

RESEARCH ARTICLE

Dietary composition regulates *Drosophila* mobility and cardiac physiology

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SUMMARY

The impact of dietary composition on exercise capacity is a subject of intense study in both humans and model organisms. Interactions between diet and genetics are a crucial component of optimized dietary design. However, the genetic factors governing exercise response are still not well understood. The recent development of invertebrate models for endurance exercise is likely to facilitate study designs examining the conserved interactions between diet, exercise and genetics. As a first step, we used the *Drosophila* model to describe the effects of varying dietary composition on several physiological indices, including fatigue tolerance and climbing speed, cardiac performance, lipid storage and autophagy. We found that flies of two divergent genetic backgrounds optimize endurance and cardiac performance on relatively balanced low calorie diets. When flies are provided with unbalanced diets, diets higher in sugar than in yeast facilitate greater endurance at the expense of cardiac performance. Importantly, we found that dietary composition has a profound effect on various physiological indices, whereas total caloric intake *per se* has very little predictive value for performance. We also found that the effects of diet on endurance are completely reversible within 48 h if flies are switched to a different diet.

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Key words: *Drosophila*, diet, exercise.

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INTRODUCTION

Endurance exercise is known to produce conserved physiological effects in humans, mice and flies. These effects include post-training improvements to fatigue tolerance (Matoba and Gollnick, 1984; Booth and Thomason, 1991; Tinkerhess et al., 2012a), speed (Chandler and Hadley, 1996; Fitts and Widrick, 1996; Piazza et al., 2009) and cardiac performance (Piazza et al., 2009; Kemi and Wisloff, 2010; El-Haddad et al., 2011). In mice and humans, endurance exercise is known to improve glucose sensitivity (Henriksson, 1995; Hayashi et al., 1997), while in mice and flies, endurance exercise is known to increase autophagy (He et al., 2012; Sujkowski et al., 2012). In all species tested, endurance exercise increases mitochondrial biogenesis (Freyssenet et al., 1996; Irrcher et al., 2003; Tinkerhess et al., 2012b). Because of these conserved changes, endurance exercise is thought to be a potential low-cost intervention that may provide increased healthspan to the human population by alleviating pathological states related to obesity and glucose insensitivity (Hopps and Caimi, 2011; Chudyk and Petrella, 2011).

However, the effects of endurance exercise have generally been observed to vary substantially among individuals, with between 8% and 13% of subjects exhibiting adverse reactions to endurance programs (Bouchard et al., 2012). Two major potential reasons for this variance are genetic background and dietary composition. The development of an endurance training model in flies makes the fly system a useful model in which to gain insight into the role of these two factors in modulating the effects of endurance training. In this study, we focused on the effect of macronutrient intake on several parameters of behavior and physiology. Laboratory flies are provided

with a diet containing all three of the primary macronutrient groups thought to be important in human energy balance: carbohydrate, protein and fat (Hall et al., 2012). In the fly diet used here, protein and lipid were provided by the inclusion of brewer's yeast, while sucrose was provided as a source of carbohydrate.

Flies have previously been used as an effective model for the role of diet in the regulation of several important physiological parameters, including sleep (Catterson et al., 2011; Linford et al., 2012), baseline locomotion (Bhandari et al., 2007; Parashar and Rogina, 2009), fertility (Skorupa et al., 2008; Lushchak et al., 2012) and feeding behaviors (Mair et al., 2005; Skorupa et al., 2008). Multiple groups have taken advantage of the relative simplicity of the fly diet and the ability to quickly generate large numbers of genetically identical subjects in order to test the effects of diet on complex, additive phenotypes such as longevity. Historically, reduction of total caloric intake (calorie restriction) has been associated with increased longevity in flies (Partridge et al., 2005; Grandison et al., 2009) and other organisms (Bishop and Guarente, 2007; Mercken et al., 2012).

More recently, the reduction of specific dietary components has been demonstrated to have equally potent effects in extending longevity in flies (Skorupa et al., 2008; Lushchak et al., 2012) and mice (Bartke et al., 2004; Miller et al., 2005; Sun et al., 2009). Use of the two-component diet has facilitated approaches in which a matrix of diets varying the two components has been employed to study the effect of balance between the two in flies (Lee et al., 2008; Skorupa et al., 2008; Lushchak et al., 2012). Generally, these findings are in agreement that caloric balance is more important for a healthy lifespan than total caloric intake, and that these effects

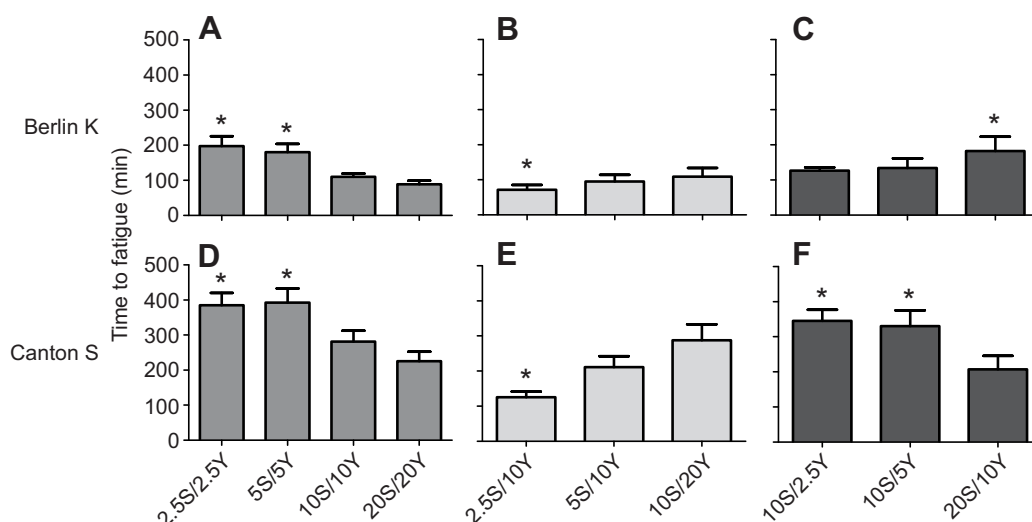


Fig. 1. Dietary composition alters endurance in 3 day old wild-type flies. Following eclosion, male age-matched Berlin K and Canton S flies were fed one of 10 diets for the remainder of their life. The average time to exhaustion for each cohort is presented as a histogram. Data are presented as means and s.e.m. Berlin K (A–C) and Canton S (D–F) flies were tested for endurance on diets with an equal (A,D), low (B,E) or high (C,F) sucrose/yeast (S/Y) ratio. Canton S flies have greater endurance than Berlin K flies, independent of diet (log-rank: $P < 0.0001$). Regardless of genotype, flies fed balanced low calorie diets run significantly longer than siblings fed balanced high calorie diets (log-rank analysis: asterisk indicates significant difference of $P < 0.05$ compared with 10S/10Y). Among unbalanced diets, flies eating food with a high sucrose/yeast ratio have higher endurance than those consuming diets with a low sucrose/yeast ratio (log-rank analysis: asterisk indicates $P < 0.05$ for all significant differences). Average fatigue time and full statistical analyses for each diet are presented in supplementary material Table S1. Data are also presented in 'runspan' form in supplementary material Fig. S1.

are at least partially mediated by alterations in feeding behavior and triglyceride storage (Lee et al., 2008; Skorupa et al., 2008). The effects of dietary composition on endurance capacity and other parameters related to vigor have not been tested in the fly model.

Here, we exposed flies of two different genetic backgrounds to diets of varying concentrations of sucrose and yeast. The impact of dietary composition on induced negative geotaxis speed, fatigue tolerance, cardiac stress resistance and feeding behavior is presented. In addition, cardiac muscle was used as a model to detect trends in lipid accumulation and autophagy levels during aging.

We found a general trend toward protective effects of balanced, low-calorie diets on most parameters, while, within unbalanced diets, diets high in sucrose promote high endurance and speed, but impair cardiac performance.

MATERIALS AND METHODS

Fly stocks, diets and husbandry

Drosophila melanogaster (Canton S and Berlin K) larvae were reared on a standard laboratory diet containing 10% sucrose, 10% brewer's yeast and 2% agar (10S/10Y). Male flies age matched within 48 h were collected under light CO₂ anesthesia, allowed to recover for 24 h in vials containing standard food, and then transferred to vials containing one of 10 experimental treatment diets at a density of 20 flies per vial. The experimental diets were created by manipulating the nutrient quantities from our standard laboratory 10S/10Y diet. Sucrose and yeast have been estimated to contain roughly the same number of calories/gram (Mair et al., 2005). Diets maintained an equal ratio of sucrose and yeast while altering total calorie content, maintained sucrose content at 10% while varying the amount of yeast, or maintained yeast content at 10% while varying the amount of sucrose. Unbalanced diets that had a higher concentration of sucrose were classified as having a 'high sucrose/yeast ratio', while those with a higher concentration of yeast were referred to as 'low sucrose/yeast ratio' diets. Throughout the

experimental time course, flies were kept at 25°C and 50% humidity with a 12 h:12 h light/dark cycle. Vials of fresh food were provided every 2 or 3 days for the entire experimental duration.

Negative geotaxis assay

Fly climbing speed was assessed using the rapid iterative negative geotaxis (RING) technique described previously (Gargano et al., 2005), with a starting sample size of approximately $N=100$ flies. The RING assay measures the average height climbed by a cohort of flies in a given time period following induction of the negative geotaxis behavior. Flies from each diet treatment were subjected to the RING assay five times per week for a 5 week time course. For each cohort, flies were transferred to polypropylene vials in groups of 20 per vial. The vials were quickly tapped four times to knock flies to the bottom and induce the negative geotaxis response; a photograph was taken after flies had been allowed to climb for 2 s. This procedure was carried out four consecutive times. The average distance climbed by flies in a given picture was calculated by taking the mean of the vials in that picture, and the daily average climbing distance was calculated by averaging the four picture means together. The images were analyzed using image processing software and the raw data converted into quadrants using Microsoft Excel. Each of the four vial quadrants is equivalent in height, and each fly was assigned a score based on the quadrant ascended to after 2 s. Flies that climbed to the highest quadrant received a score of 4, flies in the next highest quadrant received a score of 3, and so on. Flies that did not climb off the bottom of the vial were assigned a quadrant score of 0. The calculated daily quadrant averages were charted to assess the decline in climbing ability with age. Negative geotaxis results were analyzed using either one-way ANOVA analysis with *post hoc* Bonferroni multiple comparison tests or multivariate regression (supplementary material Table S1) in JMP (SAS Institute Inc., Cary, NC, USA). Genotype comparisons were also performed using standard least-squares regression.

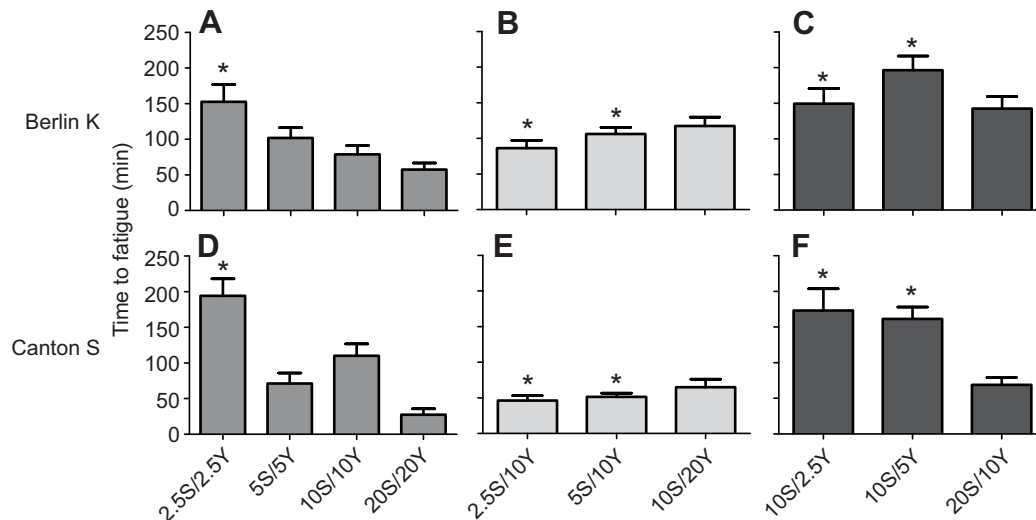


Fig. 2. Effect of diet on endurance persists during aging. Experimental flies from Fig. 1 were aged to 26 days (3 weeks) and then remeasured. The average time to exhaustion for each cohort is presented as a histogram (A,D equal S/Y; B,E, low S/Y; C,F, high S/Y ratio). Data are presented as means and s.e.m. Twenty-six day old Berlin K (A–C) and Canton S (D–F) flies exhibit the same trends as when they were 3 days old. Flies from all cohorts declined with age. When compared with Canton S, Berlin K flies have more endurance at advanced ages (log-rank: $P=0.0123$). Independent of genotype, flies fed balanced low calorie diets continue to run longer than those fed balanced high calorie diets (log-rank analysis: asterisk indicates $P<0.05$ compared with 10S/10Y). When unbalanced diets were used, flies eating food with a high sucrose/yeast ratio continue to resist fatigue better than flies eating low sucrose/yeast ratio food (log-rank analysis: asterisk indicates $P<0.05$ for all significant differences). Average fatigue time and full statistical analyses for each diet are presented in supplementary material Table S1. Data are also presented in 'runspan' form in supplementary material Fig. S2.

Endurance assay

Climbing endurance was measured using the fatigue assay described previously (Tinkerhess et al., 2012a). Eight vials of flies from each diet treatment were subjected to the fatigue assay at two time points: once in the first week of life (day 3), and once in the fourth week of life (day 26). For each assessment, the flies were placed on the Power Tower exercise machine (see Results) and made to climb until they were fatigued, or no longer responded to the negative geotaxis stimulus. Monitored at 10 min intervals, a vial of flies was visually determined to be fatigued when five or fewer flies could climb higher than 2 in (50.8 mm) after four consecutive drops. The time from the start of the assay to the time of fatigue was recorded for each vial, and the data analyzed using log-rank analysis in JMP; genotypes were also compared by log-rank analysis.

Electrical pacing

Flies were subjected to electrical pacing, as described elsewhere (Wessells et al., 2004), in order to assess heart performance in the fourth week of life. Flies were immobilized and placed between two electrodes lined with conductive electrolyte jelly. The hearts were paced using a square-wave stimulator set at 40 V and 6 Hz for 30 s, and then visually assessed for recovery or failure, defined as either fibrillation or arrest. The percentage of fly hearts that failed was recorded immediately after pacing. Sample sizes ranged from $N=50$ hearts for Berlin K cohorts, to approximately $N=100$ hearts for Canton S groups (for exact N values, see supplementary material Table S1). Results were analyzed *via* standard least-squares regression using JMP. Genotype comparisons were also performed using standard least-squares regression.

Oil Red O staining

Four week old flies were dissected ventral side up in phosphate-buffered saline (PBS) at room temperature. After exposing the heart

and removing any excess fat, partially dissected flies were rinsed three times with PBS and then fixed in 4% paraformaldehyde/PBS for 10 min. Following fixation, dissected samples were rinsed three times with PBS. Oil Red O (Sigma-Aldrich, St Louis, MO, USA), diluted 1:100 in isopropanol, was applied to the exposed hearts for 20 min at room temperature. After staining, hearts were rinsed three times with dH_2O , removed, and mounted on slides in one drop of antifade solution (Molecular Probes, Eugene, OR, USA). Slides were imaged on an Olympus BX41 compound fluorescence microscope (Olympus, Center Valley, PA, USA) using a 40 \times objective and the obtained images analyzed using ImageJ. A minimum of five samples were analyzed per diet treatment. Data were analyzed by one-way ANOVA, with *post hoc* Tukey–Kramer tests using Prism (GraphPad Software, San Diego, CA, USA). Genotypes were compared using two-way ANOVA.

LysoTracker staining

Four week old flies were dissected as for Oil Red O staining with the following changes: following dissection in PBS, hearts and fat bodies were rinsed three times with PBS and then immediately stained with LysoTracker Green (Molecular Probes), diluted to $0.01 \mu\text{mol l}^{-1}$ in PBS, for 1 min. Samples were washed three times with PBS and then removed and mounted as above. Imaging and data analysis were performed exactly as described in the Oil Red O protocol.

Feeding rate

Ten day old flies were lightly anesthetized with CO_2 and separated into vials of five flies, 24 h before the start of the experiment. Vials were placed in a randomized order for unbiased scoring, and allowed to acclimatize for 30 min following transfer to avoid disturbance of the fly feeding behavior. Each vial was observed for ~ 3 s and the number of flies feeding was recorded. Proboscis extension into the food accompanied by a bobbing motion was scored as a feeding

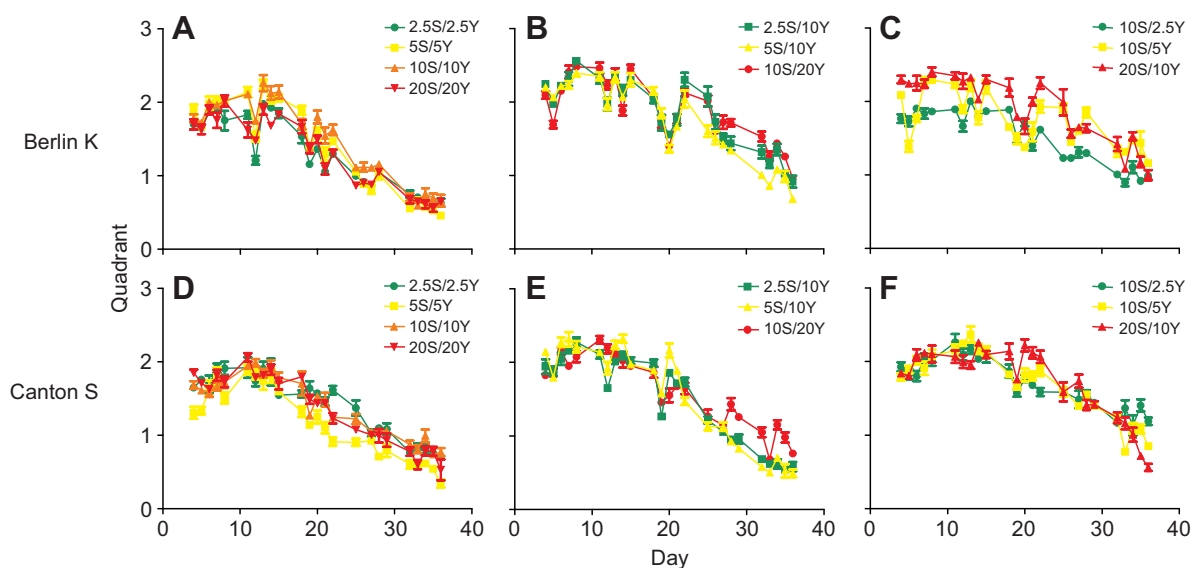


Fig. 3. Dietary composition makes small, but significant, changes in climbing speed. Age-matched Berlin K and Canton S males were fed one of 10 diets for their entire life (A,D, equal S/Y; B,E, low S/Y; C,F, high S/Y ratio). For each diet, the mean climbing height is expressed as the average quadrant that the flies were able to climb to in 2 s (climbing index). Climbing indexes are plotted over a 5 week time course. Berlin K (A–C) and Canton S (D–F) flies fed unbalanced diets climb slightly faster than those fed balanced diets (one-way ANOVA with *post hoc* Bonferroni multiple comparison tests: $P < 0.01$). Between genotypes, Berlin K flies climb significantly faster than Canton S flies (standard least-squares regression, $P < 0.0001$). Full statistical analyses for each diet are presented in supplementary material Table S1.

event (see Wong et al., 2009). Ten trials were performed, such that each vial was observed approximately every 2–5 min. The assay was repeated at the same time the following day. The feeding data were expressed as the sum of feeding events divided by the sum of feeding opportunities (number of flies in vial \times number of vials in group \times number of observations) for each individual day. In order to control for differences in bite size between the genotypes, a blue dye feeding rate experiment was also performed. Groups of five flies were transferred to fresh food containing 2.5% (w/v) blue food dye (FD&C Blue no. 1, Spectrum Chemical Manufacturing Corp., Gardena, CA, USA); a control group was transferred to fresh food containing 10S/10Y to account for absorbance by standard diet food. Vials were observed according to the proboscis extension assay procedure and then transferred to 0.5 ml centrifuge tubes and flash-frozen in liquid nitrogen. Flies were homogenized in 250 μ l of distilled water and centrifuged according to a procedure outlined previously (Vijendravarma et al., 2012). The relative amount of dye ingested was quantified at 630 nm with background optical density subtracted. As bite sizes were not significantly different, proboscis extension results were analyzed by one-way ANOVA, with *post hoc* Tukey–Kramer tests using Prism. Genotypes were compared using two-way ANOVA.

Relative caloric intake was calculated by multiplying the caloric content of the food by the number of observed proboscis extensions and the amount of dye ingested per proboscis extension (referred to as ‘bite’ size). Multinomial regression was used to estimate the percentage of variance that can be explained by relative caloric intake.

RESULTS

Experimental design

Flies from two different genetic backgrounds, Berlin K and Canton S, were collected, age matched, allowed to recover for 24 h, then placed on one of 10 different diets. These two genotypes were chosen because they represent commonly used laboratory wild-type stocks with divergent backgrounds.

The diets chosen were selected to address two distinct issues: (1) the role of caloric amount in the impact of diet, and (2) the role of the distribution and composition of calories in the impact of diet. The diets chosen can be grouped into three categories: (1) sucrose and yeast present in equal amounts, (2) high sucrose/yeast ratio and (3) low sucrose/yeast ratio.

Fatigue tolerance

Flies from each diet/genotype combination were aged to 3 days and then tested for fatigue tolerance. Flies were divided into groups of 20, then placed on an automated negative geotaxis-inducing machine, known as the Power Tower (Piazza et al., 2009; Tinkerhess et al., 2012a) until such time as fewer than five flies per vial were able to climb above a 2 in (50.8 mm) mark on the machine. At this time, the vial was considered fatigued, and removed from the machine. Fig. 1 plots the average time of removal of vials from each cohort as a histogram.

There was a general trend for Canton S flies to run longer before fatiguing than Berlin K flies, regardless of diet (compare Fig. 1D–F and Fig. 1A–C). The reasons for higher fatigue tolerance in this background are as yet unclear. However, the effect of diet on fatigue tolerance within each genotype was remarkably similar. In both backgrounds, balanced low-calorie diets promoted increased fatigue tolerance (Fig. 1A,D). In both backgrounds, diets with a high sucrose/yeast ratio promoted higher fatigue tolerance (Fig. 1C,F) than did diets with a low sucrose/yeast ratio (Fig. 1B,E). Within diets with unbalanced calorie sources, diets with higher total caloric content trended toward higher fatigue tolerance (Fig. 1B,C,E,F).

In order to find out whether long-term effects of dietary intake were similar to acute effects, we kept all cohorts of flies on the same diet for 3 weeks and then measured fatigue tolerance again. Three weeks of age is old enough for wild-type flies to display a significant age-related decline in climbing speed (Gargano et al., 2005; Piazza et al., 2009) and in fatigue tolerance (Tinkerhess et al., 2012a). As dietary intake has been proposed to have profound

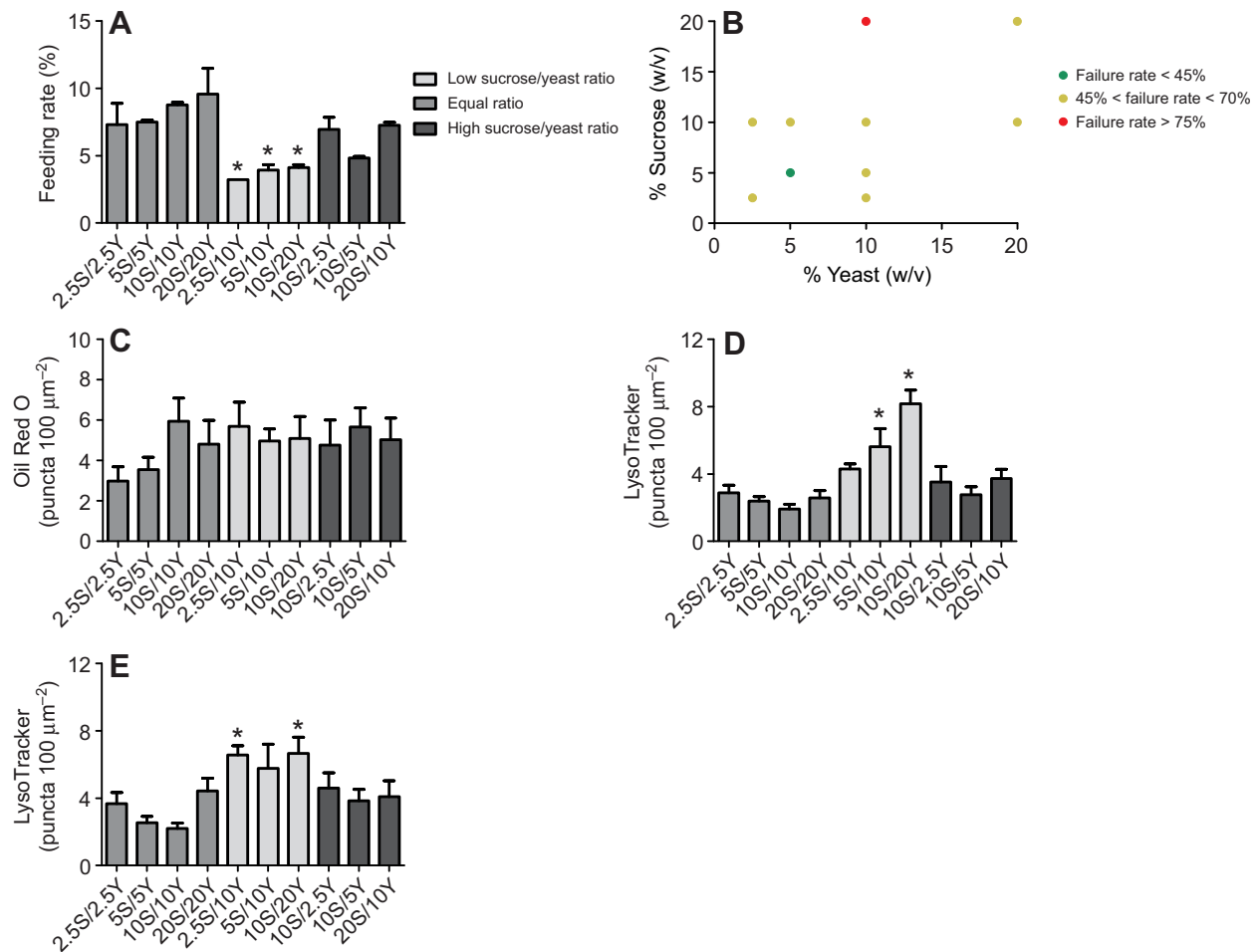


Fig. 4. Dietary composition alters feeding rate and cardiac function in male Berlin K flies. Data are presented as means and s.e.m. (A) Diets with low sucrose/yeast ratio lower feeding rate compared with other diets (one-way ANOVA: $P=0.0037$). Key also relates to C–E. (B) Percentage of flies undergoing cardiac fibrillation or arrest in response to temporary external electrical stress. Flies on the 5S/5Y diet had the lowest failure rate while flies on the 20S/10Y diet had the highest. *Full diet-by-diet comparisons are presented in supplementary material Table S1. (C) Cardiac lipid levels, as detected via Oil Red O staining, are not significantly affected by dietary make-up. (D) Flies fed low sucrose/yeast ratio food have higher cardiac LysoTracker Green staining than those fed other diets (one-way ANOVA: $P<0.0001$). (E) LysoTracker Green staining of adipose tissue also reveals higher staining in flies fed low sucrose/yeast ratio food (one-way ANOVA: $P=0.0017$). *Full statistical analyses for each assay are presented in supplementary material Table S1.

effects on the rate of normal aging (Partridge et al., 2005; Simpson and Raubenheimer, 2009), we hypothesized that diet would alter the rate of decline in fatigue tolerance. We further hypothesized that the best diet for acute fatigue tolerance might be different from the best diet for long-term maintenance of function.

We found instead that the pattern of fatigue tolerance across diets was similar at 3 weeks to the findings at 1 week of age (Figs 1, 2). Flies of both genotypes and across all diets showed a substantial decline in fatigue tolerance between 1 and 3 weeks (Figs 1, 2). Cohorts on low-calorie balanced diets again ran the longest (Fig. 2A,D), although the 2.5S/2.5Y diet was more protective than the 5S/5Y diet in both genotypes (Fig. 2A,D). Among flies on unbalanced diets, those on high sucrose, low yeast diets again performed better than those on low sucrose, high yeast diets (Fig. 2B,C,E,F). An unexpected genotype effect was observed when comparing 1 and 3 week measurements. Although Canton S flies were clearly stronger runners at 1 week across all diets than Berlin K flies, this advantage disappeared by 3 weeks of age (Figs 1, 2). This suggests that Berlin K flies have a slower rate of age-related decline in this parameter than Canton S flies. Statistical comparisons

for every pairwise combination of cohorts are provided in supplementary material Table S1.

Negative geotaxis

Negative geotaxis, a measure of how rapidly flies instinctively respond to a stimulus by moving up the sides of a vial, is a standard measure of fly vigor and mobility (Jones and Grotewiel, 2011). Negative geotaxis speed is known to decline with age (Gargano et al., 2005) and increase with exercise training (Piazza et al., 2009; Tinkerhess et al., 2012a). Here, we performed longitudinal measurements five times per week to track the changing negative geotaxis performance of aging flies on various diets.

In general, Berlin K flies were found to climb faster than Canton S flies, independently of diet (Fig. 3). The two genotypes showed similar trends in dietary responses, although the magnitude varied between genotypes. Within balanced diets, negative geotaxis tended to be slow, in comparison to unbalanced diets (Fig. 3; supplementary material Table S1). Balanced diets also showed only minor differences in the effect on climbing in comparison to each other (Fig. 3A,D). There was little difference between the effects of the

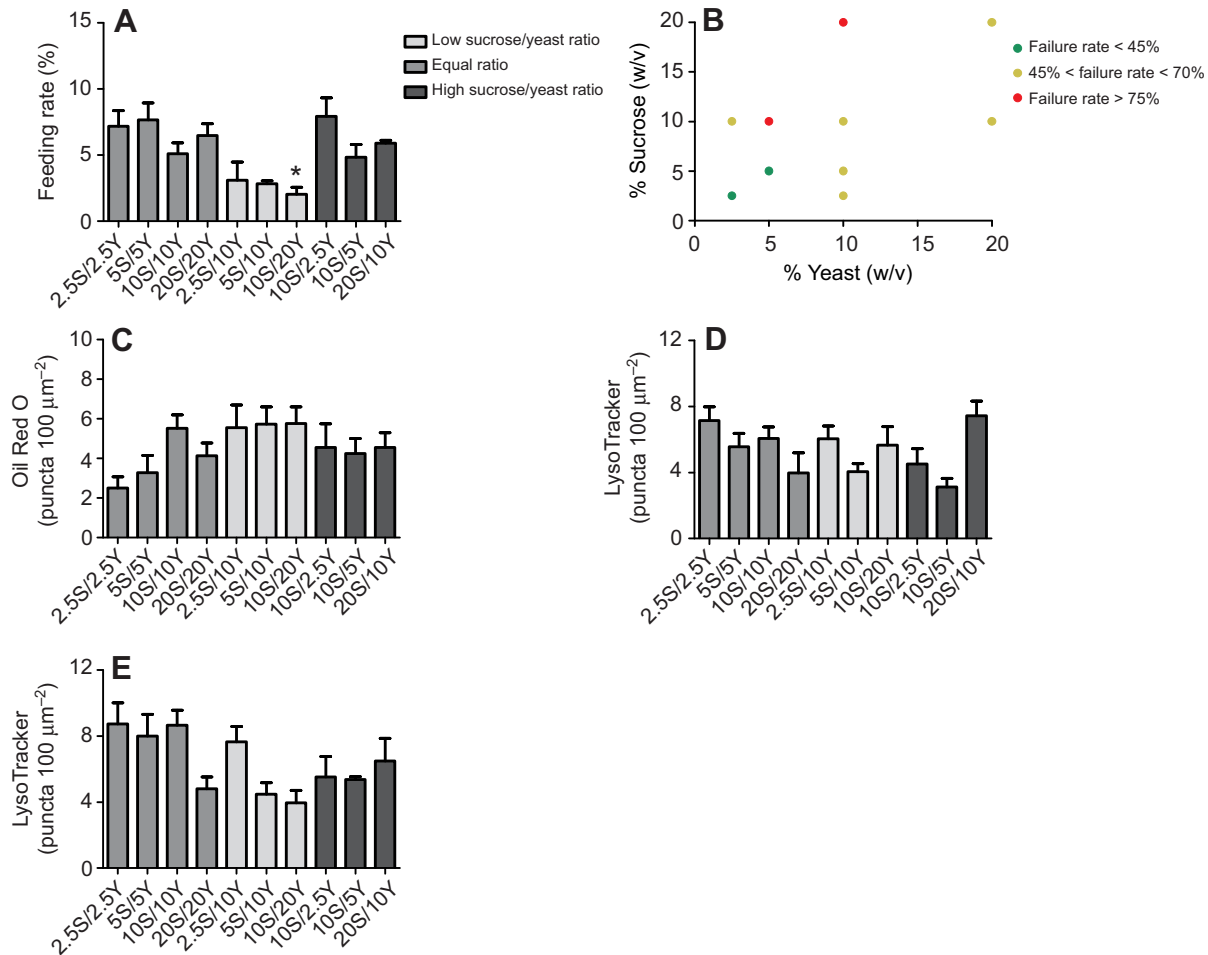


Fig. 5. Dietary composition alters feeding rate and cardiac function of male Canton S flies. Data are presented as means and s.e.m. (A) Diets with low sucrose/yeast ratio lower feeding rate compared with other diets (one-way ANOVA: $P=0.0129$). Key also relates to C–E. (B) Percentage of flies undergoing cardiac fibrillation or arrest in response to temporary external electrical stress. Flies fed balanced low calorie diets have a lower pacing-induced failure rate than those on any other diet (standard least-squares regression: $P<0.02$ for both). Flies fed high sucrose/yeast ratio food display an elevated pacing-induced failure rate when compared with those on other diets (standard least-squares regression: $P<0.05$ in all cases). (C) Cardiac lipid levels as detected by Oil Red O staining are not significantly affected by dietary composition. (D) LysoTracker Green staining for cardiac autophagy does not reveal a clear dietary trend in Canton S flies. (E) LysoTracker Green staining for adipose tissue autophagy does not reveal a clear dietary trend in Canton S males. *Full statistical analyses for each assay are presented in supplementary material Table S1.

different low sucrose/yeast diets at early ages. Differences began to become evident after 20 days, when flies on the 10S/20Y diet retained a significantly higher negative geotaxis capacity in both genotypes (Fig. 3B,E).

The largest differences were observed in Berlin K flies on high sucrose/yeast diets. These flies exhibited substantial differences at early ages, and these differences were retained across the period of observation. Within this group, the rank order of climbing speed was the same as the rank order of total caloric content (Fig. 3C). This effect was not as evident in the Canton S background (Fig. 3F), and the effect of relative caloric content was not significant when normalized to feeding rate. Relative caloric intake for each diet/genotype cohort is provided in supplementary material Table S2. Statistical comparisons for every pairwise combination of cohorts are provided in supplementary material Table S1.

Feeding rate

As dietary composition has previously been reported to influence feeding rate (Lee et al., 2008; Skorupa et al., 2008), we measured

feeding rate in both genotypes after 1 week of exposure to each diet. We found that the two genotypes displayed a similar pattern of response (Fig. 4A, Fig. 5A). Diets with equal ratios of sucrose and yeast stimulated similar feeding rates to those with high sucrose/yeast ratio (Fig. 4A, Fig. 5A). Berlin K flies showed a mild tendency toward increased feeding rate with increased total caloric content (Fig. 4A), but this tendency was not observed in Canton S flies (Fig. 5A). In both genotypes, diets with a low sucrose/yeast ratio significantly reduced feeding rate regardless of total caloric content (Fig. 4A, Fig. 5A). This is in agreement with the previously published observation that sugar/yeast ratio is a key determinant of feeding rate (Lee et al., 2008; Skorupa et al., 2008).

Cardiac stress resistance

Between 50 and 100 male flies of each genotype were placed on each diet. At 3 weeks of age, flies were connected to an external pacing device and stimulated to twice their normal heart rate for 30 s. Following pacing, the percentage of flies that exhibited a fibrillation or arrest event was scored visually and recorded as failure

rate. This parameter has been previously reported to be age dependent (Wessells et al., 2004), and to be susceptible to the effects of extreme diets (Birse et al., 2010). Across a range of different dietary concentrations, we found no significant difference in effect between most diets and our typical lab diet (10S/10Y). Furthermore, both genotypes exhibited failure rates similar to published wild-type results from this age (Wessells et al., 2004; Wessells et al., 2009). However, the 20S/10Y diet produced a significantly increased cardiac failure rate in response to pacing in both genotypes (Fig. 4B, Fig. 5B). By contrast, balanced low calorie diets significantly reduced failure rates, with 5S/5Y being significantly reduced in both genotypes, and 2.5S/2.5Y being significantly reduced only in Canton S flies (Fig. 4B, Fig. 5B). Statistical comparisons for every pairwise combination of cohorts are provided in supplementary material Table S1.

Cardiac lipid storage

Hearts from each diet/genotype combination were dissected and stained with Oil Red O to assess levels of lipid accumulation in the myocardium. High myocardial lipid content has previously been associated with impaired cardiac performance in *Drosophila* (Birse et al., 2010; Sujkowski et al., 2012). Here, we found no significant difference in effect between most diets tested (Fig. 4C, Fig. 5C). However, flies on balanced, low-calorie diets showed lower myocardial lipid levels than flies on all other diets in both genotypes (Fig. 4C, Fig. 5C). Notably, these are the same diets that promote lower cardiac failure rate in response to stress (Fig. 4B, Fig. 5B).

Cardiac autophagy

Hearts from each diet/genotype combination were dissected and stained with LysoTracker, in order to assess levels of autophagy in the myocardium. For comparison, fat bodies were also dissected and subjected to the same analysis. Significant genotype differences were observed in this assay. In Berlin K, a significant trend towards higher autophagy in both tissues was observed for all diets with a low sucrose/yeast ratio (Fig. 4D,E). By contrast, in Canton S, all diets produced similar levels of autophagy, with no obvious trends emerging (Fig. 5D,E).

Diet switch

Many effects of diet on animal physiology are acute and can be reversed by changing the animal's diet. In flies, for example, the effect of diet on age-specific mortality is known to be reversible (Piper et al., 2005). Because dietary intake has a substantial effect on fly endurance (Figs 1, 2), we asked whether this effect was reversible.

Four age-matched cohorts of flies were collected and placed on one of two diets, either 2.5S/10Y or 10S/2.5Y. These diets were chosen because they induce highly significant differences in fatigue tolerance in both Berlin K and Canton S flies. After 5 days, all four cohorts were tested for fatigue tolerance. In both genotypes, significant differences were observed, with flies on 10S/2.5Y performing better than flies on 2.5S/10Y (Fig. 6). These results are in agreement with those in Fig. 1.

Flies from two of the four cohorts were then switched onto the opposite diet, allowed to habituate for 2 days, and retested for fatigue tolerance. In each case, flies exhibited fatigue tolerance characteristic of their short-term, current diet, and never retained fatigue tolerance associated with their previous diet (Fig. 6). This was equally true of both Berlin K and Canton S flies. We conclude that the effects of diet on endurance are completely reversible within 48 h.

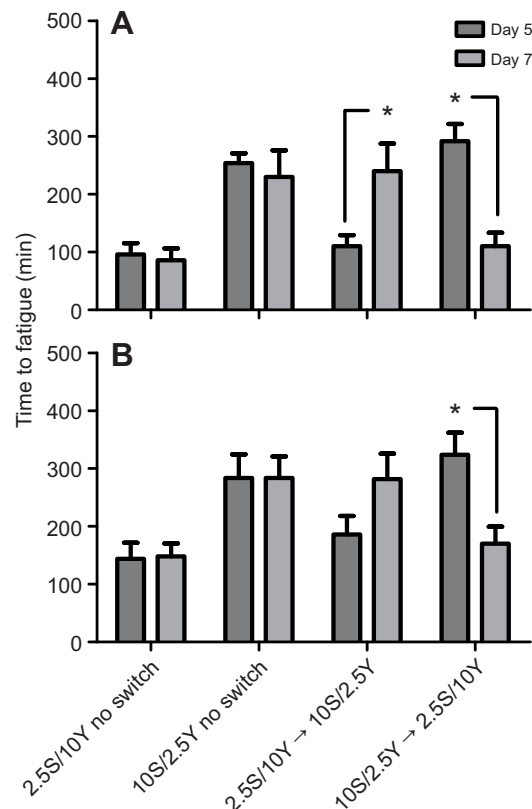


Fig. 6. Effect of diet on endurance is reversible within 48 h. Following eclosion, male Berlin K (A) and Canton S (B) flies were fed either a high or a low sucrose/yeast ratio diet. An endurance assay was performed on day 5 (means and s.e.m.). Flies fed a high sucrose/yeast ratio diet had higher endurance than those fed a low sucrose/yeast ratio diet (log-rank analysis: $P < 0.05$). On day 6, two groups were switched from one diet to the other for 24 h. On day 7, another endurance assay was performed. Within 48 h, flies that were switched displayed endurance typical of their new diet rather than of their previous diet. *Full statistical analyses are presented in supplementary material Table S1. Data are also presented in 'runspan' form in supplementary material Fig. S3.

Relative caloric intake

In principle, the impact of diet on the assays observed here could be dependent on (1) total caloric intake, (2) ratio of dietary components or (3) both. To assess the role of caloric intake, we determined the relative caloric intake for each cohort, based on dietary content and feeding rate (see Materials and methods). Relative caloric intake for each cohort is listed in supplementary material Table S2. For each physiological assay, we used regression analysis to ask whether total caloric content had a strong predictive impact on performance. Total caloric intake did not exhibit significant predictive value for any assay (Fig. 7). Therefore, our results are in agreement with longevity-based findings suggesting that dietary composition is more important than caloric intake when predicting physiological outcomes (Lee et al., 2008; Skorupa et al., 2008; Lushchak et al., 2012).

DISCUSSION

Drosophila is emerging as an excellent model for studying the impact of simple dietary constituents on various physiological indices. The short lifespan and large numbers available in the fly model make longitudinal studies of multiple non-invasive assays practical. Here, we have focused on the impact of varying two major

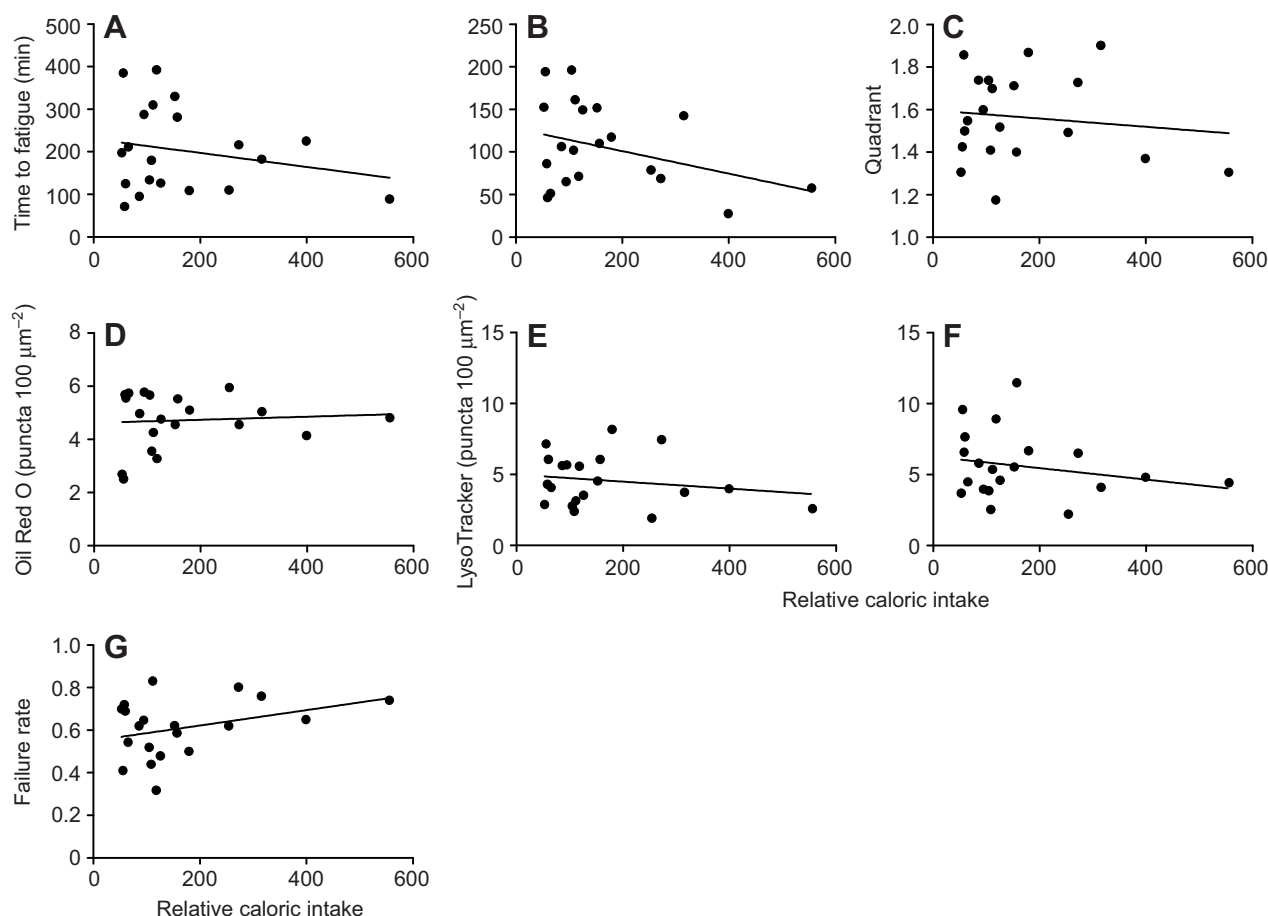


Fig. 7. Caloric intake does not sufficiently predict the variance observed in *Drosophila* mobility and cardiac assays. In order to determine whether caloric intake was a driving factor behind the observed trends, the 'relative caloric intake' of each genotype/diet treatment was determined by measuring the total caloric content of each diet, and then multiplying it by the product of the number of bites per observation and the amount ingested per bite for each cohort. The following physiological assays were plotted against relative caloric intake and a linear regression was performed: (A) week 1 average fatigue, (B) week 4 average fatigue, (C) average RING quadrant, (D) average cardiac lipid levels, (E) average cardiac autophagy levels, (F) average fat autophagy levels and (G) cardiac failure rate. In no case was caloric intake found to be a statistically significant predictor of physiological performance ($F^2 \leq 0.1227$ for all assays). Full relative caloric intake calculations for each genotype/diet are presented in supplementary material Table S2.

dietary components on locomotion, endurance capacity and cardiac performance.

When comparing relative rank orders from all assays presented (Fig. 8), several clear trends emerge. First, the two genetic backgrounds have similar rank order responses to most assays, but the magnitude of the difference is generally higher in Berlin K flies. The reason for the greater plasticity in dietary response in this background is presently unclear.

Some commonalities in diets that lead to an improvement in results for multiple assays are also evident. In particular, balanced, low-calorie diets appear to provide strong beneficial effects to both endurance and cardiac physiology. These benefits are especially apparent at later ages, where a substantial age-related decline has already occurred in these parameters. This correlates well with previous results associating such diets with extended longevity (Partridge et al., 2005; Bhandari et al., 2007; Grandison et al., 2009).

These commonalities raise the question of whether the benefits to longevity, endurance and cardiac performance all stem from similar physiological mechanisms. One proposed mechanism by which the interventions collectively known as dietary restriction improve healthspan and longevity is by increasing levels of

autophagy (Bjedov et al., 2010; Partridge et al., 2011). Genetic interventions in regulators of autophagy, such as target of rapamycin (TOR) and eukaryotic initiation factor 4e binding protein (4eBP), have previously been shown to preserve aging cardiac function (Luong et al., 2006; Wessells et al., 2009) and negative geotaxis during aging (Demontis and Perrimon, 2010). In rodent models, exercise-induced autophagy is a key component necessary for the benefits of exercise (He et al., 2012). However, increases in baseline autophagy associated with diets high in saturated fatty acids (comparable to the low sucrose/yeast ratio diet in this study) have been linked with increased pathological cardiac hypertrophy (Russo et al., 2012). We found no evidence in this study that cardiac baseline autophagy levels are consistently altered by diets that improve endurance or cardiac function.

By contrast, we found a strong correlation between low cardiac lipid accumulation and improved endurance/cardiac function. For example, the diets that lead to accumulation of the lowest levels of cardiac lipid, 2.5S/2.5Y and 5S/5Y, are also the diets resulting in the lowest cardiac failure rate during pacing (Fig. 8). The same diets also promote the highest endurance, though not necessarily the highest speed (Fig. 8). It has been previously observed that a

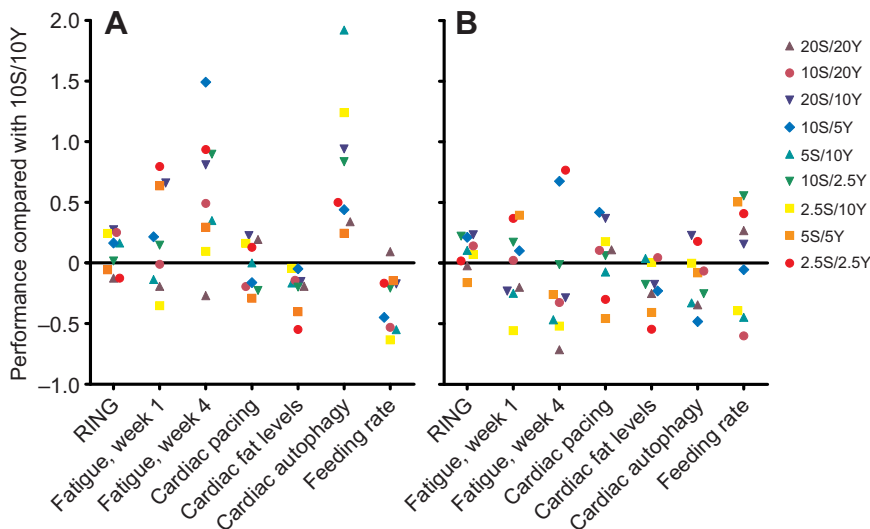


Fig. 8. Rank order summary of dietary effects on *Drosophila* mobility and cardiac phenotypes, relative to the standard 10S/10Y diet for Berlin K (A) and Canton S (B) flies. Between genotypes, dietary trends are very similar. In general, flies on balanced low-calorie diets have higher endurance across ages, lower pacing-induced cardiac failure and lower levels of cardiac lipid. Among unbalanced diets, those with a high sucrose/yeast ratio led to higher endurance but also higher pacing-induced cardiac failure than low sucrose/yeast ratio diets.

reduction in TOR signaling both reduces triglyceride storage and protects aging cardiac performance in *Drosophila* (Luong et al., 2006; Wessells et al., 2009). Conversely, flies fed a high-fat diet (Birse et al., 2010) or flies with genetic mutations that increase lipid storage in the myocardium have decreased cardiac performance (Lim et al., 2011; Sujkowski et al., 2012). Taken together, these results are all consistent with the hypothesis that reduced lipid storage in cardiac or other muscle may be an important mechanism by which balanced, low-calorie diets protect endurance and cardiac performance. Further work using flies to dissect the downstream genetics induced by dietary changes will be necessary to resolve this question with more certainty.

In the case of unbalanced diets, we found a divergence between diets that benefit endurance and those that benefit cardiac performance. Diets high in sugar/yeast ratio generally benefit endurance, but have a negative impact on cardiac performance. This may indicate a difference in energy requirements between the beating heart and the various neuromotor functions involved in endurance running. As sugars are preferentially used during endurance behaviors, especially in untrained animals (Martin and Klein, 1998; Graham and Adamo, 1999), extra dietary sugar may provide greater access to quick sources of glucose for ATP production in muscle. The mechanism by which such a diet impairs cardiac performance in flies is unclear.

In contrast to the profound effects of dietary content on endurance, the effects of diet on climbing speed were statistically significant, but small in magnitude. The most significant effects were always seen in the Berlin K background, whereas negative geotaxis in Canton S flies was less clearly influenced by diet. No strong trends emerged when comparing the slope of decline across time of climbing speed, suggesting that diet does not strongly affect the age-related decline of negative geotaxis, in agreement with previous observations (Bhandari et al., 2007).

The experiments here focus on the effect of diet on acute measures of mobile capacity. The effect of diet on the long-term improvement in climbing speed following a training regimen has previously been measured. In that context, variation of sucrose had little effect, whereas the magnitude of improvement following training was closely associated with the amount of yeast in the diet, independent of sucrose (Piazza et al., 2009). Taken together, these results clearly indicate that flies have substantially different requirements for acute energy expenditure *versus* long-term

physiological responses to training. This should be taken into account in comparing and interpreting varying results from different experimental protocols.

Lastly, we found that the effects of diet on endurance are completely reversible within 48 h of switching animals to a new diet. These findings are consistent with the acute ability of diet to alter mortality rates within the same time scale (Mair et al., 2005). Flies should serve as an excellent model for future longitudinal studies more thoroughly dissecting the long-term and acute roles of various dietary constituents on endurance and exercise response.

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