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Paul Vigne, Christian Frelin. Food presentation modifies longevity and the beneficial action of dietary restriction in. *Experimental Gerontology*, 2010, 45 (2), pp.113. 10.1016/j.exger.2009.10.016 . hal-00559141

HAL Id: hal-00559141

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Accepted Manuscript

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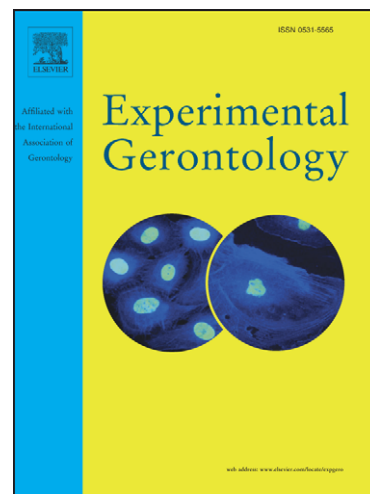
PII: S0531-5565(09)00232-0
DOI: [10.1016/j.exger.2009.10.016](https://doi.org/10.1016/j.exger.2009.10.016)
Reference: EXG 8668

To appear in: *Experimental Gerontology*

Received Date: 19 November 2008
Revised Date: 15 October 2009
Accepted Date: 29 October 2009

Please cite this article as: Vigne, P., Frelin, C., Food presentation modifies longevity and the beneficial action of dietary restriction in *Drosophila*, *Experimental Gerontology* (2009), doi: [10.1016/j.exger.2009.10.016](https://doi.org/10.1016/j.exger.2009.10.016)

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Food presentation modifies longevity and the beneficial action of dietary restriction in
Drosophila

Short title: Feeding preferences and diet restriction in *Drosophila*

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Abstract

Recent studies have indicated that flies respond to dilute food solutions by compensatory feeding. The existence of compensation mechanisms calls for a reconsideration of the relationships between diet, feeding behaviour and longevity. This study shows that flies fed on liquid diets, sense sucrose and yeast nutrients and adapt to changes in the quantity and presentation of the two nutrients. They have a marked preference for sucrose and regulate their sucrose intake more tightly than their yeast intake.

These preferences are not modified as fly age from 1 day to 30 days. Compensatory feeding suppresses the beneficial action of dietary restriction on longevity when flies are fed on liquid diets supplemented with yeast extracts. Flies which are given the choice to feed on separate yeast and sucrose food sources were longer lived than flies fed on nutrient mixtures. We conclude that flies sense and respond to specific nutrients and that food presentation is a major factor which determines the sensitivity of flies to dietary restriction.

Key words: *Drosophila*; Nutrition; Ageing; Dietary restriction; Longevity.

1. Introduction.

The relationships between diet and longevity are of increasing interest. The observation that flies fed on dilute sources of food are long lived has suggested that dietary restriction promotes longevity (Partridge et al., 2005, Piper and Partridge, 2007). This conclusion assumes that flies ingest nutrients in proportion of their concentrations in their food. Recent papers have challenged this view. First flies respond to specific macronutrients such as protein and carbohydrates and not to the calorie content of the food (Mair et al., 2005, Min et al., 2007, Vigne et al., 2007a, b, Skorupa et al., 2008). Second flies can change their feeding behaviour in response to changes in their diet (Carvalho et al., 2005, Ja et al., 2007). Feeding behaviour by small insect has long been difficult to analyze. It can now be assessed using the capillary feeding assay (Ja et al., 2007). This assay has recently been used to show that caloric restriction *per se* does not promote longevity of flies (Lee et al., 2008).

We previously reported that aged, 30 day old, flies do not respond to dietary restriction by an increased longevity (Vigne and Frelin, 2007a). Whether their unresponsiveness is due to a modified feeding behaviour or to a modified sensitivity to nutrients was not defined. This study uses a capillary feeding assay to analyse the feeding behaviour of flies and its possible alterations with ageing. The results indicate that the

feeding behaviour of flies is more complex than previously thought. They suggest that feeding behaviour must be carefully defined when considering the relationship between nutrition and longevity.

2. Materials and Methods.

2.1 Fly stocks.

*w*¹¹⁸ flies (Bloomington stock Center) were reared in 300 ml bottles filled with 30 ml of standard food medium (8.2% cornmeal, 6.2% sucrose, 1.7% heat inactivated baker's yeast and 1% agar supplemented with 3.75 g/l methyl 4-hydroxybenzoate) in humidified, temperature controlled chambers at 25°C and 60 % relative humidity and under a 12:12 light: dark cycle. Experiments were performed using male flies. Young flies were 1 day old. Aged flies were 10, 20 or 30 day old. During ageing, flies were maintained on standard food.

2.2. Dietary conditions

Diets were composed of sucrose and yeast extract (Fluka 70161). Diets were labelled using the following convention. A "10S10Y" diet means that flies were fed with a mixture of 10% sucrose and 10% yeast and had "no choice" to select their nutrients. A "10S-10Y" diet means that flies were exposed to separate sources of 10% sucrose and 10% yeast and could select which nutrient to consume. 5S and 10S diets corresponded to 5% and 10% sucrose solutions. A 10Y diet corresponded to a 10% yeast solution.

2.3 Capillary feeding assay.

30 ml plastic vials were used. A piece of paper towel wetted with 2 ml of distilled water was introduced down to the bottom of the tubes. It had two functions. It provided a source of water that was independent of feeding and moist conditions to prevent evaporation of feeding solutions. Flies in groups of 6 were added to the tubes and tubes were sealed with

rubber stoppers (SubA seal, Sigma) in which three holes had been drilled. Food was provided by one (or two) 75 μl micro capillary tube (Hirschmann). The third hole was used to insert a syringe needle for pressure compensation and gas exchanges. Capillaries were filled with 40 μl of liquid diet and inserted through the stopper via truncated 200 μl pipette tips. A mineral oil overlay (about 5 μl) was used to prevent evaporation from the top of the capillaries. In the “choice” experiments two capillaries were used. They were filled either with sucrose or yeast solutions and the flies were allowed to select which source of food to use. They will be called “self selecting flies”. Only one capillary was used in the “no choice” experiments. It was filled with pure yeast, pure sucrose or mixed yeast/sucrose solutions. Flies were allowed to recover from anaesthesia for 6 hours and maintained at 21 °C. The height of the liquid column in the capillaries was measured twice a day using callipers and during 2-3 consecutive days.

Figure 1A plots liquid intakes (using a 5S solution) as a function of time and of the number of flies used (between 3 and 9). Intakes were linearly related to time. The slopes of the representations were measured and used to calculate daily volumes intakes. Figure 1B shows that daily intakes were linearly related to the number of flies used. The representation extrapolated to a value of 2 μl / day in the absence of flies. This value represented the rate of evaporation from the bottom of the capillaries and was used to correct all intake data. This evaporation cannot be neglected. It limits the precision of ingestion rates and imposes important constraints to the design of the experiments. The conditions used (6 flies per tube fed with a single capillary) provided the best precision. Precision was lowest when the amount of ingested liquid was the lowest, for instance when rich, 10S or 10Y solutions were used. No evidence for a dependence of food consumption on fly number could be obtained, as reported by Ja et al., (2007). Different results have been reported for flies fed on agar based food (Wong et al., 2009).

2.4. Survivorship of flies fed on liquid solutions.

Flies were fed with liquid solutions as described above for the capillary feeding assay with two differences; (i) groups of 10 flies were used and (ii) two capillaries per vials were used. Vials were maintained at 21 °C and dead flies were scored every day. Liquid solutions in the capillaries were changed twice a week.

2.5. Survivorship of flies fed on semi solid media.

Three types of semi solid food mixtures were prepared: (i) 10% sucrose in 2% agar, (ii) 10% yeast in 2% agar and (iii) 10% yeast and 10% sucrose in 2% agar. Lyophilized yeast was used in these experiments. All mixtures were supplemented with 3.75 g/l of methyl 4-hydroxybenzoate. Mixtures were layered in Petri dishes and 1 cm² squares of jellified food patches (height ~ 0.3 mm) were cut with a razor blade. Two patches were glued onto the side of the plastic tubes with a 2% agar solution and at a distance of 1.5 cm from the bottom of the tubes. Male, 1 day old, flies were added in group of 10 to the tubes and tubes were left in an upright position at 25 °C. Dead flies were counted everyday. In the “choice” conditions flies were given the choice to forage on a patch of sucrose, a patch of yeast and agar (which provides an independent source of water). In the “no choice” condition, flies could only forage on patches of mixed sucrose/yeast or on agar. Flies were transferred to new patches of food twice a week.

2.6. Statistical analyses

Means were compared using unpaired t tests or two way ANOVA with Benferroni correction. Survivorship curves were compared using the log rank test. The GraphPad Prism 4 software was used. P values < 0.01 were considered as statistically significant.

3. Results

3.1 Influence of ageing on feeding behaviour

The feeding behaviour of aging male flies was analysed using a capillary feeding assay. Four age classes were selected: 1, 10, 20 and 30 days. Flies were exposed to a pure sucrose solution, to a mixed sucrose/yeast solution or to independent sources of sucrose and yeast. Figure 2 compares sucrose intakes by flies exposed to rich diets (10S, 10S10Y or 10S-10Y) or to poorer, two fold diluted diets (5S, 5S5Y or 5S-5Y). Under all conditions, we observed that flies on the diluted diet consumed larger volumes of the nutritive solutions, which indicated compensatory feeding. As a consequence, net daily ingestions of sucrose were similar (Figure 2). Compensatory feeding was observed at all ages from 1 to 30 days.

Different results were obtained with pure yeast diets. The daily intake of yeast by 1 day old flies fed on a 10Y solution was $39 \pm 2 \mu\text{g}$ ($n = 8$). It was 2 times larger ($77 \pm 6 \mu\text{g}$, $n = 12$) when flies were fed with a diluted 5Y solution ($p < 0.01$). Thus, young flies over-compensated for the dilution of a yeast solution. Aged, 30 day old flies were different. The daily intakes were $85 \pm 12 \mu\text{g}$ ($n = 6$) and $20.5 \pm 3.0 \mu\text{g}$ ($n = 7$) when flies were fed on a 10Y diet and a 5Y diet respectively ($p < 0.01$). Thus, aged flies did not compensate for dilutions of their food. We then analyzed yeast intake by self selecting flies. Figure 2B shows that flies compensated for a 2 fold dilution of their food when they were 1 day old. They did not compensate for food dilutions when they were 10, 20 or 30 day old. These results indicated that regulation of yeast intake changed between 1 and 10 days of age.

Figure 3 analyses intake data according to the nutritional geometrical model developed by Simpson and Raubenheimer (1997, 1999). This representation relates sucrose and yeast intakes under different conditions. Figure 3 first shows that flies under self selection conditions had a marked preference for sucrose. The sucrose: yeast ratio of ingested food was 1:3 to 1:5 and this value was not affected by ageing. Figure 3 also shows that self selecting flies and flies fed on a pure sucrose diet ingested similar amounts of sucrose, suggesting that flies had a better control of their sucrose yeast intake than of their yeast intake. Thus, the additional intake of yeast was not accompanied by a decreased intake of sucrose.

Flies fed on a mixed diet have no choice. They are forced to ingest yeast and sucrose in proportions that are different from those of self selecting flies. Figure 3 shows that flies fed on mixed diets ingested less sucrose than self selecting flies ($p < 0.01$) and two times more yeast ($p < 0.01$). Very similar results were reported by Lee et al., (2008) for female flies. Taken together these results indicated that flies did not regulate their daily calorie intake and responded to specific nutrients. They had a marked preference for sucrose and they regulated their intake of sucrose more tightly than their intake of yeast. This study further shows that ageing had little influence on feeding preferences, nutrient intake targets, and the capacity of the flies to respond to mixed diets.

3.2. The limit to compensatory feeding

Is there a limit to compensatory feeding when flies are exposed to highly diluted sources of food? To answer this question, we exposed 1 day old flies to serial dilutions of a 10S feeding solution and measured their daily intakes of liquid. Figure 4A shows that flies compensated for 2 to 3 fold dilutions of a 10S solution. They did not compensate for a 5 fold dilution. Thus, compensatory feeding was limited at large dilutions of a sucrose solution. As a consequence, the daily intake of sucrose is a saturable function of the dietary sucrose (Figure 4B). The curve is usually interpreted as feeding compensation; i.e. flies increase their feeding in response to food dilution. It can also be viewed as a saturable phenomenon. For instance, flies fed on concentrated sucrose solutions could be rapidly satiated and could stop feeding.

3.3. Feeding flies on dilute solutions did not promote longevity.

The existence of compensatory feeding mechanisms calls for a reassessment of the protocols of dietary restriction. It is well known that diluting food (provided as a semi solid medium) increases longevity probably because flies are dietary restricted. The previous results (Figures 2 and 3) indicated that flies fed on a liquid 5S5Y diet ingested just the same amount of nutrients as flies fed on a liquid 10S10Y diet, meaning that they were not dietary

restricted. As a consequence their longevity should not be modified by a change in diet from 10S10Y to 5S5Y. Figure 5 shows that, as expected, longevity was identical under the two conditions.

3.4. Self selection conditions promote longevity

Lee et al., (2008) recently reported that feeding flies on liquid solutions under self selection conditions promoted longevity. We therefore asked whether flies responded to self selection conditions when nutrients were presented as semi solid food. Figure 6 shows the results of an experiment in which flies were given the choice to feed on separate patches of 10% yeast and 10% sucrose or forced to feed on patches of mixed 10% sucrose/10% yeast. It shows that flies under self selection conditions had an increased median longevity. The maximum longevity was not affected however. We also observed that the first deaths occurred soon after the start of the experiments, which suggests that flies experienced more stressful conditions. It should be noted that in these experiments, flies did not sit on food and had to climb to the top of the tubes in order to reach their food. As a consequence, weak flies have probably less chance to survive than under standard conditions.

4. Discussion.

This study analyses the feeding behaviour and feeding preferences by male *Drosophila* and their relationships to longevity

In a key paper, Lee et al., (2008) showed that female flies regulate their intake of proteins and carbohydrates separately. Under self selection conditions, they select a low yeast diet that maximises fitness. This study shows that male flies have a similar behaviour. Male flies have a 3 to 5 fold preference for sucrose when sucrose and yeast are presented separately. When there is no choice, male flies control the sucrose intake more tightly than the yeast intake.

4.1. Does age modify feeding behaviour?

One objective of this study was to determine whether flies changed their feeding behaviour with age. Analysis of the data using the nutrient geometrical framework (Simpson and Raubenheimer, 1997, 1999, Lee et al., 2008) indicates that flies do not modify their preference for sucrose and their responses to mixed diets. Flies respond to a dilution of a sucrose diet by compensatory feeding and this response is little affected by ageing.

A change in the regulation of yeast intake was observed as flies reach 10 days of age. Young, 1 day old, flies respond to a dilution of a yeast solution by compensatory feeding. Older (10, 20 or 30 day old) flies do not (Figure 2). In other words, yeast intake by young flies saturates. As a consequence, compensatory feeding is observed when a yeast solution is diluted. Yeast intake by aged flies does not saturate. The more yeast is provided to aged flies, the more yeast is ingested. Compensatory feeding was observed however when aged flies were fed with mixed diets (Figure 2D). Thus, the presence of sucrose corrected for the changes in yeast intake.

4.2 Limits to compensation

This study also confirms previous reports (Carvalho et al., 2005, Ja et al., 2007, Lee et al., 2008) showing that flies respond to a dilution of their diets by compensatory feeding. We further show that compensation was nearly complete over a large range of sucrose concentrations, from 3% to 10%. Flies exposed to lower concentrations of sucrose did not compensate for the dilution of food. The reason for this lack of compensation is not known. We observed however that flies fed on a diluted 2% sucrose solution ingest about 1.2 μ l of water solution per day. This volume is 3 times larger than the water content of a fly (0.4 mg assuming an average wet weight of 0.6 mg and a mean water content of 69%, Gibbs and Matzkin, 2001). How flies handle such a water load is not known. Water balance in *Drosophila* has been studied in relation to desiccation resistance (Giggs and Matzkin, 2001, Albers and Bradley, 2004). The responses to large water loads have not yet been studied. In hematophagous insects, feeding triggers a rapid diuresis which is used to eliminate the

excess of water (Maddrell et al., 1993). It is of interest to note that flies fed on dilute solutions lay down numerous liquid droplets on the wall of the culture tubes, which indicates diuresis. Thus, water intake may limit the intake of nutrients when flies are fed on highly diluted solutions.

4.3. Influence of food presentation.

Dietary restriction, which increases longevity in *Drosophila*, is usually achieved by diluting food (Partridge et al., 2005). This and previous studies (Carvalho et al., 2007, Ja et al., 2007, Lee et al., 2008, Wong et al., 2009) clearly indicate that food presentation modifies the effect of dietary restriction.

(i) semi solid vs. liquid diet. The feeding behaviour of flies fed on agar based food was analyzed in great details by Wong et al. (2009). Briefly, flies fed on semi solid food do not change their feeding behaviour after switching to diluted food (Skorupa et al., 2008, Wong et al., 2009). They can be dietary restricted. Their longevity is usually increased as compared to flies fed on richer diets. The feeding behaviour of flies fed on liquid food is different. Flies respond to a dilution of their liquid food by compensatory feeding. They cannot be dietary restricted. Their longevity is not modified by a two fold dilution of their food.

It should be noted that different sources of yeast have been used in the two types of experiments. Soluble yeast extracts are used in capillary feeding experiments such as the ones presented here. Heat inactivated lyophilized yeast is used to prepare agar based food mixtures. Wong et al., (2009) recently reported that flies on agar based media containing yeast extracts decrease their feeding frequency as the concentration of yeast extract is increased. They do not when they are fed on agar based media containing lyophilized yeast. Thus, the source of yeast probably contributed to the different behaviours of flies exposed to liquid or agar based diets. Results presented here do show that flies which compensate for different quality of their foods and which cannot be dietary restricted, do not get any benefit from a dilution of their food.

(ii) Nutrient ratio. Female flies (Lee et al., 2008) and male flies (this study) have a 3 to 5 fold preference for sucrose. A large sucrose/yeast ratio in the food promotes maximum longevity of female flies fed on liquid food (Lee et al., 2008). This study reports similar results for male flies fed on agar based food. Self selecting flies which have the choice to feed on separate sources of sucrose and yeast are longer lived than flies which are forced to ingest sucrose and yeast in a 1/1 ratio.

4.4. Conclusions.

This study shows that the feeding behaviour of *Drosophila* is far more complex than usually considered. Flies sense sucrose and yeast nutrients independently and adapt to changes in the quantity and presentation of their foods. This study, together with recent studies (Lee et al., 2008, Skorupa et al., 2008, Wong et al., 2009) stresses that a careful analysis of feeding behaviour is necessary before drawing any conclusion the relationship between diet and longevity.

Acknowledgements

This study was funded by the INSERM, ANR and the University of Nice Sophia Antipolis. We are grateful to anonymous reviewers for their comment of on a previous version of this paper.

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Legends to the Figures

Figure 1. Correcting for evaporative losses

Flies (3 to 9 per tube) were fed with a capillary filled with a 5S solution and total liquid intakes were measured (Panel A). Daily intakes were calculated from the slopes of the representations and plotted against the number of flies used (panel B). A control experiment using fly free tubes was also performed. The daily evaporation rate was estimated to 2 $\mu\text{l/day}$. Means \pm SEM are indicated ($n = 6 - 13$).

Figure 2. Feeding preferences are independent of age.

Flies were fed with different diets as indicated and the daily intakes of nutrients were determined. Flies used at 1, 10, 20 and 30 days as indicated. Filled bars refer to the richest dietary conditions (10S10Y, 10S-10Y or 10S). Open bars refer to the poorest diets (5S5Y, 5S-5Y or 5S). All graphs have been drawn to the same scale to highlight differences.

A. Sucrose intake by self selecting flies. A two way ANOVA indicated a statistically significant effect of the sucrose concentration ($p = 0.002$); Age had no effect. Mean intakes were computed using pooled data were $51.9 \pm 3.1 \mu\text{g}$ ($n = 63$) and $43.5 \pm 1.9 \mu\text{g}$ ($n = 72$) for flies fed on 10S-10Y and 5S-5Y diets respectively. Thus, flies responded to the diluted solution of sucrose by increasing their intake of sucrose solution. Yet, compensation was not complete and flies on a 5S-5Y diet got less sucrose than flies on the 10S-10Y diet.

B. Yeast intake by self selecting flies. A two way ANOVA indicated statistically significant effects of yeast concentration and age (both $p < 0.0001$). Yeast intake by 1 day old flies fed on 10S-10Y or 5S-5Y diets were not different. Young flies largely compensated for a two fold dilution of their yeast diet. Yeast intake by older flies (10, 20 or 30 days) was significantly larger ($p < 0.001$) when flies were fed on a 10Y diet. Thus aged flies lose their capacity to compensate for a two fold dilution of their yeast diet.

C. Sucrose intake by flies on pure sucrose diets. A two way ANOVA indicated that age and the concentration of sucrose had no effect. Mean intakes were computed for pooled data. They were $39.5 \pm 2.0 \mu\text{g}$ ($n = 78$) and $43.9 \pm 1.9 \mu\text{g}$ ($n = 70$) for flies fed on 10S and 5S diets respectively. Thus, flies fully compensated for a two fold dilution of a pure sucrose diet.

D. Sucrose intake by flies on mixed diets. A two way ANOVA indicated that food concentration and age had no effect. Mean intakes were computed for pooled data. They were $27.9 \pm 2.7 \mu\text{g}$ ($n = 63$) and $34.7 \pm 1.8 \mu\text{g}$ ($n = 52$) for flies fed on 10S10Y and 5S5Y diets respectively. Thus, flies compensated for a two fold dilution of a mixed 10S10Y diet. Note that in these experiments, yeast and sucrose were mixed in the same solution. As a consequence flies ingested identical quantities of yeast and sucrose.

Figure 3. Analysis of feeding data using the nutritional geometrical framework.

Flies of different ages were fed on diets consisting of different proportions of sucrose and yeast (10Y, 5S5Y, 10S10Y, 5S-5Y, 10S-10Y, 5S, 10S and 10Y as indicated) and the daily intakes of sucrose and yeast were determined. Data corresponding to the different ages (0, 10, 20, 30 days) are clustered into intake spaces. Numbers on their sides indicate the corresponding ages. The thin lines represent the nutritional rails for different yeast to sucrose ratios. Self selecting flies had a 3 to 5 fold preference for sucrose. This preference and the intake targets were not modified as fly age from 1 day to 30 days.

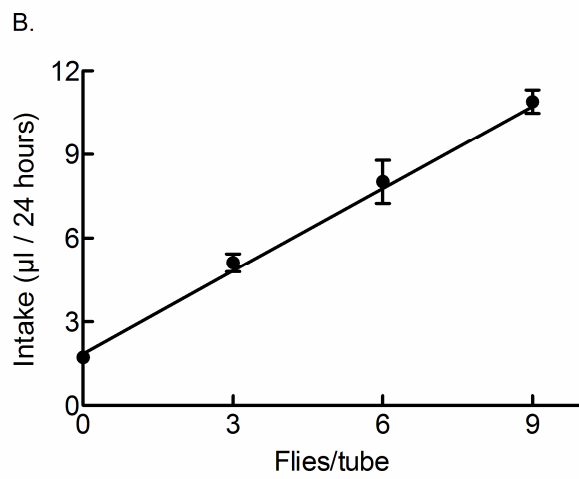
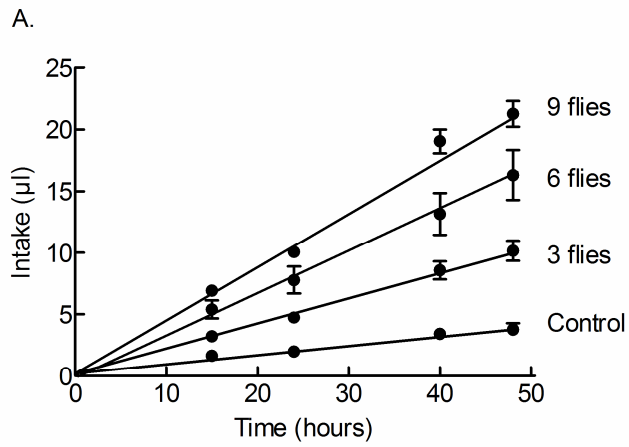
Figure 4. Limits to compensatory feeding

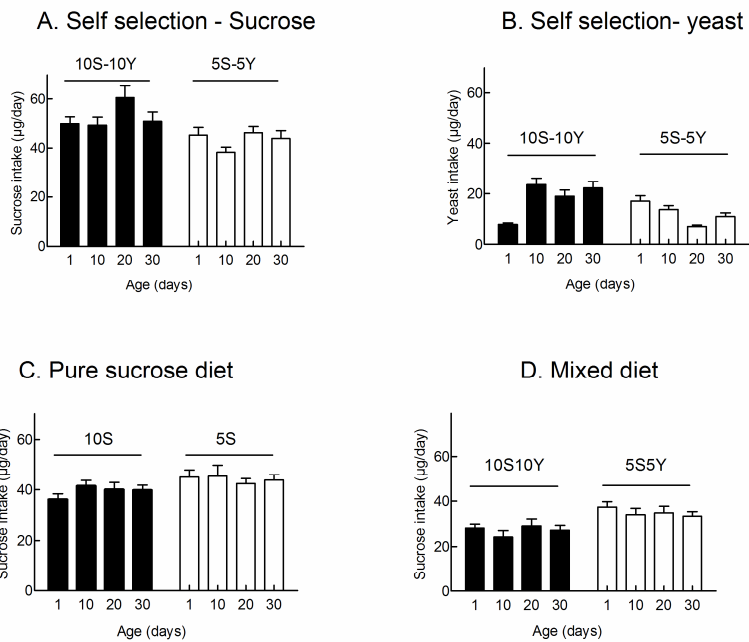
A. Flies were fed on serial dilutions of 10S solution and daily intakes of solution were determined. Means of 9-12 experiments are indicated. Error bars were smaller than the size of the symbols. The dotted line represents the volumes of solution that had to be ingested in order to maintain a daily intake of 36 μg , i.e. to fully compensate for food dilution. B. Sucrose intake is a non linear, saturable function of dietary sucrose. Daily intakes of sucrose were calculated from the data presented in panel A. Means \pm SEM are shown. The sucrose intake curve can be viewed as a saturation phenomenon (upper arrow, flies avoid highly concentrated solutions) or as feeding compensation (lower arrow, flies increase their feeding in response to food dilution).

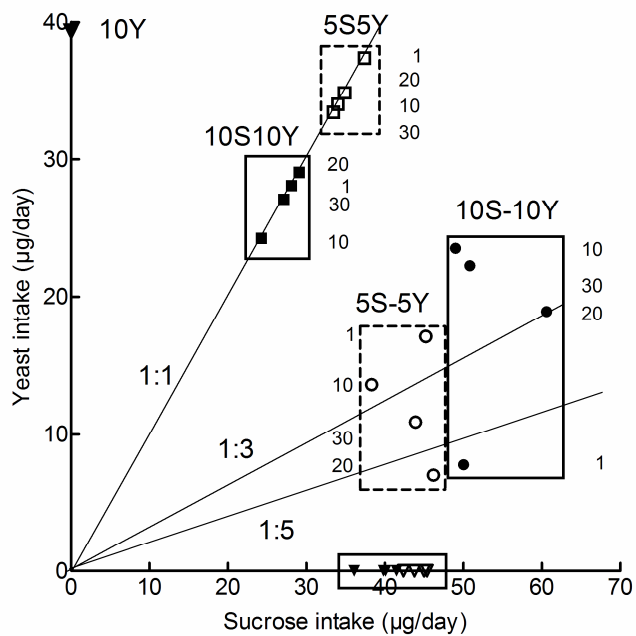
Figure 5. The beneficial effect of dietary restriction is lost when flies are fed on liquid diets. Survivorship curves of flies fed on liquid 10S10Y (N = 122, median: 26 days) and 5S5Y (N = 129, median: 31 days) diets. P = 0.80 using the Log rank test.

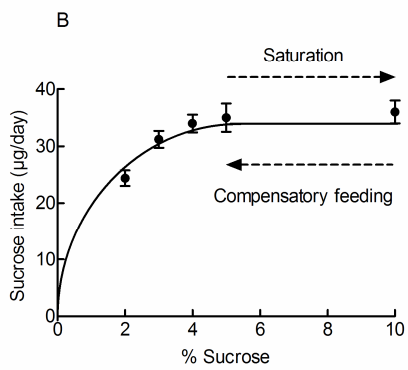
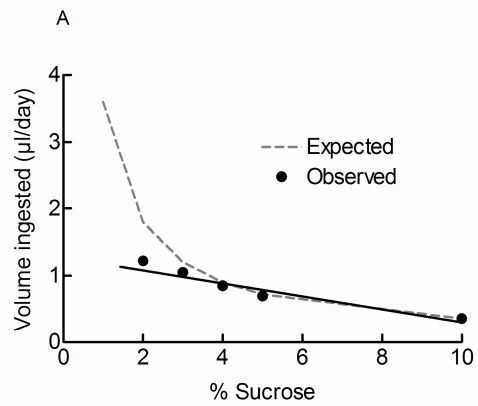
Figure 6. Self selecting flies are longer living.

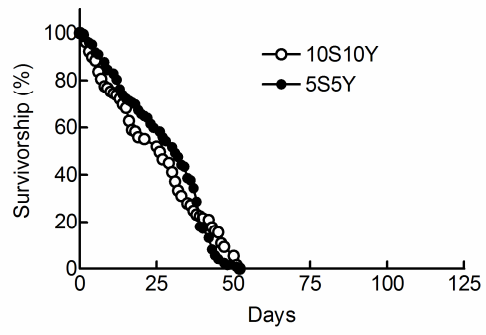
Flies were fed on a mixed 10S10Y diet under “choice” (10S-10Y) or “no choice” (10S10Y) conditions and survivorship curves were determined using conditions defined as Protocol B in the Materials and Method section. Median and maximum longevities were 41 days and 81 days under “no choice” conditions. They were 55 days and 83 days under “choice” conditions. Sample sizes were 192 and 119 under the “no choice” and “choice” conditions respectively. P < 0.01 using the log rank test.



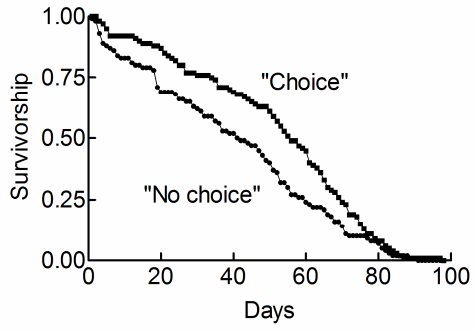








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