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## Discriminating between energetic content and dietary composition as an explanation for dietary restriction effects

Jacintha Ellers\*, Bas Ruhe, Bertanne Visser

Department of Ecological Sciences, VU University Amsterdam, The Netherlands

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### ABSTRACT

A reduction in dietary calories has been shown to prolong life span in a wide variety of taxa, but there has been much debate about confounding factors such as nutritional composition of the diet, or reallocation of nutrients from reduced reproduction. To disentangle the contribution of these different mechanisms to extension of life span, we study the effect of caloric restriction on longevity and fecundity in two species of sugar-feeding parasitoid wasps. They have a simple diet that consists of carbohydrates only, and they do not resorb eggs, which rules out the proposed alternative explanations for beneficial effects of caloric restriction. Two caloric restriction treatments were applied: first, dietary dilution to investigate the effect of carbohydrate concentration in the diet; and second, intermittent feeding to examine the effect of feeding frequency on longevity and fecundity. Only the dietary dilution treatment showed an effect of caloric restriction with the highest longevity recorded at 80% sucrose (w/v). No effect of dietary regime was found on fecundity. We also measured the weight increase of the parasitoids after feeding to obtain an estimate of consumption. A constant quantity of the sugar solution was consumed in all dietary dilution treatments, hence caloric intake was proportional to sucrose concentrations. Although the present study does not disqualify the relevance of nutrient composition in other species, our data unequivocally demonstrate that caloric restriction alone is sufficient to extend life span and invalidate alternative explanations.

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### 1. Introduction

It has become increasingly clear over the last decade that dietary restriction (DR) without malnutrition prolongs life. Reduced nutrient availability increases life span and ameliorates age-related diseases in a wide array of species (Weindruch and Walford, 1988; Nemoto and Finkel, 2004; Colman et al., 2009). Modest restriction of dietary intake was first applied in rats (McCay et al., 1935), but its effects have been found to hold for other mammals (Colman et al., 2009), invertebrates (Partridge et al., 2005; Kaeberlein et al., 2007) and micro-organisms (Skinner and Lin, 2010), with the exception of some studies finding no detrimental effect of nutrient-rich diets (Cooper et al., 2004; Bauerfeind et al., 2009; Molleman et al., 2009). Although the effect of DR seems phylogenetically well-conserved, it is less clear if common principles and mechanisms underlie the life-extending effects of DR. The relevance of understanding the costs and benefits of dietary composition is highlighted by the fact that obesity has been increasing

rapidly in the western world and the interest in the potential life-extending effects of DR in humans is growing.

The mechanisms responsible for the beneficial effects of DR have remained elusive, but proposed mechanisms can be broadly divided into three categories. First, the caloric restriction hypothesis assumes that a reduction in energetic content of the food has a life-extending effect, because of reduced oxidative stress (Harman, 1956; Sohal and Weindruch, 1996). With lower available energy, metabolic rate will slow down and reduce free radical formation. This widely held view emerged from early work on DR, but has recently been challenged by studies that suggest specific nutrients are involved in ageing and life span. Hence, a second proposed mechanism focuses on the composition of diet rather than its caloric content. Dietary components may act independently on signalling pathways such as the insulin pathway (Kimura et al., 1997) or the TOR pathway which mediates growth and cell division in response to nutrient availability and determines life span in *Drosophila* (Kapahi et al., 2004). Also, deficiencies of specific nutrients may lead to excessive consumption of others. Third, DR often reduces reproductive output, which may increase life span because of the trade-off between reproduction and longevity. The relative importance of the direct effects of nutrient composition versus the indirect effects of reallocation of nutrients, has remained largely unaddressed in DR protocols (Bass et al., 2007).

\* Corresponding author. Address: Department of Ecological Sciences, VU University Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands. Tel.: +31 20 5987076; fax: +31 20 5987123.

E-mail addresses: [jacintha.ellers@falw.vu.nl](mailto:jacintha.ellers@falw.vu.nl) (J. Ellers), [sb.ruhe@gmail.com](mailto:sb.ruhe@gmail.com) (B. Ruhe), [bertanne.visser@falw.vu.nl](mailto:bertanne.visser@falw.vu.nl) (B. Visser).

To disentangle the contribution of different mechanisms on extension of life span, it is indispensable to extend the study of DR to invertebrates, which are more amenable to experimental manipulation. Invertebrates offer the opportunity to reveal more subtle effects of diet because of their relative fast life cycle and the possibility of larger sample sizes. The recent surge in studies using *Drosophila* has made important contributions to the understanding of life span extension, such as a detailed evaluation of the effect of dietary protein to carbohydrate ratios versus energetic content (Simpson and Raubenheimer, 2007; Lee et al., 2008) and the role of olfaction and odorant receptors in life span regulation (Libert et al., 2007).

Invertebrates offer a wider range of experimental manipulations than mammals because dietary complexity is more variable among species (Slansky and Rodriguez, 1987). Elaborate diets are found in predatory arthropods, and life span in these species will probably be strongly determined by dietary composition and nutritional ratios (Toft, 1999; Hvam and Toft, 2005). On the other hand, several invertebrate species have relatively simple diets that contain no proteins or lipids and consist of carbohydrates only. For example, adult diets may exist only of nectar consumed from flowers or extrafloral nectaries (Wackers, 2001). Many of these species are towards the pro-ovigenic end of the ovigeny spectrum, meaning that they emerge with the majority of their eggs ready for oviposition (Jervis et al., 2001). If they do not resorb eggs during adult life, reallocation of nutrients from reproduction will play only a minor role in life span extension. Such species offer a yet unexploited opportunity to discriminate between the role of energetic content and dietary composition in DR.

In this study, we investigate the effect of caloric restriction on longevity and fecundity in two parasitoid wasp species, *Asobara tabida* and *Trichopria drosophilae*. During the larval stage, these parasitoids develop in *Drosophila* larvae or pupae, respectively, and kill their host to complete development. However, the adult diet of both species is simple and consists only of sugar-rich substances (Eijs et al., 1998). Adult feeding is of particular importance in parasitoid species because the majority of parasitoids are unable to accumulate lipids, even when a surplus of food is available (Ellers, 1996; Giron and Casas, 2003; Visser and Ellers, 2008). A comparative study showed that the parasitic life style has led to the evolutionary loss of this essential metabolic trait (Visser et al., 2010), which means that in order to save irreplaceable lipid reserves, they need permanent access to food sources. Indeed, carbohydrate feeding lowers lipid depletion rates and extends longevity in *A. tabida* (Ellers and vanAlphen, 1997) and other species (Wu et al., 2008), and under natural conditions parasitoids feed frequently on carbohydrates (Desouhant et al., 2010). Due to the continuous dependence on adult feeding, we expect no benefits of caloric restriction on longevity in these two parasitoid species. We test this hypothesis using a two-pronged approach to restrict calorie intake: First, a dietary dilution experiment to investigate the effect of carbohydrate concentration in the diet; and second, an intermittent feeding treatment to examine the effect of feeding frequency on longevity and fecundity of *A. tabida* and *T. drosophilae*.

## 2. Materials and methods

### 2.1. Insects

*Drosophila melanogaster* and *Drosophila subobscura* hosts were kept at 20 °C, RH 75% and a 12:12 h L:D regime on food medium containing 20 g agar, 50 g sucrose, 35 g yeast, 9 g kalmus (10 parts acidum tartaricum, 4 parts ammonium sulphate, 3 parts potassium phosphate and 1 part magnesium sulphate) and 10 mL nipagin (100 g 4-methyl hydroxyl benzoate per litre ethanol) per litre water. For the rearing of *A. tabida* 100–200 2-day old larvae of

*D. subobscura* were collected and subsequently placed in pots with wasp medium containing 20 g agar, 9 g kalmus, 5 mL nipagin and 4 mL proprionic acid per litre water, covered by a thin layer of liquid yeast. Two mated females were placed with the larvae for parasitization and allowed to feed on honey. All experimental pots were kept at 23 °C, RH 75% and a 16:8 h L:D regime for further parasitoid development. Successfully parasitized pupae were transferred individually into vials containing moist cotton wool. For the rearing of *T. drosophilae* 30 1-day old pupae of *D. melanogaster* were collected and subsequently placed in pots containing wasp medium. Conditions for parasitization and development were similar to those described for *A. tabida*, with the exception that pots and vials were kept at a 12:12 h L:D regime.

### 2.2. Feeding regimes and longevity, egg load and body size measurements

After emergence, adult female wasps were kept singly in a vial containing wet cotton wool and were randomly assigned to treatments. For dilution treatments, sucrose was diluted with water to the following concentrations in weight per volume (w/v): 0%, 20% (20 g sucrose in 100 mL water), 40%, 60%, 80% or 100%, provided *ad libitum* on a small piece of parafilm containing a droplet of the appropriate dilution. For intermittent feeding treatments, adult female wasps were allowed to feed on a 40% w/v concentration of sucrose and water (cf Hogervorst et al., 2007) placed on a piece of parafilm for the duration of 2 h. Adult females were presented with water or with food once, weekly, twice weekly, daily or *ad libitum*. Dilutions were refreshed every week to prevent fungal growth. For longevity measurements all vials were inspected daily for deaths and subsequently frozen at –18 °C. Body size was determined by placing a single adult female under a microscope (Leica DC 200; 500×) and measuring hind tibia length. After size had been determined the adult female was placed in a droplet of demineralised water, dissected and the number of eggs counted.

### 2.3. Food uptake experiments

Food uptake in the dilution treatments was determined for all six sucrose concentrations. Freshly emerged females of *A. tabida* and *T. drosophilae* were randomly assigned to feed on one of the concentrations during 2 h, after which individuals were frozen at –18 °C. Food uptake was determined by measuring the wet weight of 5 pooled individuals on a microbalance (Mettler Toledo), with 8 replicates per concentration per species.

### 2.4. Statistical analyses

Longevity data were checked for normality and homogeneity of variances. General Linear Models were used to test for an effect of treatment on egg load and longevity for each species. Tibia length was included in these analyses as a covariable. Posthoc comparisons were carried out using a Tukey test. Data on food uptake were subjected to Analysis of Variance, followed by a Tukey test. The probability level at which differences were considered significant was  $P < 0.05$ . Statistical analyses were done using SPSS version 17.

## 3. Results

### 3.1. Dilution treatment

Dietary dilution had a significant influence on longevity of both parasitoid species (*A. tabida*:  $F_{5,82} = 15.545$ ,  $P < 0.001$ ; *T. drosophilae*:  $F_{5,83} = 22.405$ ,  $P < 0.001$ ). For *A. tabida* the shortest longevity was observed at 0% sucrose (6.72 days  $\pm$  1.14SE) and the highest

longevity at 80% sucrose ( $30.93 \text{ days} \pm 2.97\text{SE}$ ; Fig. 1A). When fed the highest concentration of sucrose (100%), longevity of *A. tabida* was significantly lower than when fed 60% ( $P < 0.026$ ) or 80% ( $P < 0.001$ ) sucrose. *T. drosophilae* also had the shortest longevity when fed 0% sucrose ( $11.21 \text{ days} \pm 0.61\text{SE}$ ) and the highest longevity when fed 80% sucrose ( $86.25 \text{ days} \pm 3.72\text{SE}$ ; Fig. 1B). Similar to *A. tabida*, *T. drosophilae* had a significantly reduced longevity when fed 100% sucrose compared to 80% (Tukey test:  $P = 0.049$ ). Tibia length was not a significant variable in explaining variation in longevity among treatments in either of the two species (*A. tabida*:  $P = 0.89$ ; *T. drosophilae*:  $P = 0.80$ , Table 1).

The average egg load of *A. tabida* ( $151.3 \pm 6.91\text{SE}$ ) was higher than in *T. drosophilae* ( $73.17 \pm 2.02\text{SE}$ ), but for neither of the two species did sucrose concentration affect egg load (*A. tabida*:  $F_{5,46} = 0.728$ ,  $P = 0.606$ ; *T. drosophilae*:  $F_{5,58} = 1.565$ ,  $P = 0.184$ , Table 1).

### 3.2. Intermittent feeding

Intermittent feeding had a significant influence on the longevity of both parasitoid species (*A. tabida*:  $F_{5,92} = 23.182$ ,  $P < 0.001$ ;

*T. drosophilae*:  $F_{5,84} = 30.108$ ,  $P < 0.001$ ). In *A. tabida* there was a clear dichotomy between feeding treatments: average longevity was uniformly high when females were fed daily or had permanent access to food (Tukey:  $P = 0.506$ , Fig. 2A), while both treatments differed significantly from treatments with less frequent feeding (Tukey: all pairwise comparisons  $P < 0.001$ ). When females were fed twice a week or less frequently, longevity was consistently low. In fact, there was no significant difference in longevity between the starvation treatment and being fed twice a week ( $P = 0.477$ ). Egg load did not differ among treatments except for the daily feeding treatment which had a lower egg load than the other treatments (Tukey: all comparisons  $P < 0.05$ , Table 1). Egg load and tibia length contributed significantly to the variation in longevity (egg load:  $F_{1,92} = 4.66$ ,  $P = 0.034$ ; tibia length:  $F_{1,92} = 5.71$ ,  $P = 0.019$ ). Long-lived individuals were larger and had a higher egg load compared to shorter-lived females.

In *T. drosophilae* the highest average longevity was also observed in the daily and permanent treatment (Fig. 2B). The two most frequent feeding treatments differed significantly from the other treatments (all comparisons  $P < 0.001$ ), but not from each

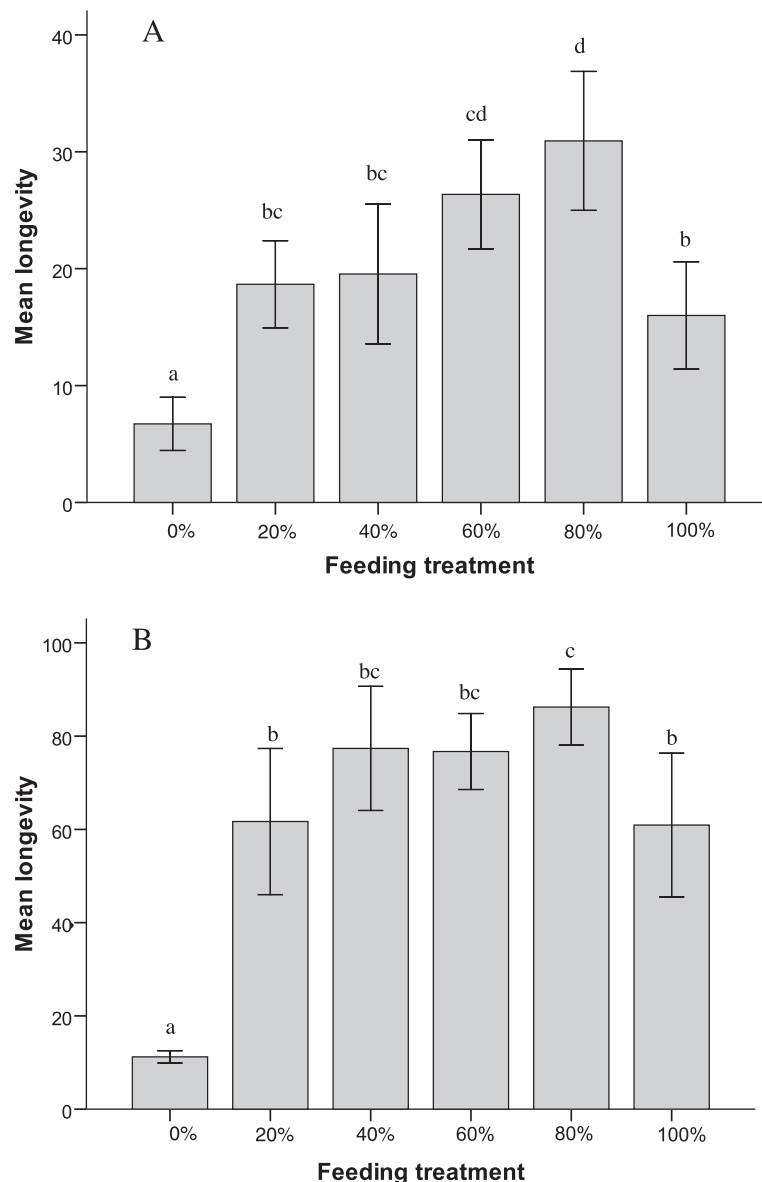


Fig. 1. Average longevity (days  $\pm$  1SE) of (A) *A. tabida* and (B) *T. drosophilae* when exposed to dietary dilution treatments with different sucrose concentrations. Different letters represent differences significant at  $\alpha = 0.05$  level.

**Table 1**

Average body size and egg load (mean  $\pm$  SE) of two parasitoid species subjected to caloric restriction through dietary dilution and intermittent feeding.

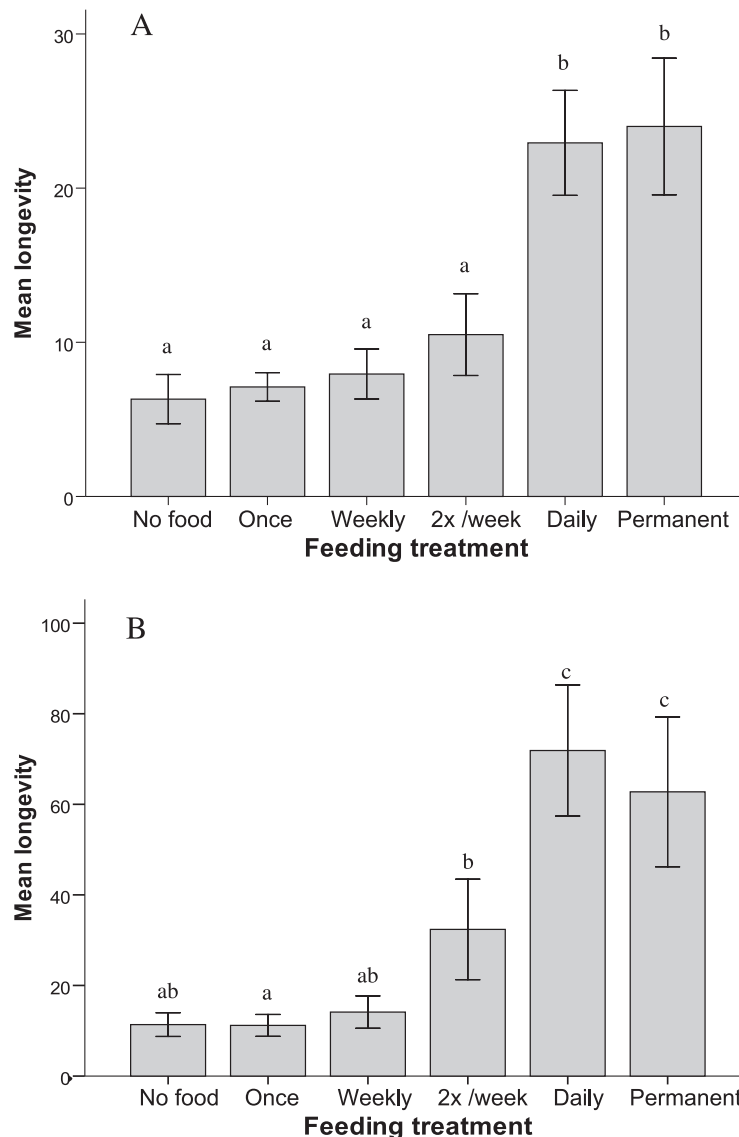
	<i>A. tabida</i>		<i>T. drosophilae</i>	
	Tibia	Egg load	Tibia	Egg load
<i>Dietary dilution (%)</i>				
0	0.980 <sup>a</sup> $\pm$ 0.021	146.9 <sup>a</sup> $\pm$ 8.64	0.786 <sup>a</sup> $\pm$ 0.014	80.00 <sup>a</sup> $\pm$ 4.01
20	0.985 <sup>a</sup> $\pm$ 0.018	139.6 <sup>a</sup> $\pm$ 18.19	0.778 <sup>a</sup> $\pm$ 0.016	74.62 <sup>a</sup> $\pm$ 2.22
40	0.993 <sup>a</sup> $\pm$ 0.032	138.00 <sup>a</sup> $\pm$ 23.67	0.767 <sup>a</sup> $\pm$ 0.012	75.44 <sup>a</sup> $\pm$ 5.17
60	0.969 <sup>a</sup> $\pm$ 0.038	145.20 <sup>a</sup> $\pm$ 9.56	0.771 <sup>a</sup> $\pm$ 0.017	75.36 <sup>a</sup> $\pm$ 4.75
80	0.995 <sup>a</sup> $\pm$ 0.029	173.00 <sup>a</sup> $\pm$ 38.25	0.760 <sup>a</sup> $\pm$ 0.020	65.20 <sup>a</sup> $\pm$ 6.12
100	0.998 <sup>a</sup> $\pm$ 0.025	173.20 <sup>a</sup> $\pm$ 19.23	0.760 <sup>a</sup> $\pm$ 0.018	65.11 <sup>a</sup> $\pm$ 7.38
<i>Intermittent feeding</i>				
No food	0.916 <sup>a</sup> $\pm$ 0.023	154.00 <sup>a</sup> $\pm$ 10.15	0.708 <sup>a</sup> $\pm$ 0.029	68.43 <sup>a</sup> $\pm$ 4.24
Once	0.956 <sup>a</sup> $\pm$ 0.024	149.89 <sup>a</sup> $\pm$ 14.60	0.759 <sup>ab</sup> $\pm$ 0.018	79.88 <sup>a</sup> $\pm$ 3.62
Weekly	0.978 <sup>a</sup> $\pm$ 0.024	172.83 <sup>a</sup> $\pm$ 11.21	0.750 <sup>ab</sup> $\pm$ 0.016	70.88 <sup>a</sup> $\pm$ 3.65
Twice weekly	0.954 <sup>a</sup> $\pm$ 0.018	167.63 <sup>a</sup> $\pm$ 10.44	0.756 <sup>ab</sup> $\pm$ 0.018	78.31 <sup>a</sup> $\pm$ 3.73
Daily	0.964 <sup>a</sup> $\pm$ 0.025	112.06 <sup>b</sup> $\pm$ 10.92	0.803 <sup>b</sup> $\pm$ 0.024	81.81 <sup>a</sup> $\pm$ 9.07
Permanent	1.009 <sup>a</sup> $\pm$ 0.018	150.42 <sup>a</sup> $\pm$ 13.21	0.750 <sup>ab</sup> $\pm$ 0.021	77.33 <sup>a</sup> $\pm$ 6.79

Different letters represent differences significant at  $\alpha = 0.05$  level.

other ( $P = 1.00$ ). Tibia length was significant as covariable in the model ( $F_{1,84} = 4.34$ ,  $P = 0.04$ ) with larger females living longer. Egg load was not a significant covariable in the model ( $F_{1,84} = 1.40$ ,  $P = 0.425$ ) and egg load did not differ among treatments ( $F_{5,84} = 0.36$ ,  $P = 0.88$ , Table 1).

### 3.3. Feeding experiments

To ascertain that the differences in longevity were not due to the quantity of food imbibed, we measured the weight gain of females after feeding for 2 h. In *A. tabida* there were no differences among sucrose concentrations in the weight gained by females after feeding (Tukey all comparisons  $P > 0.05$ ; Fig. 3A), indicative of similar feeding activity. Only in the water treatment (0% sucrose concentration) did females gain less weight than in the 100% sucrose treatment ( $P = 0.014$ ). Also in *T. drosophilae*, all feeding treatments yielded similar weight gain after 2 h (Tukey all comparisons  $P > 0.05$ ), indicating that there were no differences in food uptake among females in different treatments (Fig. 3B).



**Fig. 2.** Average longevity (days  $\pm$  1SE) of (A) *A. tabida* and (B) *T. drosophilae* when exposed to an intermittent feeding regime with different feeding frequencies of 40% w/v sucrose solution. Different letters represent differences significant at  $\alpha = 0.05$  level.

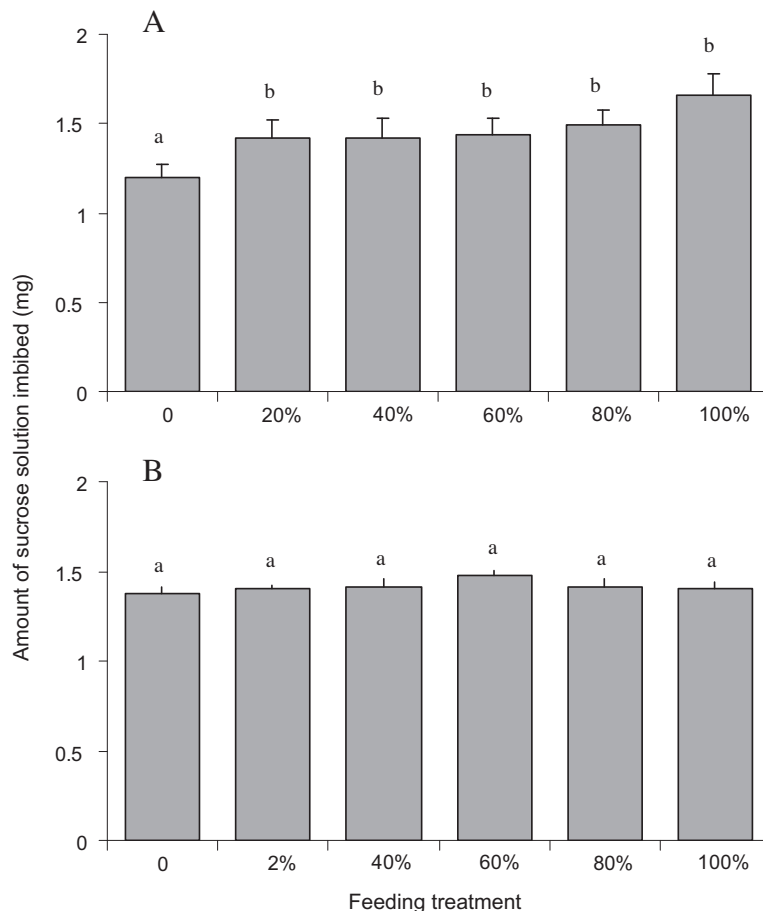
#### 4. Discussion

Dietary restriction has been thought to increase longevity due to reduced calorie intake, but there is uncertainty about the cause of the life-extending effects. Confounding effects include an unsuitable ratio of macronutrients in the richest diets and re-allocation of resources from reproduction to survival under restricted diet (Simpson and Raubenheimer, 2007). The parasitoid species investigated in this study have a simple adult diet consisting of carbohydrates only and lack egg resorption (Eijs et al., 1998), which rules out the proposed alternative explanations for beneficial effects of DR. The dilution treatments we applied showed a clear decrease in longevity for *A. tabida* and *T. drosophilae* at the highest sucrose concentration without any concomitant effect on fecundity. This finding strongly supports the hypothesis that high nutritional content can be detrimental, and that the reduction in energy intake is a key element of the dietary restriction effect (Masoro, 2005).

In contrast, we did not find evidence for a beneficial effect of caloric restriction in the intermittent feeding treatment. The feeding frequency was an important determinant of longevity, especially with frequencies of two feedings per week or more, but no decrease in longevity was detected when sucrose solution was available permanently compared to daily or twice weekly. A possible explanation for the lack of effect of caloric restriction is the relatively low sucrose concentration in the intermittent feeding experiment (40% w/v) compared to the highest concentration in the dilution experiment (100% w/v). Even if a 40% sucrose solution is available permanently it does not lead to a caloric restriction

effect in either of the two species (Fig. 1). Repeating the experiment with higher energetic content in the food could reveal an effect of DR. Alternatively, several studies have demonstrated the capability of organisms to compensate for food-deprived periods by overeating when food is available again (Anson et al., 2003; Cerqueira and Kowaltowski, 2010). Compensatory feeding can produce comparable overall food intake or growth rates compared to continuous feeding (Inness and Metcalfe, 2008), so that no life span extension is to be expected.

One of the problems that have hampered a thorough investigation of the mechanisms underlying DR is the lack of information on how much the animals actually eat (Carvalho et al., 2005). In the current experiment we measured the weight increase of the parasitoids after feeding to obtain an estimate of consumption. The data showed that a constant quantity of the sugar solution was consumed in all dietary dilution treatments; only the water control was consumed to a significantly lesser extent. Therefore, the amount of calories consumed was directly proportional with the concentration of sucrose in the food. The lack of differential intake suggests that the parasitoids are constrained by the volume of their stomach, which may prevent a compensatory feeding strategy in the low concentration treatments. In contrast, in *D. melanogaster*, individuals were found to adjust the volume they ingested to account for differences in the concentrations of nutrients (Carvalho et al., 2005; Lee et al., 2008). If *A. tabida* and *T. drosophilae* have a comparable strategy to *Drosophila*, a decreased intake at higher concentrations would have been expected. More importantly, the similarity in quantity imbibed between the two highest



**Fig. 3.** Average food intake (mg  $\pm$  1 SE) of (A) *A. tabida* and (B) *T. drosophilae* when fed different sucrose concentrations during 2 h. Values represent the gain in wet weights of five pooled individuals. Different letters represent differences significant at  $\alpha = 0.05$  level.

concentrations validates the assumption that the energy intake is higher in the highest sucrose concentrations.

A second confounding factor in the interpretation of many DR experiments is the lack of distinction between reduced energy content and changed nutrient composition (Lee et al., 2008; Grandison et al., 2009). Recent techniques in nutrient geometry have been successfully used to show that carbohydrate: protein ratio is responsible for life span reduction in *D. melanogaster* and other species (Fanson et al., 2009), and that detrimental effects can easily be remediated by addition of specific amino acids, for example (Grandison et al., 2009). An alternative approach to avoid interaction between the effect of nutrient composition and energy content is to choose study organisms with a diet containing only a single component. Carbohydrates are about the only dietary component for non-host feeding parasitoid species (Jervis et al., 2008), as they feed on honeydew, (extra) floral nectary secretions, fruit juice or fruit pulp (Eijs et al., 1998; Sivinski et al., 2006; Hein and Dorn, 2008), in which the proportion of nitrogenous material is very small (Bernstein and Jervis, 2008). The principal sugars present in these food sources are glucose, fructose and sucrose, which have been shown to have the greatest beneficial effects on parasitoid longevity compared to other, less prevalent sugar sources (Wackers, 2001; Hogervorst et al., 2007). Also, variation in the proportion of glucose, fructose and sucrose in the diet does not affect its profitability (Tompkins et al., 2010), despite earlier suggestions (Vattala et al., 2006). The DR effect we found in *A. tabida* and *T. drosophilae* occurred when provided with different concentrations of sucrose solutions, therefore nutrient composition should not be of any influence on our results.

A third mechanism to account for the increased life span under restricted dietary conditions is a reallocation of nutrients from reproduction to somatic maintenance, which is thought to be an adaptive response to aid survival of food shortages in nature (Williams, 1966; Holliday, 1989). Indeed, DR lowers fecundity in several taxa (Klass, 1977; Chapman and Partridge, 1996); for example, in the mosquito *Aedes aegypti* a restricted number of blood meals significantly extended longevity at the cost of producing fewer eggs (Joy et al., 2010). In *A. tabida* and *T. drosophilae* as well as many other parasitoids species, oogenesis and vitellogenesis starts well before female eclosion, so that a major proportion of the eggs is mature at female emergence (Jervis et al., 2001). Due to the early timing of egg development, the only possibility for reallocation of nutrients during adult life is through egg resorption, which is known to occur in periods of nutritive stress in certain species (Asplen and Byrne, 2006; Bodin et al., 2009; Wakefield et al., 2010). However, no evidence of egg resorption was found in any of our dietary treatments as the egg load of females was independent of sugar concentration or feeding frequency. Reallocation of nutrients therefore does not explain the responses to DR found in this study.

The detrimental effects of a high calorie intake put parasitoids in a particularly difficult evolutionary dilemma. Due to the inability to convert carbohydrates into lipids (Visser et al., 2010), a continuous intake of carbohydrates is necessary in order to economize on capital lipid reserves and maintain egg production potential (Ellers and vanAlphen, 1997; Giron and Casas, 2003; Desouhant et al., 2010). On the other hand, the present results prove a high sugar consumption to be disadvantageous for longevity, and suggest selection may exist against overeating. A crucial factor is the quality of sugar sources in the field, both with regard to the type and concentration of sugars as well as the availability of food sources. However, sugar concentrations in fruit pulp or concentrated honeydew may well be high enough to induce unfavourable effects.

Recently, there has been a surge of papers convincingly demonstrating the importance of nutritional balance in the diet in order to obtain maximal life span (Lee et al., 2008; Fanson et al., 2009;

Grandison et al., 2009). Although the present study does not disqualify the relevance of nutrient composition in other species, our data unequivocally support the caloric restriction hypothesis and invalidate alternative explanations. A reduced energetic content is thought to be associated with a reduction in metabolic rate that leads to decreased ROS production (Sohal and Weindruch, 1996; Hunt et al., 2006). A next step in disentangling the mechanisms responsible in life span extension in parasitoids would be to investigate the association between caloric restriction and resting metabolic rate, as has been found in stick insects (Roark and Bjørndal, 2009). In addition, measuring several indicators of oxidative stress under a full and restricted diet could resolve the role of reactive oxygen species in life span (Monaghan et al., 2009).

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