Short Report

Counting calories in *Drosophila* diet restriction

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Abstract

The extension of life span by diet restriction in *Drosophila* has been argued to occur without limiting calories. Here we directly measure the calories assimilated by flies when maintained on full- and restricted-diets. We find that caloric intake is reduced on all diets that extend life span. Flies on low-yeast diet are long-lived and consume about half the calories of flies on high-yeast diets, regardless of the energetic content of the diet itself. Since caloric intake correlates with yeast concentration and thus with the intake of every metabolite in this dietary component, it is premature to conclude for *Drosophila* that calories do not explain extension of life span.

1. Introduction

Reduced food intake without malnutrition extends life span in many organisms including yeast, nematode, fruit fly, and rodents (Koubova and Guarente, 2003; Masoro, 2000; Partridge et al., 2005). In rodents, reduced intake of total calories extends life span, yet limiting specific nutrients can also increase survivorship (Miller et al., 2005; Yu and Masoro, 1985; Zimmerman et al., 2003). It is a current debate as to whether limiting calories is a feature of nutrition responsible for extended life span in the fruit fly, *Drosophila melanogaster*. In recent work to address this question Mair et al. (2005) simultaneously manipulated dietary yeast and sugar to vary nutrient quality and caloric value. Survivorship was increased substantially on diets that restricted yeast while holding sugar constant, while life span changed little when sugar was restricted in diets with a constant amount of yeast. Notably, Mair et al. reasoned that the actual caloric intake was equal for females given diets with equal energetic value because solitary test females extend their proboscis for equal durations. Since

females on isocaloric diets with low yeast are longer lived than those on high yeast, the authors argue that diet restriction (DR) mediates life span because it limits specific nutrient components of yeast rather than calories.

The conclusion of Mair et al. requires actual caloric intake to be proportional to the energetic content of the diet media. However, the relationship between proboscis extension and nutrient intake is unknown, and recent studies confirm that food intake may not be constant across diets that vary in yeast or sugar concentration (Carvalho et al., 2005; Min and Tatar, 2006a). Females are seen to both increase and decrease the rate of intake when fed a restricted diet. It is thus possible that the actual caloric intake will differ among females as a function of yeast concentration when they are presented with diets of similar energetic value.

To test whether the energetic value of diet media is proportional to actual caloric intake, we replicated the diet manipulation experiments of Mair et al. and simultaneously measured survival and caloric flux. A complete energy budget would measure calories from metabolism, excretion, changes in body mass, and total allocation to eggs. We do not account for metabolic rate because it does not differ among fully fed and DR *D. melanogaster* (Hulbert et al., 2004). We do not account for excreta; to the extent this rate

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differs between treatments, we will at most underestimate the discrepancy between actual caloric flux and diet energetic value. In practice, therefore, we measure caloric flux as the total energetic value of eggs and of body tissue across five days, and across each of the diet types described in Mair et al. (2005).

2. Materials and methods

2.1. Demography and media

Larvae of the Canton-S strain were grown on standard cornmeal/sugar/yeast/agar medium (Elgin and Miller, 1980), supplemented with several grains of live yeast. Newly enclosed adults were collected over 48 h and were assigned to 1 L demography cages to a density of 200 individuals (100 females, 100 males). Food vials (25×95 mm) were attached to each cage via a 25 mm plastic tube and changed every 2 d, at which time dead flies were removed, sexed, and recorded. Three replicate cages were established for each of four diet treatments. Cages were maintained at 25 °C, 40% relative humidity and a 12-h light–dark cycle.

The composition of each diet follows the design of Mair et al. (2005), although our media also contains a uniform concentration of cornmeal (Table 1). Cornmeal acts as a colloid to maintain homogeneity of the nutritive sugar and yeast mixture. Dry, autolysed SAF yeast was purchased from Lesaffre Yeast Corporation (Milwaukee, WI). Energetic content of media is calculated from the individual caloric value and proportional contribution of each component. The high yeast/low sugar and low yeast/high sugar media were 'isocaloric' at about 99 kcal/100 ml.

2.2. Body and egg collection for bomb calorimetry

Larvae of the Canton-S strain were grown as described above. Upon eclosion, 250 females and 50 males were sorted into 1 L demography cages; four replicate cages plus one spare were established for each diet treatment. Density in the replicate cages was held constant by replacing dead flies with same aged adults from the spare cage of the treatment. We measured caloric value of females within each replicate as the calories of all eggs laid from eclosion (day zero) through day five, plus the calories of all adults at the end of day five. Since cages were established with adults that developed on the same diet but before they consumed any adult diet, differences in the energetic value of tissue

at day five reflects differences caused by adults feeding upon the various diets.

Eggs were collected from food media dishes (60×15 mm) that were attached by a 25 mm plastic tube and funnel to each cage. Dishes were changed daily, eggs were washed free, freeze-dried, and weighed. At day five, food dishes were removed for four hours before we collected adults to ensure that flies did not contain undigested food. All females from each cage were freeze-dried and weighed.

2.3. Calorimetry

We used combustion calorimetry (e.g., Lamprecht and Schmolz, 1999; Schmolz et al., 2005) to determine the heat content of bodies and eggs combined within replicates. Each sample was mixed with calorimetry-grade n-tetradecane (Sigma, St. Louis, MO) at a mass ratio of 1:5 (specimen:tetradecane) and combusted in a static jacket oxygen bomb calorimeter (Parr Instrument, Moline, IL; model No. 1341). We previously determined the optimal quantity of n-tetradecane to ignite the insect samples to full combustion, and we calculated the net caloric value of the sample by subtracting the known heat content of the primer.

3. Results

Life span (Table 1) and survival (Fig. 1A) was increased in females maintained on diets with dilute concentrations of yeast (Median survival: Low yeast, 46 d; High yeast, 32 d; Log–Rank test, $\chi^2 = 333.7$, $p < 0.0001$), consistent with the survival outcomes reported in Mair et al. (2005) and others (Chippindale et al., 1993; Min and Tatar, 2006a). There was a marginal but non-significant impact of reduced dietary sugar upon life span (Median survival: Low sugar, 38 d; High sugar, 34 d; Log–Rank test, $\chi^2 = 2.01$, $p = 0.16$). Low yeast/high sugar diet and high yeast/low sugar diet have the same caloric content (Table 1), but flies fed those diets have significantly different life span as reported by Mair et al. We plot our results (Fig. 1B) in the same format as Mair et al. and recapitulate their published outcomes.

In contrast to these results, flies fed isocaloric diets in fact differ markedly in assimilated calories. When energy influx is measured by counting the calories of fly soma and of eggs produced during 5 days, per fly caloric content is greater for flies fed yeast-rich diet than flies fed yeast-poor diet. There is a strong correlation between life span

Table 1
Diet composition, caloric content and conferred life span

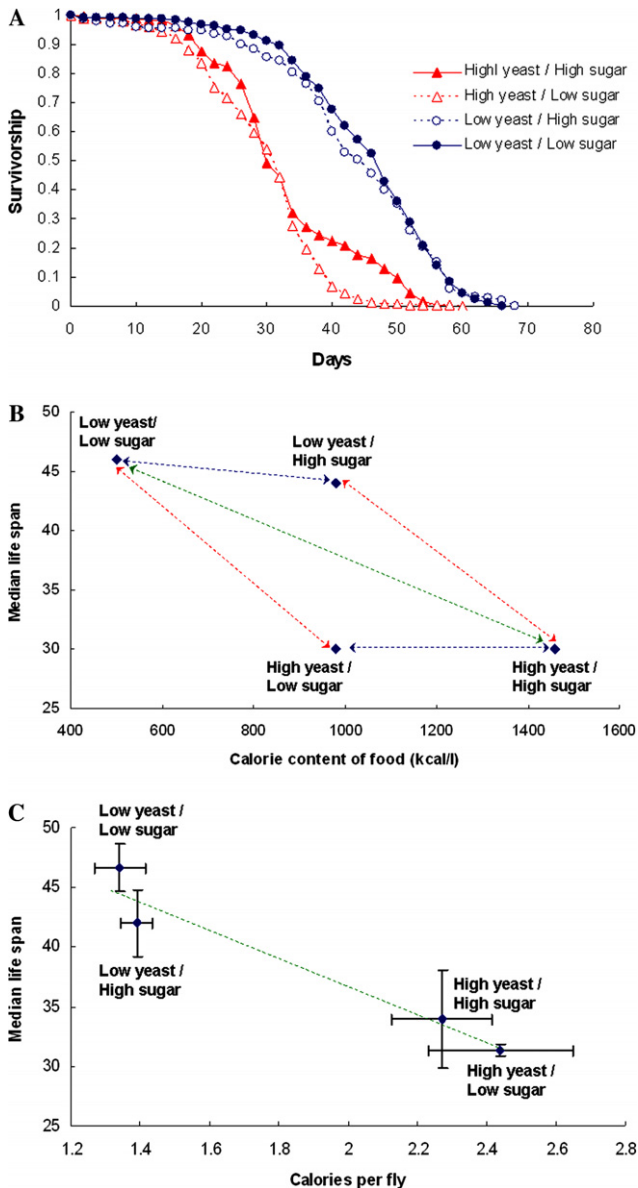| Diet treatment	Composition (in 100 ml water)	Energy in 100 ml media, kcal	Median life span, d (95% CI)
High yeast/high sugar	16 g yeast, 16 g sucrose, 5.2 g cornmeal	146.8	30 (30–34)
High yeast/low sugar	16 g yeast, 4 g sucrose, 5.2 g cornmeal	98.8	32 (30–34)
Low yeast/high sugar	4 g yeast, 16 g sucrose, 5.2 g cornmeal	98.8	46 (42–48)
Low yeast/low sugar	4 g yeast, 4 g sucrose, 5.2 g cornmeal	50.8	48 (46–48)

Fig. 1. Longevity, virtual calories, and caloric intake. (A) Longevity. Reducing yeast content of the diet had a much greater effect on life span than reducing sugar content of the diet. (B) Virtual Calories: Plot of median life span relative to diet energetic value. Data are presented with the format of figure 3 in Mair et al. (2005). (C) Assimilated Calories: Plot of median life span (among replicate mean and SE) relative to calories of body and laid eggs per capita (among replicate mean and SE). Correlation between life span and assimilated calories is strong and significant.

and caloric content ($R^2 = 0.98$, $F_{(1,2)} = 109.99$, $p = 0.009$, Fig. 1C).

We also observed patterns of nutrient assimilation that address questions of compensatory feeding. Dry weights of female body and eggs laid during 5 days were greater in flies fed yeast rich diet (Fig. 2), but the extent of this effect depended on the concentration of sugar in the diet. Flies fed high yeast diet produced more eggs when the diet had low sugar than high sugar. Similarly, flies fed high yeast/low sugar diet produced more eggs and gained more weight than flies fed low yeast/high sugar diet even though these diets were of the same energetic content.

4. Discussion

Food consumption has been explored across the decades of building *D. melanogaster* into an experimental model, although not with any diet known to extend life span (Driver et al., 1986; Edgecomb et al., 1994; Tatar, 2007). The first study to retard aging by diet restriction was reported only some dozen years ago (Chippindale et al., 1993). Females maintained with a relatively dilute solution of dietary yeast extended life span by 25–30% and concomitantly laid fewer eggs. While dilute medium presumably reduced yeast consumption, feeding was not measured. Likewise, this early design did not aim to distinguish effects of calories relative from those of specific yeast metabolites. Studies to address these questions have only appeared in recent years.

Our own group simultaneously measured life span and feeding rate of mated females maintained on a constant agar-based diet of uniform sugar with varied concentrations of autolysed yeast (Min and Tatar, 2006a). Life span was greatest upon diet of 2% yeast and progressively less as yeast concentration increased. We estimated feeding rate from the uptake of a soluble, non-digestible dye. Females on 16% yeast consumed four-fold more diet (dye) than those on 2% diet. This disproportionate difference in consumption suggests that concentrated yeast diet not only confers a high level of food intake but also stimulates feeding behavior, perhaps in response to the metabolic demands of elevated egg production.

Different outcomes were seen by Carvalho et al. (2005) where virgin females were presented with media that varied in both sugar and yeast-extract; survival was greatest upon 1% sugar-yeast diet. Consumption was measured by the uptake of a soluble nucleotide CTP[α-^{32}P] tracer. In contrast to our observations, feeding rate increased upon progressively dilute diets. Thus, females upon the 1% diet ate less food (tracer) but the quantity consumed was only 4/10th the intake measured on 15% sugar-yeast diet. Feeding was stimulated at low diet concentrations, perhaps because these media reduced both sugar and yeast, or because compensatory feeding occurs in the absence of reproduction. Our new data also suggest there is compensatory feeding when sugar content is reduced because flies fed on high yeast/low sugar diet produced more eggs than flies fed on high yeast/high sugar diet.

While the dye and tracer studies confirm that flies consume less food upon dilute diet they do not address whether life span is modulated by reduced calories or by specific metabolites of the diet. To solve this specific problem Mair et al. (2005) independently varied the concentration of sugar and of yeast to produce diets with similar caloric content but varied composition. The survival of fecund females was markedly increased on diets that restricted yeast but not when sugar was limited. Comparing
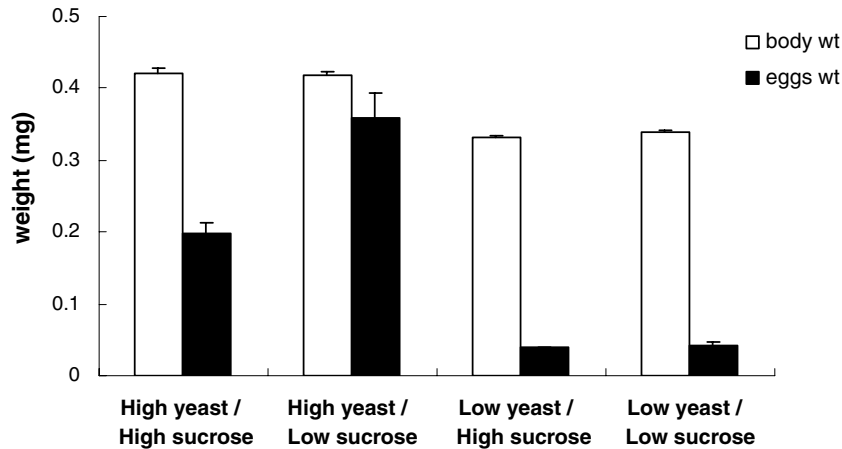
Fig. 2. Body and egg mass of female by variation of yeast and sugar contents. Mass of females (ovary and immature eggs inclusive) and produced eggs was estimated from eclosion through 5 d old. High-yeast induces high egg production and weight gain. Note that low-sugar further elevates egg production.

life span across diets that varied in quality but not in energetic content provided a way to assess the relative importance of specific nutrients and calories. The low yeast/high sugar and the high yeast/low sugar diets were energetically equivalent, yet females lived about 13 days longer on low yeast diet. Based on the frequency of proboscis extension in undisturbed conditions, Mair et al. (2005) argued that aging females feed at the same rate on each of the tested diets, and therefore that they consume the same amount of calories upon diets of similar energetic content. Since females on diets with low yeast concentration are longer-lived than those on diets with high yeast diet of the same energetic content, Mair et al. (2005) concluded that diet restriction mediates life span independent of caloric intake.

This inference requires that nutrient acquisition is proportional to nutrient concentration in the diet. Our data here do not support this assumption. As reported, life span was strongly increased on diets with reduced yeast concentration, and there was little impact on survival from diets with reduced sugar, and when we plot our life span data relative to diet energetic value we recapitulate the results of Mair et al. (2005). However, females on iso-caloric diets did not assimilate the same amount of calories. Females fed low-yeast diet consume almost half the calories of females on high-yeast diet, and life span was strongly correlated with assimilated calories. Thus, females on low-yeast diet consumed fewer calories, less yeast and aged slowly. From this experimental design it is premature to exclude calories as a determinant of Drosophila life span because the intake of calories from yeast is confounded with consumption of all metabolites within yeast.

Ultimately it may be necessary to treat the fly more like we do rodents: directly control food intake or explicitly measure assimilated nutrients. Experimental regulation of food intake, in fact, has been applied to the Mediterranean fruit fly (Carey et al., 2002) and to the housefly (Cooper et al., 2004). In these relatively large flies there was no positive effect of reduced nutrient intake on life span, contrary

to precedence. Scaling such protocols to study the effect of defined food intake with Drosophila should be a high priority.

Likewise, dyes and tagged nucleotides at best are partial proxies of consumption because they only mark solute intake. Adult Drosophila does not have chewing mouthparts and insoluble metabolites from yeast embedded below the media surface are relatively inaccessible. Measuring stable isotopes of carbon and nitrogen from diet can remedy this problem and precisely measure nutrient acquisition and metabolic flux. The butterfly Heliconius charitonius was shown through such methods to acquire essential amino acids from dietary pollen to produce eggs (O'Brien et al., 2003). We recently adapted this approach for Drosophila to determine how adults use sugar of the larval diet (Min et al., 2006). Turnover of carbon from larval acquired dietary sugar is rapid and nearly complete in the metabolite pool used to produce eggs and in adult somatic tissue itself. Applying this approach with the carbon and nitrogen from dietary yeast has the potential to identify how specific metabolites are acquired and allocated to somatic maintenance when diet restriction extends life span.

Efforts to clarify how the practice of diet restriction with Drosophila affects nutrient uptake are progressing but incomplete. It is clear that dilution of dietary yeast reduces food consumption and extends life span. On the other hand, we cannot yet resolve the effects of calories from those of metabolites specific to yeast, let alone distinguish the effects of components within yeast, such as carbohydrates, sterols, fatty acids, vitamins, minerals and amino acids. Amino acids deserve attention because reduced methionine extends life span in rats and in mice (Miller et al., 2005; Zimmerman et al., 2003). Whether D. melanogaster survival can be improved by limiting dietary amino acids remains difficult to assess because defined diets optimized for larvae are not suitable for adults (Lamb, 1978; Min and Tatar, 2006b). It is perhaps ironic that while the tools to dissect the molecular basis of metabolism and

aging in *Drosophila* have advanced tremendously in recent years we are currently stuck on a fundamental problem of gastronomy: How do we measure and control what is eaten by a fly?

Acknowledgments

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