Protein:carbohydrate ratios explain life span patterns found in Queensland fruit fly on diets varying in yeast:sugar ratios

Benjamin G. Fanson · Phillip W. Taylor

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Abstract Dietary restriction extends life span across a vast diversity of taxa, but significant challenges remain in elucidating the underlying mechanisms. Distinguishing between caloric and nutrient effects is an essential step. Recent studies with *Drosophila* and tephritid fruit flies have reported increased life span as dietary yeast-to-sugar ratios decreased and these effects have been attributed to changes in protein-to-carbohydrate (P:C) ratios of the diets rather than calories. However, yeast is a complex mix of macronutrients and micronutrients, and hence changes in yeast content of the diet necessarily alters other nutrients in lockstep. To explicitly test whether studies using yeast are justified in attributing results to diet protein content rather than correlated nutrients, we developed a chemically defined diet allowing manipulation of just the ratio of protein (free amino acids) to carbohydrate (sucrose) levels of diets while holding other nutrients constant. Mated, female Queensland fruit flies (Q-flies) were fed 1 of 18 diets varying in P:C ratios and diet concentration. Diet consumption, egg production, and life span were recorded for each fly. In close concordance with recent studies using yeast diets, flies had increased life span as

P:C ratios decreased, and caloric restriction did not extend life span. Similarly, egg production was maximized on high P:C ratios, but lifetime egg production was maximized on intermediate P:C ratios, indicating a life history trade-off between life span and egg production rate. Finally, Q-flies adjusted their diet intake in response to P:C ratios and diet concentration. Our results substantiate recent claims that P:C ratios significantly modulate life span in flies.

Keywords *Bactrocera tryoni* · Dietary restriction · Geometric framework · Holistic diet · Lifespan · Life history trade-off · Nutrition

Introduction

Dietary restriction (DR) extends life span across a vast diversity of taxa, ranging from unicellular yeast (Lin et al. 2002) to primates (Messaoudi et al. 2006; Colman et al. 2008, 2009), supporting the idea that an evolutionary conserved mechanism may underpin this phenomenon. Substantial research has been devoted to identifying the mechanisms involved, especially in the nematode worm *Caenorhabditis elegans* (Kimura et al. 1997; Lakowski and Hekimi 1998; Dillin et al. 2002), the fruit fly *Drosophila melanogaster* (Chapman and Partridge 1996; Clancy et al. 2001; Mair et al. 2003; Marden et al. 2003), and the mouse *Mus musculus* (Hursting et al. 1994; Bluher et al. 2003; Harrison et al. 2009). Insights into mechanisms underpinning the effects of DR on life

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B. G. Fanson (✉) · P. W. Taylor
Department of Biological Sciences, Macquarie University,
Sydney, NSW 2109, Australia
e-mail: bfanson@gmail.com

span have come from studies drawing on several key techniques including gene mutations (e.g., Kimura et al. 1997; Clancy et al. 2001; Marden et al. 2003), manipulations of reproduction (e.g., Hsin and Kenyon 1999; Cargill et al. 2003; Mair et al. 2004), and sophisticated dietary approaches (e.g. Bateman and Sonleitner 1967; Carey et al. 1998; Mair et al. 2003; Grandison et al. 2009). One such recent advance has been the implementation of a multidimensional dietary approach (a.k.a. ‘Geometric Framework’) to disentangle the effects of caloric and nutrient effects on life span (Simpson and Raubenheimer 2007; Lee et al. 2008; Archer et al. 2009).

Recent studies utilizing the geometric framework with *D. melanogaster*, the field cricket (*Teleogryllus commodus*) and the Queensland fruit fly (Q-fly, *Bactrocera tryoni*), have demonstrated that nutrients, rather than calories, underpin DR-associated life span extension in these species (Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009); for review, see Simpson and Raubenheimer (2009). In these studies, individual animals were fed diets differing in protein: carbohydrate (P:C) ratios and diet concentration. By measuring nutrient intake and by plotting bi-coordinate intakes of protein and carbohydrates, response surfaces for life span in relation to protein and carbohydrate intakes were fitted. These surfaces show a remarkably consistent pattern, across the three insect species studied, of life span increasing as P:C ratios decreased and as caloric intake decreased. These studies, in conjunction with others (Mair et al. 2005; Piper et al. 2005; Ja et al. 2009), demonstrate that nutrients, rather than calories, are responsible for life span extension via DR, at least for *Drosophila*, field cricket, and Q-flies.

Though these studies show that yeast-to-sugar ratios modulate life span, it remains to be conclusively shown that the specific nutrients of protein and carbohydrate are responsible in flies. Lee et al. (2008) and Fanson et al. (2009) used a hydrolyzed yeast–sucrose diet and considered only the protein and carbohydrate components of hydrolyzed yeast in their analyses. However, yeast is rich mixture of micronutrients and macronutrients and the proportion of these nutrients varies in lockstep with the proportion of protein in the diets. Hence, the life span pattern ascribed to protein is potentially due at least in part to other nutrients and/or may be due to toxicity effects of some nutrients at higher yeast levels (Piper and Partridge 2007).

Here, we explicitly test Fanson et al.’s (2009) assertion that protein and carbohydrate are responsible for the negative relationship between dietary yeast: sucrose (Y:S) ratios and life span in Q-flies. To this end, we developed a holidic (chemically defined) diet for Q-fly. Mature and mated-female Q-flies were fed 1 of 18 diets varying in protein (free amino acids) to carbohydrate (sucrose) ratios (P:C 0:1, 1:32, 1:16, 1:8, 1:4, 1:1) and solution concentrations (40, 120, 360 g/l⁻¹). We assessed the effects of diet composition on life span, egg production, and food consumption, testing whether trends attributed to protein by Fanson et al. (2009) persist in the absence of correlation between levels of protein and other nutrients.

Methods

Study animals

Q-flies were sourced as pupae from the Fruit Fly Production Facility at Elizabeth Macarthur Agricultural Institute (EMAI, New South Wales, Australia). This fly stock is maintained on a larval diet of lucerne chaff and torula yeast, and an adult diet of hydrolyzed yeast and sucrose. Within 24 h of emerging, flies were separated by sex into 5-l plastic containers in which one side had been replaced by fiberglass insect mesh. Each cage contained a 70-ml plastic specimen jar of distilled water with a cotton wick inserted through the lid to draw moisture into the cage. Each cage also contained two 55-mm Petri dishes of synthetic agar diet (0.5% agar), one that was sucrose-rich (0:1 P:C) and one that was protein-rich (1:1 P:C). Q-flies self-regulate macronutrient intake, so providing separate protein and sucrose diets facilitates optimal nutrient intake (Fanson et al. 2009). Temperature and humidity were maintained at ~24°C and ~82%, respectively. Under these conditions, Q-flies reach sexual maturity by day 10 (Meats et al. 2004; Perez-Staples et al. 2007).

Experimental protocol

Eleven days after emerging, virgin females were removed from the 5-l containers and were individually mated at dusk with virgin males of the same age. Females were considered mated if they copulated for longer than 5 min. On the following day, 90 mated females were individually transferred to clear poly-

styrene containers (70 ml). Containers had small holes drilled into the bottom and parafilm covering the top. Containers were placed upside down on an ovipositing dish. The ovipositing dish was a plastic weight-boat (35 mm×35 mm) filled with 2.5 ml of 0.7% lemon essence solution (Queen Fine Foods, Alderly, Queensland, Australia) and covered with a single layer of parafilm that had been pierced several times within an entomological pin. Finally, each container had a 200 µl pipette-tip filled with filtered water and two 50 µl microcapillary tubes (Drummond) filled with 35 µl of 1 of the 18 experimental diets. Five replicate flies were set up for each diet.

Containers were arranged evenly across a Plexiglas shelves (see below). We checked mortality daily and replaced ovipositing dishes every 2 days. To assess fly size, we removed and photographed the right wing of each fly postmortem. Using Adobe Photoshop (v11.0.2) software, we measured the distance from the intersection of the anal and median band to the margin of the costal band and the R4+5 vein (Perez-Staples et al. 2007).

Experimental diets

Our experimental diet regime followed that of Lee et al. (2008) and Fanson et al. (2009) with minor modifications. We created 18 diets regimes comprising one of six P:C ratios (0:1, 1:32, 1:16, 1:8, 1:4, 1:1) and one of three diet concentration (40, 120, and 360 g l⁻¹). To facilitate comparisons with the yeast–sucrose diet used by Lee et al. (2008) and Fanson et al. (2009), our diet concentrations matched the estimated amount of protein and carbohydrates that would be present in an analogous yeast–sucrose diet. The hydrolyzed yeast used by Lee et al. (2008) and Fanson et al. (2009) contained 21% indigestible fiber, and diet concentration was based on total yeast and sucrose amounts. Thus, as Y:S ratios increase for a given diet concentration, diets contain more fiber and less total protein+carbohydrate. Therefore, our concentrations of protein and carbohydrate decrease in the exact same pattern (Table S1).

For our diet formulation, we modified a chemically defined diet developed for Mediterranean fruit fly (*Ceratitis capitata*) by Chang et al. (2001). We used sucrose as a source of carbohydrate and free amino acids as the source of protein. All diets also contained cholesterol, Vanderzant vitamin mixture, ribonucleic

acid (RNA), and Wesson salt mixture (Tables S1, S2). Minimal thresholds for micronutrients are not known for Q-flies, and there is no information about relationships between micronutrients requirements and physiological processes such as egg production. Thus, we kept the ratio of macronutrients to micronutrients constant so each additional macronutrient consumed had a constant amount of micronutrients associated with it. All dry ingredients were thoroughly mixed, dissolved in filtered water (50°C), and vigorously mixed. Finally, we added 0.13% (v/v) of blue food dye (Queen Fine Foods) to facilitate measurement of diet consumption from digital images.

Measuring diet consumption

Diet consumption was measured from still pictures captured using an 8 MP Canon IXUS 80IS camera programmed to photograph the containers every 20 min during the light phase (Fig. S1). We corrected for barrel distortion using Adobe Photoshop CS4 (San Jose, CA, USA) and measured remaining diet (distance displaced by dyed diet in the microcapillary tube) using ImageTool (v2.0; UTHSCSA). Correlation between the photograph method and physically measuring each microcapillary tube using digital callipers (the method used by Fanson et al. 2009) was $r=0.99$. Microcapillary tubes were refilled every 4 days or sooner if diets were depleted (checked regularly during the light phase).

We maintained 18 control containers, each with two different experimental diets, to record evaporation rates for each diet-, allowing us to implement corrections to intake estimates. High humidity levels in the room greatly reduced evaporation, but some evaporative loss did occur, especially for the lower concentrations. To correct for evaporation, we measured evaporative loss from the control containers and measured humidity and temperature using Hygrochron dataloggers (iButtons, EDS, USA). We then fitted a regression model using P and C concentrations, temperature and humidity to predict daily evaporative loss (Fig. S2). Next, we corrected for evaporative loss each day by: (1) using the regression model to estimate water loss given current nutrient concentrations, (2) recalculating the diet concentration for this loss, and then (3) using this new concentration to estimate nutrient consumption (Meats and Kelly 2008).

Data analysis

All statistical analyses were conducted in SAS (v9.1). We used the mean daily C and P consumption for the first 7 days on experimental diets (trends remain consistent for shorter or longer time frames). During this time period, nearly all flies were still alive, making mean daily intakes for different diets more comparable. Furthermore, egg production rates decrease with age, and hence the loss of the high-protein groups at earlier ages makes comparisons more difficult with longer time windows. To understand the effects of nutrients on life span and reproduction, we conducted a second-order surface analysis to test the effects of mean daily C and P consumption over this 7-day period on life span, daily egg production rate, and lifetime egg production. For each model, we included the main factors (C and P consumption), their quadratic terms, and interactions between the main effects (Montgomery 2001). Additionally, we included wing length and first-order interactions with P and C, but removed any nonsignificant interactions with wing length in the final models.

Next, we performed a similar surface analysis to describe the level of compensatory feeding in relation to nutrient concentration. We tested the effect of P and C concentrations (g l^{-1}) and wing length on daily volumetric intake of diet (μl) with all second-order interactions. All final models satisfied homoscedasticity and normality assumptions. For all surface analyses, we fitted nonparametric thin plate splines to the raw data using the FIELDS package in R (v2.9.0) to facilitate visualization of the results.

Results and discussion

Validity of holidic diet for Q-fly

We used a modified version of Chang et al. (2001) holidic diet developed for another tephritid, the Mediterranean fruit fly (*C. capitata*). To ensure that this holidic diet was suitable for Q-flies, we compared the life history parameters between our study and other studies using yeast–sugar diets. On the holidic diet, mated Q-flies lived to mean ages of 47.5 and 34.2 when fed a sugar-only and 1:4 P:C diets, respectively. These life spans are 21% longer and 14% shorter than virgin Q-flies fed sugar-only diet

and yeast–sugar with 1:4 P:C (Fanson et al. 2009). Additionally, egg production rates on the 1:4 P:C diet were $27.0 \text{ eggs day}^{-1}$ at the age of 21 days, which is very similar to mated Q-flies on high yeast–sugar diet that had an egg production rate of 26.5 at 21 days (Meats and Leighton 2004). Thus, life history parameters for Q-flies on the holidic diet were in similar ranges as Q-flies fed yeast–sugar diets.

Nutrient-dependent consumption

Many animals actively adjust their dietary intake to meet nutritional targets (Simpson et al. 1989, 2004; Raubenheimer and Simpson 1993; Simpson and Raubenheimer 1993; Lee et al. 2006). As DR studies with insects often require dilution of diets to restrict food consumption, it is important to understand the extent to which intake is adjusted so that accurate estimates of caloric and nutrient intake can be derived (Carvalho et al. 2005). Q-flies adjusted their volumetric intake in response to both diet concentration and P:C ratio, although carbohydrate concentration had a stronger influence (Fig. 1a; Table 1). Diluting the diet sixfold resulted in a nearly threefold volumetric increase in consumption. These results suggest that the intake behavior of Q-flies is strongly influenced by carbohydrate and protein regulation.

Translating the volumetric intake into caloric intake revealed that Q-flies consumed more calories as diet concentration increased (Fig. 1b). Thus, on average, flies were not able to overcome diet dilution. Caloric intakes also revealed that Q-flies consumed more calories on higher P:C diets, which also had the highest egg production rates (Fig. 2b). Similar results were found with virgin Q-flies fed a matrix of yeast–sugar diets in which Q-flies increased intake on diluted diets and along the Y:S ratio that maximized egg production rates (Fanson et al. 2009). In contrast to Fanson et al. (2009) who found that Q-flies greatly restricted intake on the sugar-only diet, intakes on high carbohydrate diets in this study were only slightly reduced from higher P:C diets. This difference between the studies likely reflects differences in the state of flies used. In Fanson et al. (2009), the flies were virgins and were placed on experimental diets from the first day of adulthood, whereas in this study, the flies were on self-regulated diet for the first 11 days, allowing flies to mature reproductively, and were then mated. In Fanson et al. (2009), only those

Fig. 1 Daily liquid intake (μL) (a) and caloric intake (cal) (b) as affected by the nutrient composition of the diet. Surface colors depict intake amounts, ranging from *dark blue* (low intake) to *red* (high intake). Figure axes represent the concentration of carbohydrate and protein in the diet

flies with access to sufficient nutrition would have matured. Presumably because of prior access to a more complete diet, reproductive maturity and mating status, total egg production, was much greater in this study (Fig. 2b) than in the virgin flies studied by Fanson et al. (2009). Overall, Q-flies demonstrate a high degree of plasticity in nutrient intake in response to diet characteristics.

Nutrient and caloric effects on life span and reproduction

DR studies with insects often reduce both the caloric and nutrient composition of the DR diets (Simpson and Raubenheimer 2007). Accordingly, it can be very difficult to disentangle caloric and nutrient effects on life span. By sampling at multiple points in this two-dimensional nutrient space and through the use of a chemically defined diet, we were able to disentangle these effects. Nutrient composition strongly affected

life span (Fig. 2a; Table 2). For a given caloric intake, life span decreased monotonically as P:C ratios increased, that is, as the diet became more protein-rich. Caloric effects depended on the P:C ratio of the diet. At low P:C ratios (sugar-rich diets), increasing caloric intake extended life span, but as the P:C ratio increased, calories had almost no effect on life span (Fig. 2a). We found no evidence that caloric restriction extended life span at either P:C extreme. Overall, life span was optimized along the 1:32 P:C rail.

Our life span findings are remarkably similar to those of Fanson et al. (2009), despite flies in this study differing in receiving self-regulated nutrition for 11 days before they were switched to the experimental diets, being mature, and being mated. Mating results in >20-fold increase in egg production rates in Q-flies (Meats and Leighton 2004). Nevertheless, despite these differences, virgin and mated Q-flies showed similar patterns with higher P:C diets resulting in shorter life span.

Equally interesting is that life span patterns are independent of when the flies received the experimental diets. Q-flies mature sexually during the first 10 days after emergence when provided adequate nutrition. During this period, female Q-flies increase protein consumption, develop reproductive organs, and become sexually receptive (Meats et al. 2004; Meats and Leighton 2004). Because of these changes, we expected life span patterns for the mated flies in this study to differ from those virgin flies found in Fanson et al. (2009). However, the general life span patterns were remarkably similar between these quite different studies. These findings are concordant with other studies showing that the mortality rates for *D. melanogaster* switched from a full diet to a restricted

Table 1 Statistical results for surface fits for the effect of protein, carbohydrate, and wing length on diet intake

	Estimate $\pm$ SE	<i>T</i>	<i>P</i> value
Wing length	3.10 $\pm$ 2.38	1.30	0.1959
Carbohydrate	−0.15 $\pm$ 0.02	−9.42	<.0001
Protein	−0.043 $\pm$ 0.021	−1.98	0.0508
Carbohydrate by carbohydrate	0.00028 $\pm$ 0.00004	7.28	<.0001
Protein by protein	0.00001 $\pm$ 0.00001	0.17	0.8690
Carbohydrate by protein	0.00031 $\pm$ 0.00009	3.68	0.0004

This analysis relates carbohydrate and protein concentrations (g l^{-1}) to daily volumetric intake (μL) of Q-flies. The estimates are the regression coefficients from the general linear model

Fig. 2 Nutrient effects on (a) life span (days posteclosion), (b) lifetime egg production and (c) daily egg rates. Thin-plate splines were fitted to the data to visualize the response surfaces for each fitness parameter, with *bluer* colors indicating low

values and *redder* colors indicating higher values. Figure axes are the daily mean intake for each nutrient. *Dashed grey line* indicates isocaloric line and *dashed red line* shows the optimal P:C ratio for the fitness parameter

diet at various ages decreased in the same rates with flies permanently on a restricted diet (Mair et al. 2003, 2004). These changes in *D. melanogaster* mortality rates indicated that full diets increase acute risk of mortality, but do not increase aging-related damage (Partridge et al. 2005). Consequently, increasing P:C ratios of the diet may increase reversible mortality risk without causing irreversible damage.

Whereas life span was maximized at 1:32 P:C, egg production rate was maximized at the much more protein-rich 1:1 P:C nutritional rail (Fig. 2c; Table 2). Egg production rate was mainly influenced by protein consumption, although carbohydrate consumption also had a significant effect (Table 2). Increased

caloric intake along a constant P:C rail was associated with increased egg production rates. Because egg production rate and life span were optimized at different P:C rails, lifetime egg production was maximized at an intermediate rail at 1:4 P:C (Fig. 2b; Table 2). Similar patterns of nutrient-mediated trade-offs in maximal life span and egg production rates have been found previously in *D. melanogaster*, crickets, and virgin Q-flies (Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009).

Comparing life span and egg production rate surface plots revealed that Q-flies may experience a life history trade-off between reproduction and life span that is mediated by P:C ratios but not caloric intake. Along the

Table 2 Statistical results for effects of nutrient intakes (daily mean intake) and wing length on life span, lifetime egg production, and egg rate

	Life span		Lifetime egg		Egg rate	
	Estimate±SE	P value	Estimate±SE	P value	Estimate±SE	P value
Wing length	−8.07±26.77	0.76	414.1±345.9	0.23	4.49±4.13	0.28
Carbohydrate	29.36±10.41	0.006	483.2±137.2	<0.001	5.57±1.64	0.001
Protein	−81.5±32.99	0.015	1056±436.8	0.017	31.54±5.22	<0.001
Carbohydrate by carbohydrate	−17.9±17.46	0.30	−442±227.7	0.05	−6.08±2.72	0.028
Protein by protein	76.92±57.79	0.18	−2072±818.1	0.013	−42.0±9.78	<0.001
Carbohydrate by protein	−89.3±63.50	0.16	1277±822.3	0.12	24.52±9.83	0.014

General linear models were fitted separately for each parameter

isocaloric line, life span decreased and egg production rates increased monotonically as P:C ratios increased (Fig. 2a, c). This pattern suggests a cost of reproduction whereby increased egg production rates have a detrimental effect on life span (Williams 1966; Kirkwood and Holliday 1979). In contrast, we did not find evidence of a life span reproduction trade-off along a P:C rail, as life span remained constant or increased (Fig. 1a, c); however, a trade-off could be masked by increased caloric intake. If Q-flies do experience a cost of reproduction, then on the protein-rich diets, they may have allocated a constant caloric amount to life span across the diet dilutions and then allocated the additional calories on the richer diets to reproduction and/or repairing additional reproduction-inflicted damage. Only by holding caloric intake constant was the trade-off between reproduction and life span revealed.

Conclusions

Disentangling the effects of nutrients and calories has been an essential step toward understanding how DR extends life span. Evidence is accumulating in support of the conclusion that, at least in insects, effects of DR on life span are underpinned by nutrients rather than calories (Mair et al. 2005; Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009). To understand the physiological processes involved, we need to unambiguously identify which nutrients are responsible for life span modulation. Using a chemically defined diet, our results substantiate the claims of Fanson et al. (2009) that proportions of protein and carbohydrate are responsible for diet-related life span extension in Q-flies. Life span was extended as P:C ratio decreased. As caloric intake increased, life span increased or remained constant; caloric restriction did not extend life span. Our results contribute to the realization that, for at least some insects, the phenomenon of DR-associated life span extension reflects changing nutritional composition of diets and not restriction of dietary intake per se.

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