Dietary Protein and Sugar Differentially Affect Development and Metabolic Pools in Ecologically Diverse Drosophila1–3

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Abstract

We examined the effects of 3 diets differing in their relative levels of sugar and protein on development and metabolic pools (protein, TG, and glycogen) among sets of isofemale lines of 2 ecologically distinct Drosophila species, D. melanogaster and D. mojavensis. Our high protein:sugar ratio diet contained 7.1% protein and 17.9% carbohydrate, the EPS diet was 4.3% protein and 21.2% carbohydrate, and the LPS was only 2.5% protein and 24.6% carbohydrate. Larvae of D. melanogaster, a generalist fruit breeder, were able to survive on all 3 diets, although all 3 metabolic pools responded with significant diet and diet × line interactions. Development was delayed by the diet with the most sugar relative to protein. The other species, D. mojavensis, a cactus breeder ecologically unaccustomed to encountering simple sugars, completely failed to survive when fed the diet with the highest sugar and showed very poor survival even with the diet with equal parts of protein and sugar. Furthermore, the D. mojavensis adult metabolic pools of protein, TG, and glycogen significantly differed from those of D. melanogaster adults fed the identical diet. Thus, considerable within- and between-species differences exist in how diets are metabolized. Given that the genomes of both of these Drosophila species have been sequenced, these differences and their genetic underpinnings hold promise for understanding human responses to nutrition and for developing strategies for dealing with metabolic disease. J. Nutr. 141: 1127–1133, 2011.

Introduction

Although Drosophila have become popular for studying the effects of diet on fitness characteristics such as size and egg production (1,2), lifespan (3), and metabolic pools (4,5), most studies have focused on D. melanogaster. Those studies of D. melanogaster have provided important insights into the control of sugar and lipid metabolism (6,7). The potential of this model organism to help us understand the relationship between human nutrition and metabolic disorders is becoming widely recognized (8,9).

Individual differences in the development of metabolic responses to nutritional regimens are a function of the interaction between genotype and environment, with some families or populations of individuals being more vulnerable to dietary changes than others. Within D. melanogaster, considerable intraspecific variation exists in response to diets differing in levels of sugar and lipid (4), suggesting the presence of genetic variability in different components of the underlying metabolic pathways. Further evidence of major genotypic effects on metabolism is the significant difference observed in metabolic pools of protein, TG, and glycogen among 12 ecologically diverse Drosophila species when reared on identical diets (5). Large species differences most likely reflect the evolution of metabolic adaptations to the ecologically diverse diets utilized by these 12 species; they lead to questions about the evolutionary trajectories of their metabolic pathways. Characterizing the genetic variability in response to different nutritional conditions could prove highly useful to understand the mechanisms underlying metabolic disease and to develop interventions or treatments.

Two ecologically distinct Drosophila species in particular lend themselves to additional study of the metabolic responses to different dietary formulae. D. melanogaster is a fruit generalist in the subgenus Sophophora. Its resource ecology differs dramatically from that of the cactophilic D. mojavensis, which is a member of the subgenus Drosophila, separated by 30–40 million years. The native diet in necrotic cactus consumed by D. mojavensis and other cactophilic Drosophila is poor in nitrogen, phosphorus (10,11), and carbohydrates (12,13) relative to the...
fruit diets of *D. melanogaster* and its relatives. These long-term dietary differences can be expected to produce different metabolic adaptations enabling the flies to deal with the relative abundance or deficiencies of various micro- and macronutrients (14,15). Furthermore, the whole genomes of both species have been sequenced and assembled and many of the genetic tools, such as transgenic stocks, previously restricted to *D. melanogaster*, recently have been developed for *D. mojavensis*, greatly extending the range of manipulative experiments possible for this cactophilic species (16,17).

In the present study, we tested the survival, development time, body mass, and metabolic pools of protein, glycogen, and TG when larvae of both species were reared on 3 diets: high protein:sugar ratio (HPS), equal protein:sugar ratio (EPS), and low protein:sugar ratio (LPS). To determine within-species variation, we tested 5 isofemale lines of each species. Given that *D. melanogaster* is a fruit generalist in nature exposed to higher sugar levels relative to the cactophilic *D. mojavensis*, we predicted that diets higher in sugar (LPS) would more considerably affect the metabolic phenotype of *D. mojavensis*. Surprisingly, not only did *D. mojavensis* fail to survive on LPS, but only a few survived on EPS.

### Materials and Methods

*Drosophila species, isofemale lines, and culture conditions.* Three different diets were prepared: HPS, EPS, and LPS. The diets were composed of sucrose (VWR), active dry yeast (Genesee), yellow cornmeal (Genesee), and agar (Genesee). Ingredients were mixed and boiled and an antifungal composed of methyl paraben (Genesee) dissolved in ethanol (Sigma-Aldrich) was added once the food was cooled to 55°C. The complete composition of the 3 diets can be found in Table 1. Once the antifungal was added, the food was pipetted in 10-mL aliquots to 8-dram vials and allowed to cool until solid. Calorimetry and nutritional analyses were performed for samples of each diet by Exova and were (per 100 g) 452 kJ for the HPS diet, 456 kJ for the EPS diet, and 469 kJ for the LPS diet, as close to isocaloric as was possible. As intended from the recipes, HPS had the greatest amount of protein of the 3 diets (7.1%) and the least carbohydrate (17.9%). The EPS diet was 4.3% protein and 21.2% carbohydrate and the LPS diet was only 2.5% protein and 24.6% carbohydrate. All 3 diets contained 73% moisture and <1% lipid. Vitamin and mineral contents were not assessed in the 3 diets and their potential influence on experimental results, if they did differ among the diets, is unknown.

Five isofemale lines of *D. melanogaster* collected from San Diego County in 2008 were obtained from the Drosophila Species Stock Center located at the University of California San Diego. Five isofemale lines of *D. mojavensis* collected in 2007 in Organ Pipe National Monument, Arizona also were utilized. Several hundred flies from each isofemale line were placed in embryo chambers (Genesee Scientific) with 0.5% agar and a sprinkle of yeast to induce oviposition. The flies were allowed to oviposit for 24 h, after which the first instar larvae were collected and placed in 8-dram vials of HPS, EPS, and LPS (40 larvae/vial). Ten vials for each diet and isofemale line were prepared. For each vial, the times at which the first pupae and first adult flies were observed were recorded. The numbers of emerged female and male flies were recorded each day. For each set of 10 vials per diet and isofemale line, emerging flies were separated by sex, randomly grouped in sets of 5, and frozen at −80°C.

**Metabolic pools assays.** Groups of 5 flies were placed in a 50°C oven and dried for 3 d; 10 groups for each sex, species, and treatment combination were set up. Dry mass was determined with a Cahn Model C-31 microbalance. Dried flies were homogenized in 1 mL of phosphate buffer (25 mmol/L KHPO₄, pH 7.4) then centrifuged for 2 min at 15,871 × g. A total of 800 μL of supernatant was collected and frozen. Centrifugation of homogenates was performed to remove particulates that interfere with the colorimetric assays.

We conducted colorimetric assays for glycogen, TG, and total soluble protein. Glycogen content was measured using the glucose oxidase and peroxidase Enzymes (Sigma-Aldrich P7119) kit with the addition of 0.1 units of amylglucosidase (Sigma-Aldrich) per mL of reaction buffer. Samples (40 μL of homogenate + 200 μL of reaction buffer) were incubated at 37°C for 3 h, absorbance was measured at 445 nm. TG content in each sample was determined using the Triglyceride Reagent set (Pointe Scientific T7531) per the manufacturer’s instructions. We examined TG content rather than total lipid content, which includes such things as cuticle lipids, because our focus was on energy storage compounds. Soluble protein concentration was determined using the bicinchoninic acid assay per the manufacturer’s instructions (Sigma B9643). Each metabolic pool per sample was measured in triplicate. Means of each triplicate were normalized by dry weight before analysis.

**Statistical analysis.** Metabolic pool data were analyzed as a proportion of total dry mass and hence these ratios were arcsine transformed prior to analysis (18). When possible (see Results), the 3 metabolic pools were analyzed per species using a full factorial ANOVA with diet, line, and sex as factors. Comparisons across species were separately conducted for each diet using a nested ANOVA (see Results). A similar analysis was performed for the survival data. The survival data (number of individual eclosed) was square root transformed (18). Developmental time from larvae to first pupae, pupae to eclosion, and first instar larvae to eclosion was analyzed within species using a full factorial ANOVA with diet and line as factors and using a nested ANOVA between species. A principal component analysis was performed using isofemale line means for all 3 metabolic pools. To determine the effects of the different levels of carbohydrates and proteins on metabolic pools, we performed linear regression of each metabolic pool onto the log₁₀(carbohydrate/protein) concentration in the diet. For all statistical tests, α was set at 0.05. All statistical analyses were performed using JMP 8.0 (SAS Institute).

**Results**

**Survival.** The proportion of flies of each species surviving on each diet differed (Fig. 1). Due to counting errors, a few samples were omitted from the analysis. The mean, SE, and sample sizes can be found in Supplemental Table 1. *D. melanogaster* exhibited survival rates between 80 and 96%, with significant effects of isofemale line and sex, but not diet (Table 2). The results with *D. mojavensis* were strikingly different, with no flies surviving when fed the LPS diet, <10% when fed the EPS diet, and just over 40% when fed the HPS diet (Fig. 1). Too few (<2%) *D. mojavensis* survived on the EPS diet to permit us to perform an ANOVA that included diet as a term. With the HPS diet alone, there were no effects of sex, although there was a significant effect of isofemale line on survival (Supplemental

### Table 1 Composition of the 3 experimental diets used

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<th>EPS</th>
<th>LPS</th>
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<td>20</td>
<td>32</td>
</tr>
<tr>
<td>Active dry yeast, g</td>
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<td>8</td>
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<tr>
<td>Yellow cornmeal, g</td>
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<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Distilled H₂O, mL</td>
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<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Granulated agar, g</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Absolute ethanol, mL</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Methyl paraben, g</td>
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<td>0.45</td>
<td>0.45</td>
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<tr>
<td>Total protein:carbohydrate</td>
<td>0.43</td>
<td>0.20</td>
<td>0.10</td>
</tr>
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Abbreviations used: HPS, high protein:sugar ratio diet; EPS, equal protein:sugar ratio diet; LPS, low protein:sugar ratio diet.
Table 2). Of the 2 species, *D. mojavensis* clearly was more sensitive to the EPS and LPS diets; as protein relative to sugar decreased, larvae of this species did not survive. Even with the HPS diet, significant species and isofemale line effects were observed, whereas sex was not significant (Table 3).

**Development time.** Mean, SE, and sample sizes for development are in Supplemental Table 3. Although *D. melanogaster* survival was not affected by diet to the degree as in *D. mojavensis*, the time required for their first instar larvae to reach pupation was significantly delayed by the LPS diet (Fig. 2; Table 4). This delay in *D. melanogaster* larval development translated into an overall delay in adult emergence time of –1 d, attributable to the delay in reaching pupation rather than to the metamorphic period. The typical first instar larva to adult emergence time in *D. melanogaster* reared on either standard cornmeal or banana laboratory culture medium is ~10 d at 24°C (14), similar to what was observed in the HPS and EPS diets. The species differences when fed the HPS diet were significant for development time from first instar larvae to first pupation (*P < 0.001*), time from pupa to eclosion (*P < 0.001*), and overall development time (*P < 0.001*) (Supplemental Table 4). Significant isofemale line differences in development time were observed for *D. mojavensis* fed the HPS diet (Supplemental Table 5).

**Dry mass.** Dry masses observed in *D. melanogaster* reared on the different diets (Supplemental Fig. 1) were significant for all main effects and for the diet × line and diet × sex interactions (diet, *P* = 0.033; line, *P < 0.001; sex, *P < 0.001*) (Supplemental Table 6). Flies of *D. mojavensis* typically are larger than those of *D. melanogaster*, which is reflected in their dry mass in the current study when fed the HPS diet (species, *P < 0.001*) (Supplemental Table 7). Within *D. mojavensis*, dry mass was affected by sex and line (*P < 0.001*) (Supplemental Table 8).

**Metabolic pools.** As with survival, development time, and dry mass, the failure of *D. mojavensis* to survive in sufficient numbers when fed the EPS diet meant that diet effects could be analyzed only for *D. melanogaster* and that species comparisons was seen in *D. melanogaster*. *D. mojavensis* typically requires 12 d to develop from the first instar larva to adult at 24°C (14) when given either standard cornmeal or banana laboratory culture medium. Thus, the delay in larval development was more greatly extended by both experimental diets in *D. mojavensis*. The species differences when fed the EPS diet were significant for development time from first instar larvae to first pupation (*P < 0.001*), time from pupa to eclosion (*P < 0.001*), and overall development time (*P < 0.001*) (Supplemental Table 4). Significant isofemale line differences in development time were observed for *D. mojavensis* fed the EPS diet (Supplemental Table 5).

### Table 2

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<td>14.4***</td>
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<tr>
<td>Total</td>
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</table>

1 Some samples had to be omitted due to counting errors.

2 ***P < 0.05, **P < 0.01, ***P < 0.001.

*FIGURE 1* Survival of male and female adult flies through eclosion for *D. melanogaster* raised on the HPS, EPS, and LPS larval diets and for *D. mojavensis* raised on the HPS and EPS larval diets. Values are in mean adults eclosed per vial ± SE, *n* = 42 (HPS), 41 (EPS), and 42 (LPS) vials for *D. melanogaster* and 50 (*D. mojavensis*, HPS and EPS).

*FIGURE 2* Development times from first instar larvae to first observed pupation and first instar larvae to first eclosed adult fly (overall development) for *D. melanogaster* raised on the HPS, EPS, and LPS larval diets and *D. mojavensis* raised on the HPS and EPS larval diets. Values are means ± SE, *n* = 45 (HPS), 42 (EPS), and 42 (LPS) vials for *D. melanogaster* and 49 (*D. mojavensis*, HPS and EPS).
could be performed only for flies reared on the HPS diet. Means and SE for all 3 pools and dry mass are in Supplemental Table 9 for D. melanogaster and Supplemental Table 10 for D. mojavensis. For total mg protein/mg dry mass for D. melanogaster (Fig. 3A), significant diet and line, but not sex, effects were evident in the ANOVA (Table 5) in addition to several interaction terms. Flies reared on the LPS diet tended to have less total protein/dry mass. For D. melanogaster TG (Fig. 3B), the increasing carbohydrate levels in the EPS and LPS diets were associated with significantly higher total TG:dry mass. Again, the significant main effects were diet and line, but not sex, with just the diet × line and diet × sex terms significant (Table 5). Total glycogen/dry mass data (Fig. 3C) clearly revealed the effect of sex and diet; all main effects and all but 1 interaction term were significant (Table 5). We observed significant regressions between each metabolic pool and the ratio of carbohydrates:protein (in log scale) in the 3 diets (Table 6). In both sexes, a greater carbohydrate:protein diet was associated with greater TG and glycogen concentration and lower protein levels (Table 6).

D. mojavensis total protein, TG, and glycogen in mg/mg dry mass could be measured for only the HPS and EPS diets (Fig. 3A–C). Insufficient numbers of flies surviving when fed the EPS diet prevented ANOVA that included all 3 effects. Isofemale line, sex, and their interactions were significant for flies reared on the HPS diet for all 3 metabolic pools except TG, in which there was no sex effect (Supplemental Table 11). For the HPS diet in D. melanogaster, no sex difference was observed for protein/dry mass, whereas females were higher in glycogen/dry mass and lower in TG:dry mass. In D. mojavensis for the HPS diet, the reverse was observed: males had significantly higher levels of protein/dry mass and glycogen:dry mass, whereas no significant sex differences were observed for TG:dry mass.

Owing to the high mortality of D. mojavensis when fed the EPS and LPS diets, we were able to statistically compare the 2 species for their performance and metabolism only when fed the HPS diet (Fig. 3). Species significantly differed in the levels of protein and glycogen, with D. mojavensis having significantly higher protein/dry mass (Table 7) than D. melanogaster. The opposite was true of glycogen/dry mass, with D. melanogaster having significantly higher levels (Table 7).

In the principal component analysis for D. melanogaster (Fig. 4), the first 2 principal components explained ~84.5% of the variation in protein, TG, and glycogen across the 3 diet treatments in D. melanogaster. The principal components within each diet treatment formed different clusters (Fig. 4). Overall, within D. melanogaster, irrespective of sex and diet treatment, glycogen and TG concentrations were positively correlated (r = 0.40; P = 0.02), whereas no other pair-wise metabolic comparison was observed to be significant.

### Table 4

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<tr>
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1 Development time from first instar larvae to first pupation observed.
2 Development time from first pupation to first eclosed adult.
3 Development time from first instar larvae to first eclosed adult.
4 *P < 0.05, **P < 0.01, ***P < 0.001.

FIGURE 3 Metabolic pool concentration of protein (A), TG (B), and glycogen (C) for D. melanogaster raised on the HPS, EPS, and LPS diets and D. mojavensis raised on the HPS and EPS diets. Values are means ± SE, n = 49 (HPS), 50 (EPS), and 50 (LPS) homogenates of 5 flies for female and male D. melanogaster and 50 homogenates of 5 flies for female and male D. mojavensis (HPS and EPS).

FIGURE 4 Principal component analysis for D. melanogaster on HPS, EPS, and LPS diets.
Discussion

Both species responded to increasing sugar with lengthened development time, but *D. mojavensis* also failed to grow when fed highest sugar diet and its survival when fed the intermediate diet was very low. Development times were significantly affected by increasing sugar relative to protein in both species, largely owing to lengthening of the larval feeding period. While this difference was significant only for the LPS diet in *D. melanogaster*, where pupation was delayed by 1 d; in *D. mojavensis* fed the EPS diet, pupation was delayed by 6 d. When not lethal, diets with greater carbohydrate:protein ratios in *D. mojavensis* required longer larval feeding periods for development to progress to the premetamorphic state. Some minimum level of protein possibly must be consumed before larvae are able to pupate; this minimum appears to be less restrictive for *D. melanogaster."

Alternatively, the levels of sugar in the EPS and LPS diets could have been toxic to *D. mojavensis*. We are unable to distinguish between these 2 alternatives. Although life history data indicate that in most organisms, longer growth periods result in larger offspring (19,20), the opposite trend was observed here. The uncoupling of growth development period with final size may

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**TABLE 6** Results of regression analysis of each metabolic pool onto the log(carbohydrate:protein) for each sex in *D. melanogaster*

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<tr>
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1 *P < 0.05, **P < 0.01, ***P < 0.001.

---

**TABLE 7** Nested ANOVA of the concentration of metabolic pool (protein, TG, and glycogen) among isofemale lines of *D. melanogaster* and *D. mojavensis* reared on HPS

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>F ratio1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>1</td>
<td>0.104</td>
<td>102***</td>
</tr>
<tr>
<td>Sex</td>
<td>1</td>
<td>0.004</td>
<td>3.90*</td>
</tr>
<tr>
<td>Line (species)</td>
<td>8</td>
<td>0.062</td>
<td>7.50***</td>
</tr>
<tr>
<td>Sex × line (species)</td>
<td>8</td>
<td>0.020</td>
<td>2.50*</td>
</tr>
<tr>
<td>Error</td>
<td>179</td>
<td>0.183</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>197</td>
<td>0.373</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
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<td>0.002</td>
<td>2.10</td>
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<tr>
<td>Sex</td>
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<td>0.001</td>
<td>0.600</td>
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<tr>
<td>Line (species)</td>
<td>8</td>
<td>0.322</td>
<td>35.7***</td>
</tr>
<tr>
<td>Sex × line (species)</td>
<td>8</td>
<td>0.058</td>
<td>6.40***</td>
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<tr>
<td>Error</td>
<td>179</td>
<td>0.202</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>197</td>
<td>0.584</td>
<td></td>
</tr>
<tr>
<td>Glycogen</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
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<td>0.019</td>
<td>110***</td>
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<tr>
<td>Sex</td>
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<td>0.001</td>
<td>1.30</td>
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<tr>
<td>Line (species)</td>
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<td>0.047</td>
<td>33.6***</td>
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<tr>
<td>Sex × line (species)</td>
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<td>0.005</td>
<td>3.60***</td>
</tr>
<tr>
<td>Error</td>
<td>179</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>197</td>
<td>0.104</td>
<td></td>
</tr>
</tbody>
</table>

1 *P < 0.05, **P < 0.01, ***P < 0.001.
reflect an inability to acquire sufficient nutrients from the low-protein diets, even with prolonged larval feeding periods.

Both species also responded to increased dietary sugar (decreased protein) in the same way: less total protein and higher TG and glycogen relative to body mass (Table 6). The relatively greater amount of protein found in newly emerged *D. mojavensis* compared with *D. melanogaster* was noted in a previous study performed using a standard laboratory diet (5) as well as a similarity between species in their glycogen concentration. Unlike previous studies (5), we did not observe species differences in TG; this is likely a result of differences in food media between the 2 studies as well as a significantly large isofemale line effect on TG concentration observed in this study (Table 5; Supplemental Table 11).

The low survival of *D. mojavensis* on diets other than the protein-rich HPS diet relative to *D. melanogaster*, which survived mostly equally on all diets, is consistent with their different ecologies and thus with the metabolic processes underlying adaptation to these diets. Both species feed and breed in necrotic plant material, consuming the microbes involved in decomposing their resources in addition to the rotting tissue itself. *D. melanogaster* is a generalist found worldwide that consumes a wide range of rotting fruits and some plants. It is expected that this species would routinely encounter diets high in sugar. A recent arrival to the New World from Africa (21,22), *D. melanogaster*’s dietary flexibility has enabled it to invade a wide range of habitats. On the other hand, *D. mojavensis* is a cactophilic species restricted to the deserts of North America (23,24). Relative to the fruits consumed by *D. melanogaster*, cacti are low in available carbohydrates (12,13) and thus *D. mojavensis* is not expected to frequently encounter in nature fruit sources high in sugar content. The probability of colonizing new areas with nutritionally variable resources is thus less likely for *D. mojavensis* and could, under conditions of extreme global change or habitat destruction, lead to its extinction.

Genetic variability for response to such different diets is reflected in the genotype × environment interactions. Isofemale lines differ in their metabolic profiles and in their ability to survive on different foods. This high level of genotype and genotype × environment interactions for components of metabolism was observed in a recent study using 146 inbred *D. melanogaster* lines (4). Intraspecific genetic variability for metabolic enzymes, in the form of clines that follow environmental gradients, is well known for *D. melanogaster* (25). Sex differences in metabolic responses to dietary change are not unexpected. Sexually mature female *Drosophila* undergoing oogenesis provision their ovarian oocytes with carbohydrates to support embryogenesis. Although significant line (genotype) effects were observed, our data shows that differences in the carbohydrate:protein ratio of larval diets produce not only significant effects on individual metabolic pools (Fig. 3) but also on the overall metabolic state of *D. melanogaster* (Fig. 4). Although larval diets did not have a significant effect on survival to eclosion, adult diets differing in their carbohydrate:protein ratio have significant life history consequences (26,27). Increasing levels of carbohydrates relative to protein in the diets of *D. melanogaster* adults tends to maximize longevity, although fecundity is maximized as protein content in the diet increases (26).

In our study, we observed an overall reduction in glycogen and TG as a response to a low-carbohydrate diet (Table 6). Similarly, diets in humans that drastically restrict carbohydrate intake have been shown to reduce glycogen and TG levels (28). Some human populations have responded to rather sudden increases in dietary carbohydrates by developing metabolic disorders such as type 2 diabetes (29). In this regard, species such as *D. mojavensis*, which are unaccustomed to dietary sugar, may provide an informative model system for addressing metabolic responses to novel high-carbohydrate foods.

**Acknowledgments**

L.M.M. and T.A.M. designed the research; S.J., C.P., and G.B. conducted the research; L.M.M. analyzed data; L.M.M. and T.A.M. wrote the paper; and T.A.M. had primary responsibility for final content. All authors read and approved the final manuscript.

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