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SHORT COMMUNICATION

Flight activity and glycogen depletion on a low-carbohydrate diet

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ABSTRACT

Glycogen is a critical store for locomotion. Depleted glycogen stores are associated with increased fatigue during exercise. The reduced effectiveness of low-carbohydrate diets for weight loss over longer time periods may arise because such diets reduce glycogen stores and thereby energy expenditure via physical activity. To explore the effect of a low-carbohydrate diet on activity and glycogen utilisation, we fed adult *Drosophila melanogaster* a standard or low-carbohydrate diet for 9 days and measured patterns of flight activity and rates of glycogen depletion. We hypothesised that flight activity and rates of glycogen depletion would be reduced on a low-carbohydrate diet. Flight activity was elevated in the low-carbohydrate group but glycogen depletion rates were unchanged. We conclude that increased activity is probably a foraging response to carbohydrate deficiency and speculate that the previously demonstrated metabolic depression that occurs on a low-carbohydrate diet in this species may allow for increased flight activity without increased glycogen depletion.

KEY WORDS: *Drosophila*, Energy, Metabolism, Obesity, Plasticity, Protein

INTRODUCTION

Glycogen is a critical store of energy for supplying the energetic demands of locomotion in many animals (Hochachka, 1985). Glycogenolysis followed by glycolysis delivers the rapid supply of ATP required for the high-energy demands of muscle contraction. For locomotion of even moderate intensity, ATP produced from glucose via anaerobic pathways is required, and cannot be achieved if glycogen stores are depleted (Shulman and Rothman, 2001). For many insects, glycogen is also critical for flight; for example, lepidopteran and orthopteran species typically require carbohydrate for take-off, while flight in most hymenopterans and dipterans is exclusively fuelled by carbohydrate (Beenakkers et al., 1984). In *Drosophila*, it has been shown that the respiratory exchange ratio (RER: CO_2 produced/O_2 consumed) is 1.0 during flight (Chadwick, 1947), and that flight is impossible when glycogen stores are depleted (Wigglesworth, 1948).

The macronutrient content of an animal's diet has effects on body composition (Lee et al., 2003, 2004; Raubenheimer, 2003; Raubenheimer and Jones, 2006; Sørensen et al., 2008). Diets with reduced carbohydrate content can lead to reductions in whole-body carbohydrate (Simmons and Bradley, 1997) and glycogen (Azzout et al., 1984; Matzkin et al., 2011), and specifically muscle glycogen (Costill et al., 1981; Hultman and Bergström, 1967; Sherman et al., 1993), which has been associated with reduced locomotor performance (Bergström et al., 1967; Karlsson and Saltin, 1971). The macronutrient ratio of a given diet therefore has potential implications for behaviour and ecology via restriction of locomotor capacity. For example, locusts have been shown to alter their foraging behaviour depending on the distance between food sources deficient in different macronutrients (Behmer et al., 2003), and humans on carbohydrate-restricted diets may reduce physical activity because of increased feelings of fatigue during exercise that are not associated with any decrease in capacity (Keith et al., 1991; Stepto et al., 2002; White et al., 2007).

We previously investigated the link between a low-carbohydrate diet, glycogen stores and activity levels in *Drosophila melanogaster* to test the hypothesis that low-carbohydrate diets reduce glycogen stores and physical activity energy expenditure (Winwood-Smith et al., 2017). This was hypothesised as a possible explanation for the observation that in long-term weight-loss trials, low-carbohydrate diets lose effectiveness over time (Astrup et al., 2004; Hession et al., 2009; Johnston et al., 2014; Nordmann et al., 2006). We found that *D. melanogaster* on a low-carbohydrate diet showed no changes in walking activity, but displayed a drop and recovery of glycogen stores and a reduction in metabolic rate. We proposed that metabolic depression might be a compensatory mechanism to conserve glycogen stores, which may also explain the patterns observed in long-term weight loss trials (Winwood-Smith et al., 2017). This previous work considered walking activity, and it is not known how low-carbohydrate diets influence flight activity, but if a reduction in activity occurs on a low-carbohydrate diet in order to conserve glycogen reserves, it is most likely to manifest during flight, for which glycogen reserves are most critical.

Here, we investigated the relationship between a low-carbohydrate diet, glycogen stores and flight activity. Adult *D. melanogaster* were placed on two diets: a standard diet (1:4 protein to carbohydrate ratio) and a low-carbohydrate diet (1:1 protein to carbohydrate ratio) for 9 days. Flight activity was recorded daily, while glycogen depletion rates, protein levels and body mass were recorded on days 1, 5 and 9. The following hypotheses were tested: (i) given the reliance of dipteran species upon carbohydrate for flight and the association between low-carbohydrate diets and reduced locomotor performance, and increased perceptions of fatigue, it was predicted that flies on a low-carbohydrate diet would display lower flight activity than flies on a standard diet, and (ii) based on previous observations of metabolic depression and glycogen conservation on a low-carbohydrate diet, it was predicted that flies on a low-carbohydrate diet would show lower rates of glycogen depletion than flies on a standard diet.

MATERIALS AND METHODS

Experimental design

Fly stocks, rearing procedures, housing, dietary treatments, and methods for glycogen and protein assays were carried out as described by Winwood-Smith et al. (2017). All procedures were

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performed on adult virgin female *D. melanogaster* Meigen 1830 reared as larvae on standard laboratory media. Upon eclosion, virgin females were randomly assigned to one of two dietary treatments, a low-carbohydrate diet (LC: 1:1 protein to carbohydrate ratio) or a standard diet (ST: 1:4 protein to carbohydrate ratio) for 9 days. The ST diet was selected based on the study by Lee et al. (2008), which showed that when given the choice, female *D. melanogaster* regulate their dietary intake to achieve 1:4 protein to carbohydrate ratio, which is also the ratio that maximises fecundity. Diets were administered using a modified version of the CAFE assay (Ja et al., 2007), whereby flies were individually housed in 5 ml Falcon tubes with 1 ml non-nutritive 0.5% agar in the bottom to allow ample hydration, and fed a liquid diet via a 5 µl glass microcapillary inserted through a hole in the lid of the tube. Protein to carbohydrate ratios were achieved via altering the mix of autolysed yeast (MP Biomedicals, Santa Ana, CA, USA) and sucrose in distilled water mixed to a concentration of 400 g l^{-1}. The CAFE assay was carried out in a temperature-controlled cabinet at 25°C with a 12 h:12 h light:dark cycle.

Two main experiments were performed. In experiment one, which was replicated three times, measures of flight activity were performed. In experiment two, which was replicated twice, glycogen stores, protein levels and body mass were measured. For experiment one, measurements were taken daily from day 1 to 9, while for experiment two, measurements were taken on days 1, 5 and 9. In both experiments, equal numbers of flies were randomly taken from the main group for assays. Flies were housed within the CAFE assay on day 0 (day of eclosion), so day 1 measurements occurred 24 h after initiation of the dietary treatments.

Flight activity

On days 1–9, 20 flies from each treatment were randomly selected and individually transferred to an apparatus built from a modified LAM25H infrared (IR) activity monitor (TriKinetics), mounted horizontally around 95 mm×25 mm diameter glass vials. Individual flies were placed within vials that were positioned upside down on plastic lids filled with 0.5% agar solution to prevent desiccation, and the interior surface was coated in Fluon to prevent flies walking up the sides. The Fluon was scraped from the glass in a 2 mm wide ring around the circumference, approximately 20 mm above the surface of the agar, which allowed the nine IR beams of the activity monitor to create a detection plane (Fig. 1). This setup ensured that to interrupt the beams and register an activity count, the animal would have to fly. Forty flies were measured on each day for 5.5 h, and the apparatus was placed inside a temperature-controlled room at 25°C.

The number of beam breaks by a fly was recorded every 5 s. Flies occasionally landed on the Fluon-free zone, causing short periods of continuous beam breaks to be recorded. To eliminate this artefact, all continuous strings of non-zero values were transformed into single counts so that such events would be counted as a single flight event. To make the data amenable to analyses with linear models, the data were transformed into a 15 min running means, and running mean data were down sampled from a count every 5 s to a count approximately every 3.5 min by removing the intervening values.

Glycogen and protein

To estimate glycogen levels during the flight activity measurements, 180 flies were randomly taken from each dietary treatment. Sixty were immediately frozen to give a 0 h estimate of glycogen stores. The remaining 120 flies were placed into two mock activity monitors, which were put in a freezer after 3 and 6 h. Thus, 60 flies per dietary treatment were assayed for glycogen and protein at each time point (0, 3 and 6 h). The mock activity monitors consisted of the same Fluon-coated tubes housing individual flies, arranged in racks but without the IR beams, the purpose of which was to replicate the conditions of the flight activity monitor but allow easy freezing and a larger sample size. Once the flies were frozen, they were transferred five at a time into pre-weighed Eppendorf tubes, which were then weighed again to obtain estimates of body mass. Except for the weighing, which was done quickly with tubes kept on ice, all handling took place within a freezer to prevent the thawing of flies, which would lead to breakdown of glycogen by endogenous enzymes.

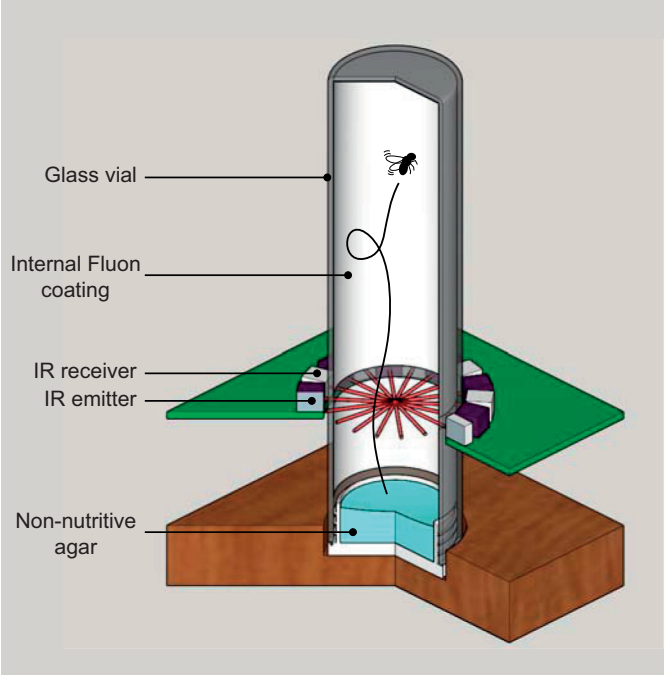

Fig. 1. Flight activity apparatus. The flight apparatus consisted of an inverted 25 mm glass vial with an inner coating of Fluon that housed an individual fly. Fluon was scraped off in a 2 mm wide ring, allowing infrared (IR) beams to cross. The fly could not cross the beams without flying as a result of the Fluon coating. Non-nutritive agar in a plastic cap at the bottom prevented desiccation.

Estimates of glycogen levels were determined using glucose oxidase and peroxidase (PGO) enzymes (catalogue no. P7719, Sigma-Aldrich) with the addition of amyl glucosidase (catalogue no. A1602, Sigma-Aldrich) to break down glycogen. Protein levels were determined using Bradford reagent (catalogue no. B6916, Sigma-Aldrich). Both assays were carried out based on the protocol described by Tennessen et al. (2014) with minor modifications as described by Winwood-Smith et al. (2017), with five flies being used for each individual assay of glycogen and protein levels.

Statistical analysis

Data were analysed with a linear mixed-effects model using maximum likelihood with the lme4 package v1.1-12 (https://CRAN.R-project.org/package=lme4), or standard linear models when random effects were non-significant, using R v3.3.0 (http://www.R-project.org/) in RStudio v0.99.902 (https://rstudio.com/). Mixed effects models were used to analyse activity and glycogen, and standard linear models were used to analyse protein and mass. The significance of random effects was examined first using likelihood ratio tests, and non-significant 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 α=0.25, as recommended by Quinn and Keough (2002), and α was set at 0.05 for tests of significance for the fixed effects. For full details of initial models, the results of tests involved in model simplification, and the final minimum adequate models used in the analysis, see Tables S1–S4.

Activity

The initial model for activity included mass, diet, day and time (of day), the two-way interaction of diet and mass, and the full-factorial combination of two- and three-way interactions among diet, day and time as fixed effects, with container, top/bottom (rack), left/right (position in rack) run as random effects along with ID. The two-way interaction of mass and diet, the single factor of mass, and all random effects except for container (χ^2_1=7.963, P=0.005), run (χ^2_1=25.296, P<0.001) and ID (χ^2_1=67,183, P<0.001) were non-significant. The final model used to explain activity was activity~diet+day+time+time×day+diet×day+diet×time+time×day×diet+container+run+id.

Glycogen

The initial model for glycogen included mass, diet, day and time (of day) and the full-factorial combination of two-way interactions between mass, diet, day and time as fixed effects, with run and plate as random effects. The two-way interactions of time×day, time×diet and mass×diet were non-significant. The final model used to describe glycogen was glycogen~diet+time+day+mass+diet×day+mass×day+ mass×time+run+plate.

Protein

The initial model for protein included mass, diet, day and time (of day) and the full-factorial combination of two-way interactions between mass, diet, day and time as fixed effects, with run and plate as random effects. All two-way interactions except for time×day and diet×day, and all random factors were non-significant. The final model used to explain protein was protein~diet+time+mass+time×day+diet×day.

Mass

The initial model for mass included diet, day, time and the full-factorial combination of two-way interactions between diet, day and time as fixed effects with run and plate as random effects. All two-way interactions, all random factors and the single effects time and day were non-significant. The final model used to explain mass was mass~diet+time+day.

RESULTS AND DISCUSSION

Activity

There was a significant three-way interaction between diet, day and time (χ^2_8=242.96, P<0.001), indicating that activity varied between diets in a way that changed with day and did not always show a consistent relationship with time. This is illustrated in Fig. 2, where the LC diet group had a consistently higher activity level, but the magnitude of the differences changed from day to day. Additionally, while the general trend was a consistent positive relationship between time and activity, on day 9 the LC group showed a decline in activity (Fig. 2, bottom right). See Table S1 for parameter estimates and test statistics for the minimum adequate model used.

Glycogen

For glycogen stores, there were three significant two-way interactions between diet and day (χ^2_1=18.31, P<0.001), time and mass (χ^2_1=15.3, P<0.001) and day and mass (χ^2_1=4.9, P=0.027). The interaction between diet and day is illustrated in Fig. 3 (top row) and shows that glycogen stores remained relatively higher in the standard diet group and declined over time (hours) in both diet groups at virtually identical rates; however, the separation between the groups diminished over days. The interactions between day and mass, and time and mass, indicate that the slope of the relationship between glycogen and mass grows steeper from day 0 to 9, but less steep through time from 0 to 6 h, but the effect of dietary treatment is independent of mass (i.e. there is no significant interaction between mass and diet). See Table S2 for parameter estimates and test statistics for the minimum adequate model used.

Protein

The final model for body protein levels showed a significant two-way interaction between diet and day (β=−2.711, P=0.004), and these two factors alone explained 64% of the variation in protein levels (R^2=0.64, $F_{6,101}$=32.7) (Fig. 3). Protein levels were relatively stable over time (hours), but varied in overall level with day, and also with diet, such that the LC group had higher protein levels, but this separation was pronounced on days 1 and 5 but minimal on day 9. See Table S3 for parameter estimates and test statistics for the minimum adequate model used.

Mass

The final model for mass had only the single significant factor of diet (β=−0.313, P<0.001) and explained just 10% of the variation in mass (R^2=0.10, $F_{3,212}$=8.62). The significant effect of diet is clear in Fig. 3 (bottom row), where the LC group had a consistently higher mass, and mass remained unchanged across the duration of the experiment. The variation in mass over time (hours) on day 1 and 9 was non-significant. See Table S4 for parameter estimates and test statistics for the minimum adequate model used.

Conclusions

The goal of the present study was to compare changes in the flight activity and glycogen depletion rates of *D. melanogaster* on a low-carbohydrate diet compared with a standard diet providing protein and carbohydrate in a ratio matching the intake target for this species. We hypothesised that flies would reduce flight activity as a compensatory response to the lowered glycogen stores associated with reduced dietary carbohydrate intake. Contrary to this, flies on the LC diet showed elevated patterns of flight activity (Fig. 2), and near-identical glycogen depletion rates (Fig. 3). The pattern of activity observed is probably explained by a compensatory response of the opposite kind: increased foraging behaviour in pursuit of an unmet carbohydrate intake target (see Lee et al., 2008, for evidence of target). Similar patterns have been described in blowflies, which show increased activity patterns and feeding frequency when feeding on more dilute sugar water (Simpson et al., 1989), and in starved *D. melanogaster*, which show increased activity (Bross et al., 2005). Thus the steady increase in flight activity is probably increased in both groups as a result of the absence of food during the 5 h measurement period, but with a greater overall activity in the LC group flies that are already further from their intake target.

As expected, glycogen levels were consistently lower in the LC diet group across all time points; however, the rate at which levels declined was equal between the two treatments for a given measurement period. This was surprising for two reasons. Firstly, our previous study showed that in the LC group, glycogen was stable over days, but decreased in the ST group. Given the lower carbohydrate intake, this led to the assumption that use of glycogen

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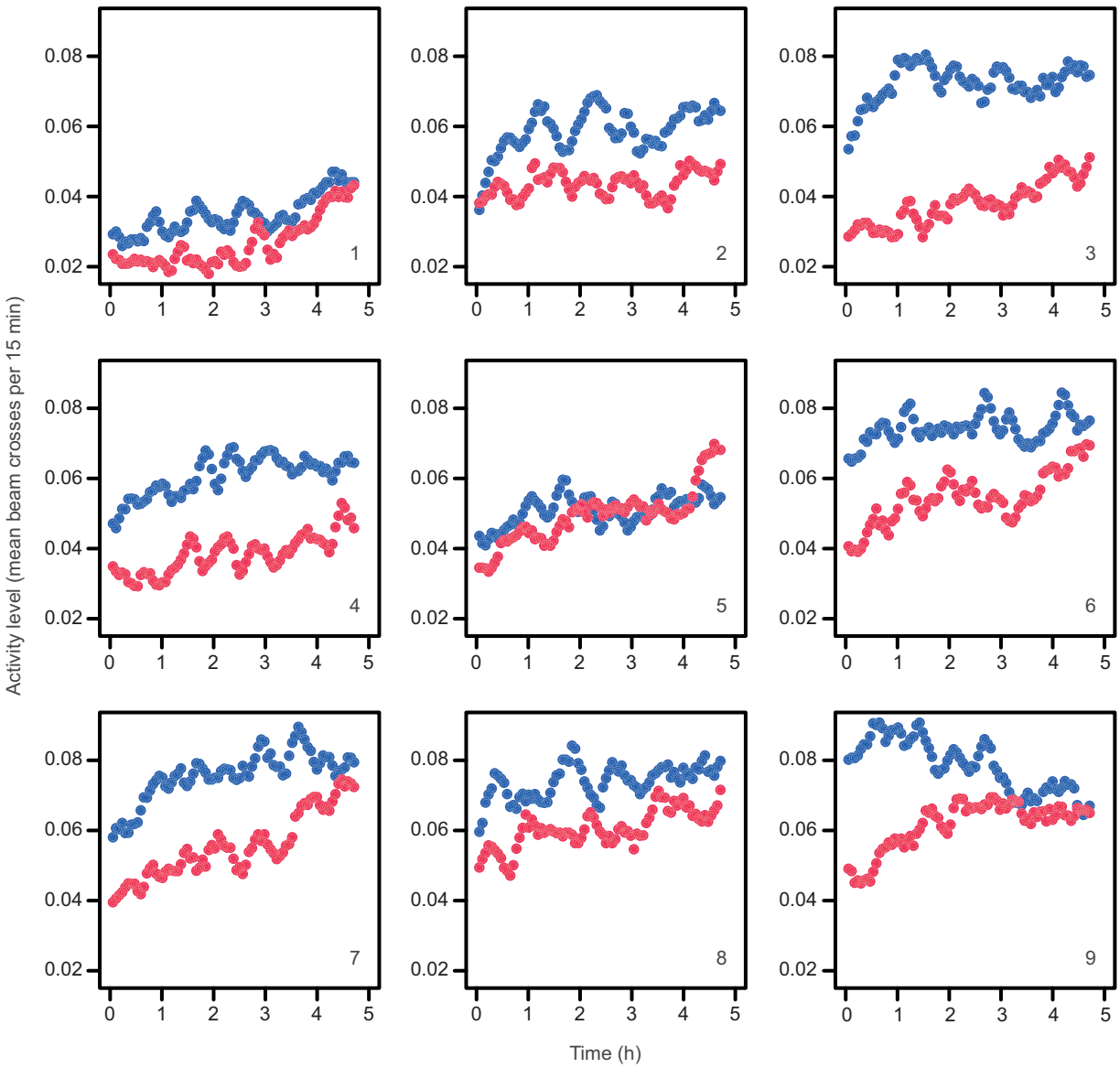

Fig. 2. Flight activity over time for *Drosophila melanogaster* on a low-carbohydrate (LC, blue) or standard (ST, red) diet on days 1–9. Each point occurs approximately every 3.5 min and is the mean number of beam crosses for the surrounding 15 min period. Panel numbers (bottom right, 1–9) represent the day on which the data were recorded. Analysis showed a significant three-way interaction between diet, time and day. Points represent mean values from *n*=40 flies.

is more sparing in the LC group. While there was a greater overall decline in glycogen levels for the ST group across the three measurement days in the present study, which is somewhat consistent with previous data, the near-identical rates of depletion during the measurement period was unexpected. Secondly, this similarity in rates of glycogen depletion was surprising because it occurred concurrently with higher flight activity in the LC group, which should deplete glycogen stores more rapidly in this group. These apparent contradictions may not be contradictions at all when considered together, however. If flies on the LC diet had lower rates of glycogen utilisation at rest, as our previous data suggest, then the absence of food and associated increase in foraging activity, which was greater in the LC group, may have resulted in similar levels of overall glycogen depletion rates in the LC and ST groups. In other words, the lower rates of resting utilisation in the LC group were offset by higher levels of activity so that glycogen was depleted at a similar rate in the two groups.

Such an interpretation is difficult to confirm, as the nature of our flight activity apparatus only delivered a semi-quantitative measure of activity. We cannot rule out the possibility that energy expended through flight is identical but the patterns of flight are altered such that different numbers of beam crosses occur in the LC and ST groups. To disentangle the contributions of resting energy expenditure and flight activity to glycogen depletion, we would require a more direct measure of the energetic cost of flight and a more accurate measure of resting energy expenditure incorporating measurements of both CO_2 production and O_2 consumption so that the RER can be determined and the relative contributions of carbohydrate and fat to resting metabolism can be inferred (Du Bois, 1924). Previous studies have demonstrated that the RER can vary with changes in dietary macronutrient ratio (Jensen et al., 2010) and between pre- and post-flight in fasted flies (Chadwick, 1947). It is also important to note that as we use a mixture of sucrose and yeast to achieve different carbohydrate to protein ratios, we are changing

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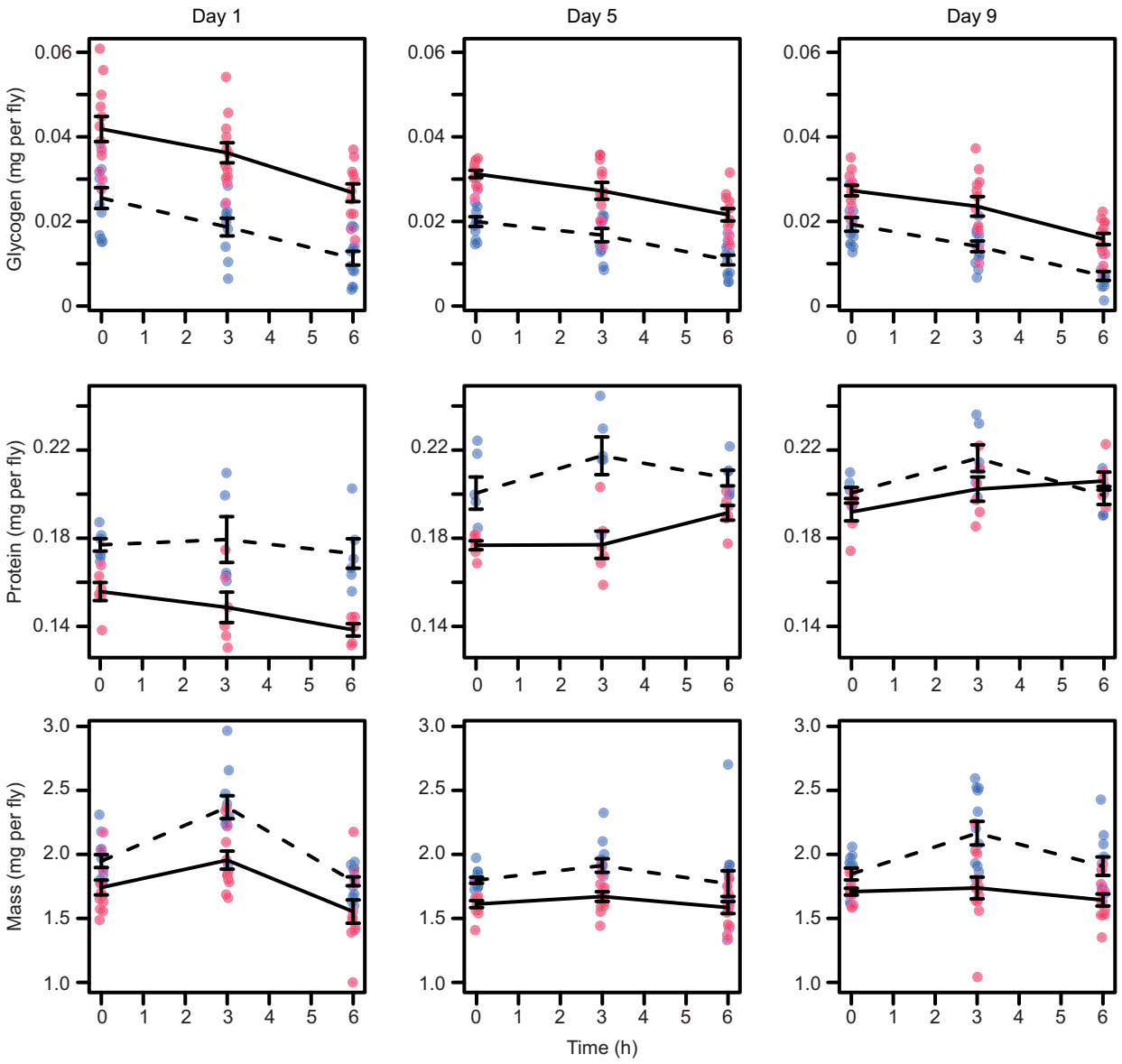

Fig. 3. Mean glycogen levels, protein levels and mass for *D. melanogaster* on a LC (blue) or ST (red) diet on days 1, 5 and 9 (left to right). Points indicate individual assay results, which were performed on multiple flies; the value has been divided to reflect the mean value for individual flies. Dashed (LC) and solid (ST) lines connect the mean values for each diet group; error bars represent s.e.m. Analysis showed: glycogen – three significant two-way interactions between time and mass, diet and day, and day and mass; protein – a significant two-way interaction between diet and day; mass – a significant effect of diet (see Results for more details). *n*=20 for glycogen, *n*=10 for protein and *n*=20 for mass.

more than just these two macronutrients. Changes in the level of yeast in the diet bring changes not only in the level of protein but also in the level of fatty acids, vitamins and minerals.

While carbohydrate is reduced in the LC diet, protein is increased. And thus unsurprisingly the protein content of flies on the LC diet was increased. The pattern over the 9 days somewhat mirrors what we might have expected based on previous data for body mass, which indicated that over the first 5 days, flies in both treatments increase in mass before stabilising (Winwood-Smith et al., 2017), with the LC group accumulating mass more rapidly, but ultimately levelling off at a similar final mass. However, this conclusion conflicts with the mass data in the present study, where mass was stable across days but with some unexpected fluctuations in the LC group, including a large increase at day 1 time 3 h. It is difficult to explain these patterns, and why they conflict with previous data. Because the discrepancy occurs in body mass but not protein, we speculate that it is explained by changes in hydration. There may be some aspects of the experimental protocol that lead to changes in water uptake; however, data from Dethier and Evans (1961) suggest that a fasted fly will not consume a greater quantity of water. The large fluctuations in body mass observed in the present study occurred in the LC flies, which were constrained to a suboptimal diet, so perhaps this leads to altered water uptake patterns.

While there is some uncertainty around flight patterns, and their contribution to glycogen depletion, what is clear is that these flies, which are maintained on diets that result in significantly different glycogen reserves and have significantly different activity patterns, converge on a remarkably similar rate of glycogen depletion. One possible explanation for this is the existence of compensatory responses for insufficient carbohydrate that allow increased flight

activity while conserving critical stores of glycogen. In recent years, our understanding of how animals respond to altered macronutrient content has expanded rapidly, but there is still much we do not know about how animals adjust behaviourally and metabolically to suboptimal diets. Such data are important for our understanding of the ecology of wild species and our own species, for which optimal nutrition is still an active area of inquiry.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: H.S.W.-S., C.E.F.; Methodology: H.S.W.-S.; Formal analysis: H.S.W.-S., C.R.W.; Resources: C.R.W., C.E.F.; Data curation: H.S.W.-S.; Writing - original draft: H.S.W.-S.; Writing - review & editing: H.S.W.-S., C.R.W., C.E.F.; Visualization: H.S.W.-S.; Supervision: C.R.W., C.E.F.; Funding acquisition: C.R.W.

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Table S1: Summary of the model used to analyse activity

```
Linear mixed model fit by maximum likelihood t-tests use
Satterthwaite
  approximations to degrees of freedom [lmerMod]
Formula: activity ~ Diet + Day + time + time:Day +
Diet:Day + Diet:time +
    time:Day:Diet + (1 | Container) + (1 | Run) + (1 |
ID)
   Data: dataFAMds

      AIC       BIC    logLik  deviance  df.resid
-333032.7 -332658.6  166556.4 -333112.7     85280

Scaled residuals:
    Min      1Q  Median      3Q     Max
-4.5020 -0.5467 -0.0512  0.5210  5.6744

Random effects:
 Groups    Name        Variance  Std.Dev.
 ID        (Intercept) 1.508e-03 0.038833
 Container (Intercept) 2.224e-05 0.004716
 Run       (Intercept) 6.122e-05 0.007824
 Residual              1.112e-03 0.033347
Number of obs: 85320, groups:  ID, 1080; Container, 4;
Run, 3

Fixed effects:
                 Estimate Std. Error         df t value
Pr(>|t|)
(Intercept)      2.707e-02  7.215e-03  1.500e+01   3.751
0.001950 **
DietN           -1.070e-02  7.223e-03  1.136e+03  -1.482
0.138631
Day2             2.610e-02  7.223e-03  1.136e+03   3.613
0.000316 ***
Day3             4.205e-02  7.223e-03  1.136e+03   5.822
7.57e-09 ***
Day4             2.649e-02  7.223e-03  1.136e+03   3.667
0.000257 ***
Day5             1.976e-02  7.223e-03  1.136e+03   2.736
0.006320 **
Day6             4.443e-02  7.223e-03  1.136e+03   6.152
1.06e-09 ***
Day7             3.921e-02  7.224e-03  1.136e+03   5.428
6.96e-08 ***
Day8             4.216e-02  7.228e-03  1.136e+03   5.833
7.10e-09 ***
```

```
Day9             6.248e-02  7.228e-03  1.136e+03   8.645
< 2e-16 ***
time             3.239e-03  3.556e-04  8.424e+04   9.106
< 2e-16 ***
Day2:time       -8.429e-04  5.030e-04  8.424e+04  -1.676
0.093786 .
Day3:time       -1.871e-03  5.030e-04  8.424e+04  -3.719
0.000200 ***
Day4:time       -2.263e-04  5.030e-04  8.424e+04  -0.450
0.652827
Day5:time       -1.465e-03  5.030e-04  8.424e+04  -2.913
0.003582 **
Day6:time       -2.034e-03  5.030e-04  8.424e+04  -4.044
5.26e-05 ***
Day7:time        6.496e-04  5.030e-04  8.424e+04   1.292
0.196503
Day8:time       -1.307e-03  5.030e-04  8.424e+04  -2.599
0.009343 **
Day9:time       -7.887e-03  5.030e-04  8.424e+04 -15.681
< 2e-16 ***
DietN:Day2      -9.348e-04  1.022e-02  1.136e+03  -0.092
0.927106
DietN:Day3      -3.005e-02  1.022e-02  1.136e+03  -2.941
0.003334 **
DietN:Day4      -1.218e-02  1.022e-02  1.136e+03  -1.193
0.233293
DietN:Day5       1.180e-03  1.022e-02  1.136e+03   0.116
0.908034
DietN:Day6      -1.618e-02  1.022e-02  1.136e+03  -1.584
0.113379
DietN:Day7      -1.509e-02  1.022e-02  1.136e+03  -1.477
0.139892
DietN:Day8      -5.191e-03  1.022e-02  1.136e+03  -0.508
0.611556
DietN:Day9      -2.628e-02  1.022e-02  1.136e+03  -2.572
0.010246 *
DietN:time       1.005e-03  5.030e-04  8.424e+04   1.998
0.045674 *
DietN:Day2:time -2.561e-03  7.113e-04  8.424e+04  -3.601
0.000317 ***
DietN:Day3:time  1.626e-03  7.113e-04  8.424e+04   2.286
0.022278 *
DietN:Day4:time -8.244e-04  7.113e-04  8.424e+04  -1.159
0.246479
DietN:Day5:time  2.262e-03  7.113e-04  8.424e+04   3.180
0.001475 **
DietN:Day6:time  1.923e-03  7.113e-04  8.424e+04   2.703
0.006870 **
DietN:Day7:time  1.363e-03  7.113e-04  8.424e+04   1.916
0.055357 .
```

```
DietN:Day8:time  7.541e-05  7.113e-04  8.424e+04   0.106
0.915574
DietN:Day9:time  7.427e-03  7.113e-04  8.424e+04  10.441
< 2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1
‘ ’ 1
```

Table S2: Summary of the model used to analyse glycogen

```
Linear mixed model fit by maximum likelihood t-tests use
Satterthwaite
  approximations to degrees of freedom [lmerMod]
Formula:
response ~ diet + time + day + mass.single + diet:day +
mass.single:day +
    mass.single:time + (1 | Run) + (1 | cplate)
   Data: EXP2gly

     AIC      BIC   logLik deviance df.resid
 -2332.0  -2294.8   1177.0  -2354.0      205

Scaled residuals:
     Min       1Q   Median       3Q      Max
-2.46901 -0.72832 -0.00607  0.57957  3.04194

Random effects:
 Groups   Name        Variance  Std.Dev.
 cplate   (Intercept) 2.629e-07 0.0005127
 Run      (Intercept) 5.615e-07 0.0007494
 Residual             9.589e-07 0.0009792
Number of obs: 216, groups:  cplate, 12; Run, 2

Fixed effects:
                   Estimate Std. Error         df t value
Pr(>|t|)
(Intercept)       2.644e-03  1.029e-03  2.241e+01   2.569
0.017373 *
dietN             3.934e-03  2.709e-04  2.188e+02  14.525
< 2e-16 ***
time              3.170e-04  1.946e-04  2.183e+02   1.629
0.104755
day              -3.414e-04  1.101e-04  1.345e+02  -3.100
0.002354 **
mass.single       1.642e-03  4.151e-04  2.173e+02   3.955
0.000104 ***
dietN:day        -1.964e-04  4.491e-05  2.140e+02  -4.373
1.91e-05 ***
day:mass.single   1.069e-04  4.793e-05  2.117e+02   2.231
0.026719 *
time:mass.single -4.214e-04  1.057e-04  2.162e+02  -3.987
9.16e-05 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1
‘ ’ 1
```

Table S3: Summary of the model used to analyse protein

```
Call:
lm(formula = response ~ diet + time + day + mass.single +
time:day +
    diet:day, data = EXP2glysubset)

Residuals:
    Min      1Q  Median      3Q     Max
-6.9473 -2.7949 -0.2395  2.3577 12.0308

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 45.66161    2.05817  22.186  < 2e-16 ***
dietN       -8.46302    1.35500  -6.246 1.01e-08 ***
time        -0.31144    0.27603  -1.128  0.26186
day          0.69796    0.21154   3.299  0.00134 **
mass.single  0.33825    0.77702   0.435  0.66427
time:day     0.08908    0.04625   1.926  0.05689 .
dietN:day    0.68380    0.22590   3.027  0.00313 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1
‘ ’ 1

Residual standard error: 3.826 on 101 degrees of freedom
Multiple R-squared:  0.6602,  Adjusted R-squared:   0.64
F-statistic:  32.7 on 6 and 101 DF,  p-value: < 2.2e-16
```

Table S4: Summary of the model used to analyse mass

```
Call:
lm(formula = response ~ diet + time + day, data =
EXP2gly)

Residuals:
    Min      1Q  Median      3Q     Max
-0.7523 -0.2160 -0.0709  0.0910  3.8743

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  2.063899   0.076074  27.130  < 2e-16 ***
dietN       -0.312971   0.062939  -4.973 1.36e-06 ***
time         0.001931   0.012847   0.150    0.881
day         -0.010197   0.009636  -1.058    0.291
---
```

```
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1
‘ ’ 1

Residual standard error: 0.4625 on 212 degrees of freedom
Multiple R-squared:  0.1088,  Adjusted R-squared:
0.09614
F-statistic: 8.623 on 3 and 212 DF,  p-value: 1.997e-05
```