

Supplemental Data

Use of stable isotopes to examine how dietary restriction extends *Drosophila* lifespan

Diane O'Brien, Kyung-Jin Min, Thomas Larsen and Marc Tatar

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Supplemental Experimental Procedures

Drosophila rearing and demography

Larvae of Canton-S wild type *Drosophila melanogaster* were reared on either unlabeled yeast diet (UY) or labeled yeast diet (LY) (4% yeast, 10% cane sugar, agar) at 25°C and 12L: 12D. Newly eclosed adults were transferred to demography cages. Cages were vented, 1L clear food-service containers with a 60mm plastic tube affixed near the bottom of the container terminated to a funnel capped with a 60mm X 15mm Petri dish of diet medium. Flies that developed on unlabeled yeast diet were either maintained as adults upon the unlabeled yeast diet (UY-UY treatment) or upon labeled yeast diet (UY-LY treatment). Flies that developed on labeled yeast diet were transferred as adults to unlabeled yeast diet (LY-UY treatment). Within isotope treatments, cohorts were maintained with 6 replicate cages on 4% (restricted diet) and with 4 replicate cages on 16% yeast (full diet). The yeast levels chosen to represent full and restricted diets were determined experimentally (Fig S8). All adult diets also contained 10% cane sugar and agar. Each demography cage was established with approximately 200 females and 50 males. Petri dishes with diet were changed daily, at which time dead flies were removed and recorded.

Fecundity was measured from counts of 4 independent 1/16th radial sectors scored from four egg-collection Petri dishes. Per capita fecundity of every other day from days 2 to days 30 for each cage was estimated as the predicted total number of eggs relative to the number of females alive in the sample

interval. Total fecundity was assessed from per capita fecundity using linear interpolation for the days not sampled.

Synthesis of labeled yeast

Labeled and unlabeled yeast were grown in batch culture (Biostat D1500, 1,000 L Fermentor) at the Center for Biocatalysis and Bioprocessing at the University of Iowa, from an isolate of active dry yeast (Fleischmann's Yeast, Fenton, MO). Unlabeled yeast was grown upon beet sugar (Baar Products, Downington, PA), and standard ammonium sulfate. Labeled yeast was grown upon cane sugar (C&H Sugar, Crockett, CA) and ^{15}N labeled ammonium sulfate diluted to 0.02 % with unlabeled ammonium sulfate (all ammonium sulfate supplied by Sigma-Aldrich, St. Louis, MO). Growth media were supplemented for vitamins, trace elements salts with 'Yeast Nitrogen Base Without Amino Acids and Ammonium Sulfate' (ForMedium, Norwich, UK). Culture was grown to early stationary phase, filtered, centrifuged and freeze-dried (1.2 kg minimum yield). IRMS analyses of products confirmed there was full incorporation of carbon and nitrogen isotopic signatures of the growth media (Unlabeled Yeast; $\delta^{13}\text{C} = -23.71\text{‰}$, $\delta^{15}\text{N} = -3.64\text{‰}$; Labeled Yeast, LY; $\delta^{13}\text{C} = -9.86\text{‰}$, $\delta^{15}\text{N} = 252.77\text{‰}$).

Sample collection for isotopic analysis

Approximately 250 eggs were collected from the surface of the medium and stored at -20°C on days 2, 4, 6, 8, 10, 14, 22 and 30. A sample of six female flies from each treatment was collected on day 0, 2, 4, 8, 14, 22 and 30; sampled

individuals were included as censored observations in the life table. The somatic tissue without the ovaries was stored at -20°C .

Stable isotope analysis

The carbon and nitrogen isotope ratios of eggs and soma were measured at the Alaska Stable Isotope Facility using continuous flow isotope ratio mass spectrometry. From 0.3 to 0.35 mg of dried sample (~ 150 eggs or six flies) were weighed into tin capsules and introduced into a Costech elemental analyzer interfaced with a Finnigan Delta XL isotope ratio mass spectrometer. Data are expressed in delta notation as $((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000\text{‰}$, where R is the ratio of heavy to light isotope; standards are Vienna PDB for carbon and Atmospheric for nitrogen. Peptone calibration standards run concurrently established accuracy of 0.05‰ for $\delta^{13}\text{C}$ and 0.01‰ for $\delta^{15}\text{N}$, precision of $\pm 0.10\text{‰}$ for $\delta^{13}\text{C}$ and 0.2‰ for $\delta^{15}\text{N}$ (std. dev.), and precision of elemental proportions as $\pm 1.1\%$ for carbon and $\pm 0.37\%$ for nitrogen (std. dev.). Isotope values for all samples are given in Table S3.

Nine egg samples collected from full diet flies (3 UY-UY, 3 LY-UY and 3 UY-LY) were run in triplicate to assess variability within a single sampling day. Standard deviations of $\delta^{13}\text{C}$ among samples were typically less than $\pm 2\%$ of the sample mean. For carbon, these ranged from 0.24‰ to 0.01‰ , within the bounds of instrument precision. Standard deviations for $\delta^{15}\text{N}$ ranged from 0.05‰ to 0.19‰ for unlabeled eggs and from 0.92‰ to 3.65‰ for labeled eggs.

Stable isotope analysis of amino acids

We measured the isotopic composition of egg amino acids using compound-specific stable isotope analysis (via gas chromatography / combustion / isotope ratio mass spectrometry (GC/C/IRMS)) of derivitized amino acids (N-trifluoroacetic acid isopropyl esters), as described elsewhere [S1-S3]. These analyses were conducted at the Alaska Stable Isotope Facility at UAF. Derivitized amino acids were analyzed using a Agilent 5860 gas chromatograph (with an Ultra -1 column) connected to a Finnigan Delta Plus XP isotope ratio mass spectrometer via the GC-III combustion interface (Thermo-Finnigan Corp).

We analyzed amino acid $\delta^{13}\text{C}$ from egg samples on days 3, 6, 9, 12 and 18 from restricted and full diet flies on all diet treatments (U-U, L-U, and U-L). We also analyzed somatic tissue (bodies minus the ovaries) from 30 day old flies from each experimental treatment; by day 30 isotopic turnover in soma was essentially complete.

We analyzed essential amino acids, four of which gave consistent, quality chromatography: valine, leucine, isoleucine, and phenylalanine. All samples were analyzed in triplicate. Calculating amino acid $\delta^{13}\text{C}$ requires correcting for the carbon added during derivitization,, following O'Brien et al [S2]. Corrected measurement error for valine, leucine, isoleucine, and phenylalanine was less than 1‰, and was often better than 0.5‰.

Calculations

To estimate carbon and nitrogen contributed to tissue (soma or egg) from yeast acquired from the larval or adult diet, we compared the tissue isotope ratios from females feeding on labeled yeast as larvae or adults to that from females continuously upon unlabeled yeast (control group) (See [S2] for derivation). We followed the same approach to estimate the dietary origin of egg and somatic essential amino acids (EAA, traced via their carbon isotopes). Each difference between isotope-varied and control group was divided by the isotopic difference between the labeled and unlabeled yeast ($\delta_{LY} - \delta_{UY} = \Delta\delta$; $\Delta\delta^{13}\text{C}$ and ΔEAA : +13.85‰, $\Delta\delta^{15}\text{N}$: +256.41‰) to estimate the percent contribution of carbon and of nitrogen derived from labeled yeast (Y_t), specifically

(Proportion C, N, or EAA in tissue from larval acquired yeast) =

$$(\delta_{LU \text{ tissue}} - \delta_{UU \text{ tissue}}) / (\delta_{LY} - \delta_{UY}),$$

(Proportion C, N or EAA in tissue from adult acquired yeast) =

$$(\delta_{UL \text{ tissue}} - \delta_{UU \text{ tissue}}) / (\delta_{LY} - \delta_{UY}).$$

We averaged the proportion EAA from the larval and adult diets from valine, leucine, isoleucine, and phenylalanine to represent the total proportion for essential amino acids.

To plot a continuous function of the proportional contribution of each diet source to eggs and soma with respect to age t (in Fig S4, Fig S6) we fit the observed Y_t to the turnover model $Y_t = (Y_{30d}) + (Y_{30d} - Y_{0d}) e^{-kt}$, where k is the fractional turnover rate (for application of turnover models to insect resource

allocation see references [S2, S4, S5]). The parameters estimated for eggs and soma are given in Table S4.

Eggs: μg carbon, nitrogen and EAA investment

The absolute daily carbon and nitrogen investment (μg) from larval or adult acquired yeast to eggs was calculated as (*per capita* fecundity)(2 μg dry-mass/egg)(element proportion within eggs)(proportional contribution of diet source to egg). Element proportion within eggs was determined empirically to be 46.9% carbon and 9.2% nitrogen and invariant among treatments. The absolute daily investment of EAA from larval or adult acquired yeast was calculated as (*per capita* fecundity)(2 μg dry-mass/egg)(% protein content of egg)(estimated % egg protein that is EAA)(proportional contribution of diet source to egg EAA). Egg protein content was calculated using average egg %N (9.2% X 6.25 g. protein per g. nitrogen = 57.7% protein) [S6]. The percent of egg protein from EAA was conservatively estimated as 40%, based on data from other insect species [S4].

Soma: μg carbon, nitrogen and EAA investment

The absolute quantity of carbon and nitrogen from larval or adult yeast in somatic tissue on each day was calculated as mean dry somatic mass (μg)/female(element proportion in soma)(proportional contribution of diet source). Somatic mass (dry) was constant with age; we used across-age, mean somatic mass for each treatment to calculate net investment (Full diet: 258 μg , Restricted diet: 215 μg). Element proportion within soma was determined empirically to be 53.1% carbon and 8.9% nitrogen and invariant among

treatments. The absolute investment of EAA from larval or adult acquired yeast to somatic tissue was calculated as $(\text{mean dry somatic mass } (\mu\text{g})/\text{female}) (\% \text{ protein content of soma})(\text{estimated } \% \text{ of somatic protein that is EAA})(\text{proportional contribution of diet source to egg EAA})$. Somatic protein content was calculated using average soma %N ($8.9\% \times 6.25 \text{ g. protein per g. nitrogen} = 55.4\% \text{ protein}$) [S6]. The percent of somatic protein from EAA was estimated as 50%, based on data from other insect species [S7].

Figure S1. Survivorship of females maintained as adults upon restricted diet (RD, 4% yeast) and full diet (FD, 16% yeast).

Within each diet, life tables of U-U, U-L and L-U treatments are combined. Kaplan-Meier survival curves estimated with censoring of females sampled for isotope analysis (RD, $N_0 = 3105$, 58 censored, median lifespan = 27 d; FD, $N_0 = 3247$, 78 censored, median lifespan = 37 d). Mortality was significantly reduced in RD to FD (log-rank test, $\chi^2 = 657.7$, $P < 0.001$). Inset: Age-specific, per capita, fecundity estimated from 200 females per experimental cohort. Gross fecundity (age 0 to 30 days): 12.9 eggs, RD; 145.1 eggs, FD.

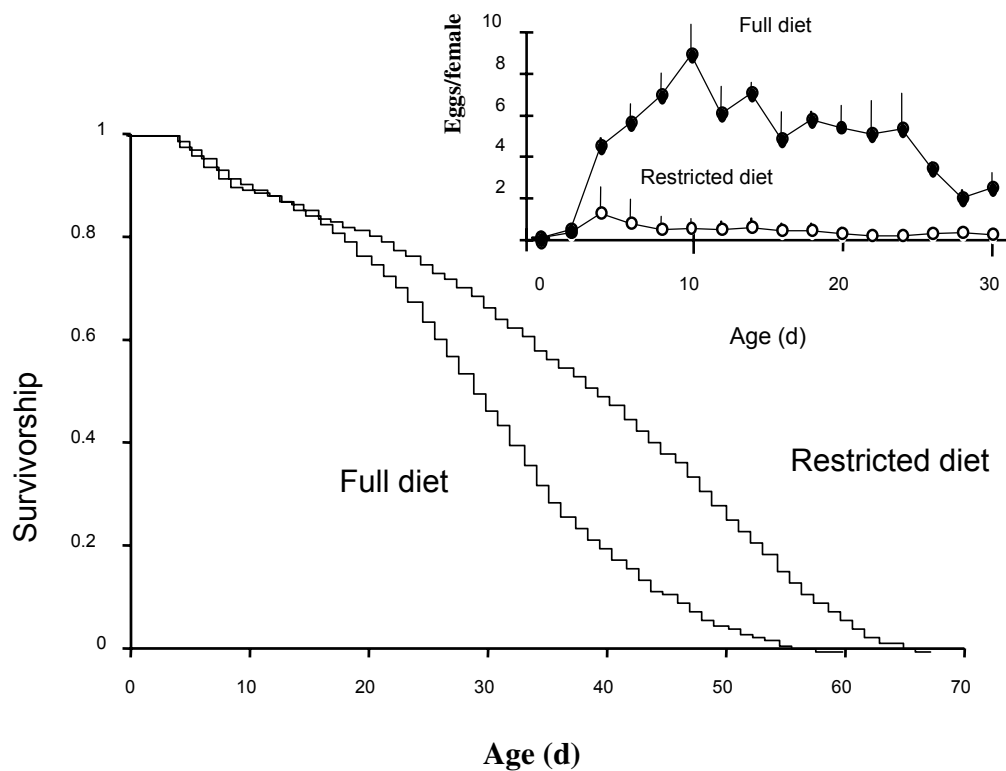


Figure S2. Allocation of larval (filled symbols) and adult (open symbols) carbon and nitrogen from yeast to eggs by full (FD) and restricted (RD) diet females.

(A,C) Proportional contribution of carbon to eggs by diet source of FD and RD females.

(B,D) Proportional contribution of nitrogen to eggs by diet source of FD and RD females.

Net reproductive investment of carbon and nitrogen from larval and adult dietary yeast by FD and RD females is presented in Table S3.

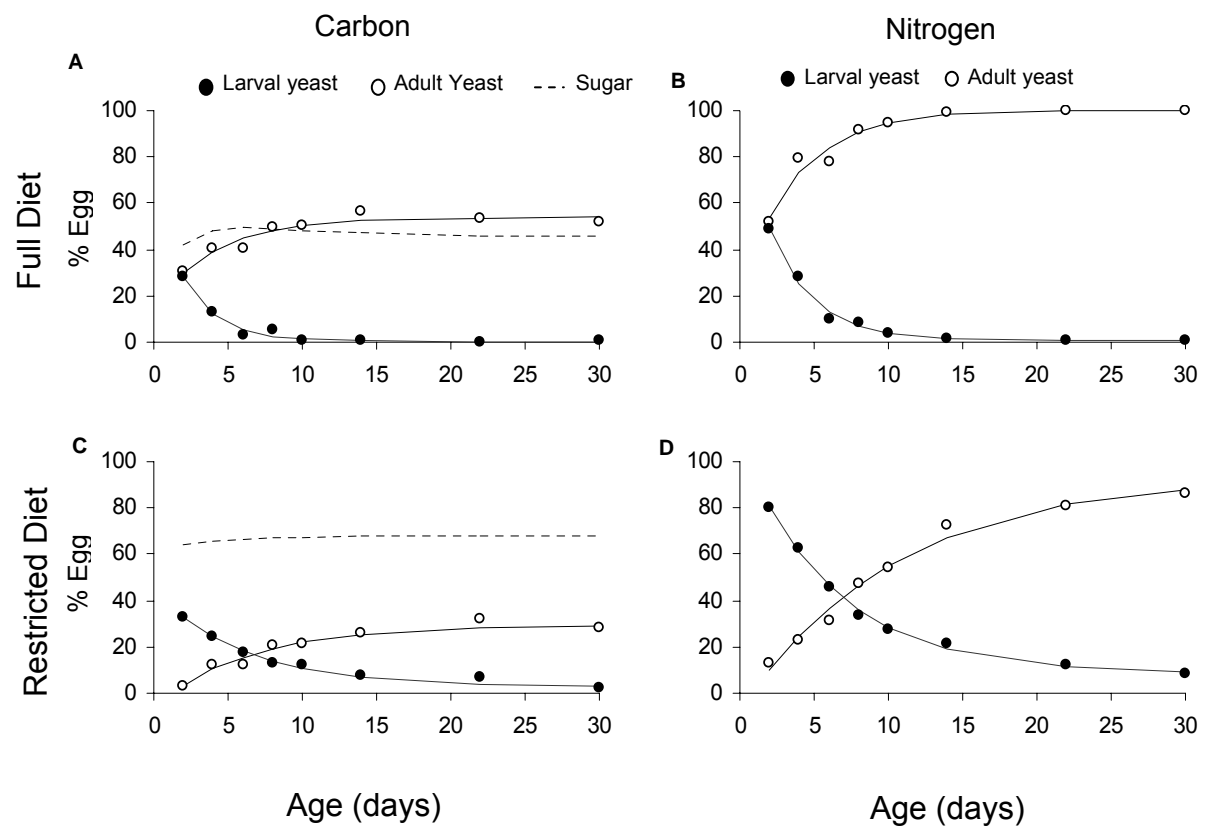


Figure S3. Allocation of EAA from larval (filled symbols) and adult (open symbols) yeast to eggs.

(A) Proportion of EAA carbon from each diet in eggs laid by full diet females. (B)

Proportion of EAA carbon from each diet in eggs laid by restricted diet females.

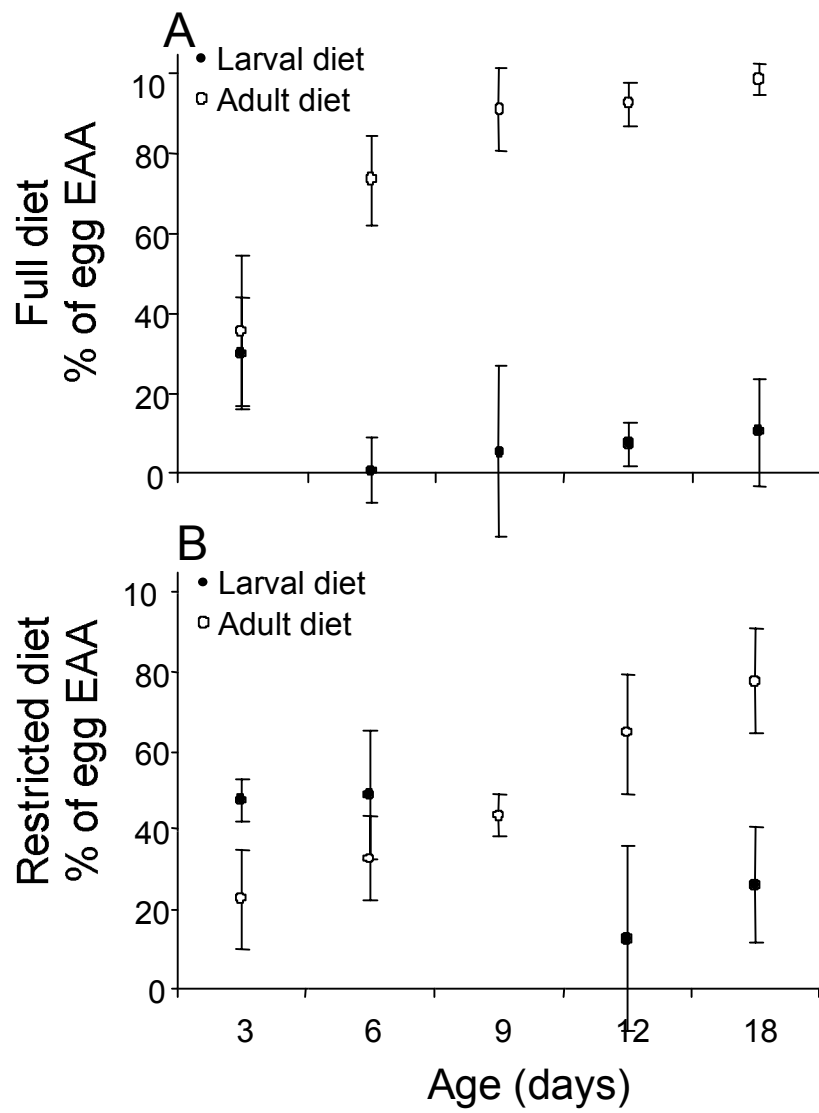


Figure S4. Somatic turnover in full-diet (FD) and restricted-diet (RD) females, evaluated as the loss from soma of carbon and nitrogen from larval dietary yeast (filled symbols) and as the gain of carbon and nitrogen from adult dietary yeast (open symbols).

(A,C) Proportional contribution carbon in soma of FD and RD females. (B,D)

Proportional contribution of nitrogen in soma of RD and RD females.

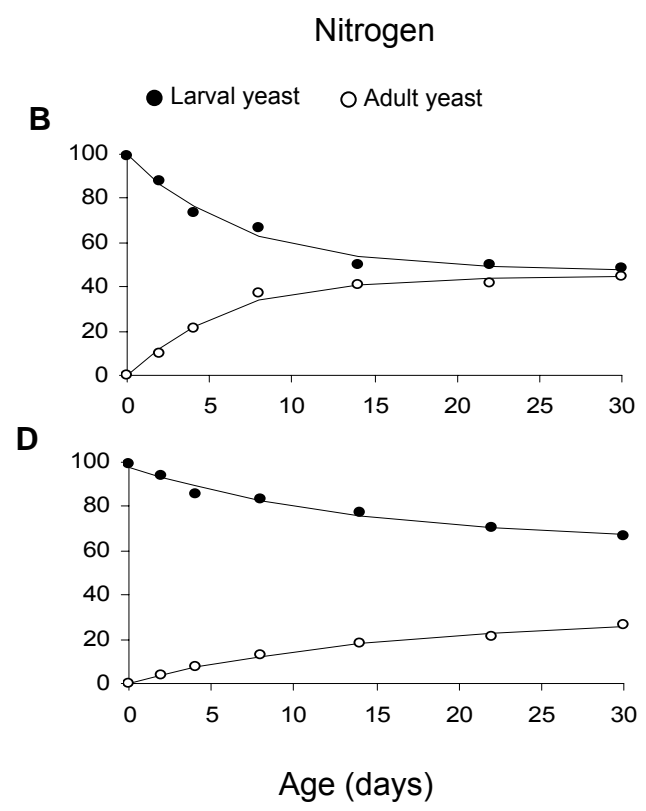
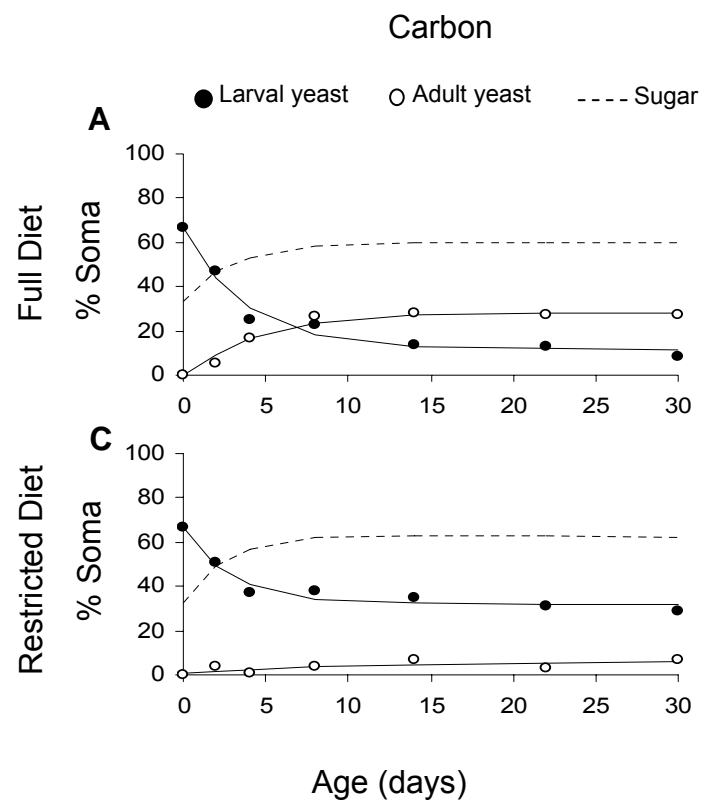


Figure S5. Source of somatic EAA restricted and full diet flies: larval dietary yeast (filled symbols) and adult dietary yeast (open symbols).

Data are from 30 day old flies only, at which point somatic turnover of carbon and nitrogen had reached a maximum (Figure S6).

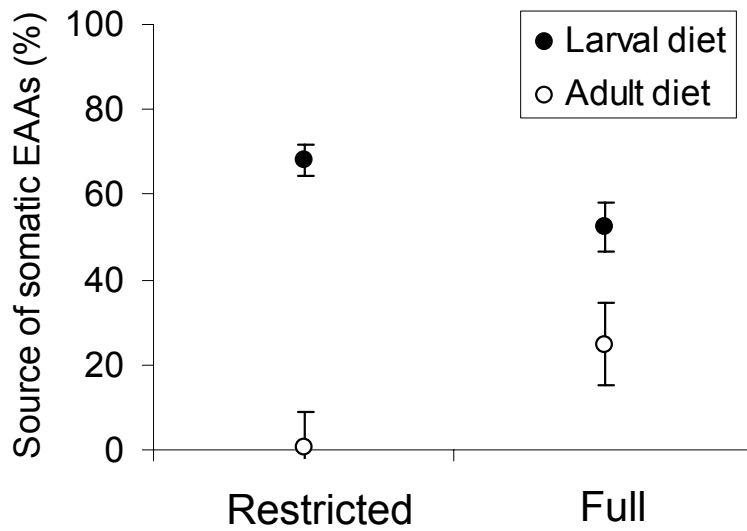


Figure S6. Body mass (dry) and C:N ratio per female (mean \pm std) based on three replicate samples of 6 females each day.

ANCOVA was used to evaluate the effect of isotope label in larval and adult diet, diet restriction (restricted vs. full), and age on somatic dry mass and C:N. (A) Diet restricted flies were lighter than full fed flies ($F_{1,31} = 19.39$, $P < 0.0001$), but age did not affect dry somatic mass. *Not shown*: label of adult diet did not effect dry somatic mass, but flies reared on unlabeled diet were lighter than flies reared on labeled yeast (mean difference = $0.047 \mu\text{g}$, $F_{1,31} = 13.48$, $P = 0.0009$). (B) Neither diet restriction nor age affected somatic C:N. *Not shown*: Label of adult diet had no effect on ratio of somatic C:N, but flies reared on unlabeled diet had slightly reduced C:N (mean difference = 0.57 , ANCOVA, $F_{1,31} = 6.06$, $P = 0.02$).

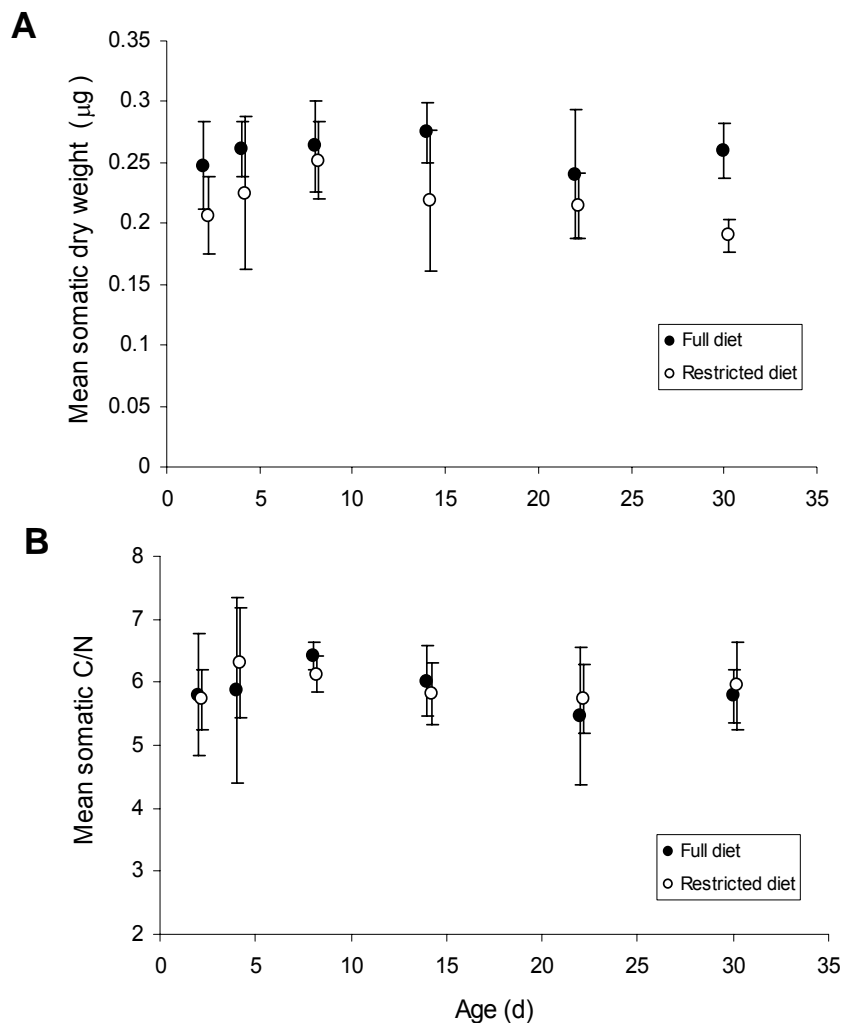


Figure S7 (A) Direct allocation model of the trade-off between somatic maintenance and reproduction. Resources when abundant are allocated to reproduction at the expense of somatic maintenance. Resources when limiting are preferentially allocated to somatic maintenance at the expense of reproduction. (B) Direct constraints model of reproduction upon the soma [S8]. Reproduction directly damages somatic functions or inhibits mechanisms of somatic maintenance. Resources when abundant increase reproductive output and somatic maintenance; life span is reduced if the damage incurred by the level of reproduction exceeds the rate of somatic repair. Resources when limited reduce reproduction and somatic maintenance. Extended survival will arise if damage induced by the reproductive effort is less than the available level of somatic repair.

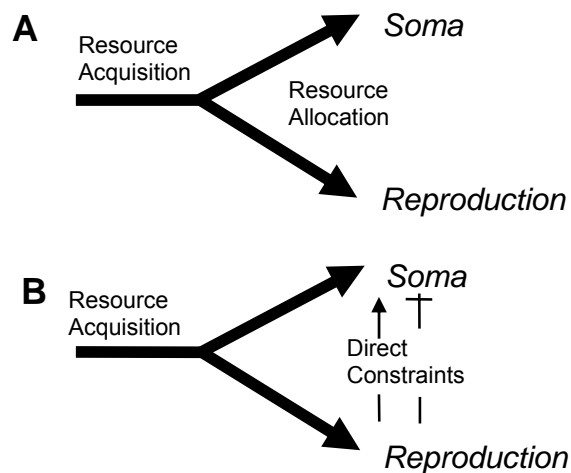


Figure S8. In preliminary tests we assessed a range of yeast concentrations with our freeze-dried yeast product to define a restricted-diet condition with optimal extension of lifespan relative to a full-diet condition. The figure plots mean life span of cohorts (197, 174, 173 and 194 females in 1%, 4%, 8% and 16%) maintained as adults upon agar-sugar diet with yeast concentrations of 1%, 4%, 8%, and 16%. Larvae were reared on standard cornmeal-sugar-yeast media. Diet of 4% yeast was selected as the optimum to represent diet restriction because females upon this medium produced eggs; females on 2% yeast with our experimental diet formulation laid few if any eggs (data not shown).

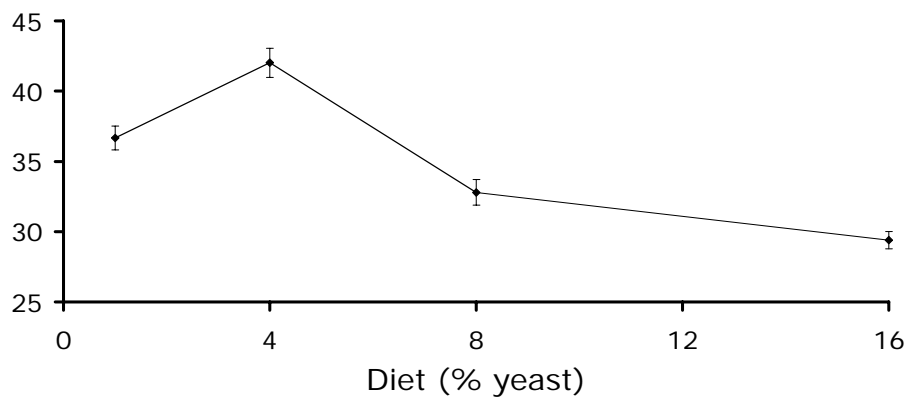


Table S1. Expected carbon and nitrogen investment in egg production.

Net mass of carbon and nitrogen from dietary yeast provisioned to eggs/female/day as function of stage acquired (diet source) and level of yeast in adult diet (yeast level: 4%, restricted diet; 16%, full diet). Mass estimated as in methods. **Bold** values are observed data, plain text are linear interpolations.

NITROGEN					CARBON				
Diet source:	Adult		Larva		Diet source:	Adult		Larva	
Yeast level:	16%	4%	16%	4%	Yeast level:	16%	4%	16%	4%
Age (d)	(μg)	(μg)	(μg)	(μg)	Age (d)	(μg)	(μg)	(μg)	(μg)
2	0.036	0.008	0.034	0.047	2	0.112	0.009	0.104	0.100
3	0.334	0.030	0.128	0.096	3	0.896	0.080	0.326	0.200
4	0.631	0.053	0.222	0.145	4	1.681	0.151	0.548	0.299
5	0.704	0.060	0.159	0.122	5	1.899	0.143	0.360	0.246
6	0.778	0.066	0.096	0.098	6	2.116	0.135	0.173	0.192
7	0.954	0.060	0.100	0.068	7	2.651	0.129	0.259	0.135
8	1.130	0.054	0.104	0.038	8	3.187	0.123	0.345	0.077
9	1.321	0.050	0.080	0.031	9	3.688	0.109	0.205	0.066
10	1.512	0.046	0.056	0.023	10	4.189	0.094	0.065	0.054
11	1.443	0.049	0.054	0.022	11	4.063	0.098	0.064	0.049
12	1.374	0.052	0.051	0.020	12	3.938	0.102	0.064	0.043
13	1.305	0.055	0.049	0.019	13	3.812	0.106	0.063	0.038
14	1.236	0.059	0.021	0.017	14	3.687	0.110	0.063	0.032
15	1.193	0.054	0.019	0.016	15	3.538	0.097	0.056	0.030
16	1.150	0.050	0.017	0.014	16	3.389	0.085	0.050	0.027
17	0.533	0.021	0.007	0.005	17	1.545	0.030	0.018	0.011
18	1.065	0.042	0.014	0.011	18	3.091	0.060	0.037	0.022
19	1.022	0.038	0.012	0.009	19	2.942	0.048	0.030	0.019
20	0.980	0.033	0.010	0.007	20	2.793	0.036	0.024	0.016
21	0.937	0.029	0.008	0.006	21	2.644	0.023	0.017	0.014
22	0.895	0.025	0.006	0.004	22	2.495	0.011	0.011	0.011
22	0.837	0.023	0.006	0.004	22	2.329	0.011	0.010	0.010
23	0.779	0.021	0.005	0.003	23	2.163	0.012	0.010	0.009
24	0.721	0.019	0.005	0.003	24	1.996	0.012	0.009	0.007
25	0.663	0.017	0.005	0.003	25	1.830	0.012	0.008	0.006
26	0.605	0.014	0.004	0.002	26	1.664	0.012	0.007	0.005
27	0.548	0.012	0.004	0.002	27	1.498	0.013	0.007	0.004
28	0.490	0.010	0.003	0.001	28	1.331	0.013	0.006	0.002
29	0.461	0.009	0.003	0.001	29	1.248	0.013	0.005	0.002
30	0.432	0.008	0.003	0.001	30	1.165	0.013	0.005	0.001
Sum	26.07	1.07	1.29	0.84		73.58	1.89	2.95	1.72

Table S2. Net assimilation of carbon and nitrogen from adult dietary yeast to soma and eggs. Minimum estimates are without mass lost to respiration; metabolic respiration is reported to be similar across diet restriction regimes in *Drosophila* [S9].

		Soma		Eggs		Net assimilation from adult dietary yeast ⁴	
		Loss ¹	Gain ²	Total investment ³		Total acquired μg	Percent allocated to soma
Adult diet	Trace d pool	From larval dietary yeast, μg	From adult dietary yeast μg	From larval dietary-yeast, μg	From adult dietary-yeast, μg		
4%	N	6.39	5.2	0.84	1.07	6.27	83%
4%	C	44.23	8.23	1.72	1.89	10.12	81%
16%	N	11.83	10.44	1.29	26.1	36.54	29%
16%	C	66.02	37.02	2.95	73.6	110.62	33%

¹ Difference in the μg somatic nitrogen and carbon derived from larval dietary yeast between Day 0 and Day 30.

² Magnitude of somatic turnover pool that turns over with carbon and nitrogen from adult dietary yeast, observed at Day 30 (P₃₀).

³ Sum of daily, per capita absolute investment in eggs through 30 d, from observed and linear interpolated values of Table S2.

⁴ Sum of somatic gain and investment to eggs.

Table S3. Isotopic signatures of eggs and soma (excluding ovaries). U-U, L-U, and U-L correspond to the isotope of the larval-adult diet group (U = unlabeled, L=labeled).

Unlabeled Yeast: $\delta^{13}\text{C} = -23.71\text{‰}$, $\delta^{15}\text{N} = -3.64\text{‰}$; Labeled Yeast: $\delta^{13}\text{C} = -9.86\text{‰}$, $\delta^{15}\text{N} = 252.77\text{‰}$. Isotope ratios are given in delta notation as $\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000\text{‰}$, where X = ^{13}C or ^{15}N , R = the ratio of heavy to light isotope, and the standards are Vienna PDB for carbon and atmospheric N for nitrogen.

		NITROGEN ($\delta^{15}\text{N} \text{‰}$)						CARBON ($\delta^{13}\text{C} \text{‰}$)							
		Full diet (16% yeast)			Restricted diet (4% yeast)					Full diet (16% yeast)			Restricted diet (4% yeast)		
diet		U-U	L-U	U-L	U-U	L-U	U-L	diet		U-U	L-U	U-L	U-U	L-U	U-L
Age (d)	EGGS							Age (d)							
2		-1.5	123.6	130.8	-1.1	204.6	32.1	2		-17.3	-13.4	-13.1	-15.7	-11.2	-15.3
4		-1.4	70.2	201.6	-1.2	159.7	57.8	4		-17.0	-15.1	-11.4	-15.7	-12.3	-14.0
6		-1.3	23.5	199.2	-0.9	117.2	79.1	6		-17.0	-16.5	-11.3	-15.6	-13.2	-13.9
8		-1.2	20.2	233.0	-1.2	84.5	119.6	8		-17.1	-16.4	-10.3	-15.7	-13.9	-12.9
10		-1.1	7.9	242.4	-0.8	69.7	138.9	10		-17.2	-17.1	-10.2	-15.5	-13.8	-12.5
14		-1.1	3.2	252.6	-0.9	53.5	184.1	14		-17.3	-17.2	-9.5	-15.2	-14.2	-11.6
22		-1.4	0.5	254.7	-0.1	31.3	208.3	22		-17.3	-17.4	-9.9	-15.6	-14.7	-11.1
30		-0.8	0.8	256.5	-0.3	20.2	221.1	30		-17.0	-16.9	-9.8	-14.9	-14.6	-11.0
		SOMA													
0		-1.3	252.9	-1.3	-1.3	252.9	-1.3	0		-18.3	-9.1	-18.3	-18.3	-9.1	-18.3
2		-1.2	239.3	8.8	-1.2	224.4	23.5	2		-16.5	-9.4	-16.0	-16.5	-10.0	-15.8
4		-1.3	217.6	18.5	-1.0	187.2	53.0	4		-16.0	-10.8	-15.9	-17.4	-12.5	-15.1
8		-0.9	213.3	32.3	-1.0	170.2	94.5	8		-15.9	-10.6	-15.3	-17.1	-12.7	-13.4
14		-1.1	197.6	45.4	-1.3	127.4	104.2	14		-15.6	-10.8	-14.7	-16.8	-13.8	-12.9
22		-1.0	179.1	54.1	-0.8	127.6	106.6	22		-15.4	-11.1	-15.0	-17.1	-13.6	-13.3
30		-1.2	169.5	66.8	-0.8	122.8	114.4	30		-15.3	-11.3	-14.3	-16.7	-14.1	-12.9

Table S4. Somatic turnover with adult acquired yeast. The enrichment of adult acquired tracer into somatic maintenance (P_t) was characterized as $P_t = P_{max}(1 - e^{-kt})$, where P_{max} is the maximum investment, k = the fractional turnover rate, and $t = 2$ to 30 days. The rate (R) at which carbon and nitrogen are allocated to somatic repair through biosynthesis is $k \cdot P_{max}$. Parameters were estimated using least squares nonlinear fitting platform in JMP IN version 5.1 (SAS Institute). RMSE: Residual Mean Square Error.

Enrichment from adult dietary yeast (exponential turnover model ⁴)					
Adult diet	Traced pool	Goodness-of-fit, RMSE	k (s.e)	$P_{max}\mu\text{g}$ (s.e)	R ($\mu\text{g}/\text{day}$)
4%	N	0.176	0.07 (0.01)	5.77 (0.41)	0.40
4%	C	2.719	0.12 (0.018)	6.73 (2.79)	0.81
16%	N	0.615	0.18 (0.03)	10.37 (0.45)	1.87
16%	C	4.003	0.23 (0.06)	38.9 (2.59)	8.95

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