

RESEARCH ARTICLE | Obesity, Diabetes and Energy Homeostasis

# Low-carbohydrate diet induces metabolic depression: a possible mechanism to conserve glycogen

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Submitted 21 February 2017; accepted in final form 5 July 2017

**Winwood-Smith HS, Franklin CE, White CR.** Low-carbohydrate diet induces metabolic depression: a possible mechanism to conserve glycogen. *Am J Physiol Regul Integr Comp Physiol* 313: R347–R356, 2017. First published July 12, 2017; doi:10.1152/ajpregu.00067.2017.—Long-term studies have found that low-carbohydrate diets are more effective for weight loss than calorie-restricted diets in the short term but equally or only marginally more effective in the long term. Low-carbohydrate diets have been linked to reduced glycogen stores and increased feelings of fatigue. We propose that reduced physical activity in response to lowered glycogen explains the diminishing weight loss advantage of low-carbohydrate compared with low-calorie diets over longer time periods. We explored this possibility by feeding adult *Drosophila melanogaster* a standard or a low-carbohydrate diet for 9 days and measured changes in metabolic rate, glycogen stores, activity, and body mass. We hypothesized that a low-carbohydrate diet would cause a reduction in glycogen stores, which recover over time, a reduction in physical activity, and an increase in resting metabolic rate. The low-carbohydrate diet reduced glycogen stores, which recovered over time. Activity was unaffected by diet, but metabolic rate was reduced, in the low-carbohydrate group. We conclude that metabolic depression could explain the decreased effectiveness of low-carbohydrate diets over time and recommend further investigation of long-term metabolic effects of dietary interventions and a greater focus on physiological plasticity within the study of human nutrition.

protein; nutrition; *Drosophila*; activity; plasticity; obesity

THE PREVALENCE OF HUMAN OBESITY worldwide has become an epidemic (84). The proposed causes of this rise are numerous and varied, and the relative contributions of these causes are not well understood (42). In the 1970s, Dr. Robert C. Atkins popularized the use of low-carbohydrate, high-protein diets for weight loss and weight maintenance (2). More recently, the protein leverage hypothesis that appetite in humans is modulated to regulate protein intake, such that total energy intake increases as the proportion of protein in a meal decreases, has been introduced (61). Several meta-analyses comparing long-term randomized weight loss trials of low-carbohydrate vs. traditional calorie-restricted (low-fat) diets concluded that low-carbohydrate diets lead to greater weight loss in the shorter term, but this advantage is either not apparent or diminished after 12 mo (1, 19, 20, 24, 47). The reduction in the efficacy of low-carbohydrate diets with time suggests that some compensatory mechanism acts to mitigate the weight loss advantage of

such a diet over longer time periods. While physiological responses to low-carbohydrate diets are well described over short time periods (40), there is a distinct lack of studies examining long-term responses. Here we propose that the reduction in the efficacy of low-carbohydrate diets over time is explained by the reduction in glycogen stores associated with low-carbohydrate diets (17, 46, 50), leading to reduced physical activity. To test this hypothesis, we used the model organism *Drosophila melanogaster*, which has become increasingly important for the study of human nutrition and obesity (70). The tractable nature of this model allowed us to quantify the physiological changes that occur in response to dietary treatments with high temporal resolution for a significant period of the organism's lifespan.

Some of the mechanisms by which low-carbohydrate, high-protein diets lead to weight loss success are well understood. It is generally reported that there is a macronutrient satiation hierarchy, whereby appetite is satisfied most easily in descending order of protein, carbohydrate, and fat (15, 72), and numerous studies on humans have demonstrated the increased satiety and satiation effects of protein (6, 21, 26, 35, 63, 66, 71, 77, 81). Additionally, because of the different pathways by which macronutrients are oxidized, net ATP yield will vary per calorie of a given substrate, with protein yielding comparatively less ATP (12, 13). This partially accounts for the increase in resting energy expenditure in individuals on a low-carbohydrate, high-protein diet (35, 43, 80). However, almost half of this increase in resting energy expenditure has been attributed to upregulation of gluconeogenesis in response to lowered glycogen stores, a result of the reduced carbohydrate intake (3, 27, 74). Enhanced satiation, lower energy yield, and increased energy expenditure, combined with a lack of evidence to suggest that low-carbohydrate, high-protein diets have adverse health effects, have led to the recommendation that such diets are an effective tool for weight loss and weight maintenance (40).

Glycogen is a major source of fuel for locomotion (16, 69); therefore, the pronounced reduction in liver and muscle glycogen stores resulting from a low-carbohydrate diet (17, 46, 50) may impact the motivation to exercise. It has been shown that endurance athletes can achieve the same performance on low- and high-carbohydrate diets (50), but they report greater perceptions of fatigue (65) and less vigor (30) on low-carbohydrate diets. Similarly, a 90-min walk resulted in increased feelings of fatigue in overweight untrained adults on a low-carbohydrate diet compared with those on a conventional diet, despite no performance difference between groups (82). Exer-

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cise performance can be attenuated by the central nervous system, despite no apparent deficiency in cardiac or skeletal muscle performance capacity (5, 48, 59), and the cessation of exercise is not always related to obvious indicators of exhaustion (29). Fatigue may arise because the brain subconsciously regulates the willingness to undertake physical activity by taking multiple cues from different organs (e.g., heart, muscles, and the respiratory system) and moderating the expenditure of energy to protect the integrity of the whole organism (29). We hypothesized that glycogen levels represent a potential cue that could feed back and modulate levels of physical activity and that low levels of voluntary activity associated with low-carbohydrate diets might arise because of the low levels of glycogen associated with such diets.

Testing such a hypothesis in humans is inherently problematic, given the difficulties of designing truly blind trials, where the experimental situation does not subconsciously influence the subject (28). For this reason, animal models may be more appropriate for assessing involuntary behavioral responses. The fruit fly *D. melanogaster* is increasingly used to study the mechanistic bases of human nutrition and obesity (70). As our reliance on this model grows, it is critical that we understand where the physiological mechanisms between humans and *Drosophila* are conserved and where they diverge. High-carbohydrate larval diets (~1:6.5 protein-to-carbohydrate ratio) have been used to produce obese, diabetic flies (45), and the physiology of carbohydrate and lipid metabolism is largely conserved between flies and humans (18, 49, 67, 70). *Drosophila* and humans have been shown to exhibit similar responses to changes in dietary carbohydrate and protein intakes. There is some evidence to suggest that diets higher in protein content induce elevations in metabolic rate (MR) and reduce whole body carbohydrate content in adult *Drosophila* (6a), and low-carbohydrate larval diets reduce glycogen stores in adults (41). Additionally, *Drosophila* rely on and rapidly consume glycogen during flight and continuously consume glycogen, although at a much lower rate, to fuel resting metabolism (85).

In the present study we investigated the possible link between glycogen levels and spontaneous activity and the effects of reduced dietary carbohydrate intake on energy metabolism. MR, voluntary activity, and glycogen levels were measured daily and food consumption was measured every 3 days to test the following hypotheses: 1) glycogen stores are associated with a reduction in physical activity; 2) glycogen stores will decline at the initiation of a low-carbohydrate diet but recover over time due to changes in energy expenditure; and 3) a low-carbohydrate diet will be associated with an increase in resting MR.

## MATERIALS AND METHODS

### Study Animals

All experiments were performed on a mass-bred population of *D. melanogaster*. Flies were wild-caught in Coldstream, Victoria, Australia, and 30 isofemale lines were created by breeding the offspring (F1) from individual females to create an F2 generation. Ten virgin male and 10 virgin female F2 flies from each line (600 in total) were placed into a bottle to create the mass-bred population. This population was kept at 25°C under a 12:12-h light-dark photoperiod on a polenta-sugar-yeast medium (105.5 g of polenta, 18 g of yeast, 91.5 g of sucrose, 11.5 g of agar, 5 ml of nipagin, and 5 ml of propionic acid in 1.1 liters of water).

### Experimental Protocol

**Experimental animals.** All experiments were performed on virgin female flies. Females were chosen for their larger body size, making all measurements slightly less challenging in terms of equipment or assay sensitivity. The flies were kept as virgins because reproduction affects allocation of carbohydrate and lipid stores in *Drosophila* (8) and, thus, is likely to affect our results. *D. melanogaster* virgin females have been observed to lay a small number of infertile eggs after a while (39); this was occasionally observed in the later days of the present experiment, but it was infrequent and not recorded. For collection of virgins for the experiment, flies were extracted from the stock population and sexed under light CO<sub>2</sub> anesthesia. Three males and three females were combined in vials on the same polenta medium used for the stock population and allowed to mate and oviposit. Eclosed offspring were collected as virgins, sexed while under light CO<sub>2</sub> anesthesia, and again combined as three males and three females into vials. The offspring from this second set of vials were collected as virgins and sexed while under light CO<sub>2</sub> anesthesia, and females were placed individually into the experimental housing apparatus. Beyond this initial setup, no anesthesia was used for handling of animals during any of the experimental procedures.

**Housing apparatus.** Experimental flies were housed individually using a modified version of the capillary feeder (CAFE) assay (23) at 25°C under a 12:12-h light-dark photoperiod. Flies were contained in 5-ml tubes (Falcon, BD Biosciences, San Jose, CA) and fed a liquid diet of distilled water, autolyzed yeast (MP Biomedicals, Santa Ana, CA), and sucrose contained within a 5- $\mu$ l capillary tube (Drummond Microcaps, Drummond Scientific, Broomall, PA or Hirschmann Mini-caps, Hirschmann Laborgeräte, Eberstadt, Germany) that was inserted through a hole in the lid of the tube. To avoid desiccation of the animals and prevent reliance on the liquid diet for hydration, each tube contained 1 ml of a 0.5% agar solution (5 g of agar, 5 ml of nipagin, and 5 ml of propionic acid in 1 liter of water). Tubes were held in a total of eight racks, two racks per one of four 48-liter plastic storage containers (Really Useful Products, Normanton, West Yorkshire, UK). Racks were mounted one above the other in an orientation that held the tubes vertically. To minimize evaporation of the liquid diets, humidified air was pumped into the container via a 20-l/min aquarium pump (Resun AC-9908, Shenzhen Xing Risheng Industrial, Shenzhen, China), which fed air through four gas-washing bottles (Schott, French's Forest, NSW, Australia).

**Dietary treatments.** Flies were fed one of two diets for the duration of the experimental protocol: a standard (ST) diet with a protein-to-carbohydrate ratio of 1:4 and a low-carbohydrate (LC) diet with a protein-to-carbohydrate ratio of 1:1. The ratio for the ST diet was chosen based on the report of Lee et al. (34) that, when given a choice, *D. melanogaster* will select a 1:4 ratio. These macronutrient ratios were achieved by varying the ratio of autolyzed yeast and sucrose, which were dissolved in distilled water and made to a total concentration of 400 g/l with 0.1% nipagin and 0.1% propionic acid to prevent microbial growth. The ST diet contained 5 g of sucrose and 10.5 g of yeast, and the LC diet contained 3.58 g of sucrose and 11.92 g of yeast; both diets contained 38.75  $\mu$ l of nipagin and 38.75  $\mu$ l of propionic acid and were made up to a volume of 38.75 ml with distilled water. A concentration of 400 g/l was chosen to avoid constraints on nutrient uptake due to the volume of ingested water, which can be a limitation with dilute diets, and because, across diet concentrations of 45–360 g/l, it was found that longevity increased with concentration (34).

### Experimental Design and Schedule

Each rack was divided in half between the two diets. To account for any confounding effect of left or right rack position with treatment, top and bottom racks within a container were divided in opposite orientation, and which rack was divided which way was randomized in each of the four containers. A total of 114 tubes were placed in each

rack (57 for each diet treatment), and two tubes for each block of 57 contained no flies and served as evaporative controls. This gave a total of 912 tubes with 880 individually housed flies. Duration of the experiment was 9 days, with MR, activity, and glycogen levels measured daily and consumption measured every 3 days. The experiment was run five times, with the position of the treatments within the racks randomized again for each run. Flies were housed on *day 0*, so that *day 1* measurements occurred 24 h after initiation of the dietary treatments. The total number of flies housed for the five runs was 4,400; the total number measured for MR and activity was 720, or 40 per treatment per day; the total number measured for glycogen levels was 1,080, or 60 per treatment per day. Consumption was measured for all surviving flies on *days 3, 6, and 9*, when food was replaced; tubes with dead flies were discarded.

**MR and activity.** On each day, 16 flies (8 from each treatment) were randomly selected for measurement of MR and spontaneous activity between 0800 and 1100. These traits were measured simultaneously on individual animals by means of a respirometry system equipped with a horizontal, cylindrical glass chamber long enough for an individual fly to walk back and forth and placed within an activity monitor (model DAM2, TriKinetics, Waltham, MA) with an infrared beam crossing the middle of the chamber that detected each time the animal crossed the beam. Activity was measured because absolute levels of activity were of interest so that correlations between activity and energy expenditure could be statistically accounted for and so that a proxy for “resting” energy expenditure could be estimated by adjustment of values to an activity level of 0 (see data presentation for more details). Rate of energy expenditure was estimated from CO<sub>2</sub> production in a four-channel, two-analyzer, open-flow respirometry system. Atmospheric air was drawn through columns of Drierite and soda lime to remove water vapor and CO<sub>2</sub>, respectively. This air was regulated to a flow rate of 30 ml/min via an air pump (model TR-SS3, Sable Systems International, Las Vegas, NV) and mass flow controllers (model GFC17, Aalborg Instruments and Controls, Orangeburg, NY). Excurrent air from the flow controllers was passed through aquarium stones in 20-ml water-filled glass scintillation vials that were immersed in a 20°C water bath before passage through a 2.2-ml glass respirometry chamber situated within four channels of a 32-channel *Drosophila* activity monitor (model DAM2, TriKinetics) and then into two CO<sub>2</sub>/H<sub>2</sub>O analyzers (model LI-7000, LI-COR, Lincoln, NE). The CO<sub>2</sub>/H<sub>2</sub>O analyzers were interfaced with a PowerLab 8/30 A/D converter (ADInstruments, Bella Vista, NSW, Australia) and recorded to 0.1 ppm at a sampling frequency of 1 Hz on a computer running Labchart software v7 (ADInstruments). The respirometry chambers and activity monitor were contained within a 25°C temperature-controlled cabinet in darkness. Air was cooled during rehumidification, so that when it rewarmed within the temperature-controlled cabinet, it would be humid, but not saturated; this was done to avoid condensation forming with the CO<sub>2</sub>/H<sub>2</sub>O analyzers, as recommended by Lighton and Halsey (36). Background CO<sub>2</sub> concentration was measured for 15 min before and after the measurement of each fly to account for baseline drift. Animals were measured for 30 min, with mean CO<sub>2</sub> production calculated from the last 15 min to allow time for the animal to settle. Activity was calculated as average beam crosses (counts/min) from the same 15-min period. Immediately after measurements were taken, live body mass was recorded using a precision microbalance (model XS3DU, Mettler-Toledo, Columbus, OH); then the flies were discarded.

**Consumption.** On *days 3, 6, and 9*, all feeding capillaries were removed and replaced with tubes containing fresh diets. For measurement of food consumed over the previous 3 days, tubes were lined up and photographed against a piece of laminated 1-mm-grid paper. Fiji image-processing software ([www.fiji.sc](http://www.fiji.sc)) was used to correct photographs for perspective, and the reduction in the size of the food column in each microcapillary was measured. To account for the effect of evaporation, for each treatment in each rack the average decrease in column size from the two evaporative controls was

subtracted from the measured decrease for each capillary from which an animal had fed. Because autolyzed yeast and sucrose have approximately equal energy content per unit of mass (38), diets were considered equally calorie-dense and, therefore, were expressed and discussed as units of volume consumed.

**Glycogen levels.** Glycogen levels were determined by a glucose assay with the addition of amyloglucosidase to break down glycogen using the protocol described by Tennessen et al. (68) with minor modifications. On each day, 25 flies (10 from one treatment and 15 from the other) were randomly withdrawn from each treatment group for glycogen assays. Which treatment donated 10 flies and which donated 15 flies alternated for each day and each run, except the final run, where only 10 flies were removed from each group per day. Flies were placed in Eppendorf tubes in groups of five and then frozen. Flies were taken straight from the freezer and homogenized in 100  $\mu$ l of 96°C PBS using a pellet pestle (model 749521-1500, Kontes) with a motor (model 749540-0000, Kontes); then 10  $\mu$ l were removed for protein quantification before samples were heated at 96°C, as recommended by Zimmerman et al. (87), for 10 min. Tubes were heated to inactivate endogenous enzymes that would prematurely degrade trehalose and glycogen into glucose and then centrifuged at 15,000 rpm at 4°C, and 30  $\mu$ l of supernatant were removed and combined with 300  $\mu$ l of PBS and kept on ice. Thirty microliters of each sample were loaded in quadruplicate into a 96-well plate along with glucose and glycogen standards. To half of the samples and the glucose standards, 100  $\mu$ l of glucose oxidase and peroxidase (PGO) enzymes (catalog no. P7719, Sigma-Aldrich) and *o*-dianisidine dihydrochloride (catalog no. D3252, Sigma-Aldrich) were added, and the same reaction mixture was added to the other half of the samples and the glycogen standards with amyloglucosidase (catalog no. A1602, Sigma-Aldrich). Reagents were mixed in the quantities and concentrations recommended by Tennessen et al. (68). Plates were then incubated in darkness at 37°C for 60 min, and the reaction was halted by addition of 100  $\mu$ l of 12 N sulfuric acid. Glucose levels were quantified by measurement of absorbance at 540 nm in a plate reader and calculated for samples without amyloglucosidase against the glucose standard curve and samples with amyloglucosidase against the glycogen standard curve. The 10- $\mu$ l portion of homogenate removed for protein quantification was combined in 10  $\mu$ l of PBS and assayed in a 96-well plate with protein standards using Bradford reagent (catalog no. B6916, Sigma-Aldrich) according to the supplier's instructions. Glycogen levels were calculated by subtraction of predigestion from postdigestion glucose levels and standardized to protein levels to account for differences in body size (68).

#### Statistical Analyses

Data for each trait were analyzed with a linear mixed-effects model using maximum likelihood with the lme4 package v1.1-12 (4) of R v3.3.0 (53) in RStudio v0.99.902 (55). Two-way interactions were included between diet and all other fixed factors. The significance of random effects was examined first using likelihood ratio tests, and nonsignificant effects were removed from subsequent models. The significance of the remaining fixed effects within this minimum adequate model was then tested using likelihood ratio tests. Random effects were retained within the minimum adequate model if significant at  $\alpha = 0.25$ , as recommended by Quinn and Keough (52), and  $\alpha$  was set at 0.05 for tests of significance for the fixed effects. Day was a common fixed factor to all traits analyzed and was treated as a categorical, rather than continuous, factor, because the changes in response variables over time did not appear to be linear. For each trait, post hoc pair-wise comparisons were made for each day by removal of the fixed effect of day and any interactions from the minimum adequate models.

**Metabolic rate.** The initial model for MR included the fixed effects of diet, day, activity, mass, and time (of day) and the random effects of analyzer, channel, container, top/bottom (rack), left/right (position

in rack), and run. The two-way interactions of diet  $\times$  time and diet  $\times$  activity, along with all random effects except run ( $\chi^2_1 = 44.2$ ,  $P < 0.001$ ) and analyzer ( $\chi^2_1 = 1.93$ ,  $P = 0.165$ ), were nonsignificant. The final model used to explain MR was MR  $\sim$ diet + day + mass + time + diet  $\times$  day + diet  $\times$  mass + analyzer + run. The model used for post hoc pair-wise comparisons for each day was MR  $\sim$ diet + activity + mass + time + analyzer + run.

**Mass.** The initial model for mass included the fixed effects of diet, day, and time and the random effects of analyzer, channel, container, top/bottom (rack), left/right (position in rack), and run. The two-way interaction of diet  $\times$  time, along with all random effects except container ( $\chi^2_1 = 6.35$ ,  $P < 0.012$ ) and run ( $\chi^2_1 = 102.42$ ,  $P < 0.001$ ), was nonsignificant. The final model used to explain mass was mass  $\sim$ diet + day + time + diet  $\times$  time + container + run. The model used for post hoc pair-wise comparisons for each day was mass  $\sim$ diet + time + container + run.

**Activity.** The initial model for activity included the fixed effects of diet, day, mass, and time and the random effects of analyzer, channel, container, top/bottom, left/right, and run. All two-way interactions, along with all random effects except run ( $\chi^2_1 = 3.8$ ,  $P = 0.051$ ), top/bottom ( $\chi^2_1 = 1.34$ ,  $P = 0.247$ ), and channel ( $\chi^2_1 = 1.64$ ,  $P = 0.201$ ), were nonsignificant. The final model used to explain MR was activity  $\sim$ diet + day + mass + time + channel + top/bottom + run. The model used for post hoc pair-wise comparisons for each day was activity  $\sim$ diet + mass + time + channel, top/bottom + run.

**Consumption.** The initial model for consumption included the fixed effects of diet and day and the random effects of container, top/bottom, left/right, and run. Although the consumption of many individuals was measured multiple times, this was not tracked, as the process was too time-consuming and logistically complicated. All fixed factors and interactions were significant, as were all the random effects of container ( $\chi^2_1 = 9.57$ ,  $P = 0.002$ ), top/bottom ( $\chi^2_1 = 76.03$ ,  $P < 0.001$ ), left/right ( $\chi^2_1 = 4.88$ ,  $P = 0.027$ ), and run ( $\chi^2_1 = 242.17$ ,  $P = 0.001$ ). The final model used to explain consump-

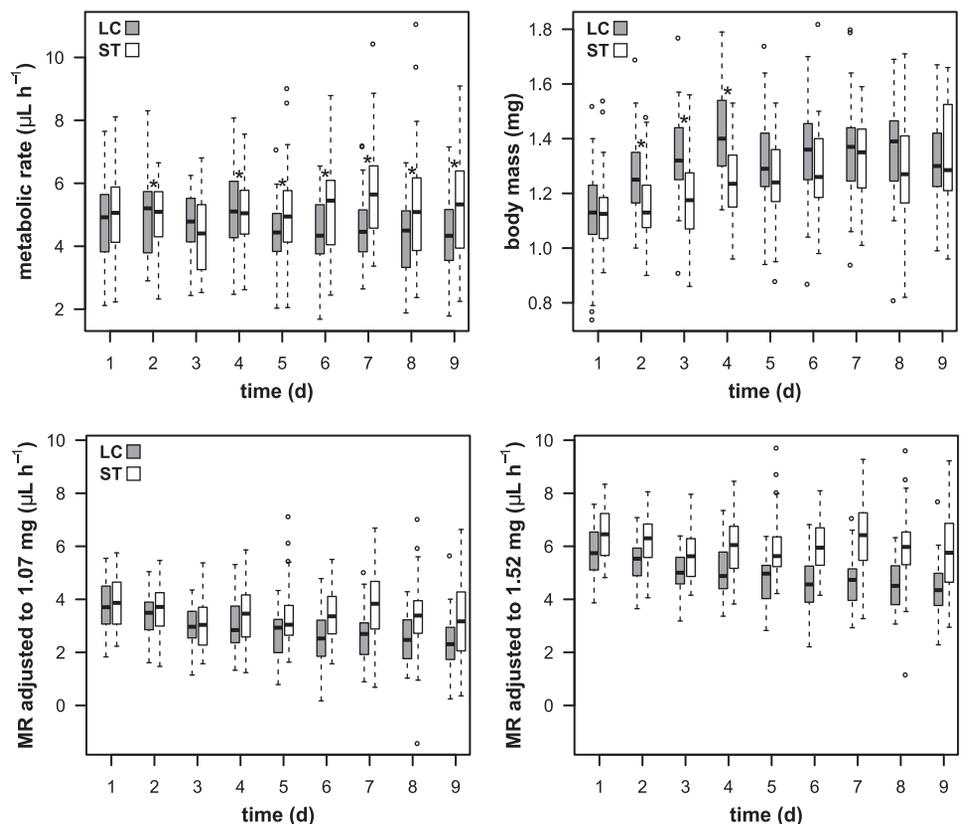
tion rate was consumption  $\sim$ diet + day + diet  $\times$  day + container + top/bottom + left/right + run. The model used for post hoc pair-wise comparisons for each day was consumption  $\sim$ diet + diet  $\times$  day + container + top/bottom + left/right + run.

**Glycogen stores.** The initial model for consumption included the fixed effects of diet and day and the random effects of G-plate (the 96-well plate for the glucose measurement) and P-plate (the 96-well plate used for the protein measurement), reagent batch, and run. Because each individual data point is produced from an assay of five flies randomly sampled from across all racks and containers, the factors of rack, top/bottom, and left/right were not relevant to the analysis. All fixed factors and interactions, as well as the random effect of run ( $\chi^2_1 = 22.762$ ,  $P < 0.001$ ), were significant. The final model used to explain consumption rate was glycogen  $\sim$ diet + day + diet  $\times$  day + run. The model used for post hoc pair-wise comparisons for each day was glycogen  $\sim$ diet + run.

#### Data Presentation

For maximum transparency, all figures are presented as box-and-whisker plots, as recommended by Weissgerber et al. (79). Where appropriate, visual presentation of MR values has been adjusted to reflect significant correlations with unrepresented cofactors so that figures more accurately represent statistical models. To represent the effect of activity, MR has been adjusted to an activity level of 0, which we interpret as a proxy for resting MR (i.e., the MR of an inactive fly; Fig. 1). To represent the effect of mass, MR has been adjusted to a mass of 1.07 mg and a mass of 1.52 mg (Fig. 1, *bottom left* and *bottom right*; see RESULTS for justification). These adjustments are achieved by subtracting from each value the mean difference between that value and the estimated value for an average animal. For example, for an animal weighing 0.57 mg with a MR of 1 to be adjusted to the mean mass of 1.07, using a parameter estimate for the effect of mass of 0.2, the difference between 0.57 and 1.07 ( $-0.5$ ) is

Fig. 1. Changes in body mass and metabolic rate (MR) in flies fed low-carbohydrate (LC) and standard (ST) diets over time. *Top left*: changes in body mass over time. *Top right*: MR adjusted to an activity level of 0. *Bottom left*: MR adjusted to an activity level of 0 and a low body mass (10th percentile of observed masses, 1.07 mg). *Bottom right*: MR adjusted to an activity level of 0 and a high body mass (90th percentile of observed masses, 1.52 mg). Analysis of MR indicates a significant effect of activity and 2 significant 2-way interactions: diet  $\times$  day and diet  $\times$  mass. Analysis of mass indicates a significant effect of the 2-way diet  $\times$  day interaction ( $n = 38$ –40 data points for each diet-day combination). \*Statistically significant pair-wise differences for each day:  $P < 0.05$  (not applicable to *bottom left* and *bottom right*).



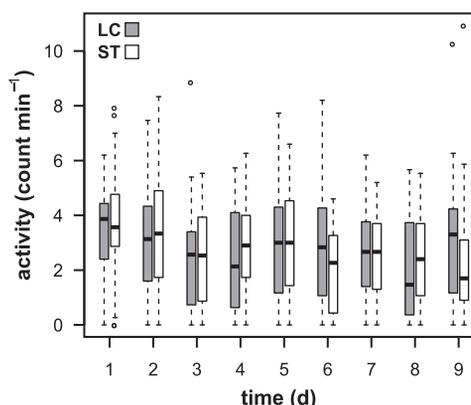


Fig. 2. Changes in activity in flies fed LC and ST diets over time. Analysis indicates a significant effect of day on activity ( $n = 38\text{--}40$  data points for each activity-day combination). Pair-wise analyses show no significant differences between diets on any day.

multiplied by the parameter estimate of 0.2 and subtracted from the animal's MR of 1 [ $1 - (0.57 - 1.07) \times 0.2$ ], yielding an adjusted MR of 1.1.

## RESULTS

### Metabolic Rate

There was a positive association of MR with activity ( $\chi^2_1 = 127.05$ ,  $P < 0.001$ ), as well as significant diet  $\times$  day ( $\chi^2_8 = 21.73$ ,  $P = 0.005$ ) and diet  $\times$  mass ( $\chi^2_1 = 6.75$ ,  $P = 0.009$ ) interactions. The diet  $\times$  day interaction indicates that MR changes with diet, but the nature of this change varies over time. This interaction is evident if we observe the values for MR for each treatment over time and notice that they diverge (Fig. 1, *top left*). The diet  $\times$  mass interaction indicates that the relationship between mass and MR differs between treatments. This interaction is evident if we observe that there is a greater increase in body mass in the LC treatment over the first 4 days than in the ST treatment (Fig. 1, *top right*), but over this same period, MR does not appear to diverge (Fig. 1, *top left*). These two visualizations demonstrate the interactions, but they fail to adequately convey the effect of diet on MR once all other factors are accounted for. The interaction with day is easy to visualize, because the values are plotted individually for each day, but the interaction with mass is more difficult to visualize. Usually, we would adjust the values for MR to a mean mass, but the diet  $\times$  mass interaction makes this inappropriate. Instead, we have adjusted MR to a small (Fig. 1, *bottom left*) and a large (Fig. 1, *bottom right*) mass, the 10th and 90th percentiles, respectively. This accomplishes two objectives: 1) the separation in MR between treatments becomes larger, because it removes the variation that can be attributed to differences in mass; and 2) we can see that the degree of separation in MR between the treatments is greater for large flies (Fig. 1, *bottom right*) than for small flies (Fig. 1, *bottom left*). Figure 1, *bottom left* and *bottom right*, shows that the LC diet leads to a decline in MR over time relative to the ST diet and that this relative decline is greater for larger animals.

### Mass

There was a significant effect of the diet  $\times$  day interaction ( $\chi^2_8 = 36.967$ ,  $P < 0.001$ ). This indicates a difference in body

mass between the dietary groups, but this difference is not consistent across days. This interaction is evident if we observe the values for mass and notice that, across the first 4 days of the experiment, the mass of both groups is increasing, but at a greater rate in the LC group (Fig. 1, *top right*).

### Activity

There was no significant effect of diet on activity, but there was a significant effect of day ( $\chi^2_8 = 27.51$ ,  $P < 0.001$ ). This indicates that both dietary groups show similar patterns of activity, and a visual assessment of the data suggests a decline over time (Fig. 2). To validate this interpretation, the minimum adequate model was refitted with day as a continuous predictor; in this model, there remained a significant effect of day ( $\chi^2_1 = 9.41$ ,  $P = 0.002$ ), and the parameter estimates showed that the slope for activity was negative and significantly different from 0, indicating a decline in activity for both diet treatments over time.

### Consumption

There was a significant effect of the diet  $\times$  day interaction ( $\chi^2_2 = 12.736$ ,  $P < 0.002$ ) on consumption. There was a reduction in the amount of food consumed in the ST group compared with the LC group. This divergence between diet groups was consistent over time, with some minor changes in the absolute values that explain the interaction (Fig. 3).

### Glycogen Stores

There was a significant effect of the diet  $\times$  day interaction ( $\chi^2_8 = 25.56$ ,  $P = 0.001$ ) on glycogen levels (Fig. 4). Although there was a significant effect of the diet  $\times$  day interaction and post hoc tests revealed that glycogen levels were significantly different between LC and ST diets on all days except *day 3*, a visual examination of the data suggests that glycogen levels are stable in the LC group but declining in the ST group (Fig. 4). To further investigate the time course of glycogen levels, the minimum adequate model was refitted with day as a continuous predictor; in this model the diet  $\times$  day interaction remained significant ( $\chi^2_1 = 13.4$ ,  $P < 0.001$ ); the

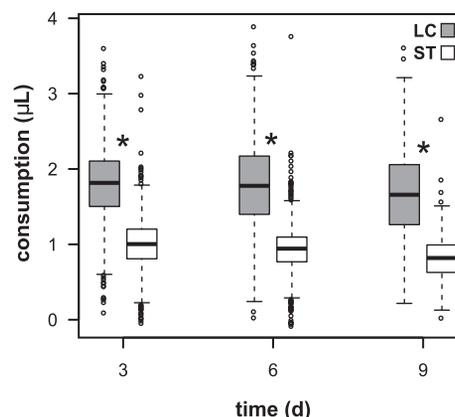


Fig. 3. Consumption level ( $\mu\text{L}$  per 3 days) in flies fed LC and ST diets over time. Analysis indicates a significant 2-way diet  $\times$  day interaction ( $n = 339\text{--}1,810$  data points for each activity-day combination). Number of data points diminishes later in the experiment, because flies are discarded after trait measurements. \*Statistically significant pair-wise differences for each day:  $P < 0.05$ .

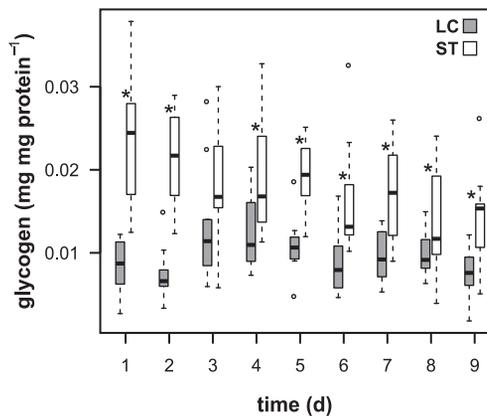


Fig. 4. Changes in glycogen levels in flies fed LC and ST diets over time. Analysis indicates a significant diet  $\times$  day interaction ( $n = 9$ –12 data points for each glycogen-day combination). \*Statistically significant pair-wise differences for each day:  $P < 0.05$ .

parameter estimates for this model showed that the slope for glycogen on the LC diet over time is not significantly different from 0 and has an intercept  $>0$ , but the slope for the ST diet is significantly more negative than that for the LC diet and has a significantly higher intercept. This indicates that glycogen levels in the LC group are positive and stable over time, while in the ST group they are initially higher but decline over time. These parameter estimates, therefore, suggest that glycogen levels would converge if the experiment continued for a longer time, although the time at which they might converge was not calculated, because doing so would require extrapolation beyond the range of measured data.

## DISCUSSION

The primary goal of this study was to test for a relationship between the level of an animal's glycogen stores and the propensity of these glycogen stores to expend energy through voluntary physical activity. It was hypothesized that, in adult *D. melanogaster*, a low-carbohydrate, high-protein diet would result in reduced glycogen stores, which would, in turn, cause a reduction in physical activity. However, while glycogen stores were lowered by the LC diet (Fig. 4), there was no difference in activity levels between the two dietary treatments (Fig. 2). This proposed link between glycogen stores and physical activity was hypothesized based on data that suggest initially superior results in humans on a low-carbohydrate diet compared with a calorie-restricted diet for weight loss, but, in the long run, this advantage is reduced or no longer apparent (1, 19, 20, 24, 47). Such patterns could be interpreted as being indicative of a long-term plastic response, which we predicted would serve to correct a glycogen deficit. In the present study, and in contrast to our hypothesis, flies fed the LC diet exhibit a reduction in MR, rather than a reduction in activity (Fig. 1). The only pattern in activity levels is a gradual decline over time that is independent of diet, which in previous studies has also been demonstrated to be an effect of aging (31, 44).

Our finding that a low-carbohydrate, high-protein diet causes a reduction in MR conflicts with previous studies of humans and a previous study of *D. melanogaster* in which increasing levels of protein led to elevations in MR (6a). This discrepancy with studies of *D. melanogaster* probably arises,

because Bradley and Simmons (6a) measured mated flies and observed a dramatic (and likely metabolically costly) increase in fecundity in flies fed high-protein diets, as has also been shown in other studies of *D. melanogaster* (34, 62). Our data, then, potentially highlight a difference in the physiological responses of humans and *Drosophila* to variations in carbohydrate and protein intake. However, it is worth considering that, for such a small animal with such a short lifespan, the duration of the experiment is significant, amounting to  $\sim 20\%$  of the adult lifespan of 50 days under optimal conditions (37). While there is an abundance of evidence demonstrating the increased energy expenditure induced by low-carbohydrate, high-protein diets in humans, this evidence is limited to relatively brief time spans of hours (25, 54), days (73, 83), or weeks (11, 22). We are not aware of studies comparing diets low in carbohydrate and/or high in protein with other diets that measure resting energy expenditure over time periods approaching  $\geq 12$  mo. Similarly, it is also possible that in our study the 24-h period between initiation of the dietary treatment and the first MR measurements may be too large a window to capture an initial rise in MR if such a response exists.

Given the significant effect of body mass on MR, the extent to which the changes in body mass explain the divergence in MR should be considered. The difference in body mass between the groups occurs over the first 4 days, whereas MR diverged from day 4. Over these first 4 days, the mass of both groups is on the rise, which is consistent with other studies that report an increase in body mass over the first days of adult life in *D. melanogaster* (8, 51). It is not surprising that this rise in body mass over the first 4 days occurs more rapidly in the LC group, as the higher protein content of the LC diet likely allows for more rapid synthesis of new tissue. It is also likely that different protein-to-carbohydrate ratios result in different body composition, with the higher-protein diet resulting in a greater lean body mass, as has been demonstrated in humans (32) and *Drosophila* (33). This effect of diet on body composition likely explains the significant diet  $\times$  mass interaction in the model accounting for variation in MR, as differing proportions of lean body mass would alter the mass-MR relationship (14, 78). We recorded and adjusted measurements of MR to live body mass, rather than lean body mass. Lee (33) reported a lower lean mass and greater fat content in adult *D. melanogaster* fed a 1:4 protein-to-carbohydrate ratio than in those fed a 1:1 protein-to-carbohydrate ratio. Adjustment to lean mass, instead of live mass, would have decreased the MR-to-tissue mass ratio of flies fed the LC diet relative to those fed the ST diet and increased the magnitude of the difference in MR between the two treatments.

If the reduction in MR in the LC diet occurs in response to depleted glycogen stores, we should expect to see a corresponding effect of diet on glycogen levels. As predicted, the glycogen stores in the LC diet group were reduced compared with the ST diet group, but the difference between dietary treatments decreases with time, as glycogen levels of flies fed the LC diet remain stable, but those of flies fed the ST diet decline (Fig. 4), presumably as a consequence of age-related decreases in metabolic efficiency (9). It could be suspected that the glycogen levels in the LC group do not decline, because the lifespan has been extended, delaying this decline, but mortality rates reported by Lee et al. (34) under similar conditions demonstrate that longevity is higher on a 1:4 than a 1:1

protein-to-carbohydrate ratio. This pattern of glycogen level stability for flies fed the LC diet can therefore be interpreted as an initial drop followed by a relative rise in glycogen stores. Similarly, endurance-trained human athletes show reduced glycogen stores after 2 days on a low-carbohydrate diet (86) but no reduction after 6 mo (75) and no difference in rates of gluconeogenesis after 8 mo (76). Considering that upregulated gluconeogenesis has been implicated as one of the major sources of MR elevation on a low-carbohydrate diet (74), the observation that gluconeogenesis does not remain upregulated over long periods of time further suggests that the increase in MR initially observed in humans on low-carbohydrate diets is not sustained over time. If glycogen stores recover over time, despite no upregulation of gluconeogenesis in the long term, then some other glycogen-sparing mechanism, which could be a reduction in resting MR, may be operating. It may be that, in both flies and humans, long-term adherence to a low-carbohydrate diet results in depression of MR as a strategy to conserve vital stores of glycogen.

It should be acknowledged that we cannot disentangle the effects of the lowered carbohydrate intake from the increased intake of protein, as well as lipids, vitamins, and other elements in the autolyzed yeast. It is possible that the responses are driven by these increases in other macro- or micronutrients. With regard to protein, it seems unlikely that the mechanisms by which its increased intake elevates MR, which relate to the biochemical pathways involved in its oxidation (12), would cease to apply with the passage of time. The downregulation of gluconeogenesis and some other metabolic pathways seems the more plausible explanation, particularly given the apparent consistency between this interpretation and the cited human studies. Nevertheless, this caveat is necessary.

The data for consumption level provide the biggest puzzle when all the measurements are considered together. While there is a significant two-way diet  $\times$  day interaction, all factors included in the model were returned as highly significant, which is likely an effect of the very large sample size of  $\sim 7,000$  consumption measurements. Of the various factors in the model, diet alone has a large effect size, and there is only minimal variation in consumption levels over time. Across the duration of the experiment, consumption levels of flies fed the LC diet are almost double those of flies fed the ST diet, and this pattern is consistent for the duration of the experiment. This increased consumption is consistent with previous studies (34, 62) and is presumed to be a response to meet a carbohydrate intake target. It could be speculated that consumption is driven by the need for hydration if the diets differentially affect the water balance of flies, but because the agar in each tube should provide adequate hydration, we assume that consumption is driven solely by nutritional requirements. Consumption has greatly diverged by the first measurement and, therefore, shows the most rapid adjustment of all measured traits. It seems likely that consumption level for the LC group is not representative of assimilation, given that it remains constant amid changes in body mass, MR, activity, and glycogen levels. Previous studies have demonstrated plasticity of digestive enzyme activity in response to altered protein and carbohydrate intake in a manner that can be both nonspecific (58) and specific (7) with respect to particular macronutrients. Changes in gut morphology (tissue mass) have also been shown in response to different carbohydrate-to-protein intake ratios, with different regions of

the gut responding in a macronutrient-specific manner (64). The level of plasticity in such responses has been shown to vary between closely related species (57) or between populations of a single species (10) and also to be constrained by dietary intake during development (56). Investigations of gut morphology and the activity of digestive enzymes, along with excretion levels and excretion contents, would be valuable to determine how assimilation changes with diet.

### Perspectives and Significance

The present study has demonstrated considerable metabolic plasticity in response to low-carbohydrate, high-protein diets in *D. melanogaster*. Such responses were hypothesized due to observations of long-term human weight loss studies and, thus, provide a possible mechanism to explain why low-carbohydrate diets may fail to produce consistent weight loss results over longer time periods. As *Drosophila* become an increasingly important model for the study of human nutrition and metabolism, it is critical to improve our understanding of how its metabolic responses are similar to or dissimilar from those in humans. In the quest to solve the puzzle of human obesity, investigations of physiological plasticity are critical. The assembled data demonstrate that, when faced with diets of substantially different macronutrient content, significant changes in intake patterns, energy stores, and resting energy expenditure can occur with little effect on body mass, which appears to be the same between the two dietary treatments by about *day 5* of the experiment (Fig. 1, *top right*). This demonstrates a remarkable level of phenotypic plasticity and highlights the importance of studies that record physiological responses over long time periods with high temporal resolution. Such future studies will be critical to expand our understanding of optimal animal nutrition and better tackle the challenge of the human obesity epidemic.

### ACKNOWLEDGMENTS

We thank Tessa Jones for many late-night hours assisting with laboratory procedures, Tirosh Shapira and Daniel Hancox for additional assistance in the laboratory, Rebecca Cramp for advice on molecular procedures, Julian Beaman and Piet Arnold for valuable discussions throughout the process, Lewis Halsey for comments on the manuscript, and Carla Sgro and Allannah Clemson for supplying the experimental animals.

### GRANTS

C. R. White is an Australian Research Council Future Fellow (Project No. FT130101493).

### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

### AUTHOR CONTRIBUTIONS

H.S.W.-S. conceived and designed research; H.S.W.-S. performed experiments; H.S.W.-S. and C.R.W. analyzed data; H.S.W.-S., C.E.F., and C.R.W. interpreted results of experiments; H.S.W.-S. and C.R.W. prepared figures; H.S.W.-S. drafted manuscript; H.S.W.-S., C.E.F., and C.R.W. edited and revised manuscript; H.S.W.-S., C.E.F., and C.R.W. approved final version of manuscript.

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