunction. The average size of progeny upon hatching was reduced and remained smaller after 48 hr of larval development. The effects on embryo quality and progeny size were not detectable in grandprogeny, suggesting they result from maternal effects of starvation as opposed to germline epigenetic inheritance (Jablonka and Raz 2009). The smallest F₁ progeny had reduced brood size, indicating that reduced fertility persists for at least one generation. We speculate that reduced progeny fertility is due to dysregulation of gene expression in the primordial germ cells of these exposed animals.

Our results are inconsistent with induction of a thrifty phenotype in animals exposed to extended L1 arrest. Dauer

larvae provide a clear example of a fitness trade-off between conditions in that they arrest development and postpone reproduction but can survive various stressors and live for months rather than weeks. If extended starvation during L1 arrest resulted in a fitness trade-off during recovery, then dauer larvae would likely develop at a detectable frequency. However, we did not observe dauer larvae among animals recovering from extended L1 arrest. We also observed a decrease in fat storage (data not shown), as opposed to the increase seen in dauers and *daf-2* insulin/insulin-like growth factor receptor mutants (O'Rourke *et al.* 2009). Moreover, we did not detect increased starvation resistance. In fact, animals exposed to extended starvation were significantly more sensitive to starvation when reexposed after recovery, providing direct evidence against a thrifty phenotype in the tested conditions. However, it is notable that we used a domesticated laboratory strain and an extended period of starvation. It is possible that milder conditions or a different genetic background could reveal a hormetic effect. Additional studies varying these parameters are necessary to determine whether *C. elegans* can display a thrifty phenotype.

Despite apparent fitness costs, our results suggest potentially adaptive responses in the animals exposed to extended L1 arrest. We discovered that smaller larvae are more heat resistant, independent of passage through L1 arrest. Slow growth in animals exposed to extended L1 arrest could provide a fitness advantage in certain adverse conditions. In addition, the majority of animals recovered from extended

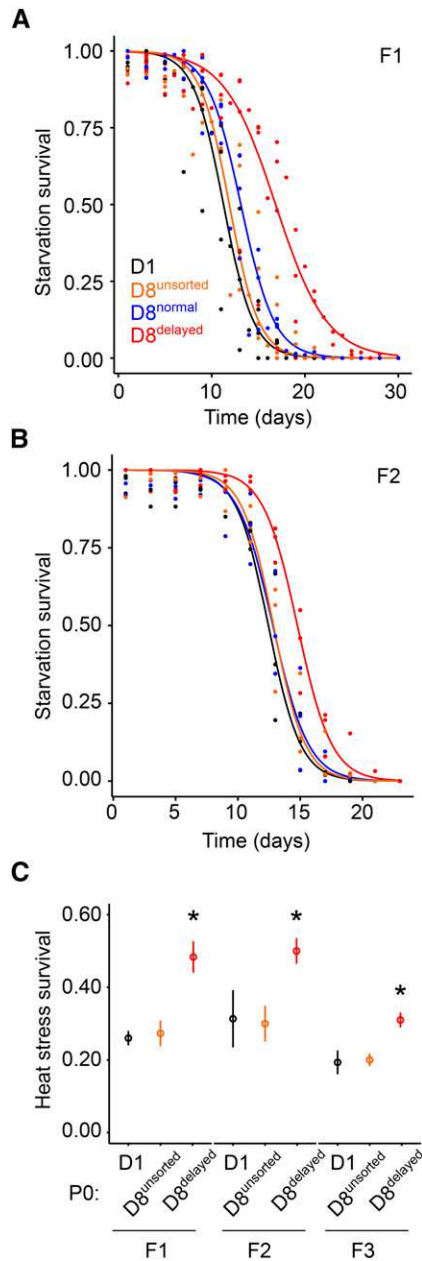


Figure 7 Extended L1 arrest increases stress resistance in descendants. (A) Starvation survival during L1 arrest is plotted for the F₁ generation (progeny). Logistic regression of mean survival from five biological replicates is shown. Half-lives for D1 and D8^{delayed} progeny are 11.0 and 16.1 days, respectively. $P = 0.005$, two-tailed t -test. (B) Starvation survival during L1 arrest is plotted for the F₂ generation (grandprogeny). Logistic regression of mean survival from three biological replicates is shown. Half-lives for D1 and D8^{delayed} grandprogeny are 12.6 and 14.7 days, respectively. $P = 0.02$, two-tailed t -test. (C) Heat stress survival is plotted for the F₁, F₂, and F₃ generations. $*P < 0.05$, two-tailed t -test.

starvation held their eggs, causing a significant fraction to die early from internal hatching (“bagging”). We were able to rescue egg laying, using exogenous serotonin in animals that had never laid, suggesting that regulation of egg-laying behavior is altered in animals exposed to extended L1 arrest. It has been proposed that bagging is an adaptive response to acute

starvation and other forms of stress (Chen and Caswell-Chen 2004). Although the egg-holding behavior we document could reflect persistent stress after recovery from extended starvation, it is plausible that this behavior would be beneficial in adverse conditions.

Our results provide evidence for a potentially adaptive response to extended L1 arrest that is manifested in the immediate descendants of the animals most severely affected by starvation. Starvation survival during L1 arrest was increased for two generations following extended L1 arrest. Moreover, heat resistance was increased for three generations. Resistance to other stressors was not tested, and so it is unclear how general or specific the effects on stress resistance are. Either way, inheritance of increased starvation survival and thermotolerance is consistent with an adaptive response to extended starvation despite reduced growth and fertility. High glucose consumption also decreases fertility but increases resistance to oxidative stress in progeny (Tauffenberger and Parker 2014), and this combination of effects could theoretically increase fitness in adverse conditions (Ratcliff *et al.* 2009). Although we argue against a thrifty phenotype in the animals exposed to extended starvation, inheritance of increased resistance to starvation and heat suggests a conceptually related example of the animal using experience to anticipate future conditions.

The transgenerational effect on thermotolerance in the F₃ generation suggests germline epigenetic inheritance of an ecologically relevant environmental effect on organismal phenotype (Jablonka and Raz 2009). Mutations affecting histone modification cause transgenerational effects on life span, but an environmental stimulus was not identified (Greer *et al.* 2011). Mild heat stress affects messenger RNA expression transgenerationally in germline RNAi-dependent fashion, but an effect on organismal phenotype was not shown (Schott *et al.* 2014). Nonetheless, these studies suggest plausible mechanisms for germline transmission. Increased life span has been reported for the F₃ descendants of animals subjected to 6 days of L1 arrest (Rechavi *et al.* 2014). This result is consistent with the epigenetic inheritance of stress resistance we describe, except that the transgenerational effect on life span was observed without sorting the exposed animals by phenotype and with significant variation between replicates. Under our conditions, inheritance of resistance to heat and starvation is detectable only in the descendants of individuals most severely affected by L1 arrest (D8^{delayed}). This discrepancy could be due to the different conditions used for L1 arrest (6 days on nutrient agar plates vs. 8 days in buffer in our study) or the different methods used for passaging between generations. Life span may also be a more sensitive assay than starvation survival or thermotolerance. Additional work is necessary to identify the salient factors among these differences as well as transmission mechanisms.

Paternal transmission of dietary effects on progeny gene expression in mice isolates epigenetic inheritance from maternal effects (Carone *et al.* 2010; Ng *et al.* 2010). Establishing

an analogous system of paternal transmission in *C. elegans* could be valuable on both theoretical and practical grounds. Male transmission of starvation-induced traits would further support the conclusion that germline epigenetic inheritance transduces ecologically relevant effects of the environment on animal physiology. Such a system could also facilitate analysis of transmission mechanisms. We describe what we believe is a combination of maternal and epigenetic effects of extended L1 arrest. These effects may be confounded, with maternal effects possibly obscuring epigenetic effects. Paternal transmission could allow maternal and epigenetic effects to be distinguished, possibly facilitating detection of epigenetic effects. *C. elegans* is hermaphroditic, and males are rare. However, males enable outcrossing, which increases fitness during adaptation to novel environments (Morran *et al.* 2009). We observed an increase in the frequency of males among F₁ progeny of worms exposed to extended L1 arrest. If males are capable of transmitting epigenetic effects, their elevated frequency following extended starvation could contribute to outcrossing while also communicating their experience epigenetically (*i.e.*, “epigenetic outcrossing”).

Our results show that the individuals most severely affected by extended L1 arrest with respect to growth rate and fecundity produce the descendants that are most resistant to starvation and heat stress. This pattern of transmission would not have been observed without manually sorting the exposed animals by phenotype and separately examining the traits of their descendants. It is intriguing to speculate that variation in the response of the exposed animals is due to decanalization and reflects a bet-hedging strategy (Waddington 1942; Gillespie 1974; Pincus and Slack 2010; Casanueva *et al.* 2012; Levy *et al.* 2012). In this speculative model, some individuals develop relatively quickly, producing more progeny, and others develop slowly, producing fewer but more stress-resistant progeny.

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Transgenerational Effects of Early Life Starvation on Growth, Reproduction, and Stress Resistance in *Caenorhabditis elegans*

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Ashley L. Lennox, and L. Ryan Baugh

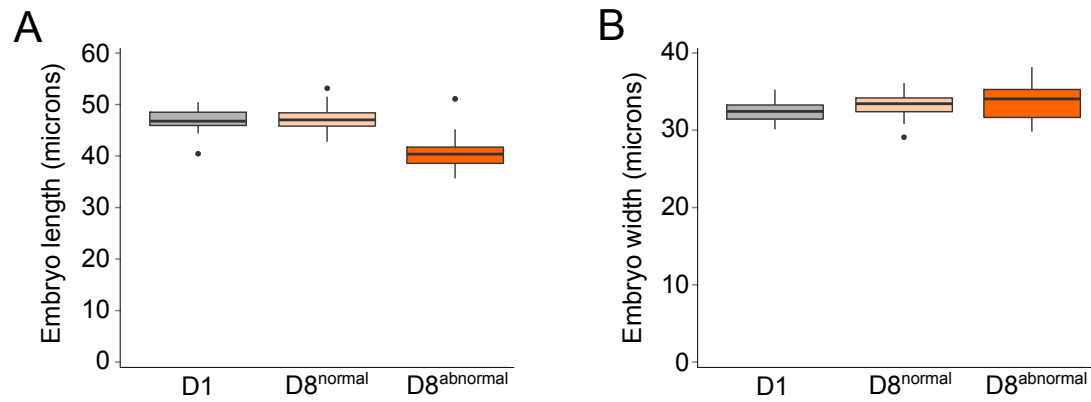


Figure S1 Extended L1 arrest leads to the production of abnormally shaped embryos. Length (A) and width (B) are plotted for D1 control, D8 "normal" and D8 "abnormal embryos. Normal and abnormal were manually classified by eye. D1, D8 normal and D8 abnormal include 36, 75 and 11 embryos, respectively. Box and whiskers show minimum, 25%, median, 75%, and maximum with outliers as dots.