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Triglyceride Pools, Flight and Activity Variation at the *Gpdh* Locus in *Drosophila melanogaster*

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ABSTRACT

We have created a set of *P*-element excision-derived *Gpdh* alleles that generate a range of GPDH activity phenotypes ranging from zero to full activity. By placing these synthetic alleles in isogenic backgrounds, we characterize the effects of minor and major activity variation on two different aspects of *Gpdh* function: the standing triglyceride pool and glycerol-3-phosphate shuttle-assisted flight. We observe small but statistically significant reductions in triglyceride content for adult *Gpdh* genotypes possessing 33–80% reductions from normal activity. These small differences scale to a notable proportion of the observed genetic variation in triglyceride content in natural populations. Using a tethered fly assay to assess flight metabolism, we observed that genotypes with 100 and 66% activity exhibited no significant difference in wingbeat frequency (WBF), while activity reductions from 60 to 10% showed statistically significant reductions of ~7% in WBF. These studies show that the molecular polymorphism associated with GPDH activity could be maintained in natural populations by selection in the triglyceride pool.

IT is well established from Drosophila studies that there is significant genetic variation for the activities of metabolic genes in natural populations (Laurie-Ahlberg *et al.* 1982; Clark 1989, 1990). Broad-based heritabilities of activity variation in Drosophila vary from a few enzymes that appear to show little genetic variation in activity to many with values >50%. The observation that the respective genes for these enzymes also show the highest codon bias among all genes in Drosophila, yeast, and *Escherichia coli* suggests that relatively strong selection for high levels of expression is a common enzyme-specific feature of the central metabolic network in diverse taxa (Futcher *et al.* 1999; Akashi 2001).

The central metabolic pathway enzymes have also been characterized for molecular variation in natural populations in *Drosophila melanogaster* dating back to the early days of allozyme screening (Singh and Rhomberg 1987; Singh 1992; Eanes 1999). From these studies we know that replacement polymorphism, as well as the heritability of activity, varies substantially across genes. However, the relative control that various steps exert over flux through the glycolytic pathway and its branches requires a systematic dissection by artificially modulating enzyme levels and measuring their consequences. This study addresses the potential metabolic control of glycerol-3-phosphate dehydrogenase (GPDH), an important and well-studied enzyme branching off the glycolytic pathway. A set of full and partial knockout alleles was derived from *P*-element excisions and used to characterize phenotypes and assess function associated with activity variation in this gene.

Numerous studies dating back over three decades have examined various aspects of the *Gpdh* gene in *D. melanogaster.* These have focused on determining the functional role of *Gpdh,* as well as assessing the potential for natural selection to act upon its wild alleles. The gene encodes two geographically widespread electrophoretic alleles, $Gpdh^F$ and $Gpdh^S$, which differ by a single amino acid substitution (Asn to Lys at residue 337 in exon 6; von Kalm *et al.* 1989). The $Gpdh^S$ variant has higher activity (Miller *et al.* 1975; Laurie-Ahlberg and Bewley 1983; Bewley *et al.* 1984; Barnes and Laurie-Ahlberg 1986; Kang *et al.* 1998) and DNA sequence variation suggests that these are relatively old alleles (Takano *et al.* 1993; Kreitman and Akashi 1995). Finally, there is a latitudinal cline for the *Gpdh* alleles that is reciprocated in both hemispheres (Miller *et al.* 1975; Oakeshott *et al.* 1984; Sezgin *et al.* 2004). This cline was first reported in 1973 (Johnson and Schaffer 1973) and our study of lines collected in 1997, or at least 240 generations later, finds the same cline in both hemispheres (Sezgin *et al.* 2004; Umina *et al.* 2005).

High frequencies of *Gpdh* null alleles (up to 2.7%) are recovered in natural populations throughout the world (Voelker *et al.* 1980; Langley *et al.* 1981; Gibson *et al.* 1991). This high frequency is difficult to reconcile on the basis of a selection–mutation balance, given the loss

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of fitness known to be associated with mutagen-derived null mutations at *Gpdh* (O'Brien and MacIntyre 1972; Kotarski *et al.* 1983). The presence of the null and low-activity alleles at such high frequencies suggests that reduced GPDH activity arising through recurrent mutation may have a fitness benefit in rare niches occupied by *D. melanogaster* or possibly that the *Gpdh* locus experiences a high mutation rate, perhaps as a preferred target for *P*-element insertion (Yamaguchi *et al.* 1994).

GPDH has two very different metabolic functions. The first is to act as the cytosolic partner in the glycerol-3-phosphate shuttle. This shuttle transports NADH/NAD equivalents in and out of the mitochondria and has an important role in Drosophila flight (see MacIntyre and Davis 1987). Homozygous null *Gpdh* genotypes are unable to fly (O'Brien and MacIntyre 1972). Given the stringent metabolic demands of flight, this suggests an important functional role for the enzyme. Barnes and Laurie-Alhberg (1986) assayed flight efficiency in constructed lines bearing the *F* and *S* alleles and reported subtle differences, where statistical significance depended on rearing and flight temperature. Using two *Gpdh* null alleles, Connors and Curtsinger (1986) estimated zero flux control associated with wing-beat frequency (WBF) for genotypes that differed by 100 and 50% relative activity. Finally, the second important role of GPDH in all organisms is to bridge glycolysis and triglyceride synthesis and degradation (see review Brisson *et al.* 2001).

Even though there are numerous studies of the GPDH protein polymorphism, no clear consensus has emerged about its phenotypic consequences (see, for example, McKechnie and Geer 1988; Barnes *et al.* 1989; Oudman *et al.* 1991, 1994). However, the general difficulty in these studies is that linked genetic background confounds both the interpretation of and the statistical power to assess phenotypic consequences. The consequences often might be subtle and difficult to statistically demonstrate, yet biologically important (Eanes 1999).

In this report we use *P*-element excisions in the 5′ region of the *Gpdh* gene to generate a series of synthetic alleles that span the range from null to full activity. To minimize linked and unlinked genetic variation, all alleles are recovered from a common progenitor chromosome and placed into a common *X* and third chromosome background. We consider the effects on two very different GPDH roles: on the control of the steady-state level of triglyceride in adults and on the role of the shuttle in affecting flight power. We examine effects across the full-activity range, including the full knockout of GPDH, but we are particularly interested in the consequences associated with the upper range, between 50 and 100% "normal" function. This is about the range of genetic variation for GPDH activity seen in nature (Laurie-Ahlberg and Bewley 1983) and represents the consequences of allele substitution of both amino acid replacements and *trans*- and *cis*-regulation.

MATERIALS AND METHODS

Stocks: The background replacement lines *w;CyO/L Bl;VT46* and *w;CyO/L Bl;VT83* were constructed in the Eanes lab from lines collected in Burlington, Vermont, in 1997 and then inbred. Line *w;6326;6326.1* is a subline from an isogenic line (6326; Hoskins *et al.* 2001) from the Indiana University Stock Center. The 10 iso-second chromosome wild lines are descended from the lines collected in 1997 along the eastern United States (Sezgin *et al.* 2004). The lines had their *X* and third chromosome backgrounds replaced using the *w;CyO/L Bl;VT83* line. All stocks and experiments were maintained at 25°.

Line constructions: In an otherwise isogenic set of second chromosomes, a series of *Gpdh* alleles varying in GPDH activity was created through *P*-element excision using the insertion *P{SUPor-P}KG02555* and the *Hopi* element (Calvi 1993) as a transposase source. This insertion is 95 bp upstream from the TATA promoter (von Kalm *et al.* 1989). From this cross we recovered 80 chromosomes with excisions (white eyes) that were balanced over the *CyO* chromosome. These lines were individually crossed against the $Gpdh^S$ electrophoretic allele (the *KG02555* progenitor *Gpdh* allele is $Gpdh^F$) and screened using starch electrophoresis (Brewer 1970). A subset of 12 chromosomes was identified with visibly reduced GPDH activity. Chromosomes from this set, as well as full-activity excision lines, were crossed into the *w;VT46;VT46* background using marker-assisted introgression (Figure 1). The alleles were retested for GPDH activity (in the direction of the back reaction) using spectrophotometric assay (Miller *et al.* 1975). The 12 alleles, along with 10 normal-activity excision lines, were subjected to molecular analysis using a combination of short- and long-range PCR and sequencing.

Relative viability crosses and design: Experiments 1A and 1B were used to examine relative viability effects of the *Gpdh* genotypes in two different activity ranges. Experiment 1A evaluated the effects of genotypic activity variation spanning ~60 to 100% "normal" activity. Using the eight lines *w;CyO/Δ9-2;VT46*, *Δ26-1*, *Δ24-1*, *Δ7-3*, *Δ10-1*, *Δ10-3*, *Δ29-1*, and *Δ15-1*, females were crossed with *w;6326;6326* males. Four crosses were set up, each with four females and four males. Every 4 days, the flies were transferred to fresh food. After the third transfer, the flies were discarded. This resulted in measurement of relative viability for 16 vials/genotype. Counts of *Cy* and +/+ flies of each genotype were made starting on day 12 and terminating on day 17 for each vial. This comparison assesses the viability of the *Gpdh* genotype relative to the *CyO/6326* genotype.

In experiment 1B, using a diallele cross, 25 crosses were set up with lines *w;CyO/Δ9-2;VT46*, *Δ26-1*, *Δ24-1*, *Δ7-3*, and *Δ15-1*. There are 10 reciprocating cross sets resulting in 15 different genotypes predicted to span 0–100% GPDH activity. Two vials were set up for each cross and transfers to fresh vials were made on days 4, 8, and 12. Thus, there were 16 vials for each genotype. Counts of *Cy* and +/+ flies of each genotype were made starting on day 12 and terminating on day 17 for each vial. This experiment tested the hypothesis that there are egg-to-adult viability differences across the entire genotype activity range.

Variation against 10 different second chromosome backgrounds: We were particularly interested to determine if any overall response in triglyceride content would be detectable across multiple genetic backgrounds. In experiment 2, a series of 20 paired crosses were made using the *GpdhΔ10-2* full-activity genotype and the *GpdhΔ9-2* null deletion genotype. Females from each of the 10 iso-second chromosome lines (third background replaced lines designated *w;CyO/i;VT83*) were crossed with males of both excision lines. Two vials (five females and five males per vial) were established for each cross genotype and these were transferred once after 4 days. Emerging flies

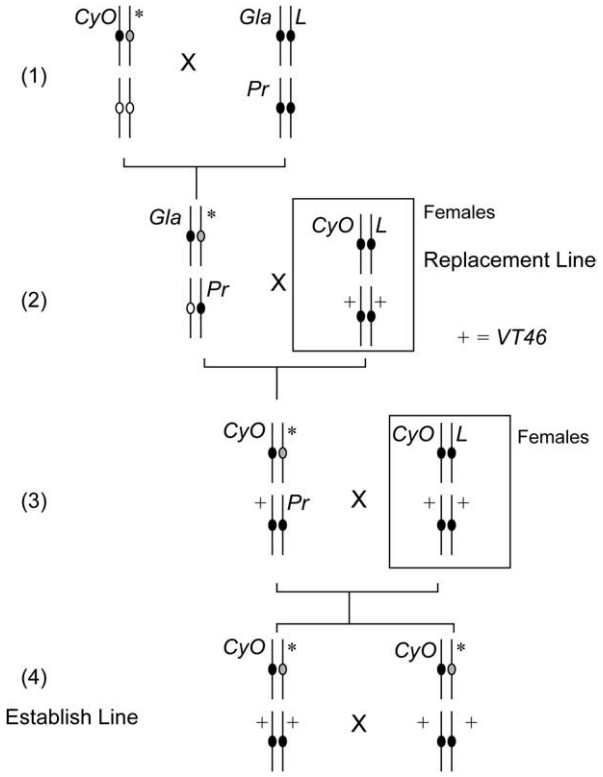

FIGURE 1.—The marker-assisted introgression crosses used to place each *Gpdh* excision chromosome in the *w*-marked *X* and *VT46* third chromosome backgrounds. The *X* chromosome is not shown in the figure, but is derived from the *w; CyO/L; VT46* replacement line. A similar scheme was used for the replacements into the *VT83* background.

were collected on day 12 and aged for 6 days before being assayed for enzyme activity and triglyceride levels.

Starvation–rebound studies: Experiment 3 was designed to assess the genotypic-dependent response of the triglyceride and glycogen pools to starvation and rebound. Interline crosses were used to establish three genotypes (Δ*9-2*/Δ*24-1*, Δ*24-1*/Δ*10-2*, and Δ*10-2*/Δ*10-2*) representing genotype-specific relative activities of 15, 66, and 100%. Emerging adults were held in groups of five on standard cornmeal for 6 days and then introduced to the starvation–rebound treatments. These consisted of a 24-hr preexposure to 2% sucrose (in 2% agar), followed by 19 hr of starvation (on 2% agar only) and then 12 hr of feeding rebound on 2% sucrose or 2% glycerol. A subset of flies was frozen at each treatment stage.

Genotype-specific triglyceride levels—experiments 4A, 4B, 5A, and 5B: This set of experiments was used to test the hypothesis that genotype sets representing different activity ranges possess different levels of triglyceride. In experiment 4A, *Gpdh*Δ*24-1* females were crossed with Δ*10-2* males and the F_1 Δ*24-1*/Δ*10-2* progeny were collected. About 200 F_1 females and 200 F_1 males, aged to 4–5 days, were mated and maintained on standard cornmeal for 5 days. The flies were then placed on standard cornmeal media in a half-pint bottle for 24 hr, with subsequent 24-hr transfers over a 7- to 10-day period. All the emerging F_2 males and females (expected to be segregating for genotypes Δ*10-2*/Δ*10-2*, Δ*24-1*/Δ*10-2*, and Δ*24-1*/Δ*24-1*) were collected over a 5-day period from each bottle, aged in groups of 10 for 5 days on standard cornmeal media, and assayed for GPDH, alcohol dehydrogenase (ADH), and malate dehydrogenase (MDH) activity, the metabolic pools of glycogen and triglyceride, soluble protein, and wet weight. A block represents all the flies collected in a 24-hr interval from a bottle and assayed on a single 96-well microplate. This experiment was designed to test in a common environment the effects of genotypes assumed to represent an activity set from 20 to 100% GPDH activity.

In experiment 4B, *Gpdh*Δ*9-2* females were crossed with Δ*10-2* males and the resulting F_1 *Gpdh* Δ*9-2*/Δ*10-2* female progeny were collected. These 200 F_1 females were crossed with *w; 6326;6326* males and transferred to bottles as in experiment 4A. These produced both *w 6326/*Δ*9-2;6326/VT46* and *w; 6326/*Δ*10-2;6326/VT46* genotypes with the anticipated 50 and 100% GPDH activities. As in experiment 4A, males were collected, aged 5–7 days, and assayed for enzyme activity and triglyceride levels. Studies on females (experiments 5A and 5B) followed the same design and were carried out in two independent crosses a month apart. This experiment was designed to test in a common environment the effects of genotypes assumed to represent an activity set from 50 to 100% GPDH activity.

Soluble triglyceride content: Soluble triglyceride was measured using a commercially available kit (Infinity triglyceride assay, Thermo Electron, Arlington, TX) following the manufacturer's suggested protocol. Ten microliters of fly homogenate was mixed with 100 μl of assay solution and incubated at 32° for 10 min. Each sample was assayed twice and the mean of these two results was used in further analysis.

Glycogen content: Total glycogen was measured by digesting glycogen to glucose and measuring total glucose using a commercially available kit (Infinity glucose assay, Thermo Electron) following the manufacturer's protocol. The digestion cocktail contained 10 μl of fly homogenate sample and 2 μl of 2.0 M Na acetate buffer, pH 5.7, containing amyloglucosidase (A-1602 from Sigma, St. Louis) at a concentration of 1 unit/sample. Samples were digested at 55° for 45 min and total glucose was measured using 10 μl of digested homogenate in 200 μl of glucose assay solution. Each sample was assayed twice and the mean used in analysis. Results are reported as micrograms of glycogen per fly. Preliminary studies showed that this protocol accurately reflected glycogen concentration and that free glucose naturally present in the flies contributed only negligibly to the results.

Soluble protein content: Soluble protein was measured using a commercially available kit (Bio-Rad, Hercules, CA) following the manufacturer's suggestions. Each sample was assayed twice and the mean of these two results was used in further analysis.

Enzyme activity measurements: Flies were homogenized in grinding buffer (0.01 M KH_2PO_4, 1.0 mM EDTA, pH 7.4) at a "concentration" of 1 fly/200 μl of buffer and centrifuged at 13,000 rpm for 5 min at 4° to pellet all solids. The supernatant was recovered and transferred to a 96-well plate and used in all enzymatic and metabolite (protein and triglyceride) assays. In experiment 2, assays were conducted on two samples per vial, where a sample is five individuals pooled in 1 ml of grinding buffer. In experiments 3, 4, and 5, assays were conducted on single flies (1 fly in 200 μl, 88 flies sampled per bottle).

Enzyme activity assays were carried out on a Molecular Designs SpectraMax 384 Plus 96-well plate spectrophotometer using 10 μl of fly extract and 100 μl of assay buffer and optical density was measured every 9 sec for 3 min. All activity assays were conducted at 25°. In all experiments, samples were assayed twice and the average was used in analysis. Enzyme activity is expressed as nanomolars of NAD^+ reduced per min per fly. The assay buffers for the three enzymes assayed in this study were as follows: GPDH—0.1 M glycine NaOH, 2.5 mM NAD^+, 15 mM α-glycerol-3-phosphate, pH 7.4; ADH—0.1 M Tris-HCL, 4.0 mM NAD^+, 0.8 M ethanol, pH 8.6; and MDH—0.1 M Tris-HCL,

TABLE 1

Eight *Gpdh* excision alleles, their molecular changes, and GPDH activity

Gpdh excision allele	Molecular phenotype	Units of activity (SE)[a]	Relative activity[b]
Δ*9-2*	1.465-kb deletion removing the first two exons and much of the upstream region	0	0
Δ*26-1*	108-bp deletion replaced by TGTTATTTCATCATG	1.14 (0.056)	0.18
Δ*24-1*	456-bp deletion replaced by 1.2 kb of P element, including TTATGTTATTCATCATG	1.31 (0.032)	0.21
Δ*7-3*	Perfect excision except ATTAGCAT replaced by TTCATCATG	4.30 (0.101)	0.70
Δ*10-3*	Perfect excision	5.30 (0.036)	0.86
Δ*15-1*	Perfect excision	5.38 (0.011)	0.87
Δ*29-1*	Perfect excision	5.62 (0.056)	0.91
Δ*10-2*	Perfect excision	6.17 (0.121)	1.00

[a] Units are nanomoles NAD per min per fly.
[b] Relative to highest-activity allele, Δ*10-2*.

4.0 mM NAD^+, 40.0 mM malate, pH 8.0. Initial values for appropriate pH, substrate, and cofactor concentrations for the reactions were taken from the literature and modified to give maximum enzyme activity.

Flight performance: Five-day-old adult male flies were gently anesthetized using CO_2 and tethered by attaching a syringe-cleaning wire hook to the dorsal surface of their mesothorax using Permount adhesive following Curtsinger and Laurie-Ahlberg (1981). Tethered flies were video recorded for 2-sec intervals using a Red Lake Imaging MotionScope high-speed video camera with a shutter speed of 1000 frames/sec. Only flies that began to beat their wings within 15 min of tethering were included in the experiment and no fly was recorded for >30 min after being tethered. Video recording and playback were done using Redlake Imagining MotionScope software. Wing-beat frequency was determined by counting the number of beats in a 200-msec interval. Each tethered fly was recorded twice and the average of the two replicates was used in analysis. Temperature was monitored, but not controlled; it was constant within days (blocks) and ranged from 26° to 28° between days.

Data analysis: Analysis of covariance (ANCOVA) analyses and Tukey's Honestly Significant Difference multiple comparison tests (Tukey's HSD test) were conducted using the JMP software package (release 5.0.1a, SAS Institute). All measures of glycogen and triglyceride were analyzed using protein and weight as covariates in the ANCOVA. The data are often partitioned into random "blocks," which was necessitated by the need in some cases to extend surveys across several days of measurement. Also the microplate reader was limited to $n = 88$ measures/plate; thus plates were treated as blocks, and some blocks combined both an assay day and plate effect. Such block effects were included in the ANCOVAs.

RESULTS

Our studies were designed to address a number of questions. These include the effect of *Gpdh* genotype-associated activity on egg-to-adult viability, on adult triglyceride pool levels, on changes in levels of glycogen and triglyceride under starvation and restored feeding, on the induction of cofactor sharing enzymes, and on flight metabolism.

Excision lines: We were able to generate a set of excision alleles spanning zero to full GPDH activity. All of the *P*-element excision lines were homozygous viable, and 12 lines were judged to possess lowered GPDH activity in the allozyme screen. In all, 22 lines (12 presumed low and 10 normal activity) were assayed for GPDH activity after background replacement with the *w*-marked *X* and *VT46* third chromosome. All normal-activity alleles possessed DNA sequences that were indistinguishable from precise element excision or gene conversion events (Nassif and Engels 1993). Many of the low-activity alleles were complex structures leaving portions of the element. Four *Gpdh* alleles were selected for the final low-activity set and four for the normal-activity set. These involved clearly defined mutations (without *P*-element LTRs) and are listed in Table 1 with estimates of the activities. The four alleles with precise excisions have slightly different activities (*e.g.*, Δ*10-2* is significantly higher than the other three) . This may reflect independent gene conversion events that have carried different portions of the *CyO* homolog. One full null, Δ*9-2*, is a large deletion removing much of the 5′ exons, and the three low-activity alleles, Δ*24-1*, Δ*26-1*, and Δ*7-3*, bear the typical 17-bp footprint of an incomplete excision of the element (Takasu-Ishikawa *et al.* 1992)

***Gpdh* excision alleles are additive with respect to GPDH activity:** We were concerned that genotypes that would be simple additive combinations of alleles with different homozygous activities could be generated. Figure 2 shows the plot of the average of homozygous parental genotypes plotted against the respective heterozygote activity for the 20 reciprocal crosses representing 10 combinations of five alleles (Δ*9-2*, Δ*24-1*, Δ*26-1*, Δ*7-3*, Δ*15-1*). The regression is highly significant (however, the points are not independent) with an estimated slope of $\beta = 1.01$. The heterozygotes possess activities that average 16% higher than their predicted values using the average pairwise homozygous activities. This suggests a low level of dominance for activity, although across the entire range most alleles combine in an additive fashion with respect to GPDH activity.

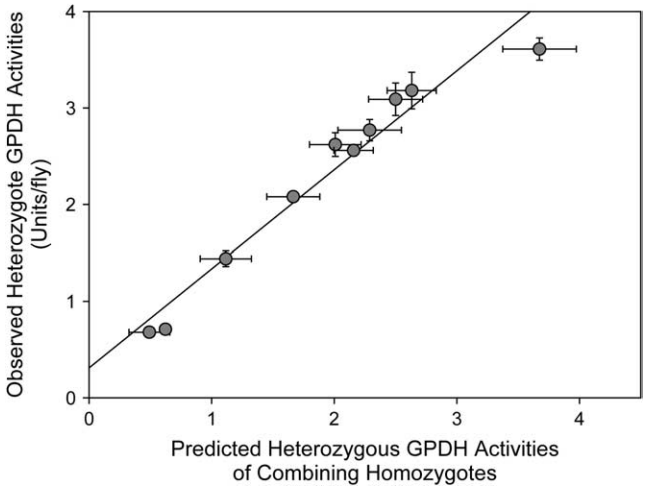

FIGURE 2.—Predicted heterozygote activities (ordinate with ±1 SE) as the average of the GPDH activities for combinations of five homozygous genotypes *vs.* the respective observed heterozygote activities for those genotypes (abscissa). Standard error bars of predicted values are approximated from the summed homozygote error variances.

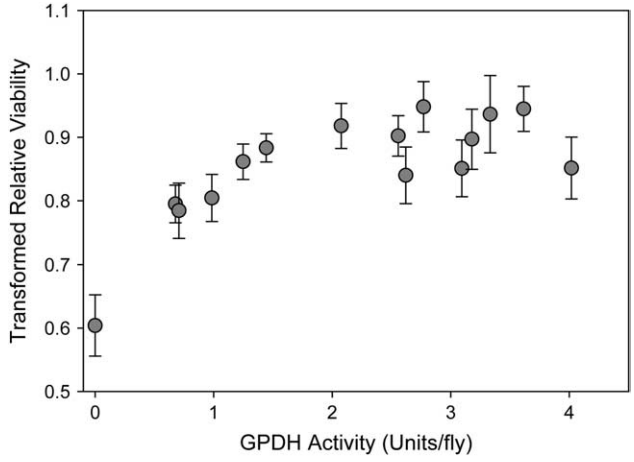

FIGURE 3.—Relative egg-to-adult viabilities (arc-sine transformed) of the 15 *Gpdh* genotypes against the respective GPDH activities (units per fly) of that genotype class. Viability is estimated as the ratio of emerging +/+ flies to *Cy* flies.

Egg-to-adult viability does not differ among genotypes ranging from 50 to 100% relative GPDH activity: The first viability experiment (1A) was designed to specifically examine the effects of reductions in activity down to 50% using the *6326* background. Egg-to-adult relative viability was then assessed relative to the standard *w;CyO/6326;6326/VT46* reference genotype emerging in each cross. The average relative viability (to *Cy* flies) was 51.6% and the means of the genotypes varied across the narrow range from 50.2 to 55.1%. After arc-sine transformation, there was no significant effect of *Gpdh* genotype on egg-to-adult viability.

Egg-to-adult viability differs among genotypes spanning 0–100% activity: Since the first viability experiment showed no significant reductions in relative viability for genotypes down to 50% wild-type activity, we can assume a constant viability for the *Cy* genotype. In experiment 1B, there is highly significant among-genotype variation in relative viability ($F_{14,219} = 17.48$, $P \ll 0.001$). Figure 3 shows the plot of relative viabilities for the 15 different genotypes. We observe a significant positive relationship between genotype-specific GPDH activity and egg-to-adult viability of the $Gpdh\Delta_i/Gpdh\Delta_j$ genotype relative to the $CyO/Gpdh\Delta_i$ genotype ($F_{1,13} = 14.20$, $P < 0.002$). This is especially apparent for the homozygous null *Δ9-2/Δ9-2* genotype, which also shows a greatly reduced male and female life span (rarely >5 days; data not shown). However, the regression is still significant with this extreme genotype removed ($F_{1,12} = 8.20$, $P < 0.014$). It is apparent that as *Gpdh* genotype activity drops below 50% there are negative effects on egg-to-adult viability.

Triglyceride pool levels are impacted by GPDH activity: Experiments 4A, 4B, 5A, and 5B were designed to assess the impact of functional variation on triglycerides and weight using individuals emerging from a common bottle. This removes the large environmental variation always associated with "vial-to-vial" differences. In all these cases, genotypes are inferred by plotting GPDH activity against wet weight. In such plots it is easy to assign individuals to the two or three genotype classes (Figure 4). In experiment 4A, the males of three genotypes (*Δ24-1/24-1*, *Δ24-1/Δ10-2*, and *Δ10-2/Δ10-2*) possessed 29, 78, and 100% relative GPDH activity (1.973 ± 0.026, 5.142 ± 0.17, and 6.603 ± 0.032 units/fly). There is some apparent dominance for activity. In experiment 4A1, we have three blocks, which are different bottles all collected on the same day of age and which include a day of assay and microplate. There is a highly significant block effect, but no significant block-by-genotype interaction effect on weight. There is significant among-genotype variation in adult male weight ($F_{2,4} = 9.410$, $P < 0.0187$) with the high-activity *Δ10-2/Δ10-2* genotype possessing a significantly lower weight by least-square (LS) means difference (Tukey HSD test; SOKAL and ROHLF 1995). This represents a 3.3% drop in weight associated with the high-activity genotype. There is also a significant among-genotype variation in triglyceride content (Figure 5, top; $F_{2,4} = 12.609$, $P < 0.0139$) again with the *Δ24-1/Δ24-1* low-activity genotype possessing lower triglyceride content by LS means difference. This represents a 16% drop in triglyceride content between the pooled higher- *vs.* lower-activity genotypes. In experiment 4A2, we have three different days of emergence from a single bottle. Weight drops significantly with day-of-emergence block, but there is no genotype effect. There is again a significant reduction of 18% in triglyceride due to GPDH genotype (Figure 5, bottom; $F = 15.57$, $P < 0.006$). Clearly large reductions in triglyceride content are associated with *Gpdh* alleles of major effect in reducing activity.

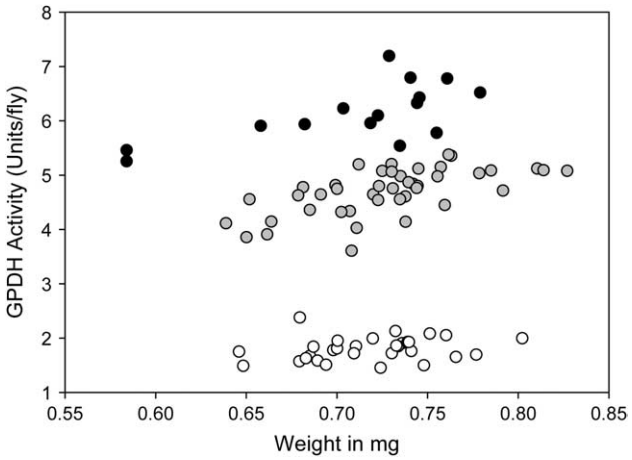

FIGURE 4.—Example of the weight and GPDH activities of male adults emerging from a single culture bottle during a 24-hr period in experiment 4A. The assigned activity genotypes are shown (Δ*24-1*/Δ*24-1*, open circles; Δ*24-1*/Δ*10-2*, shaded circles; and Δ*10-2*/Δ*10-2*, solid circles).

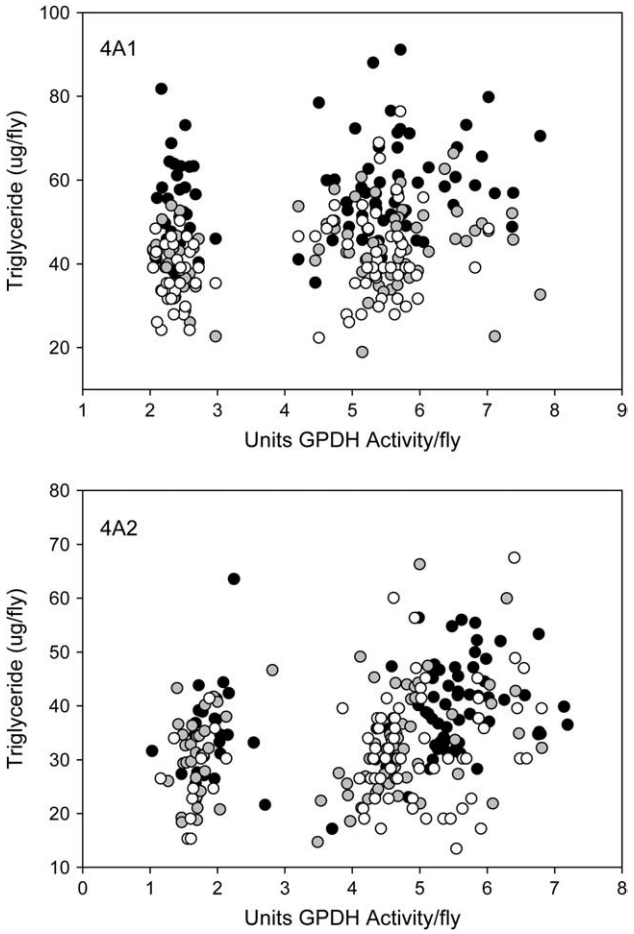

FIGURE 5.—(Experiment 4A1) Plot of whole-body triglyceride levels and weights for inferred genotypes (Δ*24-1*/Δ*24-1*, Δ*24-1*/Δ*10-2*, and Δ*10-2*/Δ*10-2*) of 5-day-old adult males emerging from day 12 in different bottles (bottles 1, 2, and 3 are open, shaded, and solid, respectively). (Experiment 4A2) Plot of whole-body triglyceride levels, genotypes (Δ*24-1*/Δ*24-1*, Δ*24-1*/Δ*10-2*, and Δ*10-2*/Δ*10-2*), and weights of 5-day-old adult males emerging from a single bottle on different emergence days (days 12, 13, and 14 are open, shaded, and solid, respectively).

For experiment 5A females, we could not easily separate the top two genotypic classes because of apparent dominance (Figure 6), so they were pooled. There is no significant difference in weight between genotypes. We observed a drop in mean triglyceride content (103.1 ± 8.9 and 107.5 ± 5.3 μg/fly), but this was not significant ($F_{1,171} = 0.0024$, $P < 0.95$).

Figure 7 shows the male weights and GPDH activities per individual emerging from a single bottle (on emergence day 3), representing block 1 in experiment 4B. This is a typical outcome and shows the unambiguous separation of the two expected *Gpdh* genotypes. The two classes represented by *w;*Δ*9-2/6326; 6326/VT46* and *w;*Δ*10-2/6326; 6326/VT46* genotypes are 66.5 and 100% GPDH relative activity classes (5.55 ± 0.017 and 8.34 ± 0.017 units/fly). There is no significant difference in body weight. There is again a statistically significant difference in triglyceride content ($F_{1,170} = 5.808$, $P < 0.017$) with the lower-activity class *w;*Δ*9-2/6326; 6326/VT46* genotype possessing ~6% lower triglyceride content (45.4 ± 1.22 and 48.4 ± 1.24 μg/fly; Figure 8). In the comparable 5B experiment with females, there is again no significant difference in body weight. The two genotypes again reflect relative levels of 74.4 and 100% activity, but while the lower-activity class again shows a lower mean triglyceride content (169.3 ± 3.4 *vs.* 173.4 ± 3.4 μg/fly), the ANCOVA is not statistically significant ($F_{1,170} = 1.0663$, $P > 0.303$). The overall result of this set of experiments is that *Gpdh* activity has a significant effect on triglyceride level in males, with lower activities associated with lower triglyceride level.

GPDH genotypes show similar triglyceride and glycogen pool responses to starvation and refeeding: This experiment was carried out to assess the differential response under conditions where glycogen and triglyceride pools were expected to change markedly. After 19 hr of starvation, the genotypes had lost an average of 12.5% of their body weight and this was nearly recovered after 12 hr of feeding on 2% sucrose in agar. There was no significant difference in body weight among *Gpdh* genotypes after any treatment and there was no significant genotype-by-treatment interaction. After 19 hr of starvation, triglyceride levels per fly dropped an average of 80% (or 4%/hr) and returned to 43% of their prestarvation levels after12 hr of feeding. The two-way ANOVA indicated a highly significant (but not unexpected) starvation treatment effect on triglyceride content ($F_{2,180} = 28.24$, $P < 0.001$), but no significant genotype or genotype-by-treatment interaction effect. If present, such an effect would have indicated a differential genotypic response to starvation and rebound on sucrose when the genotype activities are reduced to ~10% normal GPDH levels (Δ*24-1*/Δ *9-2* genotype). Upon 19 hr of

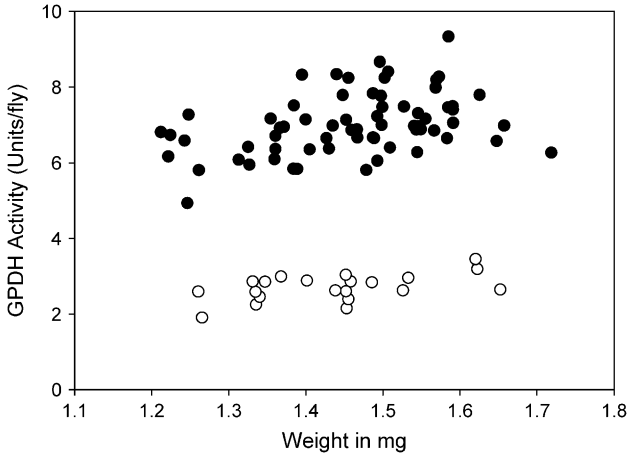

FIGURE 6.—Example of the weight and GPDH activities of females emerging during a 24-hr period in experiment 5A (Δ*24-1*/Δ*24-1*, open circles; Δ*24-1*/Δ*10-2* and Δ*10-2*/Δ*10-2*, solid circles).

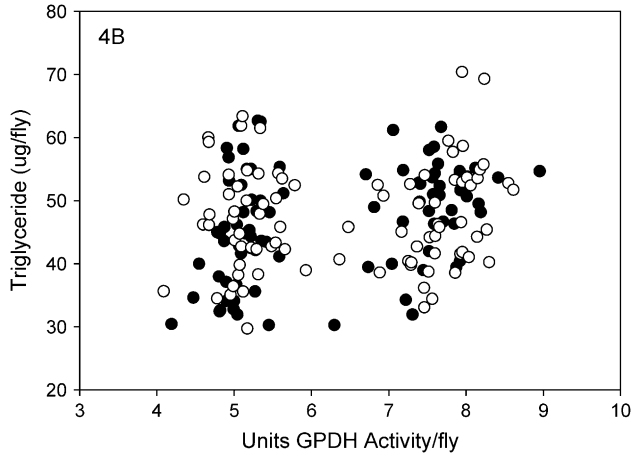

FIGURE 8.—Plot of whole-body triglyceride levels, genotypes (Δ*9-2/6326* and Δ*10-2/6326*), and weights of 5-day-old adult males emerging from day 12 in a single bottle (solid and open circles represent different assay plates) from experiment 4B.

starvation, glycogen levels dropped an average of 62% and returned to near (92%) prestarvation levels with feeding. The two-way ANOVA indicated a highly significant (but again expected) treatment effect on glycogen content ($F_{2,180} = 11.04$, $P < 0.001$), but no significant genotype or genotype-by-treatment interaction. Starvation rebound was also examined for flies rebounded on 2% glycerol, but only glycogen was studied because the triglycerol assay is strongly influenced by free glycerol in the crop and gut. With feeding on glycerol, glycogen content almost doubled and reached 78% of prestarvation levels. However, there was no significant genotype effect or genotype-by-treatment interaction. We conclude that, conditional on the power to detect such differences with our sample sizes, there is no significant effect of the *Gpdh* genotype on the response of these metabolic pools to starvation and refeeding treatments, even when the lowest genotype had only 10% normal activity.

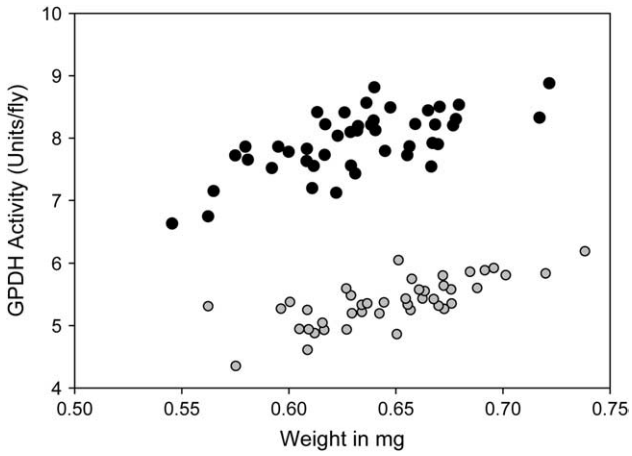

FIGURE 7.—Example of the wet weight and GPDH activities of male adults emerging during a 24-hr period from a common bottle in experiment 4B. The groups of the activity genotypes are shown (Δ*9-2/6326*, shaded circles; Δ*10-2/6326*, solid circles).

Studies with different second chromosome backgrounds: We were interested in whether general effects on triglyceride could be demonstrated across second chromosome backgrounds. We placed the Δ*9-2* (null deletion allele) and Δ*10-2* (normal) excision alleles in heterozygous condition with 10 wild-derived second chromosomes, all with the same *X* and third (*VT83*) backgrounds (Figure 9A). There were highly significant excision allele ($F_{1,8.84} = 1286.76$, $P < 0.001$) and second chromosome-line effects ($F_{9,9.19} = 6.17$ $P < 0.006$) on GPDH activity. There is no significant effect of the *Gpdh* allozyme allele. There were no significant excision allele-by-chromosome line interaction effects. Across all chromosomes the mean activities between excision alleles varied from 5.165 ± 0.068 to 8.697 ± 0.069 units/fly or about a 41% GPDH activity reduction in the null Δ*9-2* deletion background. There were significant line effects on triglyceride content ($F_{9,9.95} = 3.585$, $P < 0.032$), but no deletion allele effect on triglyceride content. However, as in all the experiments, the lower GPDH activity class has lower triglyceride content (Figure 9B; 78.5 ± 2.13 *vs.* 82.57 ± 2.13 μg/fly; for 8 of 10 chromosomes, the null Δ*9-2* deletion background has lower triglyceride content).

***Gpdh* genotypes show no induction or suppression of NAD-dependent MDH and ADH activities:** Along with the GPDH shuttle, the other important NADH/NAD shuttle is the malate-aspartate shuttle. Furthermore, under ethanol exposure joint induction of GPDH and ADH activities has been commonly observed (KHAN *et al.* 1996). We were interested in determining if reductions in GPDH activity associated with *Gpdh* excision alleles had pleiotropic effects on MDH or ADH activity. We observed no significant effects in males or females for MDH (in experiments 4A, 4B, 5A, and 5B). We estimate

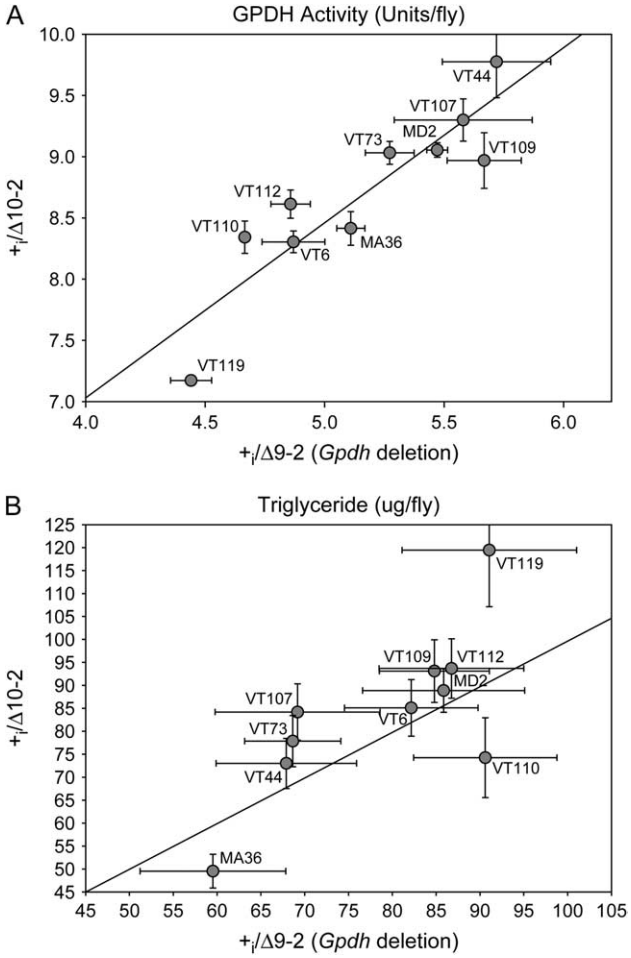

FIGURE 9.—(A) Plot of line mean activities for GPDH (±2 SE) across the 10 second chromosomes (*i*) represented by *w; +$_i$/Δ9-2;VT46/VT83* and *w; +$_i$/Δ10-2;VT46/VT83* paired genotypes. The linear regression is estimated as $Y = 1.31 + 1.43X$. (B) Plot of triglyceride means (±2 SE) for the same lines and genotypes. The diagonal line represents parity in values.

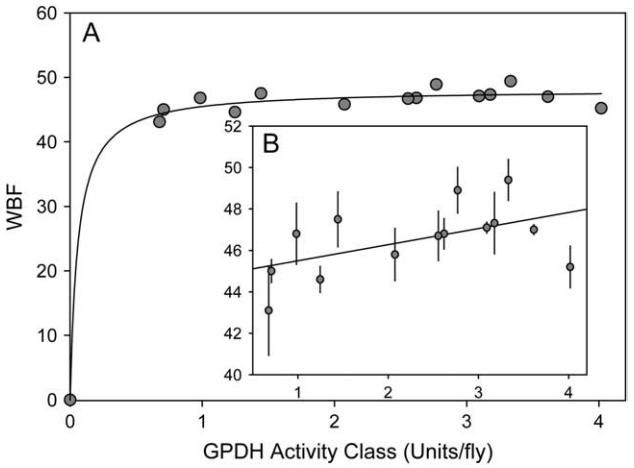

FIGURE 10.—(A) WBF per 200 msec against the *Gpdh* genotype-specific class activities (units per fly) across the entire activity range. The line fit is to a hyperbola and is meant to simply show a predicted fitted function. (B) Subset of lines in A after removing the null *Gpdh* class and applying the best fit to a linear regression model.

that such effects on MDH, if present, must be <1% in activity. In experiment 4A1 (blocks are different bottles), we observed a significant *Gpdh* genotype effect on ADH activity, with activity decreasing ~7% with a 70% *Gpdh* activity decline ($F_{2,246} = 5.29$, $P < 0.005$). In experiment 4A2 (blocks are day of emergence from same bottle), we again observed a highly significant, albeit small, drop in ADH activity ($F = 38.69$, $P < 0.0002$). No significant *Gpdh* genotype effects on ADH activity were observed in experiments 4B, 5A, and 5B.

Flight performance: We measured the wing-beat frequency in males under two different designs. In the first largely exploratory design, smaller numbers of flies were tested in each genotypic class, and each class was reared in four different vials, with two individuals assayed for WBF per vial. Excluding the homozygous null genotypes, the activity means of the genotypic classes range from 0.6 to 4.0 units/fly (Figure 10A). The *Δ9-2/Δ9-2* genotype does not beat its wings. When this point is dropped from the analysis, there is a nonsignificant reduction in WBF (Figure 10B) with a slope of $b = 0.78 \pm 0.367$ ($t_{13} = 2.12$, $P < 0.056$). This is not a particularly powerful test, but suggests a reduction in flight performance of as much as 5–6% across the genotypic classes.

In the second design, all flies to be assayed emerge from the same bottle on the same day and the block effect is the WBF assay day. We first measured the effects of the upper-range changes in GPDH activity on WBF by comparing genotypes *Δ9-2/6326* and *Δ10-2/6326*. We observed that GPDH reductions of 33% relative to normal show no significant reduction in WBF [Figure 11A; block (day): $F_{1,86} = 35.21$, $P < 0.0001$; genotype: $F_{1,86} = 0.6884$, $P < 0.409$]. In contrast, when we followed lower-range reductions (10 and 60% of normal activity) in GPDH (Figure 11B) using genotypes *Gpdh Δ9-2/Δ24-1* and *Gpdh Δ24-1/Δ10-2*, we observed a significant mean reduction of 14 beats/200 msec, or 7% WBF [block (day): $F_{1,85} = 5.83$, $P < 0.018$; genotype: $F_{1,85} = 10.67$, $P < 0.0016$]. In conclusion, we find that large GPDH reductions (to ~10% of normal) impact flight performance.

DISCUSSION

There is a large literature focused on the physiological effects of the *Gpdh* allozymes and their potential relationship to natural selection, much of which has been acknowledged to be inconclusive (see, for example, BARNES *et al.* 1989). Our creation of a set of synthetic *Gpdh* alleles varying widely and decisively in activity and placement of these into identical backgrounds (the alleles are already in identical second chromosomes) allowed us to explore subtle physiological effects of functional variation at *Gpdh*. These alleles are stripped of the

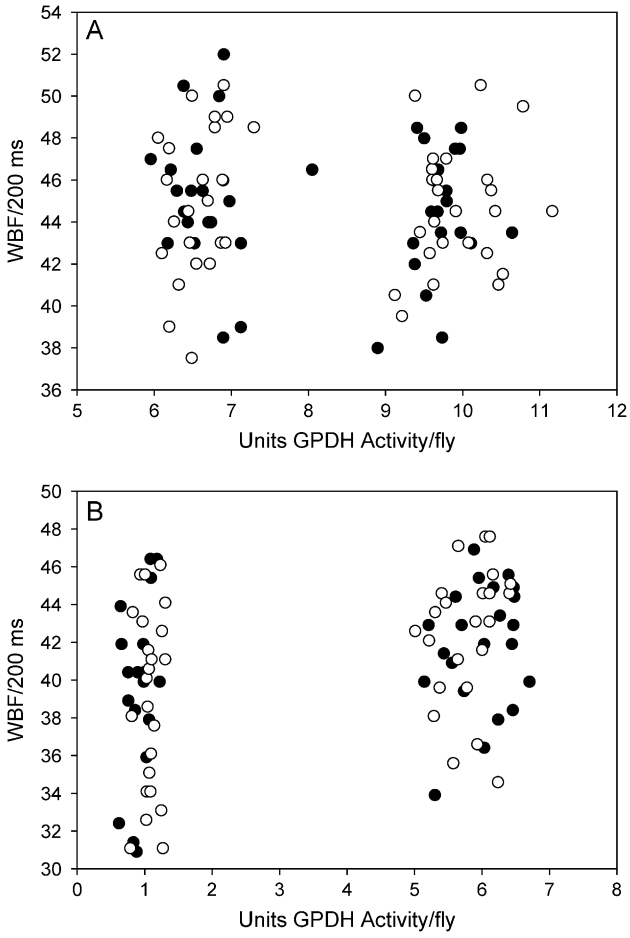

FIGURE 11.—(A) WBF of male flies all emerging on a single day from a common bottle, where the predicted classes have 66 and 100% relative *Gpdh* activities (genotypes Δ*9-2/6326* and Δ*10-2/6326*). The two blocks (different assay days) are shown with solid and open circles and the estimated block effect from the ANCOVA has been removed. There is a significant block (assay day) effect, but no significant *Gpdh* genotype effect on WBF. (B) WBF of aged male flies all emerging on a single day from a bottle, where the predicted *Gpdh* classes have 15 and 60% relative (to normal) *Gpdh* activities (genotypes Δ*9-2*/Δ*24-1* and Δ*24-1*/Δ*10-2*). The two blocks are shown with solid and open circles and the estimated block effect has been removed. There is no significant block (assay day) effect, but a significant *Gpdh* genotype effect.

encumbering and confounding effects of linked *cis*- and *trans*-acting background variation, which even in well-designed experiments is difficult to reduce, especially when phenotype differences may be measurably small (yet potentially important). Furthermore, variation in these alleles is expected to be entirely transcriptional, changing only enzyme amounts and not the catalytic properties.

In this study we are particularly interested in characterizing the effects of GPDH activity variation in the upper 50–100% range in activity. BARNES and LAURIE-AHLBERG (1986) investigated the *in vitro* activity variation for GPDH in wild chromosomes, and the chromosomal line variance component in their study can be used as an estimate of the genetic variance. The genetic standard deviation (σ_G) for second chromosomes from their study indicates a value around $\sigma_G = 0.739$ activity units/individual for their second chromosome lines with a mean of ~5.5 GPDH units/individual. Our second chromosome line variance estimate from experiment 2 is similar ($\sigma_G = 0.710$). Of course this reflects not just *cis*-acting linked *Gpdh* transcription and the allozyme differences, but second chromosome effects acting in *trans* as well. Nevertheless, it still serves as a relative scale of comparison. Therefore, ~68% of the wild chromosome lines fall within a GPDH activity span scaled to ~±26% of the mean line activity. The relative reductions of 27–33% in GPDH activity that we have generated with our excision alleles reflect the range of common genetic variation for GPDH activity seen in natural populations. We are also interested in the phenotypes of alleles of major effect because these define the extremes and further allow us to examine the physiological nature of null *Gpdh* alleles in populations.

We have examined, under rather benign environmental conditions, the two very different physiological roles of GPDH. The role of GPDH in setting a steady-state triglyceride pool level is not predictable because GPDH must be a freely reversible enzyme in the fat body. In principle, changes in activity should equally affect the forward (synthesis) and backward (degradation) catalysis of glycerol-3-phosphate and dihydroxy acetone phosphate. Therefore, it is unclear if increased activity should lead to increased or decreased pool levels, or no change at all. However, if triglycerides are removed from this pool through routes other than the path through GPDH, as in the production of eggs and sperm, then one might expect lower triglyceride pool levels associated with the lower-activity genotypes.

We observed that decreases in GPDH activity decrease the triglyceride pool and we believe that this should be the case for natural variation as well. Genotypes that represent a 33% reduction from normal GPDH activity show a modest 6% drop in triglyceride levels in males, but when scaled against the phenotypic and genetic (line) variance in triglycerides from our 10-sec chromosomes in natural populations, this is a considerable difference. Using our total phenotypic and line variance estimates for triglycerides, we estimate that these differences scale to ~22 and 60% of the total phenotypic (σ_t = 21.5 μg/fly) and genetic (or second chromosome line, $\sigma_l = 7.9$) standard deviations, respectively.

The consensus from a number of studies is that the slow-mobility allozyme allele has higher activity (MILLER *et al.* 1975; LAURIE-AHLBERG and BEWLEY 1983; BEWLEY *et al.* 1984; BARNES and LAURIE-AHLBERG 1986; KANG *et al.* 1998). Our studies predict that this difference should increase triglyceride levels and this might favor the increased frequency of $Gpdh^s$ in higher latitudes where glycerol and triglycerols are known to be involved

in winter diapause in other Drosophila (Ohtsu *et al.* 1993). In Drosophila, both desiccation and starvation have immediate and profound effects on metabolic pools (Marron *et al.* 2003). Our experiments are typical; under starvation, ~1.6–4% of the triglyceride pool is lost per hour and death occurs after ~36 hr in males. In natural populations, triglycerides could be a very labile pool whose levels should come under selection.

While GPDH is likely a physiologically reversible enzyme in the fat body, it might be considered a unidirectional enzyme in the flight muscle. Barnes and Laurie-Ahlberg (1986) examined the influence of the fast and slow *Gpdh* allozyme genotypes on tethered flight power and found suggestive, but nonsignificant, effects. In our excision genotypes, which should possess no GPDH activity variation due to genetic background, we observed no significant effects on WBF associated with a 33% reduction of GPDH. This makes the difficulty in detecting differences in the allozyme genotypes understandable, even in a well-designed project such as that of Barnes and Laurie-Ahlberg (1986). Nevertheless, there is still a statistically significant drop of ~7% in WBF associated with large GPDH activity reductions when they reach ~10% of "normal" activities. In sum, the metabolic control of flight metabolism by GPDH variation in the activity range of reported line and allozyme variation appears to be negligible and probably contributes little to fitness variation associated with this gene in natural populations. This is consistent with the report by Connors and Curtsinger (1986), who reported a zero control coefficient using a different design and stock center *Gpdh* nulls of uncertain *in vivo* activity.

The relatively small reduction of WBF for genotypes with only 10% of normal GPDH activity is surprising and suggests "excess" enzymatic capacity (see Suarez *et al.* 1996; Suarez 2003) that is not required for flight. A large amount of the soluble protein in the fly is GPDH (Lee *et al.* 1980) and it might have been suspected that this was essential for its role in flight metabolism. Possibly this extra capacity is required for flight at higher or lower temperatures, or for flies reared or acclimatized at one temperature, but challenged to fly at another temperature. This possibility is being considered in future research. Ever since its introduction by Chadwick (1939), tethered flight has been instrumental in the study of the physiology and biomechanics of insect flight. However, it is possible that tethered flight does not reflect the full power requirements of free flight, although studies claim that they reflect at least 80% of the lift requirements (Lehmann and Dickinson 1997). There is also evidence that in muscle GPDH is involved in myosin colocalization of several adjacent enzymes in the pathway (Wojtas *et al.* 1997). Perhaps GPDH is not required for flight metabolism, but does hold together a supporting scaffold. The complete loss of flight in GPDH null homozygotes could result from flux reduction, structural collapse, or both. Another possibility is that other energy sources such as proline are also significant contributors to flight metabolism, although this has yet to be demonstrated in Drosophila. Clearly, the dissection of flight metabolism using direct genetic modulation of pathway steps opens many new questions about the canalization of flight performance. This is the first demonstration of a reduction of flight metabolism associated with reduction of a pathway member, other than the effects of full knockout.

We were also interested in possible pleiotropic effects on other NAD/NADH-dependent enzymes, in particular MDH and ADH. MDH is involved in the malate-aspartate shuttle. ADH and GPDH activities covary under both ethanol and glycerol exposure (Khan *et al.* 1996) and synergistic activity variation between ADH genotypes and GPDH and MDH activities have been reported (Li 1992). A common genetic response to ethanol exposure in population cages has also been reported for these three genes and their allozyme polymorphisms (Cavener and Clegg 1981). We find that MDH shows no response, but ADH shows a positive correlation, decreasing significantly (but only 7%) as GPDH is reduced to levels of ~20% normal. This shows little compensation, but perhaps does reflect some homeostatic response since the NADH produced from ADH in ethanol metabolism may be used to generate triglycerides and some NADH will be shuttled into the mitochondria (McKechnie and Geer 1988). Overall, we conclude that genetic variation in *Gpdh* does not generate notable genetic correlations with ADH or MDH activity under these conditions. It would be of interest to examine these inductions under varying dietary changes, such as ethanol. Induction may also show a different response in the larvae.

What do our results say about the high frequencies of null and low-activity *Gpdh* alleles in natural populations? One issue was the well-established role of *Gpdh* in flight. Clearly, homozygous null flies of $\Delta 9\text{-}2/\Delta 9\text{-}2$ have low fertility, are very short lived (<5 days), and cannot fly, which is the description of the early nulls by O'Brien and Shimada (1974). It is clear that loss or near loss of GPDH has marked effects on fitness and it is likely that true null homozygous genotypes are lethal in nature. Nevertheless, it is remarkable how healthy flies with only 10–20% GPDH activity appear. Most *Gpdh* null alleles will be found in the heterozygous state in natural populations, and as adults these genotypes will possess lower triglyceride pools, but flight will not be affected.

Finally, as in the case of *Men* (Merritt *et al.* 2005), we have observed a probable case of transvection (Henikoff and Comai 1998; Wu and Morris 1999). Transvection appears to result from a homology-dependent chromosome pairing of *trans*-acting enhancers and promoters (*e.g.*, Morris *et al.* 2004). Gibson *et al.* (1999) reported a case of apparent transvection for *Gpdh* null alleles that they had collected from local populations. Their observation is based on the presence in null alleles of KP elements

inserted between the promoter and the transcription start site. Interestingly, the null allele $Gpdh^{AMB5}$ characterized by Reed and Gibson (1993) has a TATA box lesion almost identical to that of our Δ*7-3*, Δ*24-1*, and Δ*26-1* alleles, implying that the allele was probably a wild derivative of a *P*-element excision event.

It is well known that the demands on metabolic pools are set by adipokinetic hormones that are released from the central nervous system (see Van der Horst 2003; Lee and Park 2004). In the case of flight, the *demand* for ATP hydrolysis is set by these neurohormonal controls and not by the enzymes of the pathway. Blockage of these steps will certainly terminate the flow of substrate, but excess activity will not increase the flux since it is set by the distal metabolic demand. Our studies using controlled full and partial knockouts show that the activity variation associated with *Gpdh* in natural populations is probably physiologically significant and acts as polygenic variation in a fine tuning of the triglyceride pool. Scaled against the background of the phenotypic and genetic variance in triglyceride levels, these differences, even in alleles of small effect, are certainly of sufficient magnitude to respond to natural selection and to cause observations such as the frequency cline in the allozyme polymorphism.

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