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Quantifying Interactions Within the NADP(H) Enzyme Network in *Drosophila melanogaster*

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ABSTRACT

In this report, we use synthetic, activity-variant alleles in *Drosophila melanogaster* to quantify interactions across the enzyme network that reduces nicotinamide adenine dinucleotide phosphate (NADP) to NADPH. We examine the effects of large-scale variation in isocitrate dehydrogenase (IDH) or glucose-6-phosphate dehydrogenase (G6PD) activity in a single genetic background and of smaller-scale variation in IDH, G6PD, and malic enzyme across 10 different genetic backgrounds. We find significant interactions among all three enzymes in adults; changes in the activity of any one source of a reduced cofactor generally result in changes in the other two, although the magnitude and directionality of change differs depending on the gene and the genetic background. Observed interactions are presumably through cellular mechanisms that maintain a homeostatic balance of NADPH/NADP, and the magnitude of change in response to modification of one source of reduced cofactor likely reflects the relative contribution of that enzyme to the cofactor pool. Our results suggest that malic enzyme makes the largest single contribution to the NADPH pool, consistent with the results from earlier experiments in larval *D. melanogaster* using naturally occurring alleles. The interactions between all three enzymes indicate functional interdependence and underscore the importance of examining enzymes as components of a network.

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IN traits determined by a network of gene products, the phenotype is a function of the alleles present and of the relative contributions of individual network member genes. Since selection is on phenotype, the total composite genotype, not just individual loci, determines the fitness of an organism. In establishing the connection between genotype and phenotype for such networks, the first challenge is to quantify the relative contribution of each member of the network to the endpoint phenotype. By addressing function on a network-wide basis, interactions and interconnections that may not be apparent in individual gene examinations can be determined (Proulx *et al.* 2005).

In most organisms, reduction of the cofactor nicotinamide adenine dinucleotide phosphate, or NADP, to NADPH is primarily the function of four enzymes: cytosolic malic enzyme (MEN), cytosolic isocitrate dehydrogenase (IDH), and the two oxidative enzymes of the pentose shunt, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate (6PGD; recently reviewed in Ying 2008). In larval *Drosophila melanogaster*, MEN produces ∼30% of the available NADPH, IDH ∼20%, and G6PD and 6PGD the remaining ∼40% (Geer *et al.* 1979a,b). It is believed that these four enzymes interact to maintain the NADP/NADPH balance and supply of reducing power for lipogenesis and antioxidation (Geer *et al.* 1976, 1978, 1981; Wilton *et al.* 1982; Bentley *et al.* 1983; Geer and Laurie-Ahlberg 1984; Merritt *et al.* 2005; Pollak *et al.* 2007; Singh *et al.* 2007; Ying 2008). Dietary induction studies and observations of natural genetic variation have found connections between MEN activity and the activities of the pentose shunt enzymes to be generally straightforward and compensatory; reductions in one lead to increases in the other. The interactions involving IDH activity, however, have been found to be more complicated and at times counterintuitive; reductions in reducing power sometimes lead to decreases in IDH activity.

In an earlier study (Merritt *et al.* 2005), we quantified the impact of genetic variation in *Men* activity on IDH and G6PD activities and triglyceride (a strong correlate with total lipid; Clark and Keith 1989) concentration. 6PGD was not independently assayed because earlier works suggest that G6PD and 6PGD activities are highly correlated, likely because of their coupled function in the pentose shunt (Wilton *et al.* 1982). We examined both naturally occurring *Men* alleles and synthetic alleles created by *P*-element excision and found significant

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associations between MEN activity and induction of the activities of both IDH and G6PD. The apparent interactions between MEN and IDH and G6PD across these 10 different third chromosome lines were quantified as mean elasticity coefficients: $\epsilon_{MEN}^{G6PD} = -0.76 \pm 0.236$ and $\epsilon_{MEN}^{IDH} = -0.88 \pm 0.208$. Because MEN activity was reduced by 20%, both IDH and G6PD activity varied in a compensatory direction, increasing almost 1:1 with the decrease in MEN.

The significant change in enzyme activity of two members of the NADPH network in response to our genetic reduction of the activity of a third strongly suggests that a physiological mechanism coregulates the three enzymes. Such functional interdependence would mean that individual members of the network do not act in isolation and should be examined collectively, not as isolated units. In this study, we characterize the effects of the independently varying activity levels of IDH, G6PD, and MEN on the activity of each other and triglyceride concentration in adult flies. We found significant responses to changes in all three enzymes, although the responses to genetic changes in IDH and G6PD were generally small; variation in MEN caused the greatest changes in the other enzymes.

MATERIALS AND METHODS

Stocks: The background replacement line *w;VT83;TM3,Sb/Dr* was constructed in the Eanes lab. Line *w;6326;6326* is a sub-line of the isogenic line BG6326 (Hoskins *et al.* 2001) from the Indiana University Stock Center. The 10 iso-third chromosome wild lines are descended from the lines collected by Brian Verrelli in 1997 in the eastern United States (Sezgin *et al.* 2004). Stocks and all crosses were maintained on standard cornmeal food at 25°.

Line construction and characterization: Two series of *Idh* and *G6pd* enzyme activity-variant alleles were created using *P*-element excision. In construction of both series of synthetic alleles, excision chromosomes containing variant alleles were identified by visible markers (white for *Idh* and yellow for *G6pd*) and unusual enzyme activity and then crossed into the *w;6326;6326* background using marker-assisted introgression (Merritt *et al.* 2005, 2006). Chromosomes in either series are essentially isogenic, differing only in a small region at the point of *P*-element excision. The *P{Δ2-3}99B* element (Bloomington Drosophila Stock center line 2030) was used as a transposase source in both cases (Robertson *et al.* 1988). The matched set of third chromosomes varying in IDH activity was created using the *P{EP}EP3729* insertion line. The *P{EP}EP3729* insertion is at 3L:8354083, 51 bp upstream from the start of the longest *Idh* transcript (CG7176-PH). Knockout mutations derived from this far 5′ element most likely knock out all possible *Idh* gene splice products. The set of *X* chromosomes varying in G6PD activity was created using the *P{SUPor-P}KG05538* insertion line. The *P{SUPor-P}KG05538* insertion is 37 bp upstream from the start of the longest *G6pd* transcript. Background-replaced lines were retested for enzyme activity. Previous work using *P*-element-excision-derived alleles found that some allelic combinations led to nonadditive activities (Merritt *et al.* 2005). To test for such deviation from additivity, individual lines within a matched set were crossed to create heterozygotes, and heterozygote activity was compared to activities predicted from the individual alleles. The excision sites of the *Idh* and *G6pd* synthetic alleles were characterized using a combination of short- and long-range PCR and DNA sequencing. A matched set of chromosomes varying in MEN activity, which was created in similar fashion to the *Idh* and *G6pd* alleles and placed in the same *w;6326;6326* background, has already been described (Merritt *et al.* 2005).

Large- and small-scale variation in IDH and G6PD activity: Experiment 1 examined the effect of genetically varying either IDH or G6PD activity with the activity of the other NADPH-producing enzymes and triglyceride concentration. Lines from a matched set were crossed to generate allelic combinations that vary in levels of IDH or G6PD activity. For example, *w*; 6326; *Idh*Δ12/*Idh*Δ26 flies were crossed with *w*/Y; 6326; *Idh*Δ1/TM8B, *Sb* flies, and two genotypes were recovered: *w*; 6326; *Idh*Δ12/*Idh*Δ1 and *w*; 6326; *Idh*Δ26/*Idh*Δ1. Approximately 100 virgin female flies and an equal number of males were placed in 250-ml specimen bottles with standard cornmeal food and allowed to lay eggs for 24 hr. These parental flies were transferred to new bottles at 24-hr intervals for 6–8 days. The flies collected during the 2 days (in bottles) with the largest number of emerging adults were used in the analysis. In this common garden design, all test genotypes develop and emerge in the same environment (the bottles), eliminating the confounding of genotype and environmental effects (differences between bottles or vials). Individual flies were aged for 5 days after emergence and then weighed and analyzed for MEN, IDH, and G6PD activity as well as for total triglycerides and protein. Individual flies were genotyped by plotting activity by weight (see Merritt *et al.* 2006).

Variation against 10 different third chromosome backgrounds: We were interested in determining if response to enzyme activities or triglyceride concentration would be consistent in different genetic backgrounds. In experiment 2, a series of 20 paired crosses were made using the *Idh*, *G6pd*, or *Men* full-activity and null-deletion genotypes and 10 iso-third chromosome lines (second background-replaced lines designated *w;6326;i/TM8*). Females from each of 10 iso-third chromosome lines were crossed with males of the excision allele lines. For example, *w/Y*; 6326; *Idh*Δ1/*TM8*, *Sb* and *w/Y*; 6326; *Idh*Δ4/*TM8*, *Sb* were independently crossed with *w*; VT83; *i*/TM8, *Sb*, where *i* is chromosomes 1–10. Two genotypes were collected and compared: *w/Y*; 6326/VT83; *Idh*Δ1/*i* (50% IDH activity) and *w/Y*; 6326/VT83; *Idh*Δ4/*I* (100% IDH activity). *TM3*, *Sb* balanced flies were discarded. Two vials (five females and five males per vial) were established for each cross genotype, and these were transferred once after 4 days, for a total of four vials per genotype. Emerging flies were collected, aged for 5 days, and then weighed and assayed for MEN, IDH, and G6PD activity, total triglycerides, and protein.

Enzyme activity: Flies were homogenized in a grinding buffer (100 mM Tris–HCL, 0.15 mM NADP, pH 7.4), 1 fly/200 μl, and centrifuged at 13,000 rpm at 4° for 5 min to pellet all solids. The supernatant was collected and vortexed, and 250 μl was added to one well of a 96-well plate. Aliquots were taken from this source plate for each of the analyses. Activity assays were conducted on a Molecular Designs SpectraMax 384 Plus 96-well-plate spectrophotometer, using 10 μl of extract and 100 μl of buffer. In experiments involving very low G6PD activity, G6PD activity assays were conducted using 20 μl of fly extract and 100 μl of assay buffer. Absorbance was measured every 9 sec over 3 min at 25°. Samples were assayed twice and the means were used in further analysis. The assay buffers for the three enzymes assayed in this study were optimized to give maximum activity (Merritt et al. 2005) and were as follows: MEN—100 mM Tris–HCL, 0.34 mM NADP, 50 mM $MnCl_2$, 50 mM malate, pH 7.4; G6PD—100 mM Tris–HCL, 0.32 mM NADP, 3.5 mM D-glucose-6-phosphate,

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TABLE 1

Four *Idh* excision alleles, their molecular changes, and IDH activity

Idh excision allele	Molecular phenotype	Units of activity (±SE)[a]	Relative activity[b]
Δ*1*	Three single-base changes in first 100 bp of transcript	0	0
Δ*12*	1887-bp deletion replaced by TGTTATTTCATCATG upstream of start codon	1.10 (0.233)	0.24
Δ*4*	Perfect excision	4.52 (0.437)	1.00
Δ*26*	26-bp insertion 60 bp from the beginning of the *Idh* message	7.44 (0.531)	1.64

[a] Units are 10× nmol NADP/min/μg protein.
[b] Relative to normal activity allele, Δ*4*.

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pH 7.4; and IDH—100 mM Tris–HCL, 0.10 mM NADP, 0.84 mM $MgSO_4$, 1.37 mM DL-isocitrate, pH 8.6.

In experiment 1, enzymatic activities were expressed as 10× nmol NADP reduced per minute per fly. In experiment 2, enzymatic activities were graphed as 10× nmol NADP reduced per minute per microgram soluble protein to allow calculations of elasticity coefficients and comparison with earlier studies (Merritt *et al.* 2005).

Soluble triglyceride content: Soluble triglyceride concentration was measured using a commercially available kit (Infinity Triglyceride Assay, Thermo Electron, Arlington, TX) following the manufacturer's suggested protocol. The assay reactions contained 10 μl homogenate and 100 μl reagent and were incubated at 37° for 10 min. The OD_{500} of each reaction was quantified and total soluble triglyceride concentrations were determined by comparison with a commercially available standard (Sigma-Aldrich, St Louis). Each sample was assayed twice and the mean was used.

Soluble protein content and fly weight: Soluble protein concentration was measured by the Bradford (1976) assay using a commercial kit (Bio-Rad, Hercules, CA) following the manufacturer's suggested protocol. The OD_{595} of each reaction was quantified, and total soluble protein concentrations were determined by comparison with bovine serum albumin standards (Sigma-Aldrich). Individual flies were weighed to the nearest 0.01 mg using a Cahn Instruments C-32 microbalance.

Data analysis: Crosses were replicated in four vials or in two bottles and each vial or bottle was sampled twice. Initial analyses of covariance (ANCOVAs) were conducted to determine the presence or absence of a genotype effect, and Tukey's Honestly Significant Difference multiple-comparison tests (Tukey's HSD tests) were conducted to determine whether or not individual crosses could be grouped within genotype classes. Grouped data were analyzed using ANCOVA in which the activity of the modified gene is the independent variable and the activities of the other NADPH enzymes and triglyceride concentration are the dependent variables, using weight and protein as covariates. Weight accounts for differences in body size, and soluble protein content controls for differences in the degree of homogenization between samples. Ratio data were normalized by log transformation. Statistical analyses were conducted using the JMP 7.0 software (SAS Institute). In all cases, males and females were assayed in independent blocks. In cases in which ANCOVA indicated significant differences in enzyme activities, elasticity coefficients were calculated as the average of the slopes of the plots of $\ln E_2$ *vs.* $\ln E_1$ for each of the 10 different third chromosome backgrounds, where E_1 is the enzyme activity that we genetically modified and E_2 is one of the other NADPH enzymes. In calculating elasticity coefficients, enzyme activity was expressed as 10× nmol NADP reduced per minute per microgram soluble protein to allow for comparison with earlier studies.

RESULTS

Characterization of *Idh* and *G6pd* alleles: *Idh* or *G6pd* alleles, varying in IDH or G6PD activity, were created by *P*-element excision (Tsubota and Schedl 1986; Salz *et al.* 1987). Each set of alleles is created from an identical chromosome, and after replacement of the *X* and second chromosomes (*Idh* set) or the second and third chromosomes (*G6pd* set), the genetic backgrounds within each set are entirely isogenic except for a small region at the site of *P*-element excision.

Idh alleles spanning 0 to 1.64× normal IDH activity were recovered after mobilization of the *P*-element excision. Null alleles were homozygous lethal. Four excision alleles—*Idh*Δ*1* (null), *Idh*Δ*12* (low activity), *Idh*Δ*4* (normal activity), and *Idh*Δ*26* (high activity)—were selected for the experimental set. Approximately 2 kb flanking the *P*-element insertion site of each allele was sequenced, and the results and relative IDH activities of alleles after genetic background replacement are listed in Table 1. All annotation is based on the *D. melanogaster* genome sequence (Adams *et al.* 2000; http://www.fruitfly.org).

G6pd alleles that spanned zero to full G6PD activity were recovered, and all were homozygous viable. Five alleles—*G6pd*Δ*47* (zero G6PD activity), *G6pd*Δ*3*, *G6pd*Δ*53*, *G6pd*Δ*6* (all low activity), and *G6pd*Δ*10* (normal activity)—were selected, and 2 kb flanking the insertion site of each allele was sequenced. The results along with activities are presented in Table 2.

Our previous work with *Men* has shown that combinations of excision alleles do not always produce additive enzyme activities, possibly a function of transvection (Merritt *et al.* 2005). To test for a possible lack of additivity, we created excision allele heterozygotes for both the *Idh* and *G6pd* alleles and compared the observed enzyme activity of the heterozygotes to that

TABLE 2

Five *G6pd* excision alleles, their molecular changes, and G6PD activity

G6pd excision allele	Molecular phenotype	Units of activity (±SE)[a]	Relative activity[b]
Δ*47*	637-bp deletion removing 5′-UTR and the first exon	0	0
Δ*3*	45-bp *P*-element fragment at original insertion site	0.42 (0.020)	0.20
Δ*53*	63-bp deletion and a 23-bp *P*-element fragment at insertion site	0.49 (0.063)	0.23
Δ*6*	241-bp *P*-element fragment remaining at insertion site	0.53 (0.044)	0.25
Δ*10*	286-bp *P*-element fragment remaining at insertion site	2.13 (0.069)	1.00

[a] Units are 10× nmol NADP/min/μg protein.
[b] Relative to normal activity allele, Δ*10*.

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predicted by a simple additive combination of the observed homozygous activity alleles (data not shown). The observed G6PD activities were slightly lower than predicted, but in neither the *Idh* nor the *G6pd* set did we see the strong nonadditivity previously observed with the *Men* excision alleles (Merritt *et al.* 2005).

Experiment 1—effects of large-genotypic differences in IDH and G6PD activity: Experiment 1 examined the effects of variation in IDH or G6PD on the activity of the other NADP-reducing enzymes and triglyceride concentration. As in our earlier study (Merritt *et al.* 2005), 6PGD was not independently assayed because earlier works suggest that G6PD and 6PGD activities are highly correlated (Wilton *et al.* 1982). Multiple genotypes were grown in a single bottle and individuals were assigned to a genotype class using plots of enzyme activity against weight; see, *e.g.*, Figure 1. Segregating genotypes were easily distinguished in all cases.

Experiment 1A—a large reduction in IDH activity results in small changes in MEN and G6PD activity and triglyceride concentration: Flies with low IDH activity (*Idh*Δ*1*/*Idh*Δ*12*; 10% wild-type activity) were compared with flies with intermediate activity (*Idh*Δ*1*/*Idh*Δ*26*; 80%). While the complete knockout allele, *Idh*Δ*1*, was homozygous lethal, the low activity flies were fully viable and fertile, and we found no difference in average size (in weight) between the two genotype classes. In males, there was significant dependence of MEN and G6PD activity and triglyceride concentration on *Idh* genotype (Figure 2; $F_{1,167} = 4.24$ and $P < 0.04$, $F_{1,167} = 8.61$ and $P < 0.004$, and $F_{1,167} = 8.78$ and $P < 0.004$, respectively). G6PD activity was 5% higher in the low IDH genotypes. In contrast, MEN activity decreased 5% as IDH activity decreased. Triglyceride concentration was reduced 14% in the low activity genotype. Similar interaction patterns were observed in females, although only the effects on

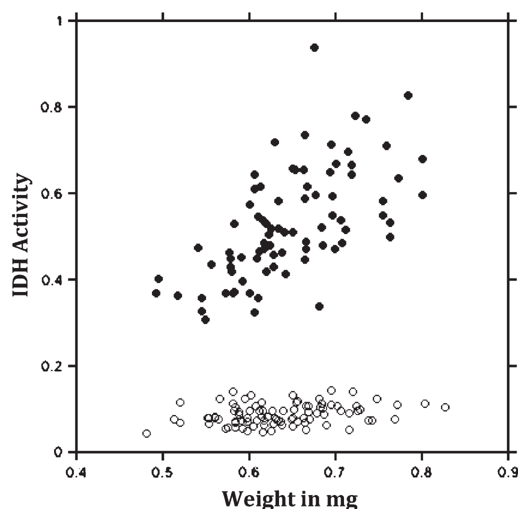

Figure 1.—Example of the weight and IDH activities of male adult flies emerging from a single culture bottle during a 24-hr period in experiment 1A. The assigned activity genotypes are shown: *Idh*Δ*1*/*Idh*Δ*12*, open circles; *Idh*Δ*1*/*Idh*Δ*26*, solid circles.

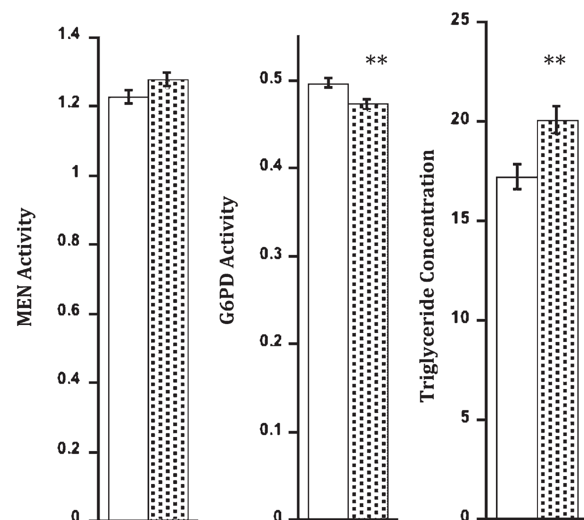

Figure 2.—Experiment 1A: plot of MEN activity, G6PD activity, and triglyceride levels for inferred genotypes of 5-day-old adult male flies with 10% wild-type IDH activity (*Idh*Δ*1*/*Idh*Δ*12*, open bar) and 80% wild-type activity (*Idh*Δ*1*/*Idh*Δ*26*, dotted bar). Enzyme activity units are in 10× nmol NADP reduced/min (mean ± 1 SE). Triglyceride levels are in micrograms per fly (mean ± 1 SE). Statistical significance is indicated by (*) for $P < 0.05$ and (**) for $P < 0.01$; exact *P*-values are given in the text.

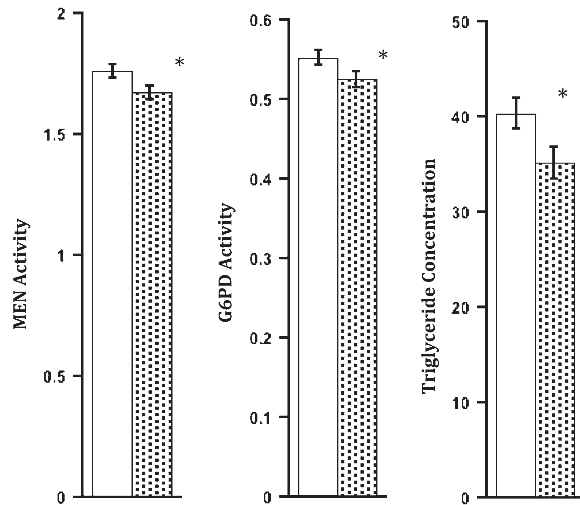

FIGURE 3.—Experiment 1B: plot of MEN activity, G6PD activity, and triglyceride levels for inferred genotypes of 5-day-old adult male flies with 90% wild-type IDH activity (*Idh*Δ*12/Idh*Δ*26*, open bar) and 160% wild-type activity (*Idh*Δ*26/Idh*Δ*26*, dotted bar). Units are as in Figure 2.

G6PD and triglyceride concentration were statistically significant ($F_{1,157} = 4.93$, $P < 0.03$; $F_{1,157} = 14.22$, $P < 0.0002$).

Experiment 1B—increased IDH activity above normal levels results in small changes in MEN and G6PD activities and triglyceride concentration: *Idh*Δ*12/Idh*Δ*26* flies with 90% wild-type IDH activity and *Idh*Δ*26/Idh*Δ*26* flies with 160% activity were compared. In males, MEN and G6PD activity and triglyceride concentration all significantly differ between the *Idh* genotypes (Figure 3; $F_{1,166} = 5.12$ and $P < 0.03$, $F_{1,166} = 4.00$ and $P < 0.04$, and $F_{1,166} = 5.08$ and $P < 0.03$, respectively). G6PD and MEN activities were 6% lower in the high IDH activity genotype. Triglyceride concentration was reduced by 15% in the high IDH activity flies. Similar patterns were observed in females, although none were statistically significant.

Experiment 1C—elimination of G6PD activity causes a small increase in MEN activity but not in IDH activity: Zero G6PD activity flies, *G6pd*Δ*47/Y*, and 30% activity flies, *G6pd*Δ*53/Y*, were compared in males. Zero activity flies, *G6pd*Δ*47/G6pd*Δ*47*, and 50% activity flies, *G6pd*Δ*47/G6pd*Δ*10*, were compared in females. In males, MEN activity was 7% higher in the null G6PD male genotypes than in the intermediate activity genotype (data not shown; $F_{1,165} = 4.55$, $P < 0.03$) while IDH activity and triglyceride concentration were not significantly different. In females, MEN activity was 10% higher in the zero G6PD activity genotype ($F_{1,161} = 6.66$, $P < 0.01$), while the variation in IDH activity or triglyceride concentration was not significant.

Experiment 1D—G6pd genotypes with smaller reductions in activity also show significant increases in MEN activity: Thirty-percent activity flies, *G6pd*Δ*53/Y*, and normal activity flies, *G6pd*Δ*10/Y*, were compared in males. Sixty-percent activity flies, *G6pd*Δ*53/G6pd*Δ*10*, and normal activity flies, *G6pd*Δ*10/G6pd*Δ*10*, were compared in females. In males, MEN activity was 7% higher in the low G6PD activity flies than in the high G6PD activity flies (data not shown; $F_{1,170} = 7.22$, $P < 0.008$), while IDH activity and triglyceride concentration were not significantly different. Female intermediate and normal G6PD activity flies showed no significant difference in levels of MEN and IDH activity or in triglyceride concentration.

Experiment 2—comparison of interlocus effects of genotypic variation in IDH, G6PD, or MEN activity across 10 third chromosome backgrounds: Experiment 2 was designed to determine and compare the response to changes in IDH, G6PD, and MEN activity across different genetic backgrounds. We placed normal and half activity *Idh*, *G6pd*, and *Men* genotypes in a common *X*, common second, but 10 different third chromosomes. We found that variation in IDH and G6PD had limited effects on the activities of the other enzymes and that there was no significant impact on triglyceride concentration. When significant differences were observed, elasticity coefficients, which quantify the magnitude of change in relation to the magnitude of variation in the controlled gene, were calculated (KASCER and PORTEOUS 1987; FELL 1996). The elasticity coefficients were used to compare the effects of variation in IDH, G6PD, and MEN. In general, changes in MEN resulted in greater (~4×) effects than changes in either IDH or G6PD.

Experiment 2A—variation in IDH activity is associated with variation in both MEN and G6PD activity: The null *Idh* allele heterozygotes (*Idh*Δ*1/i*) averaged 54% lower IDH activity compared to the full activity allele heterozygotes (*Idh*Δ*4/i*) (Figure 4). MEN activity was slightly reduced in the low IDH activity genotypes in both sexes (males: 8%, $F_{1,9.28} = 5.59$, $P < 0.04$; females: 3%, $F_{1,10.67} = 7.29$, $P < 0.02$). The reduction in MEN activity with a reduction in IDH activity is consistent with experiment 1A and other reports (BENTLEY *et al.* 1983; MERRITT *et al.* 2005). G6PD activity was 5% higher in the low IDH activity genotype for females ($F_{1,10.42} = 9.47$, $P < 0.01$), but not for males. G6PD activity was higher in the low IDH activity male genotypes for 8 of the 10 third chromosome backgrounds, although this was not statistically significant. There was no significant effect of *Idh* genotype on triglyceride concentration. The elasticity coefficient for MEN was 0.04 in both males and females (the positive coefficient reflects the parallel reduction in MEN with IDH) and was −0.07 for G6PD in females.

Experiment 2B—reduction in G6PD activity increases both MEN and IDH activity across all chromosomes: Males with the knockout *G6pd* allele (*G6pd*Δ*47/Y; 6326/VT83;6326/i*) possess no G6PD activity and ~2% (background levels) the activity of males with a normal allele (*G6pd*Δ*10/Y; 6326/VT83;6326/i*) (Figure 5). Female knockout heterozygotes (*G6pd*Δ*47/w;6326/VT83;6326/i*) averaged 43%

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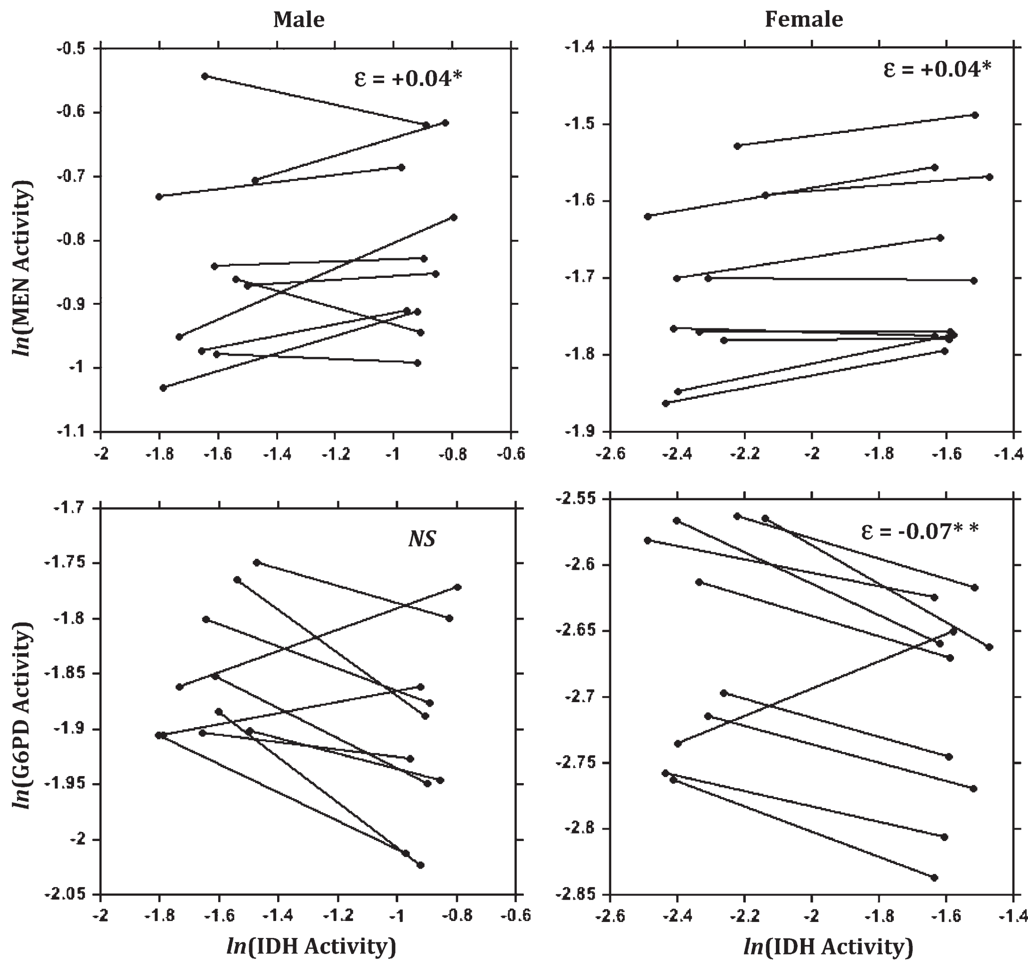

FIGURE 4.—Experiment 2A: comparison in response to changes in IDH activity across 10 different third chromosome lines. Two synthetic *Idh* alleles, one knockout, and one with normal IDH activity were crossed to 10 different third chromosome lines to produce full or partial IDH activity flies. Each pair of points, connected by a line, shows the difference in MEN or G6PD activity or triglyceride concentration in a different third chromosome background. Values from the full activity flies are on the right end of the line and those from the partial activity flies are on the left end of the line. Enzyme activity units are in 10× nmol NADP reduced/min/μg protein. Log values are plotted so that the slope of each line is an estimate of the elasticity coefficient (ε) in each line (FELL 1996). Elasticity coefficients were calculated when an ANCOVA indicated significant interactions between enzymes across the 10 different third chromosome genetic backgrounds and are shown in the top right corner of each graph. Statistical significance is indicated by (*) for $P < 0.05$ and (**) for $P < 0.01$; exact *P*- values are given in the text.

the activity of the normal activity allele heterozygotes (*G6pd*Δ*10/w;6326/VT83;6326/i*). MEN activity was 10% higher in the male *G6pd* knockout heterozygotes and 4% higher in females ($F_{1,9.74} = 13.61$, $P < 0.0005$ and $F_{1,18.53} = 5.42$, $P < 0.03$, respectively; note the genotype-dependent reduction of G6PD activity is much greater in males). IDH activity was also higher in the low G6PD activity genotypes in females (3%; $F_{1,9.56} = 11.50$, $P < 0.004$), but not in males. There were no significant *G6pd* excision allele effects on triglycerides. In females, the elasticity coefficient for MEN was −0.07, and the coefficient for IDH was −0.05. Elasticity coefficients were not calculated for males because the *X*-linked nature of the gene prevents comparison across reasonably small changes in G6PD activity in males.

Experiment 2C—genetic variation in MEN activity affects both IDH and G6PD activity: Across chromosome backgrounds, the knockout *Men* allele (*Men*Δ*9/i*) heterozygotes averaged ∼80% the activity of the normal *Men* activity heterozygotes (*Men*Δ*3/i*) in both males and females (Figure 6). The high enzyme activity (>50%) in the heterozygous flies is consistent with the earlier study of these alleles suggesting transvection effects (MERRITT *et al.* 2005). IDH activity was increased in the low MEN activity genotype in both males (5%) and females (6%) ($F_{1,8.31} = 5.83$ and $P < 0.01$ and $F_{1,10.4} = 11.48$ and $P < 0.001$, respectively). G6PD activity was also increased in males of the low MEN activity genotypes (7%, $F_{1,8.69} = 9.03$, $P < 0.004$), but not in females. The elasticity coefficients calculated in this experiment, −0.22 for IDH in both males and females and −0.28 for G6PD in males, were larger than those for *Idh* or *G6pd*, but one quarter the magnitude of those calculated in our earlier study (MERRITT *et al.* 2005). The fly lines in this study differ from the MERRITT *et al.* (2005) study in second chromosome genotype, so it is possible that this genetic difference is responsible for the variation in elasticity estimates between the two studies. There were no significant excision allele or chromosome effects on triglycerides.

DISCUSSION

Metabolic networks are interacting systems in which the functioning of any one component may depend upon the state of other components. Phenotypes determined by such networks will be the consequence of genotypes at

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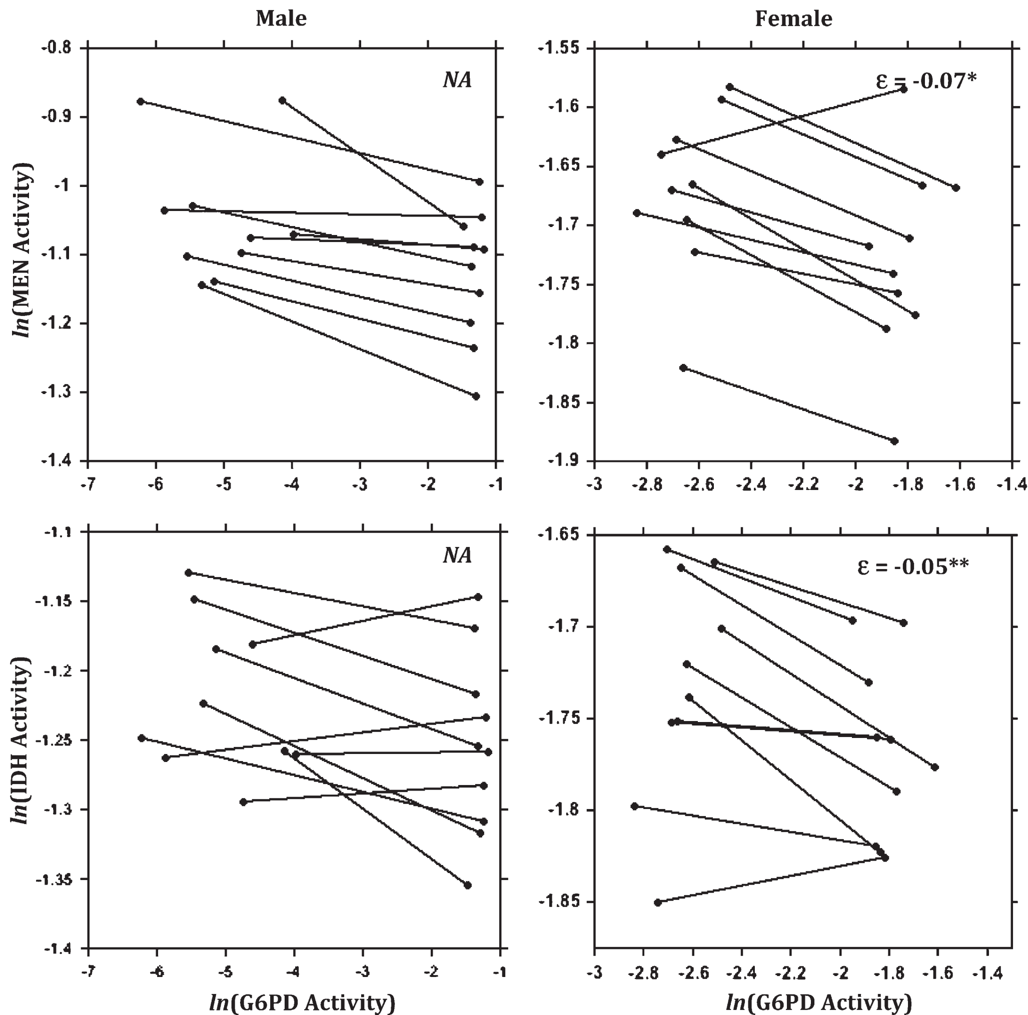

FIGURE 5.—Experiment 2B: comparison of the effects of changes in G6PD activity across 10 different third chromosome lines. Similar to Figure 4, except that the location of the *G6pd* gene on the *X* chromosome prevents construction of male *G6pd* allele heterozygotes. Comparisons in males are therefore between full and no G6PD activity flies. Elasticity coefficients for males were not computed because the *X*-linked nature of *G6pd* means that only large changes in G6PD activity could be generated in males, and it is unlikely that control would be consistent across such a large variation in enzyme activity. See the RESULTS for details of crosses. Statistical significance is indicated by (*) for $P < 0.05$ and (**) for $P < 0.01$; exact *P*-values are given in the text.

multiple loci. Understanding the function and evolution of such a network therefore requires both knowledge of the network members and quantification of their interacting contributions to the expression and control of network function. Our earlier study of the effects of variation in MEN activity suggested that *Men*, *Idh*, and *G6pd* could form such a network through a shared function in maintenance of the NADP/NADPH pool (MERRITT *et al.* 2005). In the study reported here, we have used synthetic activity alleles of *Idh* and *G6pd*, in addition to synthetic *Men* alleles, to individually vary enzymatic activity and measured responses for the other two enzymes and triglyceride concentration (a potential downstream phenotype). Using alleles spanning major and minor changes in activity for all three genes, we find significant responses among these three NADPH-dependent enzymes and, under extreme levels of activity reduction, in triglyceride concentration. This interdependence suggests that the system should be examined as interconnected members and not as isolated, individual, enzymes.

Curiously, genetic reduction of IDH activity has small but opposite effects on MEN and G6PDH activity. Lower IDH activity was associated with slightly higher G6PD activity, but with slightly lower MEN activity. The differences in G6PD are consistent with compensatory regulation of enzyme activity to maintain a relatively constant balance of NADPH to NADP, but the reduction in MEN is not compensatory. Both compensatory and parallel changes in IDH and MEN activities have been reported in the literature (GEER *et al.* 1976, 1978, 1981; WILTON *et al.* 1982; BENTLEY *et al.* 1983; GEER and LAURIE-AHLBERG 1984; MERRITT *et al.* 2005). It may be that the complex interactions observed between MEN and IDH reflect the physiological need to balance the oxidative products of these enzymes.

Reduced G6PD activity caused small increases in both MEN and IDH activity, consistent with compensation to maintain NADPH levels. We found no effect on triglyceride concentration across any of the G6PD experiments, and complete elimination of G6PD activity was not accompanied by any significant changes in triglyceride concentration. The lack of impact of G6PD activity on triglyceride concentration is in contrast with MEN (MERRITT *et al.* 2005) and IDH (above) where changes in activity cause pronounced reductions in triglyceride concentration. The less pronounced effects

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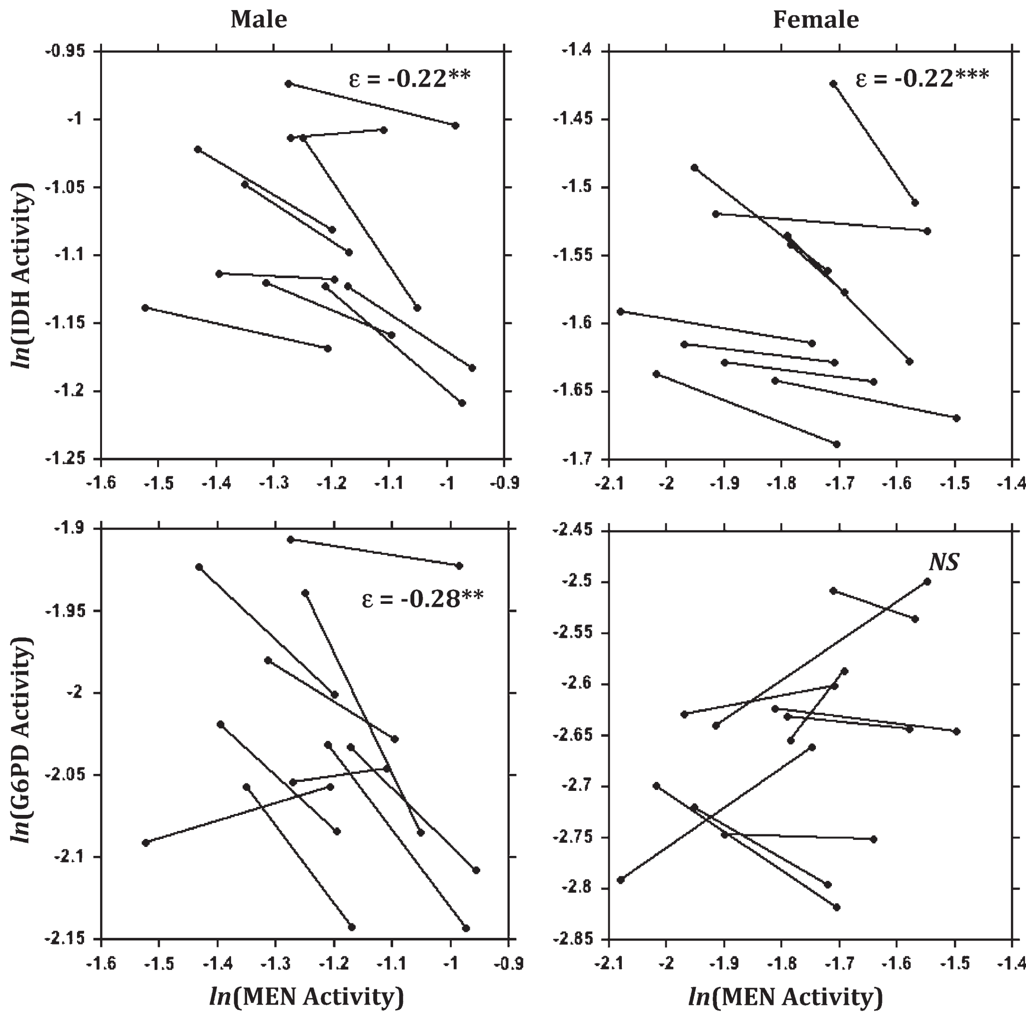

FIGURE 6.—Experiment 2C: comparison of the effects of changes in MEN across 10 different third chromosome lines. For details see Figure 4. Statistical significance is indicated by (*) for $P < 0.05$, (**) for $P < 0.01$, and (***) for $P < 0.001$; exact *P*-values are given in the text.

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of reduction in G6PD than in IDH or MEN suggest that G6PD supplies less NADPH than MEN or IDH. *G6pd*, however, does have naturally occurring alleles in *D. melanogaster* that both demonstrate significant activity differences (Eanes 1984; Eanes *et al.* 1990) and may be under selection (Flowers *et al.* 2007), suggesting that variation in G6PD likely does have a biological impact.

Our results suggest that MEN is the largest contributor to the NADPH pool in adult *D. melanogaster*, consistent with results from larval experiments using naturally occurring null alleles of *Men* (Geer *et al.* 1979a,b). We found that genetic perturbation of IDH and G6PD activities generally led to small effects, and even large reductions in the activity of these enzymes were associated only with small responses in the other member enzymes or in triglyceride content. The relative differences in contribution may reflect the multifunctional nature of the enzymes; the different oxidative role of each enzyme may limit or affect its relative role in producing reduced cofactor. A similar hierarchy for MEN, IDH, and G6PD in supplying NADPH has recently been demonstrated in bacteria and has been attributed to the pluripotent nature of pyruvate, the oxidative product of MEN (Singh *et al.* 2008).

The significant decreases in both MEN activity and triglycerides with decreasing IDH activity question our expectation of compensation for the NADPH/NADP pool. Both MEN and IDH are members of the proposed pyruvate/malate and pyruvate/citrate/ketoglutarate cycles that are associated with controlling energy balance and nutrient sensing (Guay *et al.* 2007; Pongratz *et al.* 2007). Among many candidates, citrate stands out as a metabolite signal in the secretion of insulin in response to energy balance (Farfari *et al.* 2000). Microarray studies measuring genomic responses to starvation and nutrient supply in Drosophila show that starvation and the subsequent signaling clearly lead to decreases in glucose oxidizing and fat biosynthetic enzymes (Zinke *et al.* 2002; Gershman *et al.* 2007). Likewise in mammalian pancreatic β-cells, blockage of cytosolic IDH impairs insulin secretion and pyruvate cycling (the malate/pyruvate cycle in which MEN is the central step) and leads to responses that are normally associated with starvation conditions (Ronnebaum *et al.* 2006). Our imposed experiential changes in cytosolic IDH activity in Drosophila may mimic this effect, resulting in suppression of MEN activity and reduced synthesis of triglycerides. Many features of energy

homeostasis are conserved across broad taxonomic distances, and if a pyruvate/citrate/ketoglutarate cycle is also important in energy-state signaling in Drosophila, then decoupling IDH from general NADPH/NADP compensation may be important.

These three enzymatic sources of NADPH show physiological interactions: the activity of any one often affects the activity of the other two. Although the differences in enzyme activity that we create using the synthetic alleles of all three loci are often larger than those seen between wild alleles, some closely mimic naturally occurring variation. For example, the activity variation between the synthetic *Men* genotypes resembles the variation seen between the naturally occurring *Men* and *G6pd* alleles (Eanes 1984; Eanes *et al.* 1990; Merritt *et al.* 2005). Given this similarity, interactions among enzymes likely occur between wild genotypes as well. The final phenotype, reflecting the NADPH/NADP ratio or total available NADPH, will be a multilocus or network trait. The connectivity between these enzymes indicates that selection at any one locus may be modified by the genotypes present at the other loci and that the enzyme's response under selection must be considered within the context of the network, not as a component in isolation.

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