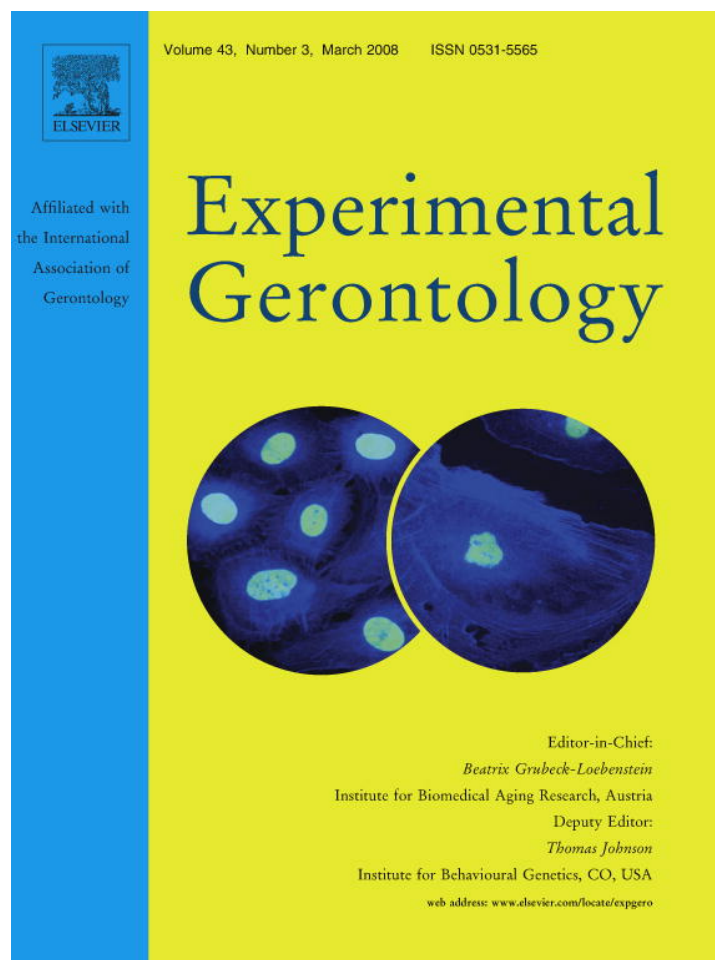


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Dietary restriction by bacterial deprivation increases life span in wild-derived nematodes

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Abstract

Dietary restriction is known to promote longevity in a variety of eukaryotic organisms. Most studies of dietary restriction have been performed on animals bred for many generations under conditions that differ substantially from their natural environment, raising the possibility that some apparent beneficial effects of dietary restriction are due to adaptation to laboratory conditions. To address this question in an invertebrate model, we determined the effect of dietary restriction by bacterial deprivation on life span in five different wild-derived *Caenorhabditis elegans* strains and two strains of the related species *Caenorhabditis remanei*. Longevity was enhanced in each of the wild-derived *C. elegans* strains, in most cases to a degree similar to that observed in N2, the standard laboratory strain. Both strains of *C. remanei* were substantially longer lived than any of the *C. elegans* isolates, produced larger brood sizes, and retained the ability to produce offspring for a longer period of time. Dietary restriction failed to increase mean life span in one *C. remanei* isolate, but significantly increased the maximum life span of both *C. remanei* strains. Thus, we find no evidence that adaptation to laboratory conditions has significantly altered the aging process in *C. elegans* under either standard or food-restricted conditions.

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Keywords: Calorie restriction; Caloric restriction; Food; Aging; Longevity; Natural environment; Laboratory; Reproduction; Diet

1. Introduction

Dietary restriction (DR), defined as a decrease in food availability in the absence of malnutrition, extends life span in a variety of eukaryotic organisms including the budding yeast *Saccharomyces cerevisiae* (Jiang et al., 2000; Lin et al., 2000), the nematode *Caenorhabditis elegans* (Houthoofd et al., 2003; Kaeberlein et al., 2006; Lakowski and Hekimi, 1998; Lee et al., 2006), and rodents (Weindruch et al., 1986). DR is also known to slow many physiological aging processes and retard age-associated pathologies (Weindruch and Walford, 1988). The mechanism(s) by which DR acts to slow aging in laboratory animals is unknown, but may involve modulating the activity of evolutionarily conserved nutrient response pathways, such as

those mediated by insulin/IGF-1-like signaling and the target of rapamycin (TOR) kinase (Kennedy et al., 2007).

Most studies involving DR have been performed using organisms bred for many generations under laboratory conditions characterized by an abundant food source, a lack of pathogens and predation, and low temperature variation (Pugh et al., 1999). Laboratory breeding protocols tend to favor individuals exhibiting rapid growth and early reproductive maturity (Harper et al., 2006). Studies comparing laboratory bred mouse strains to recently captured wild strains found that wild mice have less body mass and consume less food than those bred in the laboratory (Austad, 2001; Austad and Kristan, 2003; Bronson, 1984), suggesting that DR protocols may resemble natural feeding levels more closely than do laboratory control diets. Combined with evidence from both mice (Miller et al., 2002) and flies (Linnen et al., 2001; Sgró and Partridge, 2000) that laboratory strains have a reduced life span relative to wild strains, this raises the question of whether the beneficial effects of DR are artifacts of adapta-

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tion to a low-hazard, food-rich environment. In this scenario DR may simply return laboratory strains to an environment and life cycle more closely resembling those found in nature. A recent study using wild-derived mice (grand-offspring of mice captured in the wild) found that DR did not increase mean life span but did increase maximum life span of these animals (Harper et al., 2006).

To test the possibility that life span extension from DR in *C. elegans* is a laboratory artifact, we examined the effect of DR on longevity in wild-derived nematode populations. The natural food source of *C. elegans* is thought to be exclusively bacterial; however, the effect of dietary restriction in wild nematode populations is difficult to predict because little is known about food availability and feeding levels in the natural environment (Caswell-Chen et al., 2005; Hodgkin and Doniach, 1997; Jovelín et al., 2003). In the laboratory, nematodes are typically maintained on nutrient-agar nematode growth medium (NGM) with *Escherichia coli* OP50 at a cell density of $\sim 10^9$ cells/cm² or greater, while natural food sources are presumably not as abundant as these standard laboratory conditions.

To accomplish DR in nematodes, we utilized a optimized reduced bacterial feeding protocol in which animals are maintained in the absence of bacterial food after the fourth day of adulthood; this DR method is hereafter referred to as bacterial deprivation (BD). BD provides maximum life span extension from food restriction under the standard laboratory conditions used in a majority of prior *C. elegans* studies (Kaeberlein et al., 2006; Lee et al., 2006). Genetic epistasis tests suggest that BD promotes longevity by a mechanism similar to previously described genetic models of DR, such as mutation of *eat-2* (Hekimi et al., 1998). Specifically, BD increases life span (1) independently of the FOXO family transcription factor *daf-16*, (2) independently of the sirtuin *sir-2.1*, (3) additively with mutation of the insulin-like receptor *daf-2*, and (4) non-additively with mutation of *eat-2* (Kaeberlein et al., 2006; Lee et al., 2006). Although animals subjected to BD are unable to feed on bacterial cells after the fourth day of adulthood, the extreme longevity and motility (foraging) of BD animals indicates that energy reserves are sufficient to maintain health and maximize longevity. Thus,

BD meets the formal definition of DR, a reduction in food availability in the absence of malnutrition.

The effect of BD on survival was examined in five wild *C. elegans* isolates, and two different isolates of the related species, *Caenorhabditis remanei*. *C. remanei* is characterized by a female/male mating type, in contrast to *C. elegans*, which is primarily hermaphroditic (Baird et al., 1992; Brenner, 1974; Geldziler et al., 2006). To induce DR, we utilized a reduced bacterial feeding protocol (bacterial deprivation; BD) that has been shown to optimally increase life span under standard laboratory conditions (Kaeberlein et al., 2006; Lee et al., 2006). BD was found to significantly increase both mean and maximum life span in every strain examined, with the exception of one *C. remanei* isolate in which maximum, but not mean, life span was increased.

2. Materials and methods

All worm strains used in the study are listed in Table 1. Strains were provided by the Caenorhabditis Genetics Center or obtained from Thomas and propagated at 25 °C on solid nematode growth media (NGM) seeded with the *E. coli* strain OP50. *E. coli* OP50 cultures were seeded in liquid Luria broth (LB) from a single bacteria colony, grown overnight at 37 °C, and then concentrated fivefold. Control plates (10 ml NGM agar in 50 mm diameter Petri dishes) were seeded with 100 μ l of the concentrated OP50 stock and allowed to dry overnight. The dry bacterial food source was then killed by exposure to a UV-source. Ampicillin was added to the NGM prior to pouring the plates at a concentration of 100 μ M to ensure all bacteria were killed and to inhibit contamination by foreign bacteria. Unseeded plates used for BD experiments were also treated with UV and Ampicillin.

To initiate life span experiments, 15 hermaphrodites (*C. elegans*) or 15 females and 5 males (*C. remanei*) per plate were transferred to NGM + UV-killed *E. coli* OP50, allowed to lay eggs for 6 h, and then removed. The resulting synchronized population was allowed to reach L4, at which point approximately hermaphrodites (*C. elegans*) or females (*C. remanei*) were transferred to NGM + UV-killed OP50 supplemented 50 μ M 5-fluorodeoxyuridine (FUDR). Although developmental timing was not quanti-

Table 1
Nematode strains used in this study

Strain	Description	Source
N2	Standard <i>C. elegans</i> laboratory strain.	J. Thomas
JU262	<i>C. elegans</i> wild isolate from Le Blanc (Indre, France). Isolated from a vegetable garden garbage pile by M. Felix. Weak Phm phenotype	CGC
MY2	<i>C. elegans</i> wild isolate obtained by H. Schulenburg from compost heap in Roxel, Mnster, North-West Germany. Frozen within 5 generations after isolation; microsatellite genotype EU2.	CGC
PS2025	<i>C. elegans</i> wild isolate. Isolated by J. Demodena from a garden in Altadena, CA.	CGC
PX176	<i>C. elegans</i> wild isolate. Isolated by B. White from compost in Eugene, OR.	CGC
PX178	<i>C. elegans</i> wild isolate. Isolated by B. White from organic soil in Eugene, OR.	CGC
EM464	<i>C. remanei</i> ssp. <i>vulgaris</i>	J. Thomas
SB146	<i>C. remanei</i> ssp. <i>remanei</i>	J. Thomas

All strains were obtained from the Caenorhabditis Genetics Center (CGC) or were provided by J. Thomas (University of Washington, Seattle).

fied, no significant difference in the time from egg to L4 was noted among any of the *C. elegans* or *C. remanei* strains examined. On day 4 of adulthood, approximately 1/3 of the cohort was transferred to NGM + UV-killed OP50 + 50 μ M FUDR (CF = control fed media) and the remainder were transferred to NGM + 50 μ M FUDR (BD media). Animals on CF media were transferred to fresh plates as needed to renew the bacterial food source. Each animal was scored every 2–3 days for movement in response to gentle prodding to determine viability. Animals that crawled off of, or burrowed into, the NGM and did not return to the surface were not included in the data set. Control fed and BD life span analysis was repeated at least twice for each wild-derived nematode strain, with similar results obtained in independent replicate experiments.

The Wilcoxon rank sum test (MATLAB RANKSUM function) was used to determine statistical significance and calculate *P*-values. Maximum life span was determined by taking the mean age at death of the longest-lived 10% of a given test population.

Brood size assays were initiated in the same manner described above for life span. For *C. elegans* strains, when the synchronized population reached L4, 10 hermaphrodites were transferred to NGM + UV-killed OP50 (1 animal per plate). For *C. remanei*, 10 females and 30 males were transferred to NGM + UV-killed OP50 (1 female, 3 males per plate). Each day all worms were transferred to fresh plates. Old plates were stored at 25 °C for 2 days and then scored for number of animals present. Transfers and scoring continued until all worms from a given strain failed to produce progeny for two consecutive days. Animals that crawled off of or burrowed into the NGM agar and did not return to the surface were not included in the data set.

3. Results

Life span was measured for five wild-derived isolates of *C. elegans* and two strains of *C. remanei* on control fed

(CF) and BD regimens and compared to the most commonly used laboratory strain of *C. elegans*, N2. Subjecting worms to BD extended both mean and maximum life span relative to CF in all cases, except for the mean life span of the *C. remanei* strain SB146, which is addressed below (Table 2).

Mean and maximum life span was observed to vary within both species. Several wild *C. elegans* strains displayed life span characteristics similar to N2 (Fig. 1A). Strain PX176 was particularly comparable to N2, having similar mean and maximum life span under CF and BD conditions. MY2 and N2 animals had similar mean and maximum life span on the CF diet and similar maximum

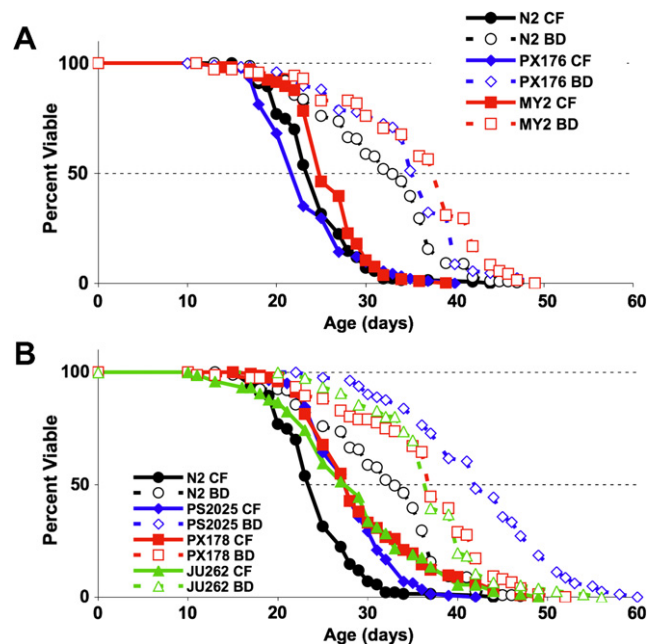


Fig. 1. BD extends life span in wild-derived *C. elegans* strains. (A) Strains MY2 and PX176 exhibit longevity comparable to N2 and significant life span extension in response to BD. (B) BD increases the life spans of strains JU262, PS2025, and PX178, which are longer-lived than N2 on both CF and BD diets.

Table 2
Survival statistics for N2, wild *C. elegans* strains, and *C. remanei* strains

Species	Strain	Brood size	<i>P</i> -value	CF				BD				Max LS ratio (BD/CF)	Mean LS ratio (BD/CF)
				Max LS	Mean LS	SE	<i>N</i>	Max LS	Mean LS	SE	<i>N</i>		
<i>C. elegans</i>	JU262	230 ± 24	1.7E–10	43.0	28.6	0.92	74	48.6	37.1	0.72	76	1.13	1.30
	MY2	184 ± 7	6.7E–16	33.1	26.1	0.40	106	45.9	36.1	0.95	71	1.39	1.38
	N2	290 ± 10	7.8E–23	32.8	24.5	0.38	143	42.6	31.8	0.45	250	1.30	1.30
	PS2025	210 ± 14	0.000	36.1	28.1	0.38	143	55.0	42.6	0.87	81	1.52	1.51
	PX176	192 ± 7	3.8E–23	33.7	23.7	0.52	91	44.6	34.8	0.61	127	1.32	1.47
	PX178	291 ± 9	1.7E–09	43.9	29.6	0.60	124	47.4	36.4	0.90	76	1.08	1.23
<i>C. remanei</i>	EM464f	469 ± 66	1.6E–06	70.8	44.5	2.15	44	98.0	62.7	2.76	59	1.38	1.41
	SB146f	409 ± 80	0.727	47.7	37.2	1.26	30	66.3	38.7	2.83	29	1.39	1.04

All life span (LS) data is given in days from the time of hatching. Maximum life span (Max LS) refers to the mean of the longest-lived 10% of each test group. Brood size indicates average total number of offspring produced per individual adult hermaphrodite (*C. elegans*) or female (*C. remanei*) and is shown as average ± standard error. *P*-value is the result of a Wilcoxon Rank-Sum test of control fed versus BD survival for each strain.

life span on the BD diet, but MY2 exhibited a longer mean life span than N2 in response to BD. Three wild *C. elegans* strains, JU262, PX178 and PS2025, were long-lived relative to N2 both on the control diet and when subjected to BD (Fig. 1B). Both JU262 and PX178 exhibited a relatively smaller extension of maximum life span by BD (8% and 13%, respectively) compared to the rest of the *C. elegans* strains tested (averaging 29% extension), while mean life span extension by BD was comparable. Only two strains of *C. remanei* were tested, and while both were long lived relative to any of the *C. elegans* strains, EM464 was also significantly longer lived than SB146 (Fig. 2).

A substantial strain-dependent variation in the degree of life span extension by BD was observed among the different *C. elegans* strains tested. The extension of mean life span in the BD experimental groups relative to control groups ranged from 23% for strain PX178 to 51% for strain PS2025, while the extension of maximum life span ranged from 8% for strain PX178 to 52% for strain PS2025 (Table 2). The average extension of mean life span is $37 \pm 10\%$ and the average extension of maximum life span is $29 \pm 15\%$. For the two *C. remanei* strains, maximum life span was extended by a similar degree while the mean life span extension was quite different. Strain EM464 exhibited a robust mean life span extension of 41% on BD relative to CF. In contrast, mean life span of strain SB146 was not significantly extended by BD.

Reproductive capacity and longevity have been linked in several different organisms, with reduced fecundity often, but not always, showing an inverse correlation with life span (Mukhopadhyay and Tissenbaum, 2007; Partridge et al., 2005). We therefore quantified the brood size for each strain to determine whether a correlation between reproduction and longevity could be discerned (Table 2). *C. elegans* strains MY2 and PX178 – those with similar life span characteristics to N2 – both displayed a substantially smaller brood size than N2 (63% and 66% of N2 brood size, respectively, Fig. 3A). Of the *C. elegans* strains that were longer lived than N2, PX178 had a nearly identical brood size, while those of JU262 and PS2025 were some-

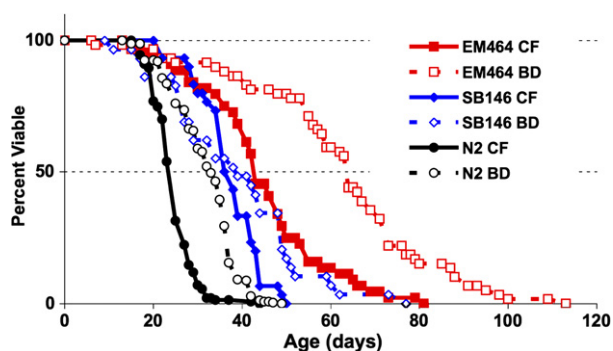


Fig. 2. BD increases maximum life span in two *C. remanei* strains. *C. remanei* strains EM464 and SB146 are long lived relative to *C. elegans* N2. EM464 exhibits significant mean and maximum life span extension by BD. BD does not extend mean life span of SB146, but does increase maximum life span.

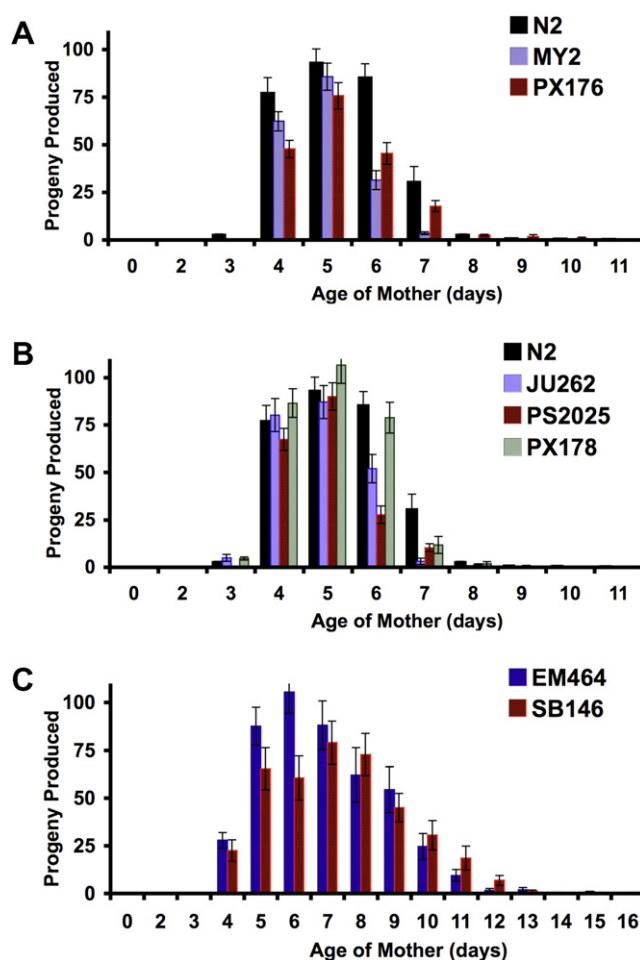


Fig. 3. Brood size analysis of wild-derived *C. elegans* and *C. remanei* strains. Number of progeny produced per animal was determined for *C. elegans* N2 and compared to wild-derived *C. elegans* isolates (A) MY2 and PX178, (B) JU262, PS2025, and PX178. (C) Number of progeny produced per animal was determined for *C. remanei* strains EM464 and SB146. Data shown is the mean number of progeny produced for 10 animals. Error bars are standard error of the mean.

what smaller (79% and 72% of N2 brood size, respectively, Fig. 3B). All three long-lived wild strains displayed an earlier reproductive peak relative to N2. Both *C. remanei* strains produced larger broods and remained reproductively active longer than the *C. elegans* strains (Fig. 3C).

4. Discussion

The data presented here demonstrate that DR by bacterial food deprivation extends life span in several wild *C. elegans* populations obtained from different locations around the world. In all five cases, wild-derived *C. elegans* exhibited significantly longer life spans when subject to BD. The survival parameters of the different strains varied substantially, but no systematic difference between wild-derived and laboratory-maintained *C. elegans* was observed. Thus, we find no evidence that adaptation to the laboratory has altered either the survival of *C. elegans* under standard conditions or the response of *C. elegans* to food restriction.

The longevity-enhancing effects of BD are also conserved among at least one related nematode species, *C. remanei*. Strain EM464 shows robust life span extension via BD. Although BD did not significantly increase mean life span in a second *C. remanei* strain, SB146, maximum life span was significantly increased. The failure of BD to increase mean life span in SB146 is reminiscent of the effect of DR observed in one strain of wild-derived mice (Harper et al., 2006). Analysis of additional *C. remanei* isolates will be necessary to determine whether EM464 or SB146 represent a typical response to BD for this species.

It is noteworthy that both *C. remanei* strains examined displayed significantly greater longevity than any of the *C. elegans* strains. Unlike *C. elegans*, *C. remanei* are not hermaphroditic. This difference makes comparative analysis of the brood size experiments presented here difficult, as the *C. remanei* females were maintained in the presence of males, while *C. elegans* hermaphrodites were not allowed to mate with males. The life span data, however, was derived solely from female *C. remanei* maintained in the absence of male animals during adulthood and is directly comparable to the *C. elegans* life span data. We attempted to measure the response of *C. remanei* males to BD, but were unable to obtain a sufficient cohort due to the loss of nearly 100% of the animals to foraging up the sides of the Petri dishes, which resulted in desiccation and death.

Both *C. elegans* and *C. remanei* showed substantial variations in longevity on a CF diet and in their response to BD. This variation could have many possible roots and should be amenable to further study. There are a number of genetic and environmental factors known to modulate nematode life span, including insulin/IGF-1 signaling, mitochondrial function, mRNA translation, and induction of stress response genes. Further exploration of the relative importance of each of these pathways in determining the longevity of different *C. elegans* isolates would be of interest.

Although laboratory adaptation in *C. elegans* does not appear to be a significant confounding factor for studies of basic mechanisms of aging in this organism, our data should not be interpreted to demonstrate that life span extension from DR is not specific to laboratory conditions. Particularly in the case of *C. elegans*, it seems likely that the natural environment experienced by this organism could often be more similar to BD conditions, compared to the extreme abundance of food and nutrients provided under standard laboratory conditions. Further complicating this issue, many *C. elegans* in the natural environment exist as dauer-arrested larvae, rather than reproductive adults. In the laboratory, animals are able to thrive in food-deprived conditions because they are provided with 5-fluorodeoxyuridine (FUdR), a drug commonly used in longevity studies to prevent eggs from hatching. If FUdR is not provided (such as in the natural environment), then hermaphrodites will die rapidly from internal hatching of eggs, a known starvation response. Additional environmental variables, such as temperature and the presence of pathogenic bacte-

ria or fungi, are also absent in the laboratory and may influence the interplay between nutrient availability and aging.

In summary, the ability of DR to increase life span in some organisms may be influenced by inadvertent selection for growth under laboratory conditions, but in nematodes it appears to be a trait of wild-derived populations. Future studies will be required to address the possibility that nutrient-rich laboratory environments accelerate aging, and DR represents a return to more natural conditions that are compatible with longer life spans. As DR is among the most widely studied and best characterized longevity interventions, efforts to address the relevance of DR in natural populations are of interest.

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